PREDICTING PROGNOSIS AND DRUG RESPONSE IN BREAST CANCER

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by

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PREFACE

The problems associated with the current management of breast cancer are reviewed in a general introduction. The first part of the thesis presents a survival study of a large group of women with primary breast cancer who have been treated by mastectomy. The significance of established prognostic indicators is defined, as is the role of steroid receptor status. In a further investigation the in-vitro Thymidine Labelling Index was measured and values related both to other prognostic variables and to the rates of recurrence and survival following mastectomy. Finally all prognostic information was combined in a form of multivariate analysis to produce a statistical model which could then be used to predict the fate of individual women with breast cancer.

In Part II the evolution of a short-term in-vitro drug sensitivity assay is described. This assay was then tested on organ cultures of breast carcinomas and their in-vitro behaviour related to oestrogen receptor status. In a small clinical study the responses of patients with advanced breast cancer treated with systemic agents were compared with the rates of in-vitro inhibition by the same drugs.

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PREDICTING PROGNOSIS AND DRUG RESPONSE IN BREAST CANCER

Introduction

Chapter 1

Incidence

Despite advances in earlier diagnosis and treatment both the incidence and mortality of female breast cancer have risen over the past 50 years. In 1980 more than 20,000 new cases were diagnosed in England and Wales and the annual mortality now exceeds 12,000 (Silverberg, 1981). It has been suggested that the increasing incidence of malignant breast disease reflects an ageing population, however in this country breast cancer remains the commonest cause of death in women between the ages of 35 and 55 years.

Aetiology

The aetiology and epidemiology of breast cancer remain obscure. However, certain risk factors have emerged which may enable us to focus our attention on certain groups of women who are at maximum risk of developing the disease. Breast cancer is most prevalent in Europe and North America (Waterhouse, 1976). Within these populations the most potent risk factors appear to be age (Doll, 1975), a positive family history (Anderson, 1974), and a history of contralateral breast cancer (Lewison, 1971). In other malignant diseases the recognition of causative factors, e.g. cigarette smoking and bronchial carcinoma, has led to significant progress in disease prevention. Such an approach does not seem likely to be successful in the prevention of breast cancer in the forseeable future.

Early Detection

It has been shown that women with smaller tumours have a more favourable prognosis (Fisher, 1969). On this basis it would seem desirable to detect tumours at an early stage in their natural history. Two approaches are possible; the first is to screen an asymptomatic "at risk" population and the second to educate women to examine themselves and promptly report abnormal findings. The relative value of these two approaches has not yet been fully evaluated scientifically.

In 1963 the Health Insurance Plan of New York began a study of 62,000 women, half of whom were randomly allocated to receive screening evaluation every 3 years. A total of 584 breast cancers were diagnosed in the first 5 years of a study - 132 of which were detected on screening. Of these 132, 33% were detectable only on mammography and 45% were noted by palpation alone. Patients with cancers detected by screening had a lower incidence of axillary lymph node involvement, and at 9 years had an improved survival in comparison to their non-screened counterparts (Shapiro, 1977).

The benefits from screening must be balanced against the physical and psychological hazards. It has been estimated that 18-30 new cancers would be induced by mammography of 100,000 women under 50 years. In contrast 10-14 lives may be saved by early detection if a similar group of women over 50 years were screened (Breslow, 1977). It would seem sensible, therefore, to restrict routine screening to women over the age of 50 years.

In theory self-examination may be as fruitful and less costly than screening by mammography. In a retrospective study of over 200 women with breast cancer from the Georgia Cancer Management Network the practice of breast self-examination was compared with the stage of

presentation of diagnosis (Huguley, 1981). They found that women practising self-examination presented at an earlier stage with smaller tumours and a lower incidence of axillary lymph node involvement. These pathological differences might eventually be expected to result in different death rates. This hypothesis is currently being evaluated by a multicentre D.H.S.S. trial comparing mortality rate for screened populations and populations intensively instructed in self-examination (U.K. Trial, 1981). Despite all of these encouraging trends there is evidence that many of these small tumours detected early by screening may be multifocal and have rapid growth rates (Schwartz, 1980). In addition to early detection, therefore, there is still a need for more effective primary treatment of breast cancer.

Chapter 2

Local Therapy

(i) Surgery: Traditionally the aim of local treatment for breast cancer was to rid the patient of all disease. The Halsted radical mastectomy evolved specifically from the hypothesis that breast cancer spread always by direct contiguity; en bloc resection of the breast and axillary nodes therefore appeared to be a logical approach. The results from this radical operation were impressive in terms of local control, when compared with simple mastectomy alone, as employed in the 19th century (Mansfield, 1976). It was felt that the development of metastases resulted from tumour cells dislodged at the time of primary surgery, or retained due to an inadequate surgical clearance.

The concept of "cure" in breast cancer remains controversial (Brinkley, 1975; Duncan, 1976). Brinkley presented a 25 year follow up of 704 women who had been treated by mastectomy. The evidence from this study suggests that breast cancer is curable, in that after 20 years there is a group of surviving patients whose overall life-expectancy is similar to that of an age-matched normal population. However, despite this, the probability of dying from breast cancer within this group is still greater than that of the normal population. Bond (1968) has therefore suggested that breast cancer should always be regarded as a disseminated disease, the metastases remaining dormant, or growing at a very slow rate until they present clinically. Although the condition is not necessarily lethal, cure in the sense of total elimination, would be impossible by local surgery alone. The impact of local therapy on survival in primary breast cancer has only been investigated scientifically in recent years.

Historically, radical mastectomy has been considered the yard-stick by which other forms of treatment should be judged. The efficacy of any treatment should be determined by comparing survival following treatment with that of contemporary untreated controls - the basis of the randomised controlled clinical trial. When women treated with Halsted radical mastectomy are compared with untreated women presenting during the same era - there is no clear survival advantage (Lewis, 1932). However, such comparisons should be considered invalid due to patient selection and the use of historical controls.

When contemporary untreated controls are not available the next best controls are those selected in a similar manner, but treated by different techniques. When patients treated by classical radical mastectomy were compared with groups treated by simple mastectomy (Haagensen, 1969) there appeared to be no survival advantage when stratified by clinical stage.

(ii) Radiotherapy In 1948 McWhirter advocated simple mastectomy plus radiotherapy to the chest wall, axilla and supraclavicular nodes. It has since been shown that the addition of radiotherapy to any surgical procedure results in a lower incidence of local or regional recurrence (Easson, 1968). There is no evidence that delay of radiotherapy, until local disease recurs, compromises survival or eventual local control (Chu, 1976). Radiotherapy does not appear to influence survival in breast cancer (Brady, 1977).

The apparent failure of radical, mutilating surgery to cure primary breast cancer has rekindled an interest in conservative surgery supplemented by radiotherapy - removing the tumour, but

preserving the breast. The pioneering work of Sir Geoffrey Keynes in 1924 was limited by the practical problem of delivering an adequate radiation dose to the tumour bed without damaging normal tissues.

Wide local excision (tylectomy) plus radiotherapy has only once been tested in a randomised control trial. Hayward and Atkins (1972) found that radical mastectomy plus DXT conferred no advantage over tylectomy plus DXT in patients with clinical stage I disease. Stage II patients treated by radical mastectomy did survive significantly longer. Although local recurrence was seen more frequently in patients treated with conservative surgery, the incidence of distant metastases was similar in both treatment groups amongst stage I and II patients. This observation would suggest that the survival of these patients is unrelated to the type of local treatment given.

Local excision plus radiotherapy is now widely practised in France and Italy and excellent results have been reported both in terms of survival and cosmesis (Calle, 1978; Pierquin, 1976). However such studies have been criticised as the patients are often self selected and the rate of salvage mastectomy following initial local therapy appears unacceptably high.

In 1981 Veronesi reported the results of a large randomised trial comparing Halsted radical mastectomy with "quadrantectomy" plus axillary dissection and radiotherapy. Only patients with small tumours (less than 2cm diameter) and impalpable axillary lymph nodes were included in the trial. At 7 years there were no significant differences between the two treatment groups in terms of recurrent disease or survival.

At present it is therefore impossible to assess precisely the contribution of local therapy to the cure of patients with breast cancer. No single type of local therapy appears to be superior to any other in terms of patient survival. If the failure of local treatment to influence survival is due to the widespread dissemination of micrometastases before the time of diagnosis - it would seem logical to treat this systemic disease with systemic treatment.

Chapter 3

Systemic Therapy

For the purposes of this review I will discuss endocrine therapy and cytotoxic chemotherapy. Immunotherapy has been of no proven value in the management of human breast cancer, though recent work suggests that linkage of drugs to the target-specific or monoclonal antibodies may be of some therapeutic value and will be discussed later (Gregoriadis, 1981).

(i) Endocrine Therapy

(a) Ablation It has long been appreciated that the gonads influence a variety of other tissues and that these influences could be altered by extirpation of the gonads. In 1835 Sir Astley Cooper made reference to variations in the size of breast carcinoma that occur during the pre- and post-menstrual period. In 1889 Schinzinger noted that younger patients with breast carcinoma had a worse prognosis and suggested that young patients may be made a "little older" by having their ovaries removed. This hypothesis was first tested in Glasgow in 1895 by George Thomas Beatson when he performed bilteral salpingo-oophorectomy on a 33 year old woman who had developed chest wall recurrence three months after radical mastectomy for breast carcinoma. A dramatic clinical response encouraged him to repeat the operation on two further women with local advanced disease, one of whom responded. Oophorectomy was adopted by several other surgeons and in 1900 Boyd reported on 54 collected cases. He concluded that clinical response occurred more frequently in premenopausal women but was otherwise unpredictable and of varying duration. Thereafter surgical oophorectomy fell into disrepute following the introduction of

irradiation castration. Adair (1945) noted approximately equal response rates (30 percent) when comparing women treated by castration surgically or by irradiation.

The role of prophylactic oophorectomy as an adjuvant to mastectomy in primary operable breast cancer is not established. In 1968 Cole reported the findings of a trial started 20 years previously in which patients were all treated by radical mastectomy following which they were randomised to receive either no further therapy or an irradiation-induced menopause. The patients given radiotherapy had a prolonged disease-free interval, but there was no significant survival difference between the two groups. In other trials significant survial advantages have only been found within smaller subgroups, for example premenopausal women with involved axillary lymph nodes (Bryant, 1981). similar benefits were found when premenopausal women received ovarian irradiation followed by prednisolone for five years after mastectomy (Meakin, 1977).

The adrenal glands were known to be a second source of oestrogens in the body and it was thought that a further decline in circulating oestrogens in castrated or postmenopausal women might induce tumour regression in women with oestrogen dependant cancers. Furthermore it was known that pituitary hormones were necessary for regrowth of breast tissue in hypophysectomised rats and perhaps hypophysectomy for breast cancer might induce tumour regression over and above that obtained from oestrogen suppression.

Trials of adrenalectomy and hypophysectomy were not possible until 1950 when synthetic steroids became availble for adequate mineralo- and glucocorticoid replacement therapy. A prospective

randomised study comparing hypophysectomy and adrenalectomy in advanced breast cancer (Hayward, 1970) showed a definite superiority for hypophysectomy suggesting that pituitary hormones may be stimulating some tumours.

Major endocrine ablative surgery is now rarely used due to the much lower morbidity and comparable efficacy of additive hormone and anti-hormone therapy.

(b) Additive

Hormones: In 1935 synthetic oestrogens became available and were used empirically in the management of advanced breast cancer. In retrospect pharmacological (rather than physiological) doses of ethinyl oestradiol were used and found to induce tumour regression in about one third of women. Response rates tended to increase with age and the number of postmenopausal years. It has been suggested that pharmacological doses of a hormone may produce tumour regression by down regulation of a "hormone receptor" thus impeding the entry of the hormone into its target tissue.

Work over the past two decades has led to understanding of the biochemical mechanisms by which oestrogens, and other steroid hormones, promote their biological effect. Experiments by Jensen (1968) established that oestrogen target tissues such as the uterus, vagina and breast were able to concentrate oestradiol. This led directly to the discovery of intracellular oestrogen binding components, termed receptors, which bound the steroid with high affinity and were responsible for the concentration effect.

The details of steroid receptor assays and their clinical applications as prognostic indicators in early breast cancer and predictors of response to drug treatment in advanced disease - will be discussed in Parts I and II.

Androgens result in tumour regression in some tumours but are now rarely used due to the unacceptably high incidence of virilising side effects. Synthetic progestogens have variable androgenic and oestrogenic activities. Trials have reported up to 40% response rates in women with advanced disease treated with high doses of Medroxy Progesterone Acetate (Cuna, 1978; Mattson, 1978), though most of these trials have been uncontrolled and many of the patients have received other forms of endocrine therapy.

Anti-hormones: Synthetic anti-oestrogens were developed as infertility drugs but were found instead to induce ovulation in infertile women and cause regression of advanced breast carcinomas. The most widely studied of these compounds is Tamoxifen which has been found to produce remission rates in advanced breast cancer similar to those achieved by other endocrine measures.

Tamoxifen is a transisomer of a triphenylethylene derivative 1-(P-B-dimethylaminoethyoxyphenyl) 1,2-diphenylbut-1-ene; the cis-isomer being a conventional oestrogen (Harper, 1966). The drug is most effective against tumours in which specific intra-cellular receptors for oestradiol can be detected and is thought to act primarily through an interference with the association of oestrogens with these binding proteins. The pharmacokinetics of Tamoxifen and its metabolites will be discussed in more detail in Part II in relation to in vitro studies.

The role of Tamoxifen as an adjuvant to mastectomy in primary breast cancer is currently under evaluation (Baum, 1983), and a recent study (Preece, 1982) suggests that Tamoxifen may be effective as the initial sole treatment for localised carcinoma of the breast in older women.

The most striking advantage of Tamoxifen is its very low incidence of side effects in comparison to other forms of anti-cancer drug therapy. Flushing attacks are uncommon, and often dose related.

Tumour "flare" and hypercalcaemia have been attributed to Tamoxifen (Plotkin, 1978) and indeed most other forms of endocrine therapy (Nesto, 1976). Tamoxifen has been shown to have partial agonist properties in some animals and marginal partial agonism at some tissues in man (Ferazzi, 1977).

In summary, Tamoxifen is considered by most to be the drug of choice in postmenopausal women with breast cancer in whom endocrine therapy is indicated.

Bilateral adrenalectomy and high dose corticosteroid therapy have both been shown to be effective treatments in advanced breast cancer, but both are associated with appreciable morbidity and mortality. Aminoglutethimide, a derivative of the hypnotic agent glutethimide, was first marketed as an anti-convulsant in 1960 and subsequently withdrawn due to the high incidence of associated adrenal insufficiency. This side effect has been shown to be due to suppression of adrenal biosynthesis of cortisol, aldosterone and endrogens by inhibition of the enzymatic conversion of cholesterol to delta 5-pregnanolone (Santen, 1979).

Initial clinical trials of aminoglutethimide were restricted to patients refractory to previous hormone manipulation or unfit for surgical adrenalectomy. However one trial suggested that response

rates for medical adrenalectomy were similar to those with surgical adrenalectomy (Newsome, 1978). When compared with Tamoxifen (Smith, 1981) aminoglutethimide produced similar response rates but at the expense of increased morbidity due to the concurrent steroid replacement therapy. More selective and potent aromatase inhibitors have been discovered (Furr, 1982), but their clinical use is limited by poor oral potency and short half lives.

There is experimental evidence that prolactin influences the growth of mammary carcinoma in rodents. On the basis of this prolactin inhibitors such as laevodopa and ergot alkaloids have been investigated in the treatment of human advanced breast cancer (Stoll, 1972). Laevodopa has resulted in relief of bone pain in some patients though objective tumour regressions have been rare and of short duration.

Anti-gonadotrophins have been shown to have significant anti-tumour activity in the hormone-sensitive DMBA induced rat mammary tumour (Peters, 1977) - their action is thought to be due to a chemical castration due to suppression of FSH and LH secretion. These compounds have not been evaluated clinically in human breast cancer.

The theoretical benefits of using combination hormone therapy have not yet been realised in clinical practice. Palshof (1978) found no benefit when comparing Tamoxifen plus medroxyprogesterone acetate with Tamoxifen alone in advanced breast cancer - though the dosage of MPA would be considered low by current standards. Since aromatase inhibitors have a different locus of action from anti-oestrogens, combinations of these two agents would seem rational but have not yet been tested clinically.

(ii) Cytotoxic Chemotherapy

Historical Review During the trench warfare of the first World War, leucopenia was consistently noted in soldiers dying following exposure to mustard gas (Krumbhaar, 1919). In 1940 its nitrogen derivative, prepared for use in chemical warfare, was found to have a similar effect. Workers in England and U.S.A., independently, applied these findings therapeutically in the treatment of leukaemias and related disorders. Their findings, published in 1946, led directly to the development of cancer chemotherapy (Gilman, 1946; Rhoads, 1946). Since then many diverse chemotherapeutic agents have been manufactured and by 1967, 88,550 compounds had been screened for anti-neoplastic activity in the U.S.A. alone (Scott, 1970). This prodigious output bears testament to the inadequacy of the currently available anti-neoplastic drugs. It would be neither feasible nor pertinent, within this thesis to list the numerous chemotherapy agents and their complex pharmacokinetics. I will therefore briefly outline the theoretical basis for chemotherapy and desribe its role in the management of human breast cancer.

Theory

Until recently it was thought that cancers grow because malignant cells divide more rapidly than their healthy counterparts. On the basis of this it was hoped that cancer cells could be selectively damaged by exploiting simple differences in the cell cycle kinetics of normal and neoplastic cells. Increased understanding of the biology of cell division has clarified concepts of tumour growth and indicated new approaches to more effective treatment.

In tumour growth, increased volume must be due either to increased mitosis or decreasing cell loss (Baserga, 1981). The potential volume doubling times of human malignant cells range from less than 24 hours for Burkitts lymphoma (Cooper, 1966) to 90 days for human breast cancer (Johnson, 1966). It is now clear that tumour cells do not necessarily proliferate faster than normal cells, and cells of normal rectal mucosa have doubling times of 24 hours (Cole, 1961). Iverson (1970) has suggested that cancer should be regarded as a disease of cellular accumulation rather than proliferation. The importance of tumour growth and cell kinetics in relation to prognosis and response to treatment will be discussed in detail in Part I, Chapter 3. Observations on animal tumours (Skipper, 1964) have shown that a given dose of an anti-neoplastic agent kills a constant proportion of malignant cells irrespective of their total number, and that surviving cells continue to divide at the previous rate. Many chemotherapy regimens have taken no account of the effects of anti-mitotic drugs on healthy proliferating tissues - the most vulnerable being bone marrow and alimentary tract mucosa. If high doses of drugs are given, therapy must be intermittent to enable normal tissues to recover.

It is known that different drugs exert their effects at different points in the mitotic cycle (Hill, 1975). There is thus a theoretical advantage in using several different agents simultaneously. The value of this approach was first established in the treatment of acute lymphoblastic leukaemia in children (Freireith, 1964).

Cytotoxic agents were first used in patients with locally advanced, "inoperable", tumours or with widespread metastatic disease. Although chemotherapy may result in objective regression and palliation, survival is often unchanged. It is known that cytotoxic

therapy is more effective in the presence of minimal tumour burden rather than massive disease (Schabel, 1975). Tannock (1968) has shown that small tumour masses have a relatively greater blood supply and therefore effective drug delivery may be more efficient. On this basis it would seem logical to give chemotherapy to patients following excision of the primary tumour in an attempt to erradicate occult disseminated micrometastases. This hypothesis has been tested in animal models and adjuvant chemotherapy has been shown to improve survival rates following excision of the primary tumour (Martin, 1961). Adjuvant chemotherapy has been shown to result in substantially increased survival in certain childhood tumours such as nephroblastoma and osteogenic sarcoma, but its value in breast carcinoma is not yet proven (Smith, 1983).

There is some evidence that failure of cytotoxic therapy may be due to genetic mutation of tumour cells. As many anti-cancer agents and their metabolites are known to be mutagenic in some instances they could in fact facilitate tumour progression (Kerbel, 1982).

The ideal anti-cancer drug is one which can selectively destroy cancer cells sparing normal tissue, has no harmful side effects, and should not lead to the development of drug resistance by cancer cells.

Applications of Cytotoxic Therapy

(a) Advanced Disease

Advanced breast cancer can rarely be cured by any means, but useful palliation can be achieved using a variety of cytotoxic agents. In a recent evaluation of single agents, Carter (1976) found 15 drugs that induced regression in more than 20 percent of advanced breast

cancers. Many of the patients had received previous endocrine therapy and the two most effective single agents were reported to be cyclophosphamide and doxorubicin (Adriamycin).

In 1969 Cooper reported complete regression of tumour in 90% of patients using a regime of cyclophosphamide, methotrexate, 5-fluouracil, vincristine and prednisolone (CMF-VP) in an intensive weekly induction course followed by a less intensive maintenance schedule. These impressive early results have not been reproducible and most investigators have reported complete response in less than 20% of cases.

There is a theoretical advantage to intermittent therapy - giving drugs at higher doses and less frequent intervals. However a randomised trial comparing intermittent (monthly) and continuous (weekly) Adriamycin found no significant difference in response rates, survival or toxicity (Hoogstraten, 1976).

(b) Adjuvant Therapy

In 1958 the use of adjuvant systemic chemotherapy was tested scientifically for the first time in a controlled, prospective, randomised clinical trial (Fisher, 1959). Women with primary breast cancer were randomised to be treated by radical mastectomy alone or by radical mastectomy plus adjuvant thiotepa (the drug being given intravenously on the day of operation and for 2 days thereafter). There was no overall difference in survival rates between the two treated groups. However in the drug treated group, premenopausal women with four or more involved axillary lymph nodes, had a significantly longer disease-free interval. The same group went on to investigate the role of 5 F.U. (5-fluouracil) but found it did not benefit the patients in terms of recurrence or survival.

In 1978 the Scandinavian Adjuvant Chemotherapy Study Group reported the results of a trial in which 1026 patients were randomised to receive either no adjuvant therapy following mastectomy, or intravenous cyclophosphamide for 6 postoperative days (Nissen-Meyer, 1978). The drug treatment conferred a significant 10% benefit in survival at 10 years (this benefit was manifest in both pre- and postmenopausal groups).

Bonadonna (1981) has recently reported the 5 year follow up of a controlled trial of adjuvant chemotherapy in women with primary operable breast cancer and histologically involved lymph nodes. Women were randomly assigned to have radical mastectomy alone or mastectomy followed by 12 monthly courses of adjuvant cyclophosphamide, melphalan and 5-fluouracil. Both disease-free interval and total survival appear to be significantly improved by adjuvant therapy. The benefits were most marked in premenopausal women, and some feel that the action of these drugs is by inducing chemical castration. However in this series the disease-free interval was independent of drug-induced amenorrhoea, and in the Nissen-Meyer study benefits were similar in both pre- and postmenopausal groups. The Bonadonna trial has been criticised with regard to the method of randomisation of patients which resulted in unequal proportions of control and treatment groups. Such discrepancies may be important when interpreting the results of this and other clinical trials.

It has been shown that most tumour cell populations are heterogenous, composed of clones with different drug sensitivities (Selby, 1983). If this is the case then it might be expected that combinations of cytotoxic agents and hormones would be more effective than if either modality were used alone. In practice combination of cytotoxic and endocrine therapy have not improved the response rates

or surival of patients with advanced breast cancer (Brunner, 1977; Rubens, 1978). Several studies are now underway to assess the value of such combinations as an adjuvant to mastectomy for primary breast cancer.

Many clinicians are still reluctant to use adjuvant chemotherapy due to the high incidence of disabling side effects (Palmer, 1981). The severity of side effects varies with different drug combinations and dosages but nausea, vomiting, myelosuppression, thrombocytopaenia and alopecia have been frequently reported. Nausea and vomiting may be controlled with anti-emetic and sedative drugs, the potential haematological sequelae need vigilance and careful monitoring during therapy. Alopecia occurs frequently with cyclophosphamide and inevitably with Adriamycin - although this may be avoided or reduced by scalp hypothermia (Gregory, 1982). In addition to these reversible side effects the anthracycline antibiotics (e.g. Adriamycin) may induce a cumulative permanent cardiomyopathy (Lefrak, 1973).

The chronic toxic effects of chemotherapy in breast cancer are not well documented. Cancerogenesis following prolonged immuno-suppression has been reported following renal transplantation but the Fisher and Bonnadonna studies found no increased incidence in second tumours in women treated with adjuvant therapy for breast carcinoma. However it is alarming to note that 3 out of 13 breast cancer patients given more than 4 years of continuous chlorambucil for breast cancer died of acute myelogenous leukaemia (Lerner, 1978).

Summary

This general introduction has reviewed the current management of breast cancer. Surgery and radiotherapy have been shown to provide effective local control of disease, although modifications in local treatment appear to have little or no effect on overall survival. In advanced breast cancer useful palliation may be achieved by endocrine manipulation or cytotoxic chemotherapy; the use of these modalities however, remains controversial in the adjuvant situation. Adjuvant therapy may result in modest prolongation of the disease-free interval in some specific sub-groups of women - although this is at the expense of considerable short term, and as yet unknown, long term toxicity.

The natural history of breast cancer is poorly understood. In common with other human solid tumours it has been shown that the clinical and pathological stage at the time of first presentation is the most accurate indicator of the prognosis of a woman with breast cancer. Nevertheless, of patients presenting with Stage I tumours, approximately one quarter die of their disease within 10 years of mastectomy. These deaths are usually as a result of distant metastases despite adequate local and regional control of disease. Paradoxically about one quarter of women with poor prognostic features, such as histological involvement of axillary lymph nodes, are alive 10 years after mastectomy (Fisher, 1975).

Clearly, conventional staging is a crude means of stratifying patients into different prognostic groups. At present, however, most clinical trials comparing different forms of treatment are conducted on this basis. The type of treatment given in the future to patients

will, in turn, be influenced by the results of such trials. The correct interpretation of results may be impossible if the patients under study cannot be accurately stratified into different prognostic groups.

The general aims of this study were twofold. In Part I the role of prognostic indicators in primary operable bresat cancer is examined. In particular the role of Steroid Receptor Assays and Thymidine Labelling Index will be defined. These more recently described parameters will be combined with more conventional prognostic information in an attempt to produce an overall prognostic index for each individual patient.

If it proves possible to accurately identify patients who have a poor prognosis, then many clinicians would feel justified in administering some form of adjuvant systemic therapy to these women. Tumours vary widely in their responses to endocrine or cytotoxic therapy. Consequently if systemic treatment is given empirically, some women will endure unpleasant side effects of the drugs unnecessarily whilst others will be denied an effective treatment. Overall response rates may improve, therefore, if systemic treatment were given knowing the sensitivity of an individual tumour to a variety of drugs.

Part II presents a review of past experience with in-vitro drug-sensitivity assays and describes the development of a short term assay using organ culture of breast carcinomas, and its application in a laboratory and clinical study.

PART I

Chapter I Conventional Prognostic Indicators

Section I. Introduction

The clinical assessment of tumour size and the presence or absence of enlarged axillary lymph nodes have been used for many years to stratify women with breast cancer into prognostic groups. Conventional staging using the Manchester classificaiton relies solely on simple, clinical criteria. In an attempt to reduce any subjective bias the International T.N.M. classification was introduced, and has been shown to improve the accuracy of staging (Charlson, 1973).

It is also recognised that prognosis relates to the degree of histological differentiation of the primary tumour, however this information is often considered in isolation and not in conjunction with other clinical or pathological data. It is not known what, if any, is the prognostic significance of patient's age, menopausal status or parity.

In this introduction previous experience with each of these prognostic variables will be reviewed in turn.

(i) Tumour size

Increasing tumour size at presentation is associated with a worse prognosis following mastectomy (Alderson, 1971, Palmer, 1971). The clinical estimation of tumour size is unreliable tending to over-estimate due to the distortion of surrounding breast tissue. Although Fisher (1979) found a constant association between clinical and pathological diameter, most recent studies have relied on the measurement of maximum tumour diameter of the pathological specimen.

It may be considered naieve to express tumour size as a single diameter as tumours are three dimensional structures of varying shape. In a recent study (Cuschieri, 1983) the tumour volume was calculated from three diameters, assuming the tumour to be elliptical. This more complex approach however did not yield any more useful prognostic information.

(ii) Axillary lymph node status

Axillary lymph node status has always been regarded as the most reliable prognostic indicator. However the determination of axillary lymph node status is not always simple.

Clinical examination of the axilla is often misleading. It has been shown that 50% of impalpable axillary lymph nodes contain metastatic deposits (Cutler, 1969) and conversely 50% of clinically enlarged lymph nodes may be free of tumour (Wallace, 1972). Enlargement of lymph nodes may be due to sinus histiocytosis - a histological feature identified as a favourable prognostic indicator (Black, 1955).

A preliminary report by Agwunobi in 1978 suggested that axillary lymphoscintigraphy may be valuable in identifying involved axillary lymph nodes. It was claimed that the uptake of labelled sulphur colloid was suppressed by lymph nodes containing tumour. Black in 1981 performed a more detailed study using an identical technique. No correlation was found between the uptake of isotope and lymph node involvement; indeed many scans were difficult or impossible to interpret.

The absolute number of axillary lymph nodes containing tumour is the single most potent prognostic indicator in primary operable breast cancer (Fisher, 1970). A formal axillary clearance provides the largest yield of nodes but this is at the expense of significant morbidity when compared to lesser surgical procedures involving "sampling" of the axillary nodes. Forrest (1976) suggested that biopsy of the pectoral and axillary tail nodes gives a reliable estimate of axillary lymph node status. It has recently been shown, however, that axillary sampling alone underestimates the extent of nodal involvement (Kissin, 1982). In addition "skip lesions" may be found, in that the lower axillary nodes are tumour free, whereas the apical nodes contain metastatic deposits.

Blamey et al (1979) have proposed a more limited procedure - namely the biopsy of a lower axillary, apical axillary and internal mammary node. The prognostic information resulting from this triple node biopsy is similar to that produced by a formal axillary dissection. However these comparisons were made retrospectively and these two surgical approaches have not been tested in a prospective, randomised trial.

At present therefore it appears that surgical removal and histological examination of axillary lymph nodes provides the most accurate and objective information.

(iii) Histological grade

In 1890 Von Hausemann suggested that the nuclear morphology of tumour cells could have important implications for their biological behaviour. He considered that both the mitotic rate and presence of abnormal mitoses were important characteristics that could influence the metastatic potential of a tumour.

Saloman (1913) studied the relationship between clinical behaviour of breast cancer and morphology and found that the degree of anaplasia was of prognostic importance. Greenhough in 1925 emphasised the importance of cytological and histological changes and his method was modified first by Patey and Scarff (1928) and later by Bloom and Richardson (1957). This classification is the basis of the World Health Authority grading system and has been widely used in clinical and pathological studies.

More recently Hultborn and Tornberg (1960) claimed that the manner of tumour growth was the most important criterion for "individualised group prognosis" and the system of Hartveit (1971) is based on factors such as nuclear crowding, lobulation, and relative diameter as well as an assessment of cell borders. Though Shivas (1972) has shown that the degree of elastosis in breast tumours is related to prognosis, this factor has not been incorporated into any tumour grading classification.

Most methods of tumour grading have been shown to be of some prognostic significance in that women with poorly differentiated tumours have the worst prognosis. Bloom and Richardson (1957) reported on a large series of 1409 women with breast carcinoma and found that the 5 year survival rate following mastectomy varied from 75 percent in women with grade I tumours to 31 percent in women with grade III tumours. This study has been criticised on the grounds that the patients were not uniformly treated and that the analysis used

crude survival rates rather than the conventionally more acceptable Life Table Analysis. Nevertheless similar results have been reported by other workers (Wolff, 1966; Champion, 1972).

Histological grading is, of necessity, subjective and Stenkvisk (1979) found unacceptably low intra and inter observer reproducibility when using these different grading systems.

It would, therefore, seem desirable to make a more objective quantitative assessment of tumour differentiation. Some workers have found correlation between histological grade and level of oestrogen receptor protein (Rosen, 1975; Maynard, 1978). Poorly differentiated tumours having lower levels of oestrogen receptor protein. However other groups have been unable to identify such a relationship (Leclerq, 1973; Longo, 1976).

Although the classification of Bloom and Richardson is by no means perfect it is still the most widely used almost 30 years after its introduction and was therefore used throughout this study.

(iv) Age, menopausal status and parity

For many years it was thought that breast carcinoma was a more aggressive disease in younger women; in 1970 Brightmore reviewed 101 women with breast cancer under 35 years of age and found survival rates lower than in a comparable older population. However more detailed analysis revealed a greater proportion of anaplastic and locally advanced tumours in the younger patients and when these factors were allowed for the survival rates were identical. A more comprehensive review of 807 patients from the same institution in 1979 (Redding) confirmed these findings.

In a 19 year survival study of 3558 women, Mueller (1978) concluded that breast cancer was more lethal in older women and suggested that this could reflect a declining immune response in the elderly. More complex forms of multivariate analysis have not confirmed these findings (Alderson, 1971), though recently Palmer (1982) reported that women in the age group 40-49 years had the best prognosis of all.

Menopausal status is, of course, closely allied to age. Previous studies have shown no clear association between menopausal status and prognosis, and at present there is no widely accepted definition of the time of menopause.

Estrogen receptor positive tumours are commoner in postmenopausal women and women with such tumours are known to have a better prognosis irrespective of patients age (Croton, 1981). This highlights the difficulty in interpreting survival data in isolation, and demonstrates the need for more complex multivariate analysis.

Nulliparity is known to be a risk factor in the development of breast cancer (Wainwright, 1931) but there have been few previous studies relating parity to prognosis following the diagnosis and treatment of primary breast cancer. Pappasetas (1980) found that parity did not significantly influence prognosis. McKay (1965) found that in general, nulliparous women had a less favourable prognosis but that parous women with more than 5 pregnancies had the worst prognosis of all groups.

(v) Aims

The aim of this part of the study was to examine these conventional prognostic variables in a population of women with primary operable breast cancer treated by mastectomy. The distribution of prognostic variables within the population and their inter-relationships will be defined, as will their influence on the survival of the women following mastectomy.

Section II Patients, Materials and Methods

The patients studied in this section all presented to surgeons within the Mersey Region from 1975 to 1982. All women were treated by mastectomy following the histological confirmation of primary operable breast carcinoma (T^{1-3} $N_{0,1}$ M_0). Excluded from the study were cases of bilateral breast carcinoma, patients who subsequently received adjuvant systemic therapy, and those treated by surgical procedures other than mastectomy.

The clinical, operative and pathological details of each patient was recorded at the time of first hospital admission and later transferred to simple punch cards. All data was then stored on computer prior to the final statistical analysis (Appendix I.1).

Follow-up data were retrieved from the hospital case records and the Regional Cancer Registry. If information was unavailable from these sources, the family practitioners were contacted directly.

The development of recurrent carcinoma was accepted only if there was histological or unequivocal radiological evidence to support the diganosis. Although the treatment of recurrent carcinoma was documented, in all cases the type of treatment was given at the discretion of the clinicians involved.

It should be emphasised that this study was designed to examine breast carcinoma as it presents to all general surgeons within a region, rather than to a single unit with a specific interest in breast carcinoma.

The documentation of each of the conventional prognostic variables was made as follows:

(i) Tumour size

The maximum tumour diameter, as measured by the hospital pathologist, was recorded and classified using the T.N.M. criteria.

T1 < 2cms

T2 2-5cms

T3 > 5cms

Tumour size was documented in all cases.

(ii) Axillary lymph node status

Although most patients were treated by mastectomy with full axillary clearance some patients had only sampling of the lower axillary nodes, and some had mastectomy alone with no excision of nodal tissue.

As the type of operation was not standardised this information was recorded as follows:

N_0 = Axillary lymph nodes excised and examined histologically but found to be tumour free.

N_1 = Axillary lymph nodes excised and examined and found to contain tumour.

N? = Axillary lymph nodes either not excised by surgeon or not examined by pathologist.

(iii) Histological grade

Grading was performed independently and retrospectively by a single pathologist (Dr. I. McDicken) on prepared haematoxylin and eosin stained slides which were retrieved either directly from the parent hospital or from the Mersey Regional Cancer Registry. All of the specimens graded were of invasive ductal carcinoma. Lobular carcinomas, medullary carcinomas, one sarcoma, and one squamous

carcinoma were excluded from the study.

The method of grading used was that described by Bloom and Richardson (1957). This system awards points according to the presence of three histological features:

1. Differentiation of tubule formation

Greater part of section shows well marked tubule formation	1 point
Moderate tubule formation	2 points
Little or no tubule formation	3 points

2. Nuclear pleomorphism (assessed at tumour periphery)

Nuclei uniform in shape, size and staining	1 point
Moderate pleomorphism	2 points
Marked pleomorphism	3 points

3. Hyperchromatic and Mitotic Nuclei (periphery)

An occasional hyperchromatic or mitotic nucleus per high power field	1 point
2-3 Hyperchromatic or mitotic nuclei per high power field	2 points
> 3 hyperchromatic or mitotic nuclei per high power field	3 points

The points allocated to each of the three histological fatures are added together and the grade determined accordingly.

Points		
3 4 5	6 7	8 9
Low Grade	Intermediate Grade	High Grade
I	II	III

Examples of a well differentiated (grade I), moderately differentiated (grade II) and poorly differentiated tumour (grade III) are illustrated (Figs. I.1, 2, 3).

(iv) Age, menopausal status, parity

The age of the patient was recorded at the time of mastectomy and was known in all cases. The patients were also stratified by the age decade.

Accurate information regarding the menopausal status and parity of patients was not known in all cases but was documented wherever possible. Women having had a hysterectomy with ovarian conservation were considered to be premenopausal until the age of 50 years. Patients were classified as either parous or nulliparous but more detailed information relating to number of pregnancies, miscarriages and numbers of live births was not recorded.

The details regarding the coding and storage of all the above data are presented in Appendix I.1).

(v) Statistical Analysis

In all cases the age of the patients and the size of the tumour were known. For other categories of prognostic variable data was incomplete. The significance of these "missing values" was assessed by cross-tabulating the distribution of other prognostic variables within groups of known and unknown values; assessing the differences by a simple chi squared test.

Survival curves were plotted by the method of Life Table Analysis (Peto, 1976, 1977) and the differences between the curves analysed by the Lee Desu Statistic (Lee, 1972).

The influence of each prognostic variable on the site of first tumour recurrence was analysed by a chi squared test.

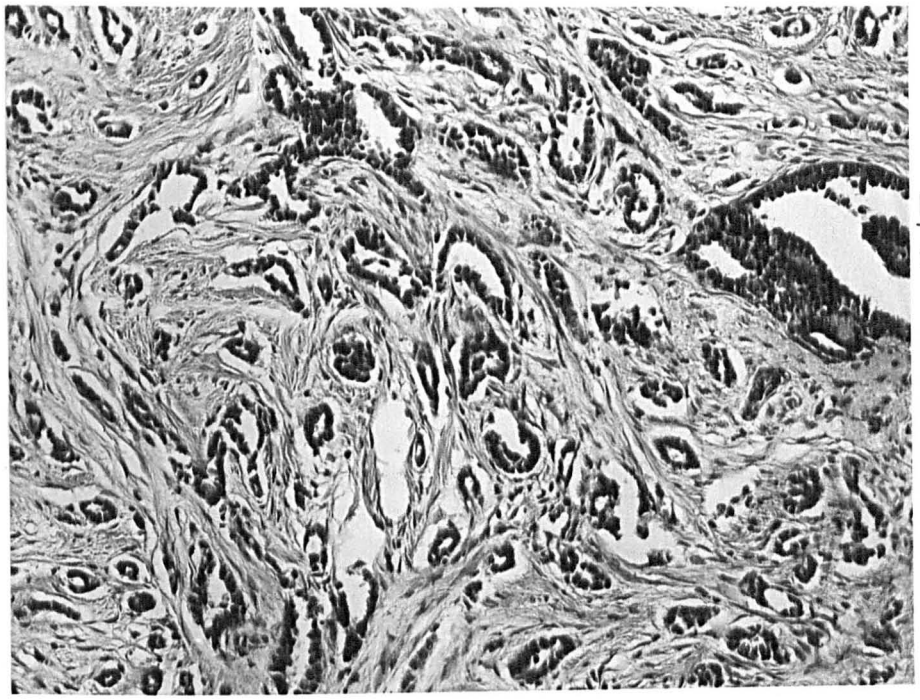


Fig. I.1
Grade I tumour

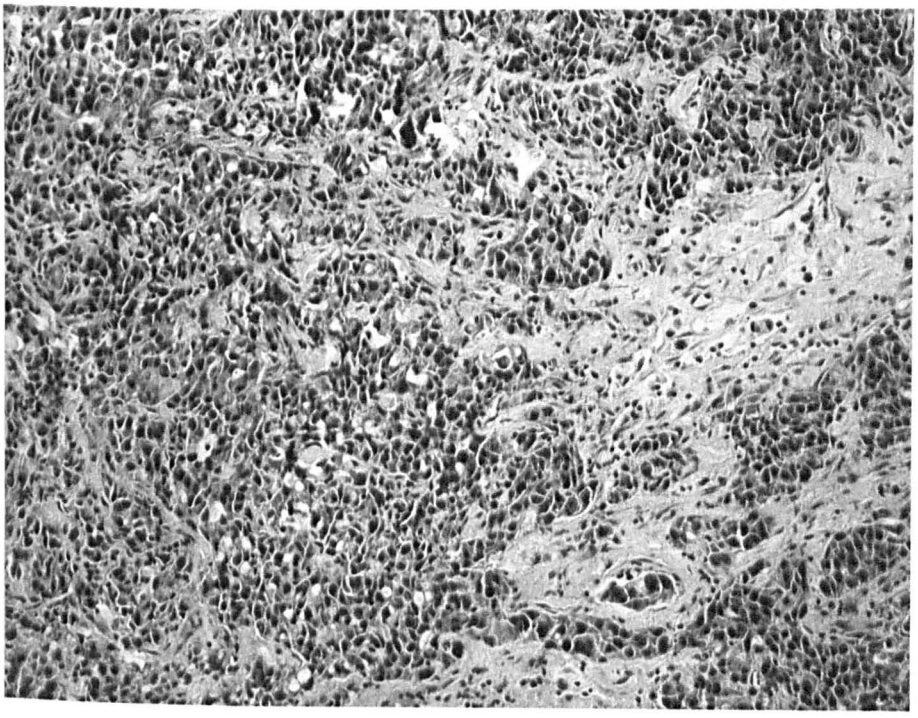


Fig. I.2
Grade II tumour

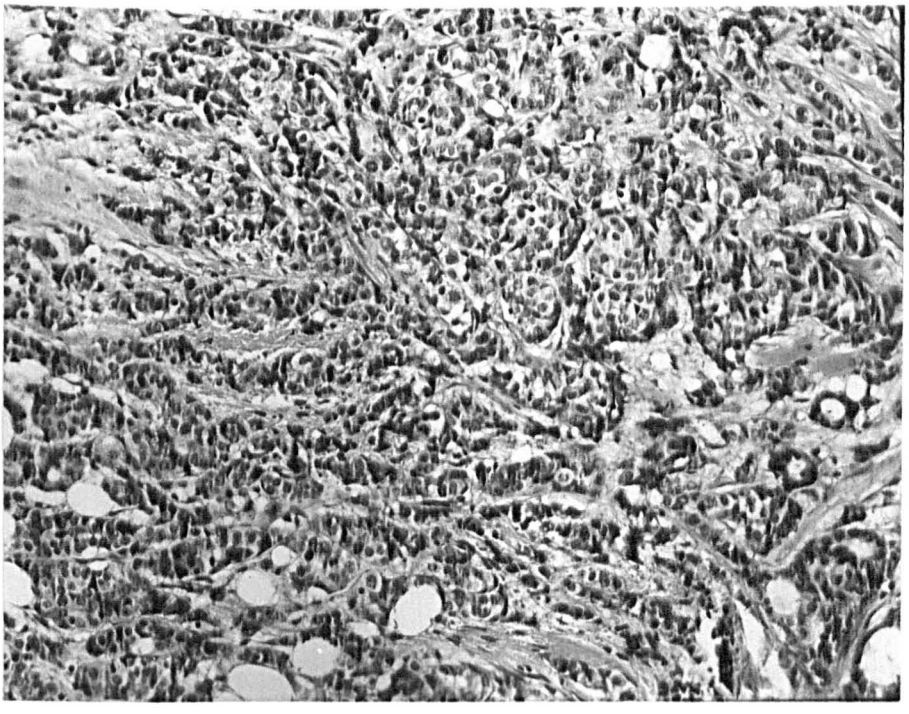


Fig. I.3
Grade III tumour

Section III Results

A total of 670 patients have been studied in this way. The data base, and code, for all of these patients appears in Appendix I.1.

The results are divided into two parts. In Part (A) the overall distribution of variables and the recurrence and survival data of the whole population are presented. In Part (B) each individual prognostic variable is examined in detail.

A. Overall distribution of variables, recurrence and survival

(i) Distribution of variables:

As described previously, documentation of prognostic variables was incomplete in some cases. The numbers of known and unknown values for each variable are presented in Table I.1. The possible significance of these missing values will be discussed later.

Table I.1

Prognostic variable	Known	Unknown
Tumour size	670	0
Axillary L.N. status	556	114
Histological grade	351	319
Age	670	0
Menopausal status	622	48
Parity	470	200

(ii) Recurrence and survival:

The fate of all patients at the time of statistical analysis 11.9.83 is presented in Table I.2.

Table I.2

Fate	No.	Adjusted Frequency %
Alive and well	418	62.4
Alive with cancer	99	14.8
Dead from breast cancer	140	20.9
Dead from other causes	13	1.9

Of the 670 patients, 140 (21%) have died of breast cancer and 13 (2%) have died of other causes. The survival curve (Fig. I.4) shows a survival rate of 60% at 5 years.

During the study period 241 patients (36%) have developed recurrent carcinoma. The distribution of first recurrence by anatomical site is shown in Table I.3.

Table I.3

Site of First Recurrence	No.	Adjusted Fequency %
Local	66	27.4
Nodal	41	17.0
Bone	33	13.7
Visceral	34	14.1
> 1 site	67	27.8

Recurrence occured most frequently locally (27%) or at more than one site (28%).

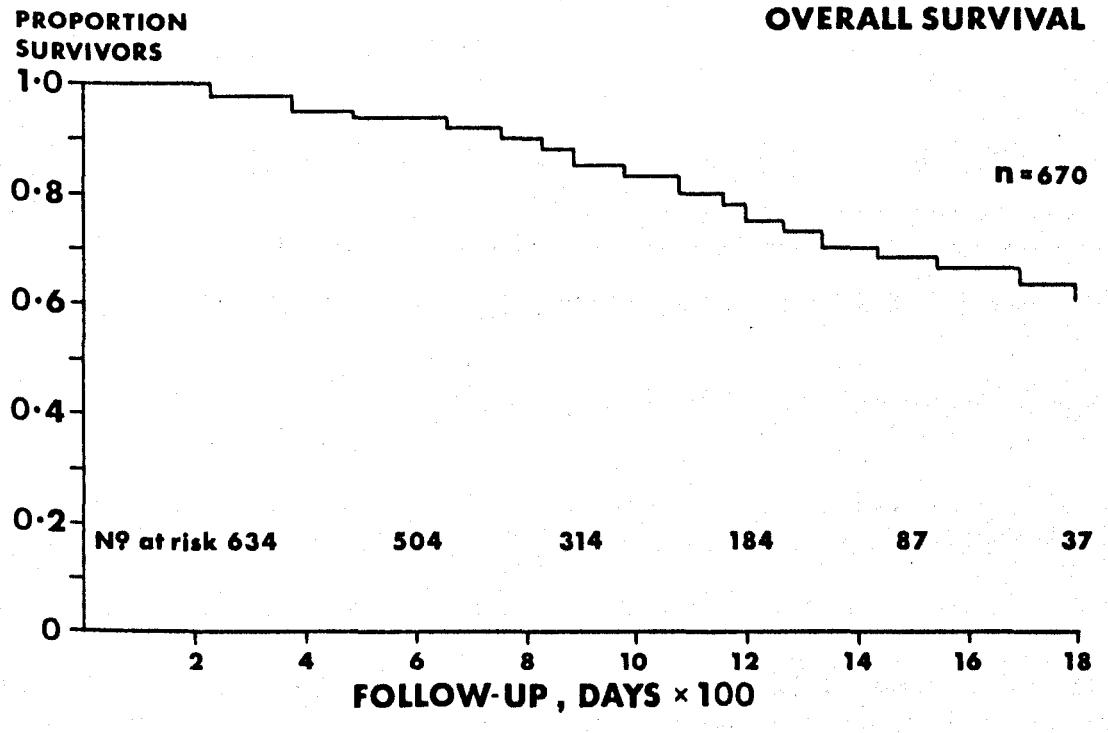

PROPORTION SURVIVORS
OVERALL SURVIVAL
1·0
0·8
0·6
0·4
0·2
0
n=670
Nº at risk 634
504
314
184
87
37
2
4
6
8
10
12
14
16
18
FOLLOW-UP, DAYS × 100

Fig. I.4

(iii) Treatment:

Hormone manipulation was the most common form of treatment for recurrence and was given to 45% of the cases with recurrent cancer. Cytotoxic chemotherapy was given as first line treatment to only 10% of patients with recurrent carcinoma (Table I.4). These figures relate only to the 166 cases of recurrence where specific treatment was known to be given. A further 75 cases developed recurrence for which no treatment was documented.

Table I.4

Treatment at First Recurrence	No.	Adjusted Frequency %
DXT	47	28.3
Hormone	74	44.6
Cytotoxic	17	10.2
Combination	28	16.9

The type of treatment given was significantly associated with the site of recurrence when these two variables were cross-tabulated. $x^2 = 73.3$ $p < 0.0001$ (Table I.5).

Of the 66 patients developing local recurrence 25 (38%) were initially treated with radiotherapy. Conversely only 1 of the 34 patients with visceral metastases was treated in this way.

Hormonal manipulation was used most commonly in those patients with regional and bone recurrence, being given to 17 out of 41 (42%) and 15 out of 33 (45%) patients respectively. This form of treatment was given to only 7 out of 34 patients (21%) with visceral metastases.

Table I.5

Cross Tabulation Treatment 1st Recurrence by Site

Treatment	Local	Nodal	Bone	Visceral	>1 site	Row Total
None recorded	16	2	4	18	35	75
DXT	25	12	7	1	2	47
Hormone	19	17	15	7	6	74
Cyto	4	5	1	4	3	17
Combination	2	5	6	4	11	28
Column total	66	41	33	34	67	241

B. Individual Prognostic Variables

(i) Tumour size:

The size of all 670 tumours was known. Only 10% of the 670 tumours were less than 2cms in diameter. Of the remaining tumours 19% were more than 5cms in diameter and the majority (71%) were of intermediate size (T_2) (Table I.6).

Tumour Size	No.	Adjusted Frequency %
T_1	67	10.0
T_2	473	70.6
T_3	130	19.4

The prognosis of patients following mastectomy was progressively worse with increasing tumour size. Of the 67 patients with T_1 tumours 9 (13%) have died during the period of study. Conversely 85 (18%) and 44 (34%) of patients with T_2 and T_3 tumours have died during the same period of follow up.

The survival curves were plotted using Life Table Analysis (Fig. I.5) and the differences between the curves were analysed using the Lee Desu statistic. The overall comparison between the curves was significant ($p = 0.0001$). The pairwise comparison between T_1 and T_2 tumours did not achieve statistical significance ($p < 0.2$) but comparisons between T_1 and T_3 and T_2 and T_3 revealed significant differences ($p < 0.005$ and $p < 0.0005$ respectively) (Table I.7).

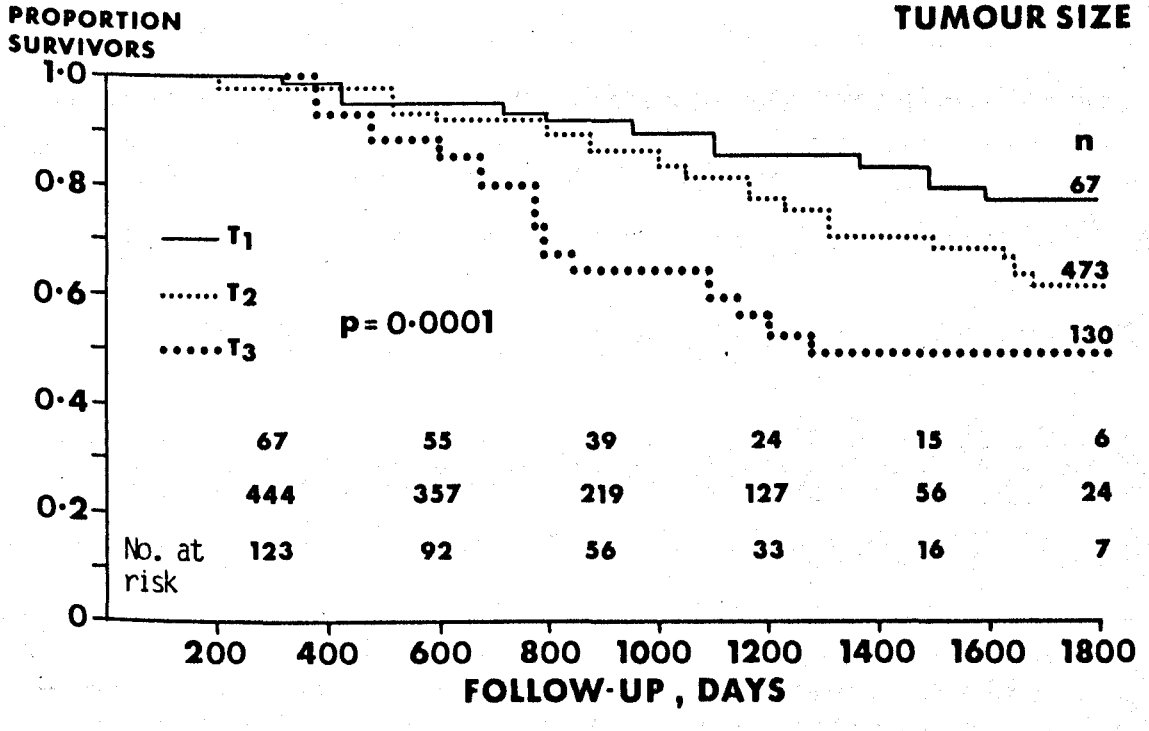

PROPORTION SURVIVORS
TUMOUR SIZE
1·0
0·8
0·6
0·4
0·2
0
T1
T2
T3
p = 0·0001
n
67
473
130
67 55 39 24 15 6
444 357 219 127 56 24
No. at risk 123 92 56 33 16 7
200 400 600 800 1000 1200 1400 1600 1800
FOLLOW-UP, DAYS

Fig. I.5

Table I.7

Tumour size and survival	x^2	DF	p
Overall comparison T_1 v T_2 v T_3	17.8	2	<0.0002
Pairwise comparison T_1 v T_2	1.65	1	<0.2
Pairwise comparison T_1 v T_3	9.92	1	<0.002
Pairwise comparison T_2 v T_3	13.8	1	<0.0005

No significant association was found between tumour size and the site of first tumour recurrence when these two parameters were cross-tabulated (x^2 = 6.69 p > 0.5) (Table I.8).

Table I.8

Site	T_1	T_2	T_3	Row Total
Local	7	39	20	66
Nodal	2	30	9	41
Bone	1	24	8	33
Visceral	3	21	10	34
> 1 site	4	38	25	67
Column total	17	152	72	241

(ii) Axillary lymph node status:

In 17% of cases the axillary lymph node status was unknown, because lymph nodes had either not been excised or not examined histologically. Of those patients with known axillary lymph node status 271 (49%) had involved lymph nodes and 285 (51%) were found to be tumour free (Table I.9).

Table I.9

Axillary lymph node Status	No.	Adjusted Frequency %
N_0	285	51.3
N_1	271	48.7
N?	114	

The possible significance of those 114 missing values was examined by comparing the distribution of other prognostic variables within the groups of known and unknown axillary lymph node status. This was done by cross-tabulating these variables with patients of known and unknown axillary lymph node status using a simple chi square test. The results are summarised below (Table 10).

Table I.10

Prognostic Variable	x^2	Degree of freedom	p
Tumour size	2.83	2	<0.5
Histological grade	1.74	2	<0.5
Age (group)	3.40	5	>0.5
Menopausal status	0.55	1	<0.5
Parity	3.16	1	<0.1

When survival was examined the patients with involved axillary lymph nodes were found to have a significantly worse prognosis when compared to node-negative patients.

The survival curves of the 2 groups are represented in Fig. I.6.

In the node positive group there were 92 (34%) deaths during the period of study compared to only 35 (12%) deaths in the node-negative group. When the 2 survival curves were compared using the Lee Desu Statistic this difference was found to be highly statistically significant ($x^2 = 39.2$ 1 DF $p < 0.0001$).

No significant association was found between the site of first recurrence in these patients and the presence or absence of axillary lymph node metastases ($x^2 = 9.02$ $p < 0.1$) (Table I.11).

Table I.11

	Axillary L.N. Status		
Site of First Recurrence	N_0	N_1	Row Total
Local	26	29	55
Nodal	7	25	32
Bone	6	22	28
Visceral	11	20	31
> 1 site	17	41	58
Column total	67	137	204

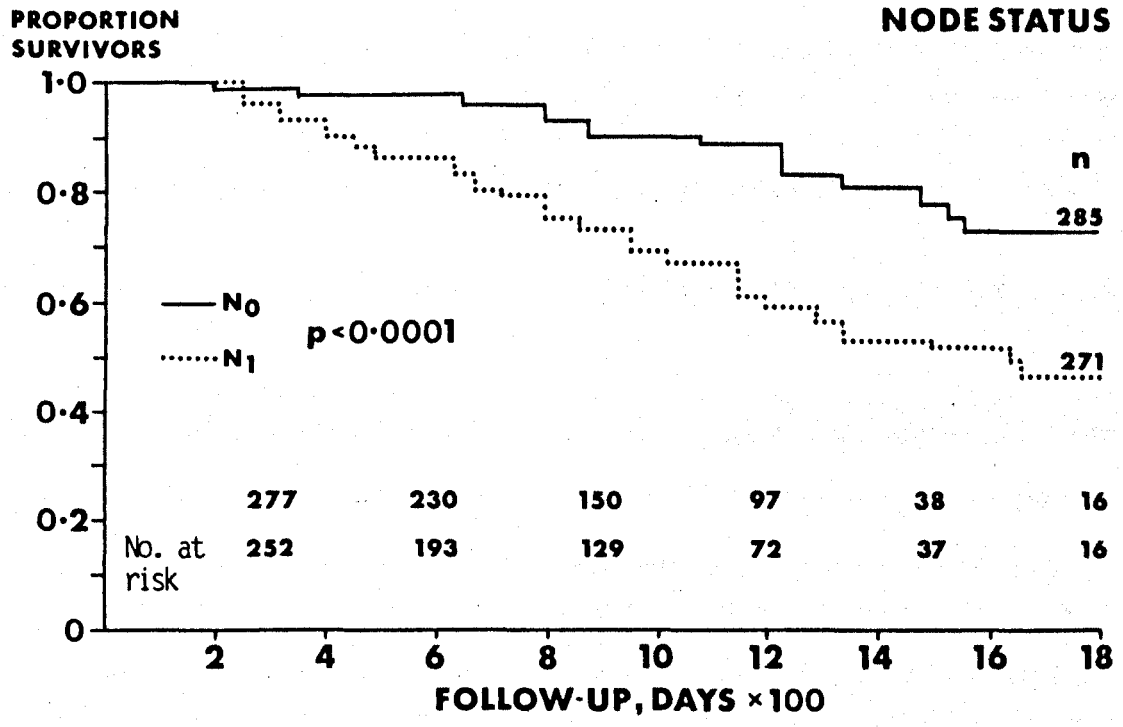

PROPORTION SURVIVORS
NODE STATUS
1·0
0·8
0·6
0·4
0·2
0
N0
N1
p<0·0001
n
285
271
277
230
150
97
38
16
No. at risk
252
193
129
72
37
16
2
4
6
8
10
12
14
16
18
FOLLOW-UP, DAYS ×100

Fig. I.6

(iii) Histological grade:

Histological grade was determined for 351 (52%) of the total 670 tumours. Of these 351, 106 (30%) were classified as grade I, 131 (37%) were grade II, and 114 (32%) were grade III (Table I.12).

Table I.12

Histological grade	No.	Adjusted Frequency %
I	106	30.2
II	131	37.3
III	114	32.5
Unknown	319	

The possible significance of the 319 missing values was examined by comparing the distribution of other prognostic variables within groups of known and unknown histological grade. This was done by cross-tabulating each variable in turn with the groups of known and unknown histological grade. The results of the chi squared analysis are summarised in Table I.13.

Table I.13

	x^2	DF	p
Node status	3.55	1	<0.1
Tumour size	2.54	2	<0.5
Age (group)	8.37	5	<0.5
Menopausal status	2.03	1	<0.5
Parity	1.18	1	<0.5

Survival was significantly related to the histological grade of the tumour. Of the 106 patients with well differentiated grade I tumours, 9 (8%) have died. Conversely 38 (33%) of the 114 patients with poorly differentiated tumours have died. The 131 patients with grade II tumours exhibit an intermediate prognosis with 33 (25%) deaths during the study period.

Survival curves were plotted using Life Table Analysis (Fig. I.7) and the differences between the curves were detrmined using the Lee Desu Statistic.

The overall comparison between all grades was highly significant ($p < 0.01$) as were the pairwise comparisons between grades I and II ($p < 0.02$) and grades I and III ($p < 0.001$). There was no significant survival difference between groups II and III (Table I.14).

Table I.14

Histological grade and survival	x^2	DF	p
Overall comparison I v II v III	11.4	2	< 0.01
Pairwise comparison I v II	6.34	1	< 0.02
Pairwise comparison I v III	11.3	1	< 0.001
Pairwise comparison II v III	1.07	1	< 0.5

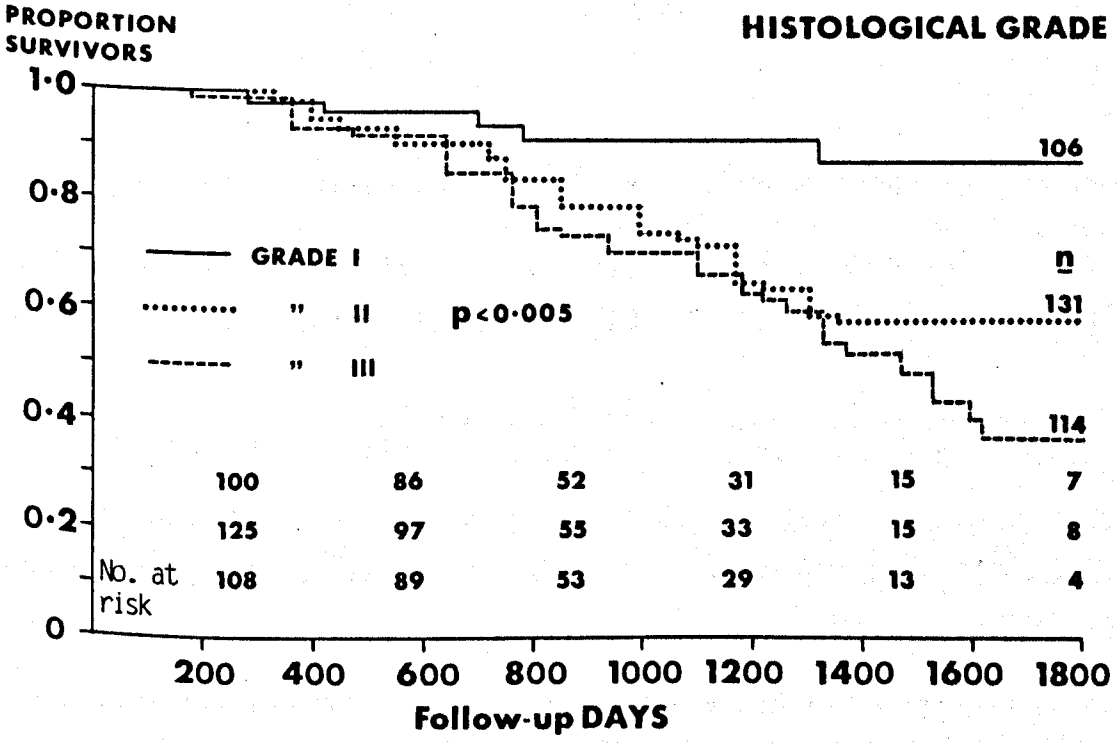

PROPORTION SURVIVORS
HISTOLOGICAL GRADE
1·0
0·8
0·6
0·4
0·2
0
GRADE I
" II
" III
p<0·005
106
131
114
n
100 86 52 31 15 7
125 97 55 33 15 8
No. at risk 108 89 53 29 13 4
200 400 600 800 1000 1200 1400 1600 1800
Follow-up DAYS

Fig. I.7

The pattern of tumour recurrence was examined by cross-tabulating the site of first recurrence with histological grade (Table I.15).

Table I.15

Site of First Recurrence	I	II	III	Row Total
Local	11	13	13	37
Nodal	1	9	13	23
Bone	3	14	3	20
Visceral	4	3	9	16
> 1 site	4	14	16	34
Column Total	23	53	54	130

The site of first recurrence was significantly related to histological grade ($x^2 = 19.1$ $p < 0.02$). Local recurrence was found in 11 out of 23 (48%) patients with well differentiated tumours compared to only 13 out of 53 (24%) grade II and 13 out of 54 (24%) grade III tumours. Conversely patients with grade II and grade III tumours more frequently developed distant metastases.

(iv) Age, Menopausal Status and Parity:

The mean age of patients studied was 57 years. In common with other studies the patients were stratified by age decade (Table I.16).

Table I.16

Age (yrs)	No.	Adjusted Frequency %
29-39	47	7.07
40-49	151	22.5
50-59	182	27.2
60-69	190	28.3
70-79	85	12.6
80 +	15	2.33

mean age 56.96 $\pm$ 11.8 years

Most patients were in the 4th, 5th or 6th decade - 22%, 27% and 28% respectively. Only 7% of patients were under 40 years and 2% were over 80 years of age.

The survival curves of the different age groups were plotted but have not been reproduced as all the curves are to a large extent superimposed and cannot be distinguished visually one from another. When the survival curves are compared using the Lee Desu statistic the overall comparison statistic is not significant. However, several significant differences do emerge when comparing individual sub-groups. The total of 16 pairwise comparisons have not been reproduced and only the overall comparison and the 3 significant pairwise comparisons are presented (Table I.17).

Table I.17: Age Group and Survival

		x^2	DF	p
Overall comparison		8.24	5	<0.5
Pairwise comparison	30-39 v 60-69	4.62	1	<0.05
	40-49 v 60-69	6.41	1	<0.02
	50-59 v 60-69	4.04	1	<0.05

Patients in their 6th decade have a significantly better prognosis than patients in the 3rd, 4th or 5th decade. Only 14% of patients in their 6th decade died from breast cancer during the study period. Of the patients in the 3rd, 4th and 5th decades - 28%, 27% and 23% respectively, died during the same period.

The menopausal status was unknown in only 7% of the total study group. Of those patients with known menopausal status 183 (29%) were premenopausal and 439 (71%) were postmenopausal (Table I.18).

Table I.18

Menopausal status	No.	Adjusted Frequency %
Pre	183	29.4
Post	439	70.6
Unknown	48	

As the menopausal status of 622 (93%) of the 670 patients was known, it was not thought necessary to analyse the distribution of other variables within the small group of unknown menopausal status.

The survival curves of pre and postmenopausal women were compared (Fig. I.8). There were 47 (26%) deaths in the premenopausal group and 79 (18%) deaths in the postmenopausal group. This difference was not statistically significant (Lee Desu Statistic $x^2 = 3.08$ 1 DF $p < 0.1$).

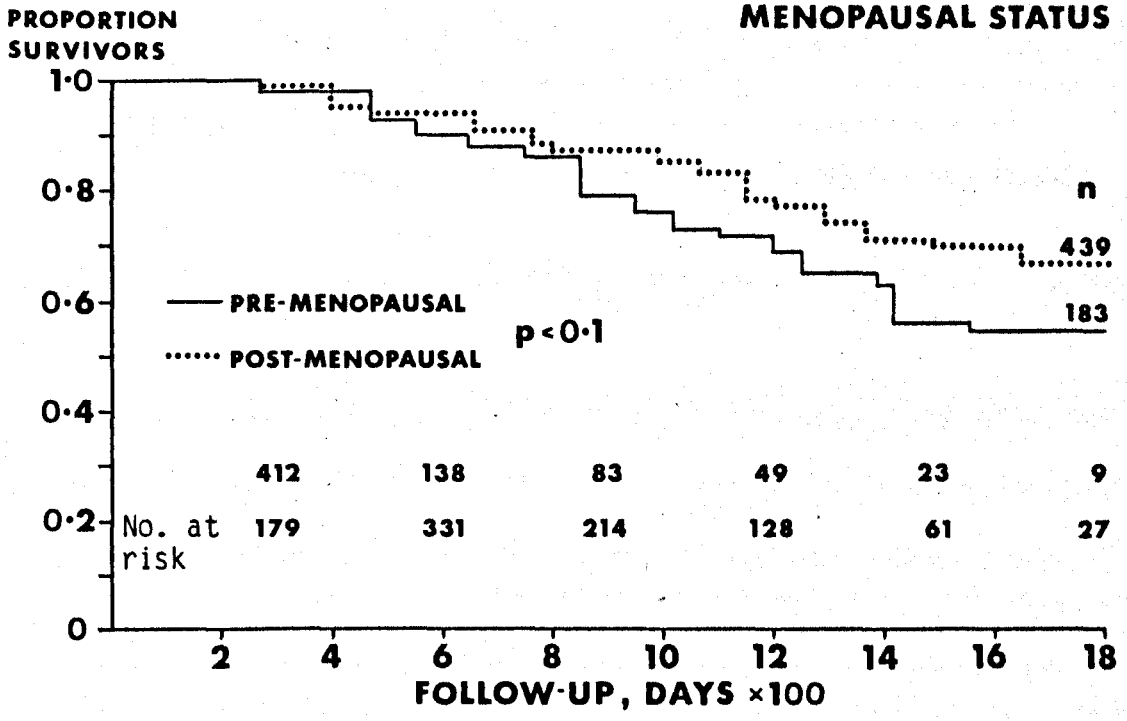

PROPORTION SURVIVORS
MENOPAUSAL STATUS
1·0
0·8
0·6
0·4
0·2
0
PRE-MENOPAUSAL
POST-MENOPAUSAL
p < 0·1
n
439
183
No. at risk
412 138 83 49 23 9
179 331 214 128 61 27
2 4 6 8 10 12 14 16 18
FOLLOW-UP, DAYS ×100

Fig. I.8

In those patients who developed recurrent disease the site of first recurrence was not sigificantly influenced by menopausal status ($x^2 = 0.491$ 4 DF $p > 0.5$) (Table I.19).

Table I.19

Site of First Recurrence	Pre MP	Post MP	Row Total
Local	22	39	61
Nodal	15	23	38
Bone	11	17	28
Visceral	10	20	30
> 1 site	22	42	64
Column total	80	141	221

The reproductive history of 45% of the patients was not documented and of the remainder 104 (22%) were known to be nulliparous and 78% were parous (Table I.20).

Table I.20

Parity	No.	Adjusted Frequency %
Nulliparous	104	22.1
Parous	366	77.9
Unknown	300	

The distribution of the other prognostic variables within the group of unknown parity was compared with the group in whom the parous state was recorded. The distribution of menopausal status was significantly different within these two groups when they were cross-tabulated along with all other prognostic variables. The results of this, and all other non-significant cross-tabulations are summarised in Table I.21.

Table I.21

	x^2	DF	p
Tumour size	1.67	2	< 0.5
Node status	2.67	1	< 0.5
Grade	1.06	2	> 0.5
Age	2.71	5	> 0.5
Menopausal status	6.20	1	< 0.02

Of the 468 women of known parity 150 (32%) were premenopausal compared to only 21% of the group of women of unknown parity. This difference in distribution of menopausal status within the two groups was statistically sigificant (Table I.22).

Table I.22

	Menopausal Status		
	Pre	Post	Row Total
Parity unknown	33	121	154
Parity known	150	318	468
Column total	183	439	622

The survival of patients following mastectomy was significantly related to their parity. Of 104 nulliparous patients 15 (14%) died during the period of study which was significantly fewer than the 84 (23%) of 366 parous women who died (Lee Desu statistic x^2 = 6.11 1 DF $p < 0.02$) Fig. I.9).

The site of recurrent carcinoma was not significantly related to the parity of the patient (x^2 = 4.30 4 DF $p < 0.5$) (Table I.23).

Table I.23

Site	Nulliparous	Parous	Row Total
Local	7	39	46
Nodal	4	24	28
Bone	7	19	26
Visceral	6	16	22
> 1 site	7	43	50
Column Total	31	141	172

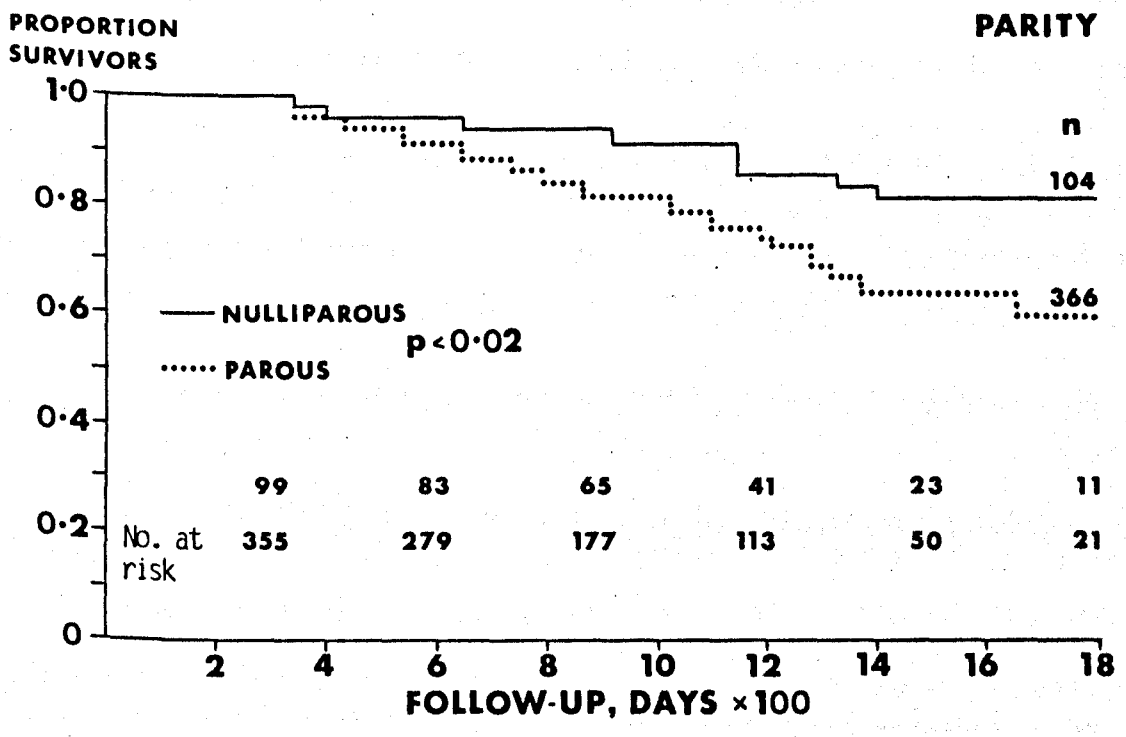

PROPORTION SURVIVORS
PARITY
n
1·0
0·8
0·6
0·4
0·2
0
104
366
NULLIPAROUS
PAROUS
p < 0·02
99 83 65 41 23 11
No. at risk
355 279 177 113 50 21
2 4 6 8 10 12 14 16 18
FOLLOW-UP, DAYS ×100

Fig. I.9

Section IV Discussion

The distribution of variables within this population is similar to that found in other comparable series of primary breast carcinomas. Most tumours (71%) were of intermediate size (2-5 cms) at the time of diagnosis. Only 10% of the tumours in this series were less than 2cms in diameter and the overall distribution of tumour size is almost identical to that reported by Fisher in 1970.

The absolute frequency of axillary lymph node involvement in this series was 49%. It has been shown that the accuracy of axillary lymph node staging is influenced by operative technique and the pathological examination and interpretation of the excised specimen. Cant (1975) reported a prevalence of 31% using simple mastectomy and axillary node sampling compared to 35% using standard radical mastectomy. This contrasts with the finding of Fisher (1969) who found that 51% of radical mastectomy specimens contained histologically involved axillary lymph nodes.

The distribution of tumours by histological grade is very similar to that reported by Bloom and Richardson in their original series in 1957, although the proportion of grade II tumours in this current series was slightly lower (Table I.24).

Table I.24

Histological grade	I	II	III	
Bloom and Richardson	26	45	29	%
Current series	30	37	32	%

The mean age of patient studied was 57 years and as one would expect the majority of women were postmenopausal (71%) and parous (78%).

Of the 153 patients dying during the period of study almost 92% have died of breast carcinoma - a slightly higher proportion than has previously been reported. Although strenuous efforts were made to trace all patients, deaths from causes other than breast cancer may have been underestimated as necropsies were seldom performed on patients dying at home.

Conversely the number of women with recurrent disease may also have been underestimated as we did not routinely screen for metastases post-operatively, investigating only those patients with signs or symptoms suggestive of recurrent disease. We felt that this policy was justified as there is no scientific evidence that more rigorous follow up improves either the quality of life or duration of survival (Redding, 1983). Although the classification of the site of recurrence is arbitrary, this classification has been used by other workers.

Hormonal manipulation was given as the initial treatment for almost half of the women treated for recurrent disease - taking the form of the anti-oestrogen Tamoxifen in almost all cases. Although objective response rates to cytotoxic chemotherapy are greater than to hormone manipulation; many clinicians are reluctant to inflict the toxic side effects of cytotoxic therapy on their patients. This could explain why only 10% of patients in this series received chemotherapy for recurrent carcinoma.

Of the 75 patients who developed recurrence and had no specific treatment, 53 (71%) had either visceral or multiple metastases. The prognosis for patients with this type of disease is so poor that many were given only symptomatic treatment. Unfortunately decisions such as those on patient management were not always clearly documented in hospital case notes.

The type of treatment given was significantly related to the site of first recurrence. Most patients developing local recurrence were treated by radiotherapy whereas of patients with bone metastases almost half were treated initially by endocrine manipulation. I believe that this therapeutic approach is based on the belief that bone metastases are predominantly hormone-sensitive and this hypothesis will be examined further in relation to oestrogen-receptor studies.

The site of first recurrence was not significantly associated with tumour size, axillary lymph node status, age, menopausal status or parity.

However the site of first recurrence was significantly related to histological grade. Well differentiated tumours were more likely to recur locally and anaplastic tumours more often metastasised to distant sites. This finding supports the belief of many pathologists that histological grading is the only prognotic variable to accurately reflect the biological aggression of the primary tumour.

In common with all other studies tumour diameter and axillary lymph node status have been confirmed as significant prognostic indicators following mastectomy. The overall comparison of survival between tumours of different histological grade was statistically significant. However, pairwise comparison revealed no significant difference between grades II and III in terms of survival. This finding may be important as grades II and III comprise the majority (69%) of the study population. A significant survival difference may have emerged if larger numbers of patients were followed up for longer but this failure of discrimination may simply reflect the problems of using any subjective grading system.

When patients were stratified by age decade the overall comparison of survival revealed no significant difference. However minor differences did emerge within subgroups in that women in their 3rd, 4th and 5th decades all had a significantly worse prognosis than women in the 6th decade of life. These results concur with the findings of Brightmore (1970) and Redding (1979). Similarly although postmenopausal women survived slightly longer than premenopausal women this difference was not statistically significant.

Parous women have a significantly worse prognosis than do nulliparous women with estimated 5 year survival rates of 57% and 72% respectively following mastectomy. The influence of parity on prognosis has not been clearly defined from previous studies, although nulliparous women are known to be at higher risk of developing breast cancer. In view of this it may be thought that parous women would develop breast cancer later in life. However, in this series the mean ages of parous and nulliparous women were almost identical at 58 and 59 years respectively.

Parity and nulliparity are discrete variables but should not be considered in isolation. Nulliparity may be elective or enforced due to female (or male) infertility; Vessey (1979) has suggested that infertile women are less likely to develop breast cancer and this in turn may influence prognosis. Furthermore, endogenous and exogenous hormones may also control the development and growth of breast cancer. Unfortunately a detailed gynaecological and drug history were not taken from all patients in this studies and it would therefore be impossible to draw any meaningful conclusions from the data presented.

Summary and Conclusions

Maximum tumour diameter and axillary lymph node status have been confirmed as significant indicators of survival following mastectomy for primary breast cancer. Histological grade was also a significant prognostic indicator but the survival of groups of patients with moderately differentiated (grade II) and anaplastic (grade III) tumours were not significantly different.

Minor survival differences were observed in relation to menopausal status and age - older, postmenopausal women having a slightly better prognosis. Nulliparous women also have a better prognosis and the possible explanations for this have been discussed.

Of those women who developed recurrent disease, the site of first recurrence was significantly associated with histological grade - patients with poorly differentiated tumours were found to develop multiple metastases more frequently.

Chapter 2. Steroid Receptors

Section I. Introduction

Following the pioneering work of Schinzinger and Beatson in the late 19th century it has been accepted that the growth and functional activity of some female reproductive tissues are influenced by oestrogens. It is only in the past two decades that the precise biochemical mechanisms by which oestrogens act have been evaluated.

The level of steroid receptor protein in malignant breast tissue may now be accurately determined. It is not yet clear if the routine performance of such assays will be of value in the management of primary breast cancer.

A. Estrogen Receptor Assay

(i) Background:

In 1961 Folca injected tritiated hexoestrol into women with advanced breast cancer about to undergo adrenalectomy. The uptake of labelled oestrogen into adrenal tissue was found to be significantly greater in those women who subsequently responded to this form of ablative treatment. Later, work by Jensen (1968) showed that the capacity for uptake of oestrogen by a breast tumour was due to the presence of a cytoplasmic protein which appeared to bind oestrogen.

It is now known that in steroid responsive target tissues the hormone functions through an integrated series of intracellular events primed by the association of oestradiol with its cytoplasmic receptor protein. The binding of hormone to receptor forms a complex which undergoes a structural change facilitating transfer to, and retention on, specific nuclear acceptor sites. Once bound the nuclear ER complex initiates a series of transcriptional events on the DNA template which, in turn, leads to the enhanced production of

components necessary for cell maintenance and function. An effective intracellular concentration of oestrogen receptor complex may therefore directly influence gene expression. The steps of the oestrogen receptor mechanism are represented diagramatically in Figure I.10.

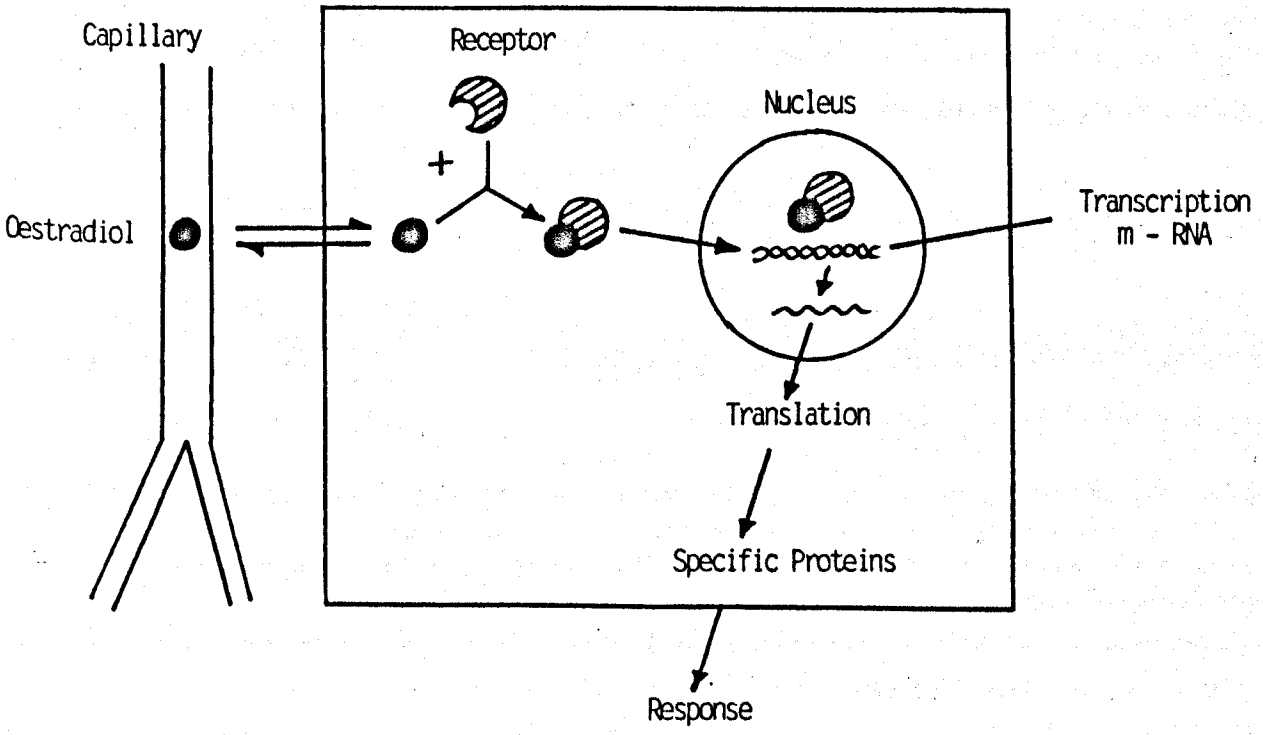


Fig. I.10 Oestrogen Receptor Mechanism

(ii) Measurement of ER:

Most methods for detecting ER activity employ the same basic principle - the incubation of cytosol with radioactive oestrogen and determination of the radioactivity bound to receptor protein. Tissue is homogenised in buffer and centrifuged to yield a tissue extract which is then incubated with radioactive oestrogen to saturate the empty ER sites. The radio-hormone protein complex (bound hormone) and the radioactivity in the bound fraction is measured by scintillation counting.

The separation of bound and free hormone may be done by:

(a) Addition of charcoal suspension to adsorb unreacted free hormone (Korenman, 1968).

(b) Addition of hydroxyapatite suspension to absorb bound hormone (Garola, 1978).

(c) Precipitation of bound hormone by protamine sulphate (Godefroi, 1973).

(d) Gel filtration (Teulings, 1975).

(e) Electrophoresis (Teulings, 1975).

(f) Sucrose density gradient analysis (Wittliff, 1971).

This latter method is able to distinguish between the two molecular subfractions of receptor (4S and 8S) - this may be important, as some workers have suggested that only the 8S form is clinically relevant. The Dextran coated charcoal technique is the most popular method because expensive equipment is not required and large numbers of samples may be analysed simultaneously. Four new methods of ER assay are currently under evaluation.

(a) Histochemical detection by immunofluorescence (Pertschuk, 1978).

(b) Radio-immunoassay (Greene, 1977).

(c) Iso-electric focussing - a form of electrophoresis (Wrange, 1978).

(d) Estrophilic binding to controlled Pore Glass Beads (Hospelhorn, 1978).

Despite many refinements in the technique of ER assay there is increasing evidence that the levels of receptor protein may vary greatly at different sites within a tumour (Tilley, 1978), and at different times in the course of the disease (Poulsen, 1981; Holdaway, 1983; Harland, 1983).

Not all methods yield the same value for receptor concentrations and different workers calculate and express results in different ways. The British Breast Group (BBG) has promoted an interchange of specimens between laboratories and have shown reasonable qualitative agreement, though significant quantitative differences (King, 1978).

(iii) Applications of ER Assay:

Estrogen receptor status is firmly established as a sensitive predictor of response to hormone therapy in advanced breast cancer. Approximately 60% of ER+ve tumours are hormone-sensitive whereas less than 10% of ER-ve tumours will respond to endocrine manipulation (McGuire, 1978). Response rates appear to be consistent, irrespective of whether ablative surgical (Jensen, 1975) or additive hormonal or antihormonal treatment is given (Westerberg, 1978).

It has been suggested that ER status may be useful in predicting response to cytotoxic chemotherapy. Lippman (1978) and Jonat (1978) have found that women with ER-ve tumours are more likely to respond to

cytotoxic chemotherapy. However, studies by Webster (1978) and Kiang (1978) were unable to confirm such an association.

In the past 5 years ER status has been shown to be a significant independent prognostic indicator in primary operable breast cancer. Tumours containing oestrogen receptors have a more favourable prognosis both in terms of disease-free (Knight, 1977; Maynard, 1978; Cooke, 1979), and absolute survival (Croton, 1981). This finding appears to be independent of clinical stage at presentation.

In addition it has been suggested that ER status may also influence the site at which recurrent disease occurs. Both Stewart (1981) and Campbell (1981) have shown that ER-ve tumours are more likely to metastasise to the viscera and soft tissue, whereas ER+ve tumours have a predeliction for bone. This latter finding may have implications for the treatment of recurrent disease and will be discussed further.

B. Progesterone Receptors

Many cytoplasmic oestrogen receptor positive (ER+ve) tumours do not respond to endocrine therapy. This discrepancy has prompted investigators to search for additional markers to discriminate between ER+ve hormone responsive and non-responsive tumours. McGuire (1978) has demonstrated the induction of a progesterone receptor. This event follows the binding of a cytosol oestrogen receptor and subsequent translation and binding as a nuclear oestrogen receptor. These findings suggest that the presence of progesterone receptors (PGR) may be an indication of a more accurate predictor of endocrine responsiveness than ER assay alone (Fernandez, 1982).

The method for PGR assay is similar to that of ER assay using a dextran coated charcoal technique. PGR activity has been found predominantly in ER+ve tumours (Forrest, 1981). (Table I.25).

Table I.25

		%
ER+	PR+	52
ER+	PR-	24
ER-	PR+	5
ER-	PR-	19

Approximately 75% of women with advanced ER+ve tumours will respond to endocrine therapy (Young, 1978; King, 1980). (Table I.26).

Table I.26

		Response Rate %
ER+	PR+	77
ER+	PR-	27
ER-	PR+	46
ER-	PR-	11

However at least 25% of ER+ PR- tumours also responded, and this finding is difficult to reconcile on the basis of the McGuire hypothesis.

Although PR assays may provide additional predictive information in advanced breast cancer, there has been no published work defining the role of progesterone receptor status as a prognostic indicator in primary operable breast cancer.

Aims of the Study

The aims of this part of the study were to perform steroid receptor assays routinely on the tumours of women undergoing mastectomy for primary breast carcinoma. Steroid receptor status was related to the other conventional prognostic indicators described in Chapter I, and was examined as an independent prognostic indicator.

Section II. Patients, Materials and Methods

The patients studied were those described in Chapter 1 and were treated and followed up in an identical manner. At the time of mastectomy a representative portion of tumour was excised and stored immediately in liquid nitrogen. Steroid receptor assays were performed on batches of tumour at the Tenovus Institute Cardiff using a Dextran Coated Charcoal technique with a cut off value of 5 f.mol oestradiol/mg cytosol protein. Details of the assay appear in Appendix I.2.

Section III Results

(i) Overall distribution of steroid receptor status

Oestrogen receptor status was determined in the tumours of all but 6 of the 670 patients studied. Approximately 54% of primary tumours were found to be ER+ve and 46% ER-ve.

Table I.27

ER Status	No.	Adjusted Freqency %
ER+ve	360	54.2
ER-ve	304	45.8
ER unknown	6	

Progesterone receptor status was not measured routinely, but was performed on an almost consecutive series of 318 tumours. Of these, 61 (19%) were PR+ve and 257 (81%) were PR-ve.

Table I.28

Progesterone Receptor Status	No.	Adjusted Frequency %
PR+ve	61	19.2
PR-ve	257	80.8
PR unknown	352	

In order to determine if this sample was biased with regard to other prognostic variables their distribution within the populations of tumours with known and unknown progesterone status were compared by cross-tabulation and analysed using a chi square test.

The results of these cross-tabulations are summarised below:

Table I.29

Prognostic Variable	x^2	DF	P
Tumour diameter	3.76	2	<0.5
Axillary node status	0.26	1	>0.5
Histological grade	1.71	2	<0.5
Age (group)	4.25	5	>0.5
Menopausal status	2.52	1	<0.5
Parity	6.15	1	<0.02
ER status	1.42	1	<0.5

The only significant difference between the two groups was the distribution with regard to parity ($p < 0.02$). In all other respects the patients of known and unknown progesterone receptor status were comparable.

Only 16% of the patients of known PR status were nulliparous, whereas significantly more of the unknown PR group (26%) were nulliparous.

Table I.30

	Parity		
	Nulliparous	Parous	Row Total
PR unknown	72	202	274
PR known	32	164	196
Column Total	104	366	470

(ii) Relationship of ER to PR status

Of the 318 tumours in which ER and PR assays had been performed 46 tumours (14%) were double receptor positive. Most tumours were either ER+ve PR-ve (42%) or ER-ve PR-ve (39%). Only 15 tumours (5%) were found to be ER-ve PR+ve.

Table I.31

	PR+ve	PR-ve	Row Total
ER+ve	46	134	180
ER-ve	15	123	138
Column total	61	257	318

The association between ER status and PR status was highly statistically significant (corrected x^2 = 9.41 1 DF $p < 0.002$).

(iii) Relationship of ER status to other prognostic variables

Estrogen receptor status was cross-tabulated with conventional prognostic variables. There was no significant association with tumour size (corrected x^2 = 2.63 2 DF $p < 0.5$).

There was a significant association with histological grade. Of 106 well differentiated (grade I) tumours 61% were ER+ve. Conversely only 43% of the 114 poorly differentiated (grade III) tumours were ER+ve.

Almost identical proportions of the tumours of intermediate grade were ER+ve (49%) and ER-ve (51%).

Table I.32

Histological grade	I	II	III
ER+ve	65	64	49
ER-ve	41	66	65

corrected $x^2 = 7.61$ DF2 $p < 0.005$

There was a significant association between ER status and patient age group. In all cases as the age increased so did the proportion of ER+ve tumours. For example only 32% of women in their 3rd decade had ER+ve tumours compared to 73% of women in their 7th decade.

Table I.33

	Age Group (decade)						
ER Status	3rd	4th	5th	6th	7th	8th	Row Total
ER+ve	15	74	88	113	61	9	360
ER-ve	32	75	92	76	23	6	304
Column Total	47	149	180	189	84	15	

x^2 for trend = 21.6 1 DF $p < 0.0001$

raw x^2 = 26.7 4 DF $p < 0.0001$

ER and menopausal status were also significantly related. Only 75 (41%) of the 182 tumours of premenopausal women were found to be ER+ve. A significantly greater proportion of postmenopausal women had ER+ve tumours (59%).

Table I.34

	Menopausal Status		
	Pre	Post	Row total
ER+ve	75	258	333
Er-ve	107	177	184
Column total	182	435	517

corrected x^2 = 16.2 1 DF $p < 0.0001$

Of the 364 parous women, almost equal proportions had ER+ve (51%) and ER-ve (49%) tumours. However significantly more nulliparous women (63%) had ER+ve tumours.

Table I.35

	Parity		
	Nulliparous	Parous	Row Total
ER+ve	64	185	249
ER-ve	38	179	217
Column total	102	364	466

corrected x^2 = 4.08 1 DF $p < 0.05$

(iv) Relationship of progesterone receptor status to other prognostic variables

In the same way PR status was cross-tabulated with all of the previously described conventional prognostic indicators. No significant associations were found, and the results are summarised below.

Table I.36

Prognostic Variable	x^2	DF	P
Tumour diameter	0.921	2	> 0.5
Axillary Node Status	0.581	1	< 0.5
Histological grade	3.65	2	< 0.5
Age (group)	4.37	5	< 0.5
Menopausal status	0.364	1	> 0.5
Parity	0.375	1	> 0.5

(v) Estrogen Receptor Status and Survival

Survival curves of the groups of women with ER+ve and ER-ve tumours were plotted using life table analysis. Of the 360 women with ER+ve tumours 57 (19%) died during the period of study.

In comparison 81 (27%) of the 304 women with ER-ve tumours died during the same time. The difference in survival rates was statistically significant when the two curves were compared using the Lee Desu statistic (x^2 = 13.6 1DF $p < 0.0002$) (Fig. I.11).

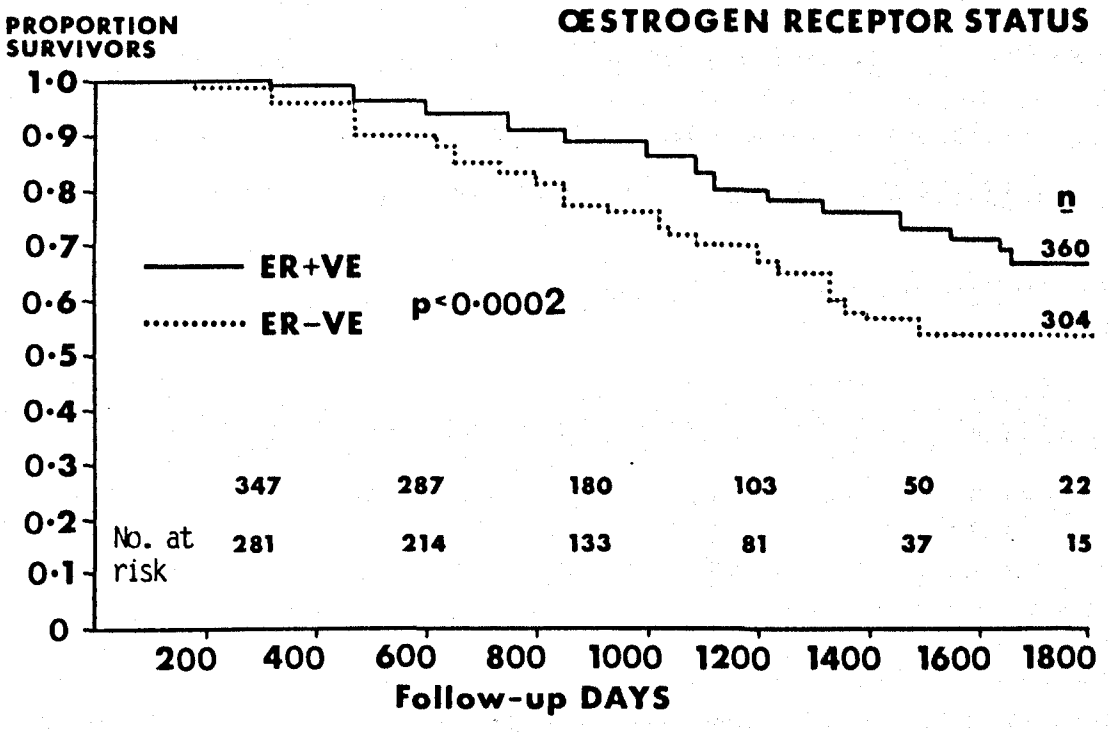

PROPORTION SURVIVORS
ŒSTROGEN RECEPTOR STATUS
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
ER+VE
ER-VE
p<0·0002
n
360
304
No. at risk
347 287 180 103 50 22
281 214 133 81 37 15
200 400 600 800 1000 1200 1400 1600 1800
Follow-up DAYS

Fig. I.11

(vi) Progesterone Receptor Status and Survival

In the same way the survival of groups of women with PR+ve and PR-ve tumours were compared. Of the 61 women with PR+ve tumours 7 (11%) died compared to 42 (16%) of the 257 women with PR-ve tumours. This difference in survival rates was not statistically significant. (x^2 = 2.77 1DF $p < 0.1$) (Fig. I.12).

(vii) Steroid Receptor Status and Pattern of Recurrence

In order to determine if the steroid receptor status influenced the site at which first recurrence appeared, receptor status was cross-tabulated with the site of recurrence using the same classification described in Chapter I. The findings are summarised in Tables I.37 and I.38.

Table I.37

ER Status and Site of First Recurrence

Site of First Recurrence	Estrogen Receptor +	-	Row Total
Local	29	37	66
Nodal	19	22	41
Bone	19	14	33
Visceral	20	12	32
> 1 site	25	41	66
Column total	112	126	238

x^2 = 7.03 4 DF $p < 0.5$

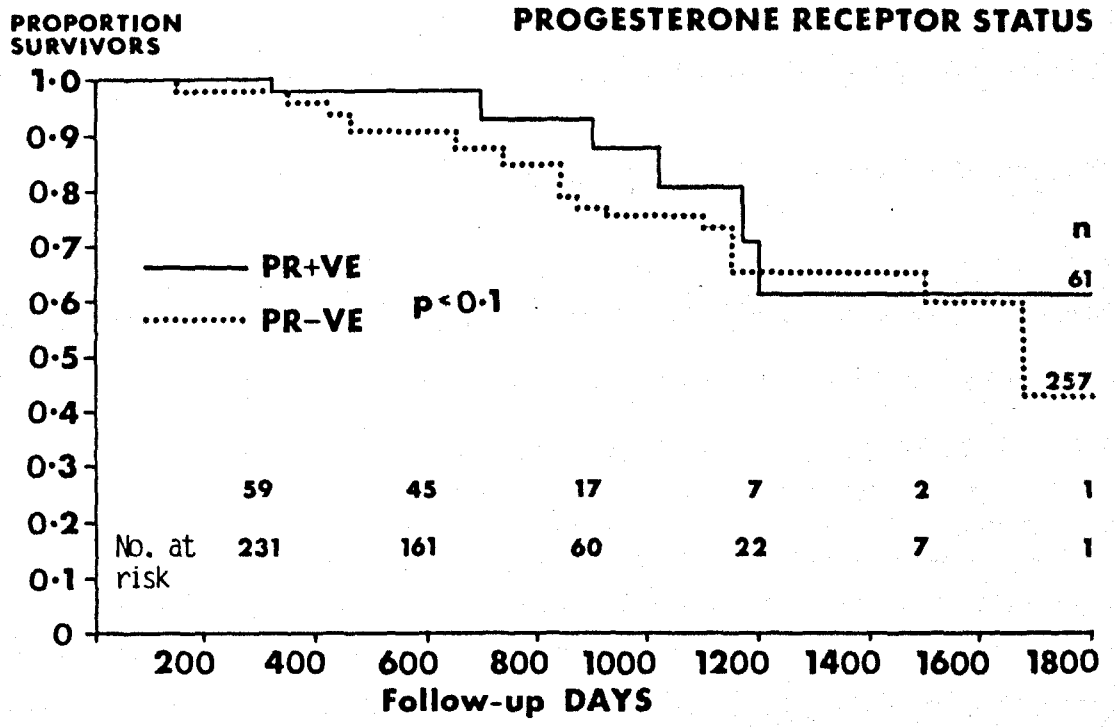

PROPORTION SURVIVORS
PROGESTERONE RECEPTOR STATUS
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
PR+VE
PR–VE
p<0·1
n
61
257
59 45 17 7 2 1
No. at risk 231 161 60 22 7 1
200 400 600 800 1000 1200 1400 1600 1800
Follow-up DAYS

Fig. I.12

Table I.38

Progesterone Status and Site of First Recurrence

Site of First Recurrence	Progesterone Receptor +	-	Row Total
Local	3	29	32
Nodal	3	19	22
Bone	1	9	10
Visceral	3	11	14
> 1 site	7	21	28
Column total	17	89	106

$x^2 = 3.39$ 4 DF $p < 0.5$

Of 112 women with ER+ve tumours who developed recurrence 19 (17%) metastasised first to bone whereas only 11% of ER-ve tumours first recurred at that site. However this, and other, minor differences were not statistically significant when the group as a whole was analysed.

When the site of recurrence was considered in relation to progesterone receptor status the first detected recurrence occured at multiple sites in 41% of the PR+ve tumours compared to only 24% of the PR-ve tumours. Conversely local, nodal and bone metastases were found more commonly in PR-ve tumours. Again, these differences were not statistically significant.

Discussion

The proportion of women with ER+ve tumours in this current series (54%) is slightly less than that quoted in the current literature. In a review article, using the pooled data from 2250 primary breast tumours, Hawkins (1982) found that 67% of women had ER+ve tumours. The type of assay used may influence results, although most laboratories now use an almost identical Dextran Coated Charcoal technique.

Some of the tumours in this series were stored in liquid Nitrogen for up to 6 months prior to receptor assay. Such prolonged storage results in a decline in levels of receptor protein and consequently some ER+ve tumours with low levels of receptor protein may have been falsely assigned as ER-ve. This may also explain why only 19% of the tumours in this series were found to be progesterone receptor positive. Recent publications by Fernandez (1982) and Nashimura (1982) reported a prevalence of 48% and 33% respectively.

Only 5% of ER-ve tumours were found to be PR+ve - an identical proportion to that reported by Forrest in 1982. However there are other significant discrepancies between the two series. Of the tumours in this study only 14% were double receptor positive compared to 52% in the Edinburgh series. Conversely 42% of tumours were ER-ve PR-ve compared to 24% in the Edinburgh study. These differences cannot be explained solely by the prolonged storage of tumour tissue, but may reflect differences in the two population samples. The current series consists only of primary operable breast cancers whereas the Edinburgh data was derived from a mixture of primary, recurrent and advanced tumours.

Estrogen Receptor positive tumours were found to be better differentiated than ER-ve tumours which were more often anaplastic. This observation has been previously made by Maynard (1978) who has gone so far as to suggest that accurate histological grading may obviate the need for steroid receptor assay. This argument has been refuted by other workers who claim that an objective biochemical assay is preferable to the more subjective interpretation of histological grade.

Significantly more postmenopausal women had ER+ve tumours than did the premenopausal group. This finding is consistent with the clinical observation that the tumours of postmenopausal women are more likely to respond to hormone manipulation. Similarly increasing age was associated with a significantly greater proportion of ER+ve tumours.

The relationship of parity to steroid receptor status has not previously been defined. Significantly more nulliparous women had ER+ve tumours than did ER-ve counterparts. This association may be responsible for the observed survival advantage for nulliparous women described in Chapter I.

Estrogen receptor status was confirmed as a highly sigificant prognostic indicator in primary operable breast cancer following mastectomy. Although progesterone receptor status was independent of other prognostic variables, its measurement yielded no additional prognostic information. The failure of progesterone receptor status to influence prognosis may be due to the much smaller group on whom the assays were performed.

The finding that steroid receptor status does not influence the patterns of recurrence following mastectomy may have important therapeutic implications. The likelihood of response to hormone therapy cannot be deduced from the site at which recurrence is first detected. Response to therapy may be more accurately predicted by measuring the estrogen receptor status of both the primary, and preferably the secondary tumour.

The significance of the prognostic variables described in Chapters 1 and 2 are inevitably influenced by their association with each other. For example the significant association between histological grade and ER status may explain why histological grade influences the site of first recurrence whereas ER status does not. ER status may merely be a further expression of tumour differentiation.

It is clear that this simple form of statistical analysis may be inadequate as many prognostic variables are inter-related. This highlights the importance of multivariate analysis which takes into account all available information. This more complex form of analysis will be presented in Chapter 4.

Summary and Conclusions

The estrogen receptor status of 666 primary breast cancers was determined and 318 of these tumours were also asayed for progesterone receptor status. Of the tumours assayed 54% were ER+ve and 19% were progesterone receptor positive. There was a significant association between estrogen receptor and progesterone receptor status.

Histologically well differentiated tumours were more likely to be estrogen receptor positive. Postmenopausal and nulliparous women also more often had ER+ve tumours. There were no other significant associations between steroid receptor status and tumour size, axillary lymph node status or patient's age, menopausal status and parity. There was a significant association between ER status and histological grade; ER+ve tumours were more often well differentiated.

Women with ER+ve tumours had a sigificantly improved prognosis following mastectomy. Although women with PR+ve tumours had a better prognosis, their rate of survival was not significantly different from that of the PR-ve group.

In those patients who developed recurrent disease, the site of first recurrence was not influenced by the steroid receptor status of the primary tumour.

CHAPTER 3

In-Vitro Thymidine Labelling Index

General Introduction

In a further attempt to identify those women with rapidly growing breast cancer and a poor prognosis, the use of cell kinetic techniques was studied.

In Section I the theoretical background and practical applications of such techniques will be reviewed. In Section II the use of one specific technique, the in-vitro Thymidine Labelling Index (T.L.I.) is reviewed and the study of T.L.I. in a series of primary breast cancers is described. T.L.I. will be analysed both in relation to other prognostic variables within the study population, and also as an independent prognostic indicator.

The practical problems associated with the routine measurement of in-vitro T.L.I. will be discussed as will the potential applications in the management of primary operable breast cancer.

Section I Cell Kinetics

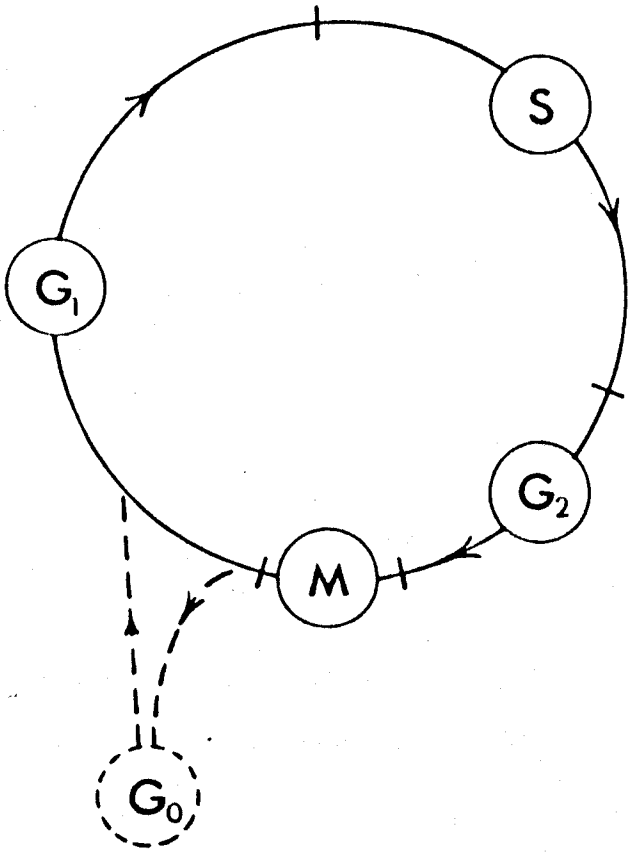
Introduction

It seems likely that variation in the rates of growth of human breast carcinomas could account for some of the marked differences in the behaviour of apparently morphologically identical tumours. The growth rates of untreated breast tumours may be estimated either by direct measurement (Heuser, 1979) or serial mammography (Fournier, 1980). From such studies volume doubling time may be calculated, and the estimated tumour life span projected. Most clinicians, however, would regard such studies as ethically unacceptable. A further theoretical objection isthe fact that volume growth takes no account of the greatly variable rates of cell loss which may be in excess of 90% of the total rate of cell production (Refsum, 1967).

One alternative approach to the direct measurement of growth has been to study tumours in-vitro using cell kinetic techniques. Various methods have been used and these are based on the identification of cells at different stages of the cycle.

The cell cycle may be represented diagramatically thus:

Fig. I.13
THE CELL CYCLE



G1 is the most variable period of the cell cycle and its duration largely influences the rate of cell proliferation. In general, rapidly growing cell populations have short G1 periods.

S phase is the period during which D.N.A. replicates and chromosomal proteins are laid down on the newly synthesised D.N.A.

G2 is the interval between the completion of D.N.A. synthesis and mitosis.

G0 or resting cells, are neither cycling nor dividing and can be induced to re-enter the cycle by appropriate stimuli.

M Mitosis

Part A presents the various techniques that have been used to examine these different components of the cell cycle. In part B the development and applications of Thymidine Labelling Index, in particular, are described.

A. Cell Kinetic Techniques

(i) Mitotic Index

Increased frequency of cells undergoing mitosis is recognised as a histological feature of malignancy (Fulker, 1981). Mitotic activity may be quantified - the mitotic index being the number of mitoses per 100 cells.

Mitotic index correlates poorly, however, with observed growth rates of tumour, this being possibly due to variations in the duration of mitosis. The arrest of genetically damaged cells in mitosis means that the mitotic index may also reflect the accumulation of dead cells rather than the true proliferative activity of a tumour.

(ii) Stathmokinetics

The rate of cell entry into mitosis can be measured in vivo by inducing metaphase arrest by administration of spindle poisons such as colchicine or vincristine.

Using serial biopsies a plot of Mitotic Index against time is produced. From this curve the rate of cell entry into metaphase is derived (Lala, 1971).

This technqiue assumes that all metaphases are arrested at the same dose level, that interphase cells are unaffected, and that degeneration does not occur before fixation. In practice it is difficult to ensure the fulfillment of these three criteria.

Stathmokinetics have been studied extensively in animal models and correspond well with data from Thymidine Labelling (Iverson, 1974). Human studies have been restricted by the difficulty of obtaining serial in-vivo biopsies (Camblejohn, 1979).

(iii) Growth fraction

The growth fraction is an estimate of the proportion of proliferating cells in a cell population. This assumes the existence of discrete compartments within the cell population - one proliferating and one quiescent.

Growth fraction has been shown to be a reliable index of proliferative activity (Malaise, 1973). However measurement is complex involving in-vivo thymidine labelling using either repeated labelling or labelled mitosis techniques (Nelson, 1973).

(iv) Potential doubling time and cell loss factor

Cell loss may account for discrepancies between potential and observed growth rates. The rate of cell loss may be inferred by comparing a computed rate of cell production (in the absence of cell loss) with observed tumour growth rate. It may be important to identify not only where cell loss occurs in relation to the cell cycle but also the mode of cell loss. At present cell kinetic techniques are unable to distinguish potential losses due to necrosis, phagocytosis and metastatic spread (Lala, 1971).

(v) D.N.A. Distribution Techniques

Individual tumour cells may be segregated on the basis of their nuclear D.N.A. content. Rapid, automated analysis of D.N.A. distribution is possible using Flow Microfluometry (Kute, 1981; Barlogie, 1978).

D.N.A. content divides breast cancer cells into diploid and aneuploid groups and in some preliminary work aneuploidy has been shown to correlate with prognosis and histological grade (Hinton, 1983).

(vi) Thymidine Labelling Index

Following in-vitro or in-vivo exposure of tissue to tritiated thymidine, the isotope is selectively incorporated by those cells in the D.N.A. synthetic (S) phase of the cell cycle. The uptake of isotope may be identified and recorded permanently by Autoradiography. Using light microscopy the percentage of cells in S phase may be calculated - this is the Thymidine Labelling Index (T.L.I.).

Various methods of assessment of T.L.I. have been described:

1. Systemic regional perfusion of isotope and subsequent preparation of autoradiographs from biopsied material (Clarkson, 1967).
2. Local injection of isotope into tissue followed by needle aspiration or biopsy (Nordenskjold, 1974; Muggia, 1972).
3. In vitro incorporation of isotope by fresh tissue removed from the host (Packard, 1975; Danekamp, 1973).

All methods have been applied in the study of animal models but there have been few applications of in vivo techniques in human malignancy. Regional perfusion of breast tumours is technically difficult and invasive. The experimental administration of isotopes to patients with malignant disease would be unacceptable ethically to many clinicians.

In vitro T.L.I. should not be interpreted as a simple, direct measurement of cell proliferation. T.L.I. relates the ratio of S phase to intermitotic time, and theoretically cycling cells with a long S phase may have a higher T.L.I. than cells with a short S phase - despite having similar proliferation rates. Further assumptions are that cells in S phase continue D.N.A. synthesis in vitro at the same rate as in vivo, and that incubation and processing do not perturb the progression of cells through S-phase. In common with most other cell kinetic techniques T.L.I. takes no account of the rate of cell loss (Steel, 1967).

Despite these many theoretical reservations T.L.I. has been shown to correlate with growth rates of human breast carcinomas (Meyer, 1979) - tumours with rapid growth rates having a higher T.L.I. than slowly growing tumours.

B. In vitro Thymidine Labelling Index

(i) Introduction

In 1896 Becquerel recognised that uranium salts blackened the emulsions of silver salts. This finding contributed to the discovery of radioactivity by the Curies in 1898. Autoradiography produces a permanent record of the tracks of radioactive particles and relates them to the structural patterns within a specimen.

The measurement of T.L.I. has only been made possible since high specific activity isotopes became available in the 1950s. These isotopes are essential for the production of high resolution autoradiographs.

In 1961 Johnson reported a method of labelling tissues with tritiated thymidine in vitro to compare rates of cell proliferation in benign and malignant breast tissue. Wolberg (1962) found great variations in the rate of nucleoside uptake when this technique was applied to breast tumours maintained in organ culture for 24 hours.

Coons (1966) suggested that this variation was due to tumour heterogeneity and hoped to overcome this problem by disaggregating the tumours into their component cells. These isolated cells were exposed to the isotope for only 2 hours. It was claimed that by using this technique the autoradiographic development time would be reduced and also that labelled cells could be identified and counted more efficiently. In practice, however, many human breast carcinomas are very difficult to disaggregate and it is perhaps worthy of note that only one of the 12 tumours studied by Coons was a breast carcinoma.

In 1968 and 1969, two major publications from Fabrikant described an in vitro method to incorporate tritiated thymidine into human tissues and this technique has been used, essentially unchanged, by most workers who have subsequently studied T.L.I. in breast cancer. This method is summarised below.

Fresh tissue is incubated for one hour at 37^{o}C under hyperbaric conditions in a serum-enriched tissue culture medium containing tritiated thymidine. Autoradiographs are prepared from fixed, wax-embedded tissues and exposed for 3-4 weeks. Following haematoxylin and Eosin staining the labelled cells are counted under a light microscope. The number of labelled nuclei expressed as a percentage of the total tumour nuclei is the labelling index.

Using this method Fabrikant studied a variety of murine and human tissues including benign and malignant breast tumours. The in-vitro distribution of thymidine within normal tissues resembled that reported from in-vivo studies (Edwards, 1961). The in vitro pattern of thymidine labelling of human tumours also corresponded to that of previous in vitro studies (Steel, 1965). Although it was suggested that the growth rate of a tumour could be estimated from the in-vitro T.L.I., the authors stressed that this must be a crude estimate in the presence of inevitable cell loss from emmigration, differentiation and death.

Several groups of workers have related T.L.I. of breast carcinomas to clinical, pathological and biochemical features. Findings have often been contradictory even from the same groups of workers at different times using identical techniques. Meyer in 1975 and 1979 reported an association between high T.L.I. and involvement of axillary lymph nodes but he refuted this in 1977 as did Silvestrini in 1974 and Gentili in 1981. Both groups of workers, however, found that T.L.I. was higher in premenopausal women and found that tumours with a low T.L.I. were more likely to be estrogen receptor negative (Meyer, 1977, 1978; Gentili, 1981).

In 1975 Murphy performed serial in-vitro T.L.I. on the tumours of 48 patients receiving cytotoxic chemotherapy and suggested that a fall in T.L.I. was associated with a favourable response to treatment.

This finding was confirmed by a further report from the same institution (Sulkes, 1979), but in a larger study of 148 patients Wolberg (1971) had found no such association between in vitro T.L.I. and response to cytotoxic therapy.

Only in recent years has T.L.I. been investigated as a prognostic indicator in primary operable breast cancer following mastectomy. Both Silvestrini (1981) and Meyer (1979) have found that tumours with high (above median) T.L.I's are associated with higher rates of early relapse. One of the many problems of analysing and comparing such data is that patients, even from the same institution, are rarely treated and followed-up in a uniform way.

Despite these investigations from a few specialist centres it is not yet possible to clearly define the role of T.L.I. in the management of breast cancer.

(ii) Aims of the Study

The aims of this study were to:

1. Measure in vitro Thymidine Labelling Index (T.L.I.) in a series of primary operable breast cancers.
2. Relate T.L.I. to conventional prognostic indicators and steroid receptor status.
3. Establish if the routine measurement of T.L.I. provides any useful prognostic information.

Section II

Patients, Materials and Methods

(i) Patients

The patients in this study form part of the larger group of 670 women with primary operable breast cancer described in Chapters 1 and 2. The treatment and follow-up of the patients was identical to that described previously, as was the measurement of clinical, pathological and biochemical parameters.

Although this series was not consecutive the mastectomies were all performed at hospitals within the Mersey Region from 23.5.78 to 17.4.82.

(ii) Materials and Methods

At the time of mastectomy a specimen of fresh tumour was transported, on ice, from operating theatre to the Department of Surgery laboratory within one hour of excision. Under sterile conditions in a laminar flow cabinet the tumour was cleared of fat and necrotic debris and sectioned into ten approximately 2 x 2 x 2mm cubes. Residual tumour was retained for in vitro drug-sensitivity assay (Part II), steroid receptor assay (Part I chapter 2) and histological grading (Part 1 chapter 1).

The tumour cubes were randomised and divided equally between 2 vacutainer tubes containing 5mls RPMI tissue culture medium. To one tube was added 125 μci tritiated thymidine, the second tube acting as a control. The tubes were stoppered and injected via the rubber bungs with 10mls of a 95% O_2/10% CO_2 mixture. Following fixation in a perspex clamp the tubes were incubated in a shaking water bath for 2 hours at 37^{o}C.

After incubation the gas from each tube was aspirated and the tumour cubes were washed with Phosphate Buffered Saline before fixation in 5mls 10% buffered formalin.

The blocks of fixed tissue were embedded in wax and 5μ sections cut. The sections were mounted on histological slides coated with chrome alum. The histological preparations were dewaxed using progressive dilutions of xyelene and absolute alcohol and finally washed with double distilled water.

Kodak AR10 stripping emulsion was applied to the slides in a photographic dark room and dried gently at 25°C to prevent cracking of the film. The slides were placed in light-tight boxes and exposed at 4°C for 28 days.

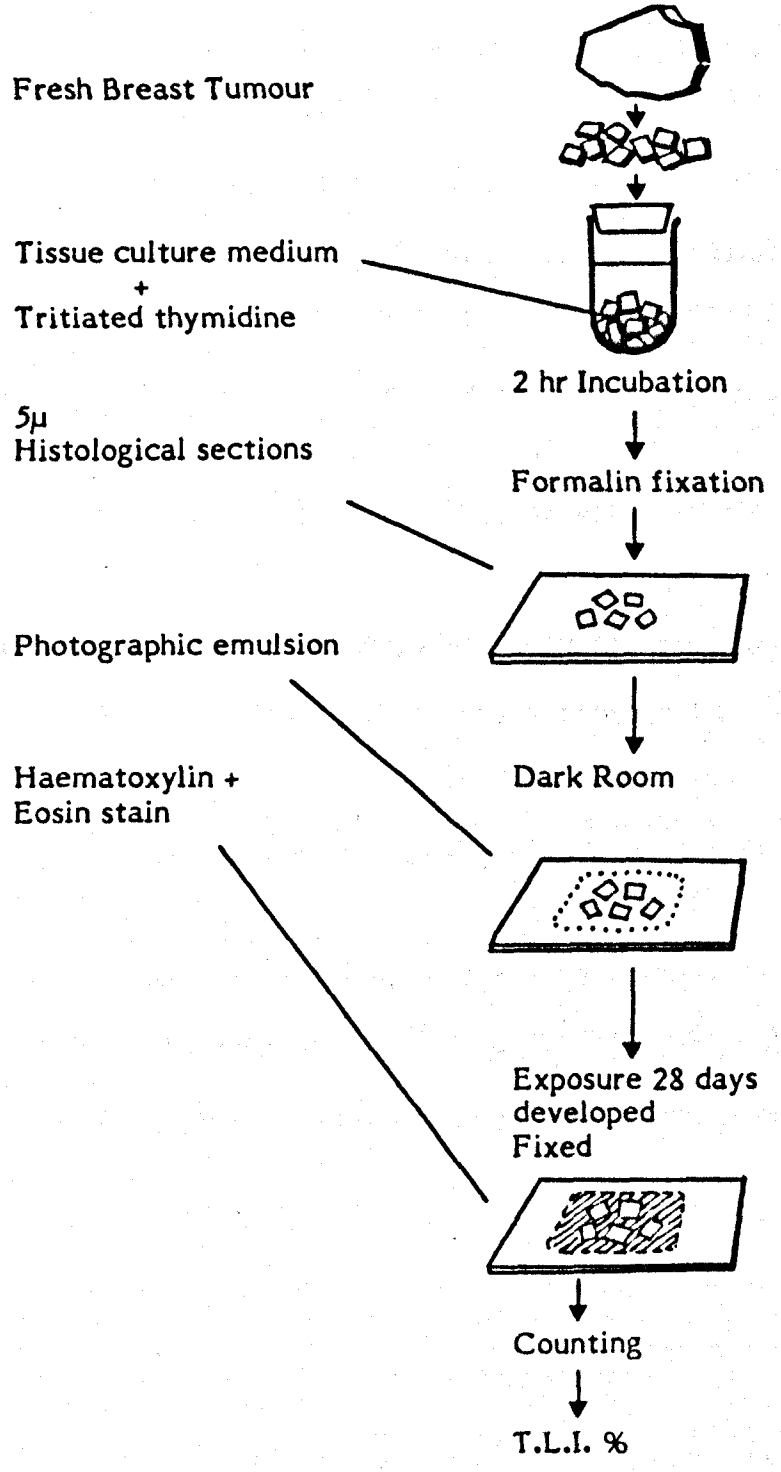
Autoradiographs were developed using Kodak D19 developer, washed in double distilled wter and fixed with Kodak rapid fix. Finally the specimens were stained with haematoxylin and eosin, and cover slips applied prior to histological examination.

The principle steps in the measurement of T.L.I. are presented diagramatically as a flow chart (Figure I.14).

The equipment and reagents used are listed in Appendix I.3.

Fig. I.14

FLOW DIAGRAM T.L.I. METHOD



(iii) Interpretation of Specimens

Contaminants and pressure artefacts may mimic labelling by following structural patterns within the specimen. It is not possible to attribute developed silver grains to radio-activity without the use of suitable controls. In view of this a control preparation was produced for each tumour by incubation without labelled thymidine followed by identical processing.

Four labelled slides were prepared from each tumour and a minimum of 2000 tumour nuclei were examined under the high power of a light microscope. A nucleus was considered to be labelled if it contained at least 10 reduced silver grains (England, 1973).

The distribution of isotope within the specimens was uneven. Although it has been shown that hyperbaric incubtaion enhances the penetration of isotope, in many of the specimens labelling was confined to the surface of the tumour. In common with the practice of other workers counting was restricted to those areas containing labelled cells.

The labelled areas were identified under low power and the nuclei counted under high power using hand counters to register the numbers of labelled and unlabelled cells. The number of labelled nuclei was expressed as a percentage of the total tumour nuclei - the resultant percentage being the Thymidine Labelling Index.

Low and high power views of tumour no. 674 (T.L.I. 19.1%) are illustrated in figs. I.15 and I.16.

(iv) Reproducibility of results

The interpretation of autoradiographs is, of necessity, subjective and is likely to be influenced by tumour heterogeneity and the counting technique of the microscopist. For this reason the intra and inter observer reproducibility were assessed in a study of 20

Fig. I.15 Autoradiograph. Low Power. Tumour No. 674

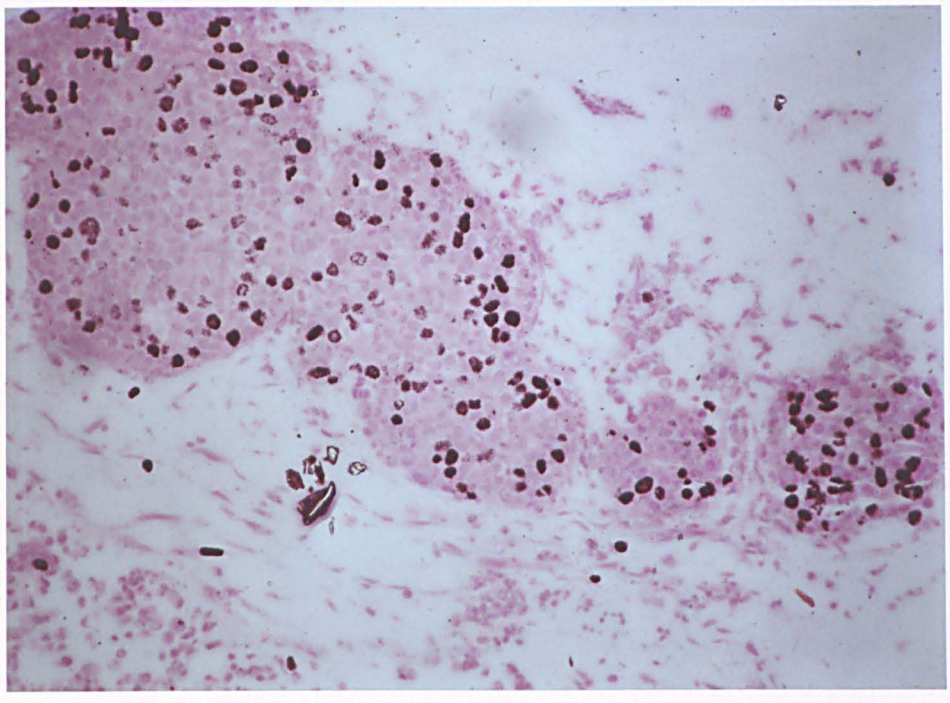
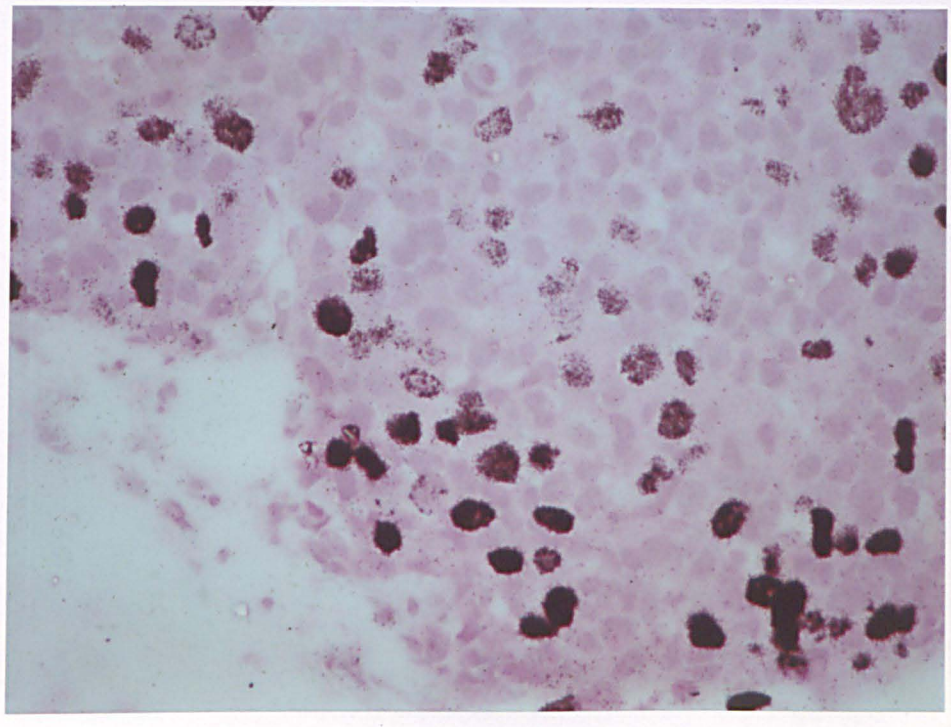


Fig. I.16 Autoradiograph. High Power. Tumour No. 674



consecutive tumours. The T.L.I. of each tumour was assessed independently by myself and a second observer (Mr. Roger Croton) - also experienced in the interpretation of autoradiographs.

One month later I repeated the counting of the same 20 tumours examined in a random order. From these three sets of observations the intra and inter observer errors were calculated.

(v) Statistics

The relationship of T.L.I. to other prognostic variables was assessed following log transformation of the raw data. The dependency of T.L.I. on the other variables was analysed using the Kruskal Wallace one way analysis of variance or Mann Whitney U-test as appropriate. The relationship of T.L.I. to patient's age was examined by the Spearman Rank Correlation Coefficient.

Patients were stratified into groups with above and below median T.L.I. tumours and these groups were cross-tabulated with other prognostic variables. Similarly these two groups were examined in relation to recurrence and survival by the method of Life Table Analysis; comparisons between curves being made using the Lee Desu Statistic.

Section III Results

(i) Overall distribution of T.L.I.

The T.L.I. of 164 primary breast cancers was measured. The values ranged from 0-20.7% with a mean of 4.54% (S.E.$\pm$ 0.305 S.D. $\pm$ 3.90%), median 3.22%. As the raw data were log normally distributed the tranformed values were mean T.L.I. 0.484% (S.E. $\pm$ 0.32 S.D. 0.410%) and median 0.505%. The individual T.L.I. values of each of the 164 tumours are contained within Appendix I.1.

(ii) Reproducibility study

From the T.L.I. values of 20 tumours the mean intra and inter observer errors were calculated. The mean intra observer error was 12.6% (S.D.$\pm$16.7 S.E.$\pm$3.71%). The mean inter observer error was 12.6% (S.D.$\pm$23.4 S.E.$\pm$5.25%). The detailed results of these 20 tumours are presented in Appendix I.3.

(iii) Distribution of Prognostic Variables

Before analysing the relationship of T.L.I. to other prognostic variables the 164 patients with tumours of known T.L.I. were compared with the 506 women in whom T.L.I. was not measured, in an attempt to identify any significant differences in the distribution of these prognostic variables within these two groups.

This was done by analysing the distribution of each prognostic variable within the groups of known and unknown T.L.I. using simple chi squared tests.

Significant differences were found only with regard to distribution of tumour size and menopausal status. The results of all statistical comparisons are summarised in Table I.39. The two significantly different distributions are presented in Tables I.40 and I.41.

Table I.39 Distribution of Other Prognostic Variables Within Groups of Known and Unknown T.L.I.

Prognostic variable	x^2	DF	p
Tumour size	6.93	2	<0.05
Axillary node status	3.41	1	<0.1
Histological grade	2.66	2	<0.5
E.R. Status	3.21	1	<0.1
P.R. Status	0.158	1	>0.5
Age (group)	9.42	5	<0.1
Menopausal status	6.21	1	<0.02
Parity	2.95	1	<0.1

The distribution of T_2 and T_3 tumours was similar within the population of known and unknown T.L.I. However a significantly smaller proportion of known T.L.I. tumours were less than 2cm in diameter (5%) when compared to the T_1 group of known T.L.I. (12%). Table I.40.

Table I.40

		Tumour Size			
		T_1	T_2	T_3	Row Total
T.L.I.	Unknown	59	354	93	506
	Known	8	119	37	164
Column Total		67	473	130	670

Of the 146 women where tumour T.L.I. was measured 91 (62%) were post-menopausal. Significantly more of the unknown T.L.I. group were post-menopausal (73%).

Table I.41

		Menopausal Status		
		Pre	Post	Row Total
T.L.I.	Unknown	128	348	476
	Known	55	91	146
		183	439	622

(iv) The relationship of T.L.I. to other prognostic variables

The distribution of T.L.I. values within each prognostic category is represented in Figs. 17, 18,19. In each case the continuous horizontal line denotes the median T.L.I. value. The numbers in each group and the mean and median values are summarised in Table I.42.

The relationship between T.L.I. and each prognostic variable was examined by the Kruskal Wallace one way analysis of variance (K.W.A.) or Mann Whitney U test (M.W.U.) as appropriate. The relationship between T.L.I. and the continuous variable age was defined by the Spearman Rank correlation coefficient and was found to be not significant. The results of all other statistical comparisons appear in Table I.42.

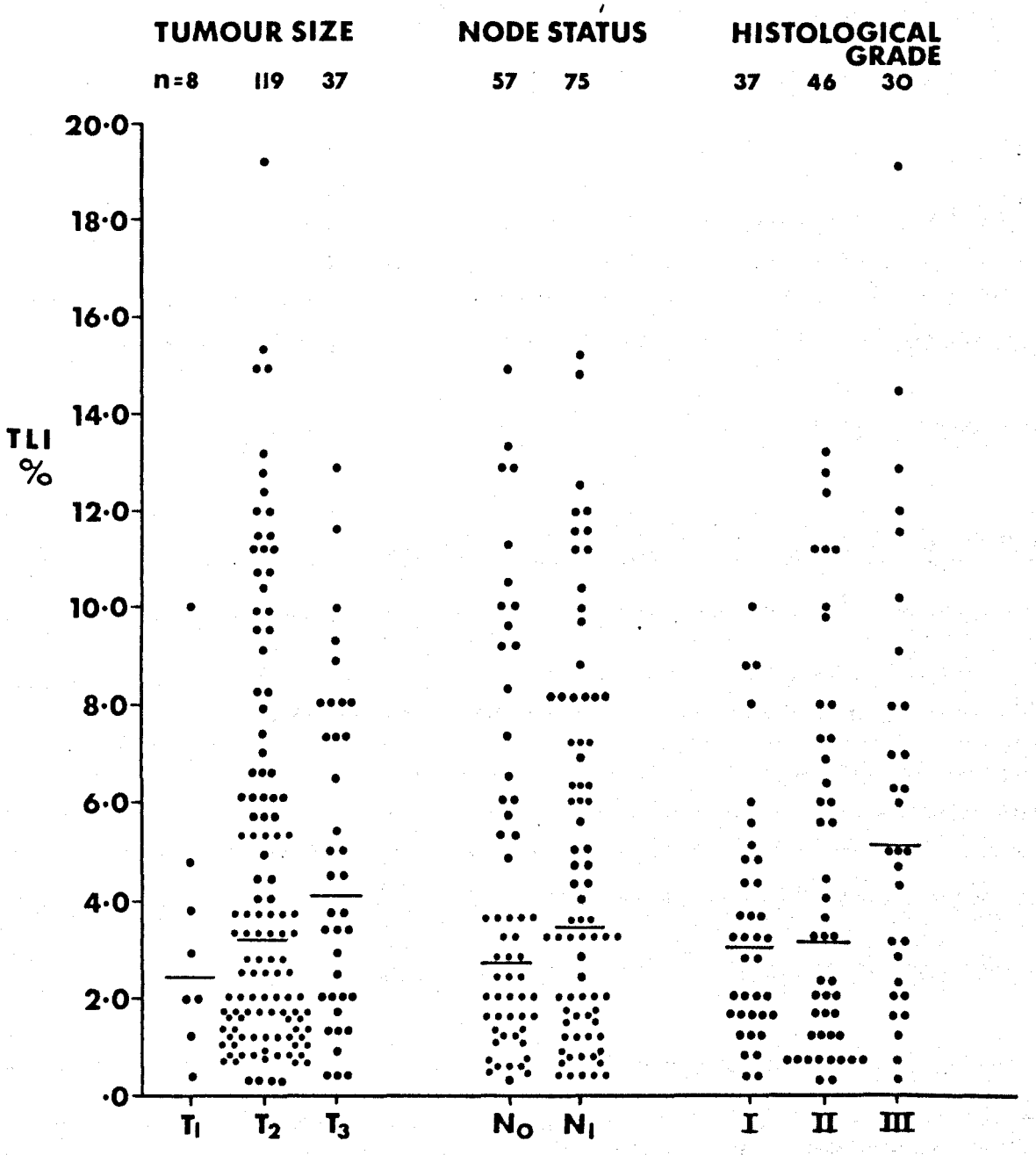


Fig. I.17 Distribution of TLI by Tumour Size, Axillary Lymph Node Status and Histological Grade

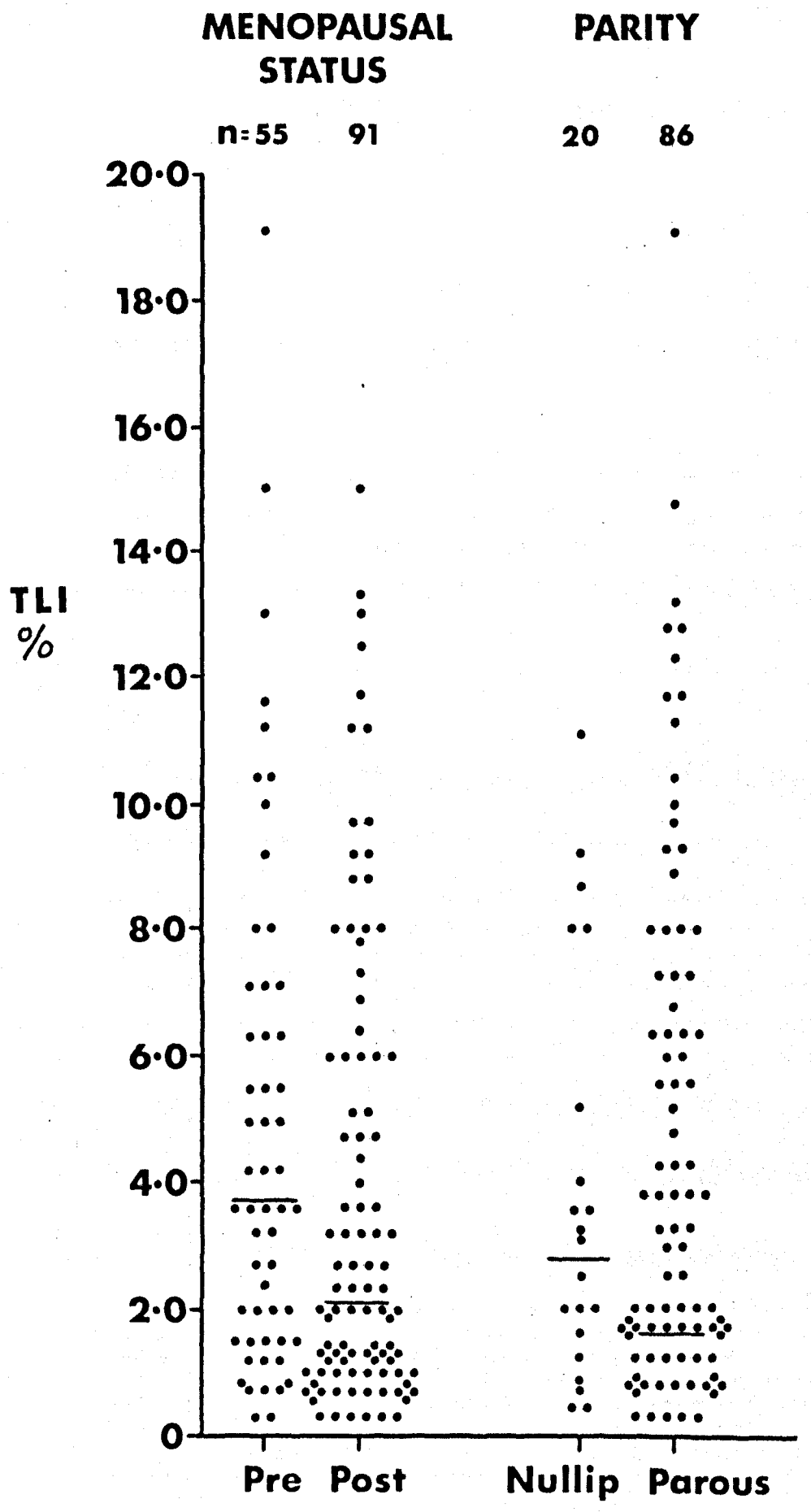


Fig. I.18 Distribution of TLI by Menopausal Status and Parity

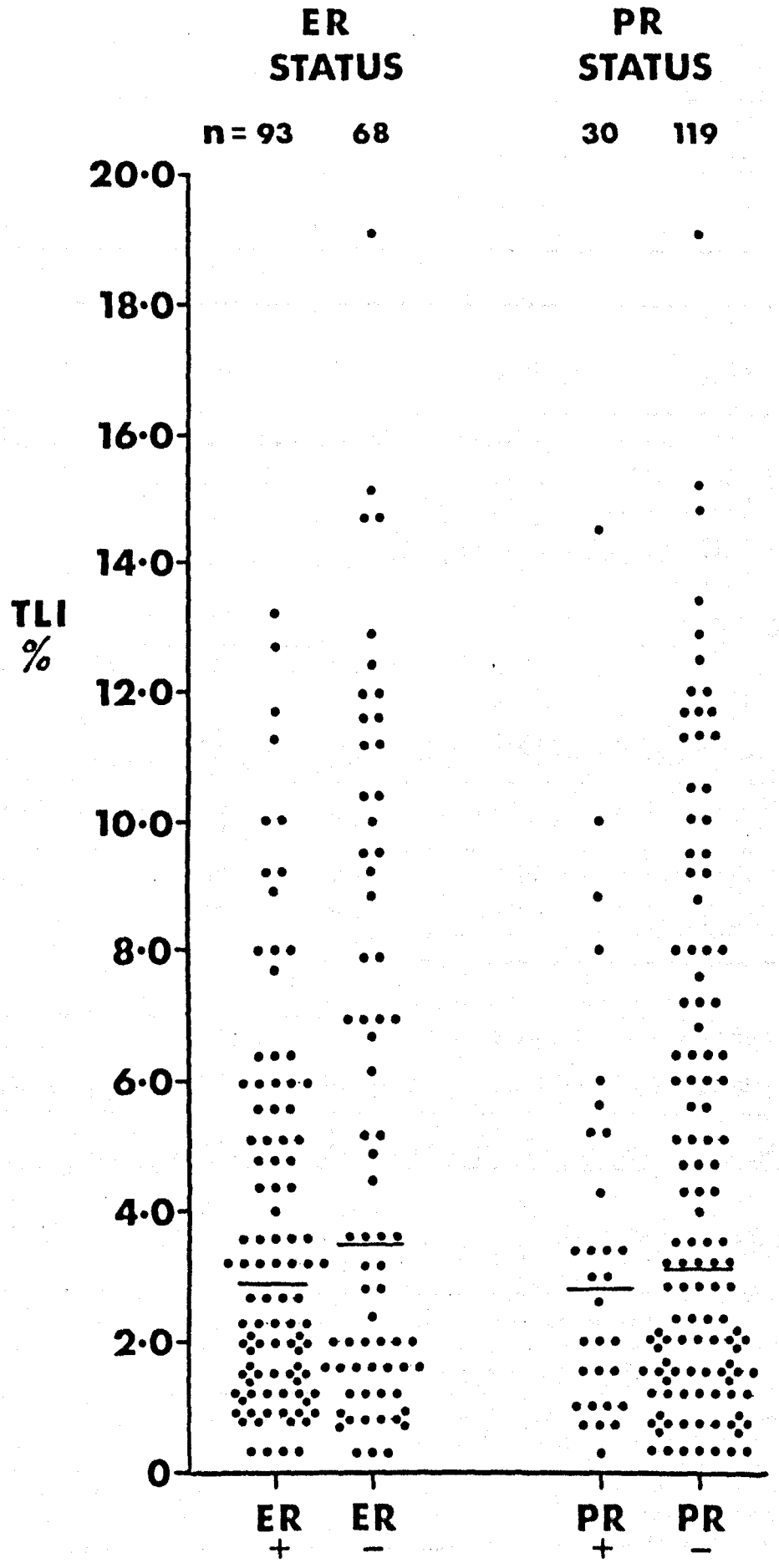


Fig. I.19 Distribution of TLI by Steroid Receptor Status

TABLE I.42 Distribution of other Prognostic Variables

	No.	Mean%	± SD%	Median %
Tumour Size p = 0.529 (KWA)				
T_1	8	3.37	3.00	2.45
T_2	119	4.55	4.11	3.20
T_3	37	4.74	3.37	4.18
Node Status p = 0.407 (MWU)				
N_0	57	4.43	3.91	2.62
N_1	76	4.96	3.88	3.45
Histological Grade p = 0.032 (KWA)				
I	37	3.40	2.47	2.98
II	46	4.55	3.97	3.10
III	30	6.21	4.53	5.15
E.R. Status p = 0.123 (MWU)				
+ve	93	3.86	3.06	2.74
-ve	68	5.47	4.71	3.31
P.R. Status p = 0.357 (MWU)				
+ve	30	3.71	3.27	2.88
-ve	119	4.76	4.09	3.12
M.P. Status p = 0.168 (MWU)				
Pre	55	4.94	4.06	3.78
Post	91	4.09	3.58	2.10
Parity p = 0.856 (MWU)				
Nulli-parous	20	4.08	3.27	1.70
Parous	86	4.68	4.11	2.85

There is a consistent trend within each category. Both mean and median T.L.I. are higher in the prognostic groups which have already been shown to have a less favourable prognosis. For example the median T.L.I. of T_3 tumours (4.18%) is higher than that of T_1 tumours (2.45%). The same trend is apparent when T.L.I. is examined in relation to axillary lymph node, steroid receptor, and menopausal status.

However the only significant association demonstrated is that between T.L.I. and histological grade. The median T.L.I. of the 37 well differentiated tumours is 2.98%, whereas the median T.L.I. of the 30 poorly differentiated tumours is 5.15%. Correspondingly the 46 tumours of intermediate differentiation (grade II) have a median T.L.I. of 3.10%.

(v) T.L.I. and rate of recurrence

In common with the practice of other workers (Meyer, 1979; Gentili, 1981) patients were then stratified into groups with tumours above and below median T.L.I. values (>< 3.22%).

The disease free intervals of the 84 patients with high (above median) T.L.I. tumours were plotted against those of the 80 patients with low (below median) T.L.I. tumours using Life Table Analysis (Fig.I.20).

Of the 84 women with high T.L.I. tumours 35 (42%) developed recurrence during the period of follow up whereas only 23 (29%) of the group with low T.L.I. tumours were found to have recurrent disease during the same time. This difference, however, did not achieve statistical significance when the two curves were compared using the Lee Desu statistic ($x^2 = 3.43$ 1 D.F.$p<0.1$).

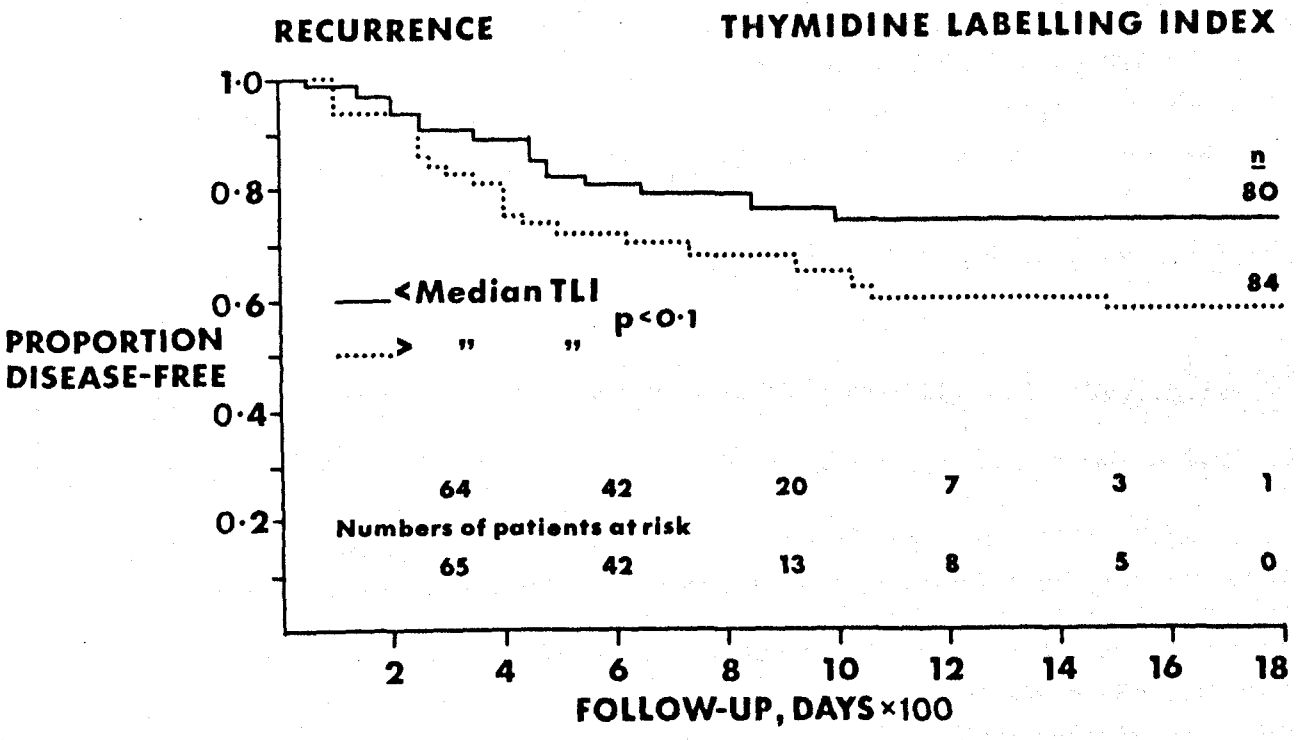

RECURRENCE
THYMIDINE LABELLING INDEX
1·0
0·8
0·6
0·4
0·2
PROPORTION DISEASE-FREE
n
80
84
<Median TLI
p<0·1
> " "
64 42 20 7 3 1
Numbers of patients at risk
65 42 13 8 5 0
2 4 6 8 10 12 14 16 18
FOLLOW-UP, DAYS ×100

Fig. I.20

(vi) T.L.I. and site of recurrence

The influence of T.L.I. on the site of first recurrence was then examined. The classification of site of recurrence was as described previously in Chapters 1 and 2. High and low T.L.I. values were cross-tabulated with the site of first recurrence and a significant association was found (x^2 = 9.69 4 D.F. $p<0.05$). Table I.43.

Table I.43

		Site of First Recurrence				
		Local	Nodal	Bone	Visceral	2 or more sites
T.L.I.	< median	8	2	5	5	3
	> median	5	9	2	8	11
		13	11	7	13	14

Local and bone recurrence occured more frequently with low T.L.I. tumours. Conversely of 11 patients developing nodal recurrence, 9 of these had high T.L.I. tumours. Similarly 8 of the 13 patients who developed visceral metastases had high T.L.I. tumours. Multiple metastases were detected in 14 patients and of these 11 had high T.L.I. tumours.

(vii) T.L.I. and survival

The survival curves of women with high and low T.L.I. tumours were plotted by Life Table Analysis (Fig.I.21). During the period of study there were 24 deaths in the high T.L.I. group compared to only 11 deaths in the low T.L.I. group. However once again this difference in survival was not statistically significant when the two curves were compared (x^2 = 2.25 1 D.F. $p<0.2$).

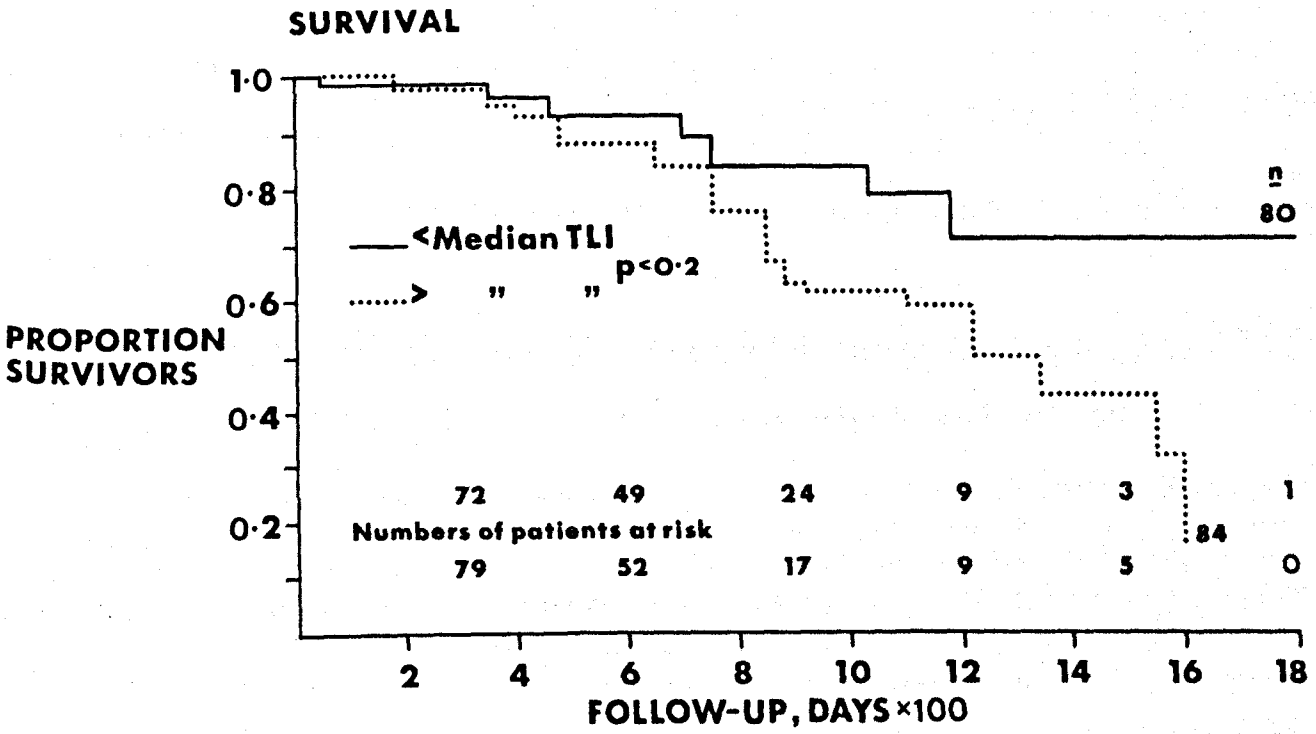

SURVIVAL
1·0
0·8
0·6
0·4
0·2
PROPORTION SURVIVORS
<Median TLI
p<0·2
n
80
72
49
24
9
3
1
Numbers of patients at risk
79
52
17
9
5
0
84
2
4
6
8
10
12
14
16
18
FOLLOW-UP, DAYS ×100

Fig. I.21

Section IV Discussion

1. The Method

Although it has proved possible to measure the T.L.I. of all the tumours submitted, there are many aspects of the production and interpretation of autoradiographs which must be considered.

Autoradiography is technically demanding and time consuming and each of the steps outlined is fraught with potential problems. The isotopes used must be pure and of high specific activity. Processing of tumours must be performed with great care to avoid contamination and the production of pressure artefacts.

To illustrate the importance of this precaution one batch of control autoradiographs were found to be densely labelled. Each stage of the processing was retraced and it was discovered that the laboratory water source had been impregnated with Chlorine due to suspect contamination of a storage tank. The preparations were reprocessed using double-distilled water, and this problem did not recur.

It is necessary to apply the photographic emulsion in a dark room with careful control of temperature and humidity to avoid cracking of the emulsion. Using the method outlined the autoradiographs are exposed for 28 days. Allowing a further week for development, staining and reading of the preparation this means a delay in approximately 5 weeks from the time of biopsy until the T.L.I. result is available. Such a delay may be important if T.L.I. is to be used in the management of breast cancer.

The distribution of labelling within each preparation is often uneven. In addition tumours are composed areas of variable histological differentiation. For these reasons the interpretation of tumour specimens is, of necessity, subjective.

In common with the practice of other workers in each case a minimum of 2000 tumour nuclei was examined. This requires an average of 20-30 minutes of intense concentration. However despite the problems of counting and heterogeneity the labelling index was found to be relatively reproducible with an inter and intra observer variability of approximately 13%.

In summary therefore the measurement of Thymidine Labelling Index was found to be time consuming and also demanded considerable technical expertise and experience in the preparation and interpretation of autoradiographs.

2. The Results

The population of patients studied was typical with regard to the overall distribution of conventional prognostic variables. Most tumours were of intermediate size (73%) and moderately differentiated histologically (41%). Of those patients of known axillary lymph node status 57% had histologically involved nodes. This proportion is greater than would be expected from a general population of women presenting with primary operable breast cancer (CANT 1975). This predominance of node positive tumours may have influenced both the relationship of T.L.I. to other variables and the survival of patients following mastectomy.

In order to identify any potential bias the patients who had T.L.I. measured were compared with the remainder of the study population described in Chapter 1.

Essentially the distribution of variables within groups of known and unknown T.L.I. was similar. However, minor, but statistically significant differences, were found in terms of the distribution of tumours by size and menopausal status. In all other respects the two groups were comparable.

When the relationship between T.L.I. and other prognostic variables was examined, high T.L.I. values were found to be associated with the unfavourable prognostic features identified by this and other studies. Nevertheless the only significant association was between T.L.I. and histological grade - well differentiated tumours having a lower T.L.I. than moderately differentiated or anaplastic tumours. This finding would support the hypothesis that T.L.I. may be a further expression of tumour differentiation rather than cellular proliferation.

Patients with high T.L.I. tumours have a worse prognosis when compared to patients with low T.L.I. tumours, both in terms of disease-free, and absolute survival. However the difference between these two groups is not significant when the two are compared using conventional statistical methods. This may be due to the fact that too few patients have been studied for too short a time; in this study only 25% of the women have been followed-up for more than 3 years. Secondly there may be important differences between the two groups that have been obscured by the subtle inter-relationship that exists between all prognostic variables. Identificaiton of these relationships involves multivariate analysis and this will be discussed further in Chapter 4.

Local and bone recurrence occured more frequently in patients with low T.L.I. tumours. Conversely visceral, nodal and multiple recurrences were significantly associated with high T.L.I. tumours. Although this may represent the aggressive nature of high T.L.I. tumours, this would be expected to result in a significantly worse prognosis for this group of patients.

There have been only 2 other major published studies of in vitro T.L.I. in primary breast cancer (Mayer, 1979; Gentili, 1981). The method used by both groups of authors was similar to that described

and the authors allude to the problems entailed in the production and interpretation of autoradiographs. In both series the values of T.L.I. were log normally distributed, although median T.L.I. was found to be lower by both Meyer (2.21%) and Gentili (2.8%) in comparison to this current series (3.22%).

It is difficult to compare this series with others as the patients have been selected and treated in different ways, and alternative forms of statistical analysis have been used. Meyer (1977, 1978) has reported a consistent association between low levels of Estrogen Receptor Protein and High T.L.I. values. This trend was noted in the current series but the association was not statistically significant. In vitro T.L.I. has been previously related to tumour type but never to histological grade.

Survival data is even more difficult to interpret as the patients in other major series have been treated by a variety of operations and many have also received adjuvant therapy which may have influenced the outcome. Nevertheless Meyer (1979) observed that relapse free survival was significantly prolonged in patients with below median T.L.I. tumours (although recurrence was not precisely defined). The same finding was reported by Silvestrini, though the survival advantage was only apparent in the premenopausal group.

In several other series significant survival differences emerged only when patients were stratified into smaller subgroups, for example by tumour size and menopausal status. Such arbitrary subdivisions were deliberately avoided as it was felt that resultant survival data would be less meaningful.

Interpetation of this and other studies must be circumspect as the majority of patients have been followed up for such a short time. Prolonged follow up of all patients is required before reaching any firm conclusions.

Summary and Conclusions

The in vitro T.L.I. of 168 primary breast cancers has been measured. This measurement is both difficult and time consuming requiring considerable skill in the preparation and interpretation of autoradiographs.

Tumours with high T.L.I. values were associated with other features indicative of a poor prognosis. In particular poorly differentiated tumours had a significantly higher T.L.I.

Although patients with high T.L.I. tumours had a less favourable prognosis following mastectomy the discrimination between patients with high and low T.L.I. tumours was not statistically significant.

Of those patients who developed recurrent disease, visceral, nodal and multiple metastases were found more often in those with high T.L.I. tumours.

From this study I feel it is unlikely that the routine measurement of T.L.I. will yield any additional prognostic information that cannot be gained by the measurement of more simple parameters.

CHAPTER 4

An Individualised Prognostic Index

Section I General Introduction

The prognosis of an individual woman with breast cancer is a complex function expressing the inter-relationship of many biological variables. Attempts have been made to define the relative importance of prognostic factors studied either in isolation or after dissecting survival data into subgroups. Within a fixed population as the number of subgroups increases, the number of patients within each subgroup diminish proportionately (Myers, 1966).

Multivariate analysis makes the most efficient use of latent information and has been applied clinically in a variety of diseases, for example in predicting the outcome of patients with severe head injury (Teasdale, 1979), identifying groups at high risk of developing post-operative DVT (Clayton, 1976) and defining the severity of acute pancreatitis (Imrie, 1978).

Logistic regression lends itself well to the study of breast cancer (Cox, 1972; Anderson, 1972), making the most efficient use of all available data and patients with a wide range of follow up times. This approach was first used by Meyers in 1965 studying 371 women treated by radical mastectomy. A mathematical model was constructed and used to predict survival on the basis of axillary lymph node status, tumour size, sinus histiocytosis and nuclear grade. Alderson (1971) analysed 21 prognostic factors and found that the combination of risk scores predicted survival more accurately than clinical stage alone.

Haybittle (1982) in a study of 387 patients with primary breast cancer in Nottingham constructed a prognostic index based on tumour size, lymph node stage and pathological grade. Estrogen receptor

status, though measured did not produce any additional significant information and was omitted from the index.

Data relating to 1022 women were analysed in a recent survival study from Manchester (Palmer, 1982). Although clinical stage was found to be the major prognostic determinant, the pathological stage, histological grade and oestrogen receptor status were not recorded.

At present there is no regional, national or international agreement on the significance of prognostic indicators. An accurate prognostic index would be useful in the design, stratification and analysis of clinical trials of treatment.

Aims

It was the aim of this part of the study to utilise all of the data presented in the preceding chapters to define, as precisely as possible, the relative importance of each of the prognostic variables described. This required a complicated form of statistical analysis to unravel the subtle relationships existing between these variables.

In Section II I will describe how this survival information was used to compute a theoretical model which makes the most efficient use of all the available data. This model is described in detail in Section III with a worked example of how this can be applied to predict the prognosis of an individual. The model is finally tested to see how accurately it can predict the observed fate of the entire study population.

Section II Patients and Methods

The data used for this analysis was derived from the survival study of the 670 patients described in Chapters 1, 2 and 3.

The prognostic significance of each variable previously described is summarised below.

Table I.44

Prognostic Variable	x^2	DF	p
Tumour size	17.8	2	<0.001
Axillary L.N. status	39.2	1	<0.0001
Histological grade	11.4	2	<0.005
Age (group)	8.24	5	<0.2
Menopausal status	3.08	1	<0.1
Parity	6.11	1	<0.02
E.R. status	13.6	1	<0.0002
P.R. status	2.77	1	<0.1
T.L.I.	2.25	1	<0.2

The data base relating to each of the 670 patients is reproduced in Appendix I.1.

The computer package used was G.L.I.M. (Genralised Linear Interactive Modelling) first described by Baker in 1978. This programme produces estimated probabilities of recurrence or death from breast cancer, using the method of maximum likelihood (Nelder, 1972). The probabilities will, in all cases, be underestimated as the analysis relates only to the study period and takes no account of future events within the study population.

A binomial outcome is selected - in this case recurrence or no recurrence; death or no death. The probability of the binomial outcome is expressed by the equation.

$$\text{Logit } p = \text{Log } \frac{\hat{p}}{1-\hat{p}} = \mu + \alpha_i + \beta j + \gamma_k + \delta_L + \text{Binomial Error}$$

where $\hat{p}$ = estimated probability

μ = constant

$\alpha \beta \gamma \delta$ = factor variables

The programme selects the most significant factor variables and it then becomes possible to estimate the probability of the binomial outcome within each category. Therefore with a simple calculation the probability of recurrence or death from breast cancer can be estimated for an individual, from the known characteristics of that patient.

Section III Results

(i) The Model

To explain the data from these 670 women, four significant factor variables were identified - age group, tumour size, axillary lymph node status and histological grade.

The programme examines the relationship of each prognostic variable to all others and this accounts for the difference in the levels of significance summarised in Table I.45. For example although ER status was highly significant as a prognostic indicator when considered in isolation the significant association with histological grade means that overall, this prognostic effect disappears. Conversely the patients age group becomes relatively more important when estrogen receptor status is omitted.

The effects of the four variables are assumed to be additive on the logit scale since interactives do not improve the fit significantly and increase the standard errors of estimation.

The significance of each of the four effects is summarised below.

Table I.45

	Recurrence			Survival		
Effect	x^2	DF	P	x^2	DF	P
Age group	17.4	5	<0.01	11.7	5	<0.05
Tumour size	29.2	2	<0.001	17.2	2	<0.001
Node status	37.6	2	<0.001	42.6	2	<0.001
Grade	11.6	3	<0.01	14.9	3	<0.01

The categories within each factor variable were represented as follows:

α_i = age group = 1(<40 years), 2 (40-49 years),
3(50-59 years), 4(60-69 years)
5(70-79 years) 6(>80 years)

β_j = tumour size j = 1 (T_1), 2 (T_2), 3 (T_3),

γ_k = Node status k = 1 (N_0), 2 (N_1), 3 (N = unknown)

δ_l = Histological grade l = 1(I), 2(II), 3(III), 4 (unknown)

The estimated probabilities within each category are presented in table form (Table I.46) and graphically (Figs I.22, 23).

In each case the first level of each factor is taken to be zero i.e. <40 years, T_1, N_0, Grade I.

As the value of logit $\hat{p}$ increases the less favourable is the outcome. Conversely a negative value of logit $\hat{p}$ denotes a better prognosis.

Table I.46

	Recurrence	Survival
Group	Logit $\hat{p}$	Logit $\hat{p}$
40-49 yrs	0.4047	0.0194
50-49 yrs	-0.1111	-0.3613
60-69 yrs	-0.4702	-0.8383
70-79 yrs	-0.6650	-0.6350
>80 yrs	-0.3104	-0.3992
T_2	0.2807	0.2123
T_3	1.184	0.9008
N_1	1.136	1.249
N?	0.4846	-0.1666
Grade II	0.8515	2.215
Grade III	0.9850	1.451
Grade ?	0.7760	1.093
Constant μ	-2.173	-2.964

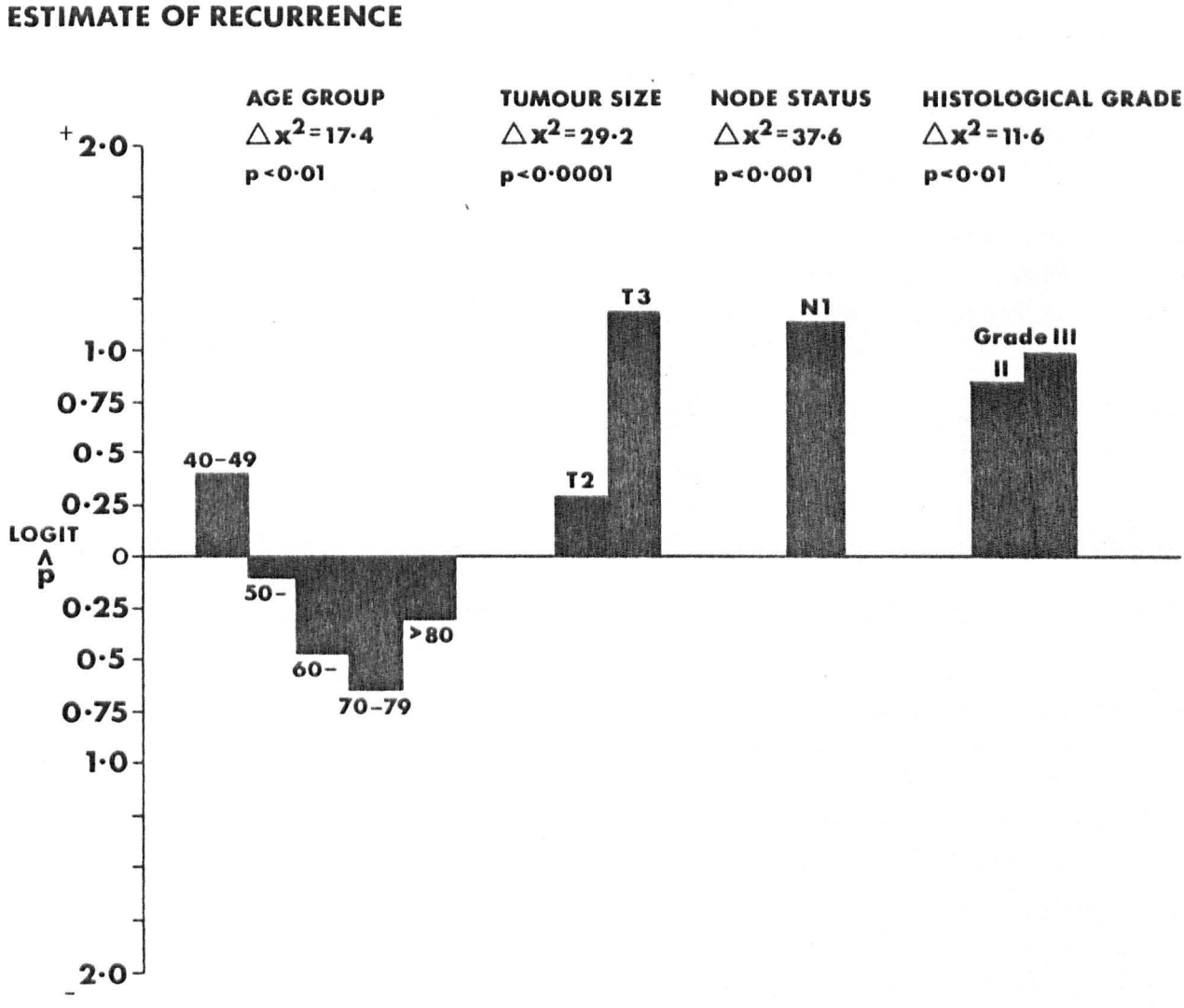

ESTIMATE OF RECURRENCE
AGE GROUP
$\triangle x^2 = 17{\cdot}4$
$p < 0{\cdot}01$
TUMOUR SIZE
$\triangle x^2 = 29{\cdot}2$
$p < 0{\cdot}0001$
NODE STATUS
$\triangle x^2 = 37{\cdot}6$
$p < 0{\cdot}001$
HISTOLOGICAL GRADE
$\triangle x^2 = 11{\cdot}6$
$p < 0{\cdot}01$
+2·0
1·0
0·75
0·5
0·25
LOGIT $\hat{p}$
0
0·25
0·5
0·75
1·0
-2·0
40–49
50–
60–
70–79
>80
T2
T3
N1
II
Grade III

Fig. I.22

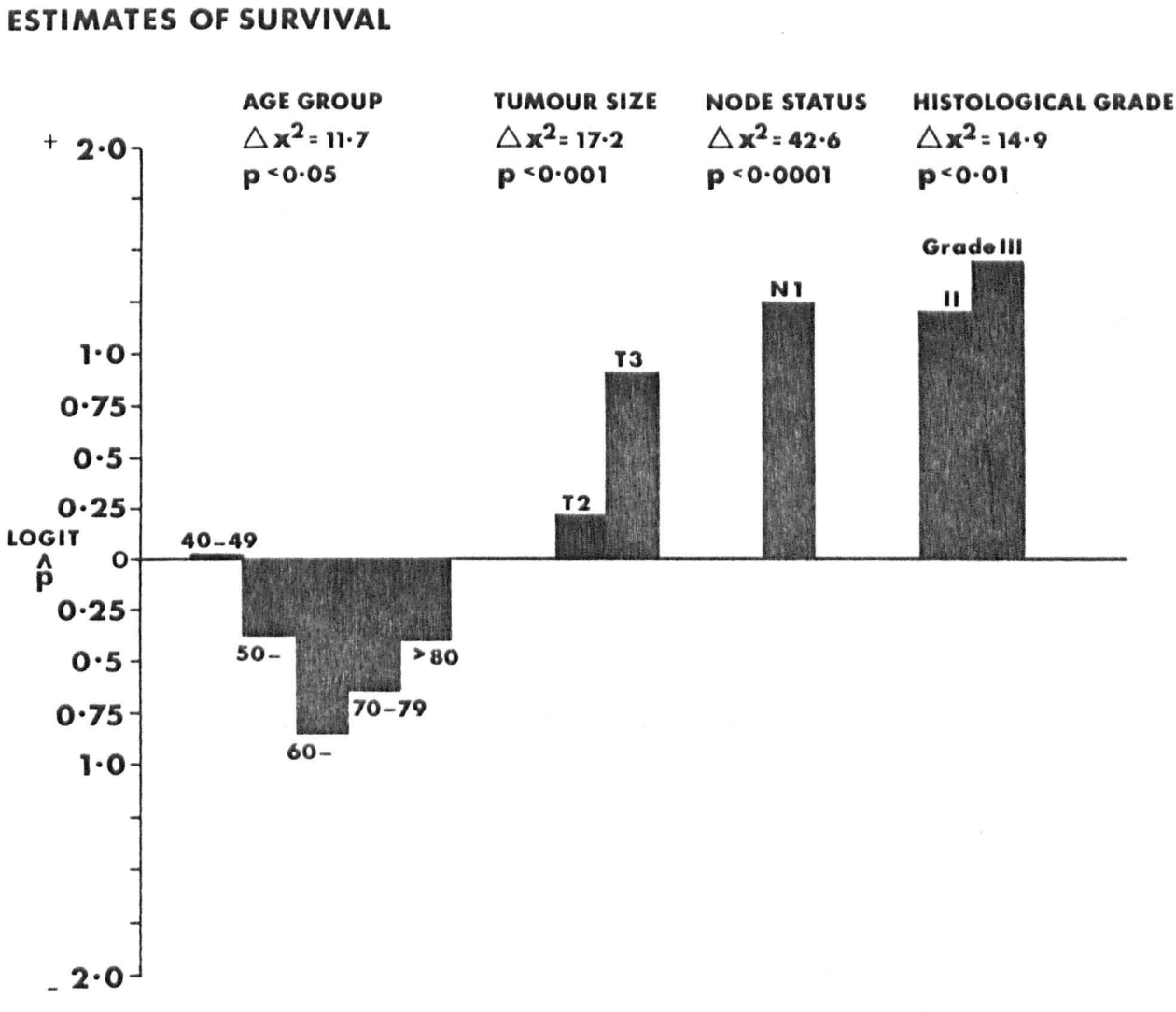

ESTIMATES OF SURVIVAL
AGE GROUP
$\triangle x^2 = 11{\cdot}7$
$p < 0{\cdot}05$
TUMOUR SIZE
$\triangle x^2 = 17{\cdot}2$
$p < 0{\cdot}001$
NODE STATUS
$\triangle x^2 = 42{\cdot}6$
$p < 0{\cdot}0001$
HISTOLOGICAL GRADE
$\triangle x^2 = 14{\cdot}9$
$p < 0{\cdot}01$
+ 2·0
1·0
0·75
0·5
0·25
LOGIT $\hat{p}$
0
0·25
0·5
0·75
1·0
- 2·0
40–49
50–
60–
70–79
>80
T2
T3
N1
II
Grade III

Fig. I.23

(ii) A worked example

The actual probability of recurrence and death of an individual woman within the study population may then be calculated.

e.g. Patient No. 46 (Tumour No. 56)

Age = 49 years

Tumour - T_2N_1

Histological grade III

For probability of recurrence

$$\text{Logit } \hat{p} = \mu + \alpha_i + \beta_j + \gamma_k + \delta_L$$

$$= -2.173 + 0.4047 + 0.2807 + 1.136 + 0.9850$$

$$= +0.633$$

$$= \log\left(\frac{\hat{p}}{1-\hat{p}}\right)$$

p = estimated probability of recurrence = 65.3%.

Similarly, estimated probability of death = 49.2% Therefore using this regression model a woman with these characteristics has a 65% chance of developing recurrence and a 49% chance of dying of breast cancer during the study period.

Approximated 95% lower and upper confidence limits may be calculated from the equation:

$$\log p_l = \text{logit } p - 2 \times SE$$

For this example

probability of recurrence = 65.3% (52.5 - 76.2%)

probability of death = 49.2% (35.3 - 63.2%)

(ii) Testing the model

The regression model was then tested by comparing, for each patient within the population, the observed fate with the predicted fate.

Of the 241 patients who developed recurrence during the study period this was predicted by the regression model in 140 cases. Conversely it was predicted that 530 patients would develop no recurrence and only 429 of the study group remained disease-free. With regard to recurrence the regression model has correctly classified 71% of the population.

Table I.47 Comparison of observed and predicted recurrence

		Predicted		Totals
		+	-	
Observed	+	94	147	241
	-	46	383	429
Totals		140	530	670

Observed + = recurrence - = no recurrence

Predicted + = $p > 0.5$ - = $p < 0.5$

Looking at survival, 140 patients died during the study period but this was predicted in only 32 cases. However 530 patients were alive at the end of the study period and this had been predicted in 638 patients. Therefore overall the regression model has correctly classified the survival of 79% of the population.

Table I.48

Comparison of Observed and Predicted Survival

	Predicted		Totals
	+	-	
Observed +	16	124	140
-	16	514	530
Totals	32	638	670

Observed + = death - = alive

Predicted + = $p > 0.5$ - = $p < 0.5$

Section IV Discussion

It is important to appreciate that this regression model provides an explanation rather than the explanation of the data presented. Theoretically any survival study of breast cancer is incomplete until all of the patients have died - either from breast cancer or other causes. For this reason any model will tend to underestimate the probability of recurrence or death. Nevertheless the fate of more than 70% of the patients in the study population has been correctly classified.

From a review of the literature it would appear that this exact form of multivariate regression analysis has not been applied to a survival study of breast cancer patients. However a similar type of analysis (Cox, 1972) was applied to a study of 387 women treated by mastectomy and followed up by the Nottingham Breast Cancer Group (Haybittle, 1982). The two studies differ in several respects. The patients in the Nottingham study were all treated by a single surgical team and were staged on the basis of a triple node biopsy (Blamey, 1979). The patients in the Liverpool study were treated by many surgeons within the region, and lymph node staging was performed on the basis of axillary dissection alone. Secondly a group of patients from the Nottingham study received adjuvant drug therapy, whereas such patients were excluded from the current study. In all other respects the two populations seem comparable.

The results of the two studies are almost identical. Tumour size, lymph node stage and histological grade are the most significant prognostic variables. Women in the Liverpool study were stratified by age decade, and the age group 40-49 years had the worst prognosis. Although the Nottingham women were not stratified by age the pre-menopausal gorup had a less favourable prognosis.

Two independent studies have shown, therefore, that the regression analysis of multiple variables identifies only four significant prognostic factors - all of which can be easily measured. The fact that these results are reproducible in two different populations may be important if this information is to be used in the management of breast cancer. If the model is reproducible within all similar populations then it could be applied in the stratification of patients for trials of treatment.

Perhaps the most surprising common finding of these two studies was the failure of E.R. status to contribute to the overall prognosis of a patient. Steroid receptor status is now firmly established as an accurate predictor of hormone-sensitivity in advanced breast cancer. More recently some workers have claimed that E.R. assay has become mandatory for the design and interpretation of clinical trials of therapy in primary operable breast cancer. In the light of the Liverpool and Nottingham studies it appears the routine measurement steroid receptor status may be uneccesary.

The purpose of the randomised clinical trial is to ensure that both "arms" of the trial are composed of equal proportions of good and bad prognostic factors (Dudley, 1983). It is becoming increasingly difficult to conduct such trials as society, reasonably or unreasonably, demands that patients give fully informed consent before entering a trial (Schafer, 1982). Many patients are unable or unwilling to accept randomisation. Under these circumstances a prognostic index, as outlined above, could be used to predict the prognosis of an individual patient. The effects of different forms of treatment could be assessed by observing the fate of groups of patients with an identical predicted prognosis.

Another application of a prognostic index would be in designing a follow up strategy for patients following primary treatment of breast

cancer. It may be unnecessary to follow up patients with a low probability of developing recurrence; conversely patients with a high probability of recurrence may be followed up more intensely, thereby utilising resources more efficiently.

Summary and Conclusions

A form of multivariate statistical analysis was applied to the data from a survival study of 670 women with primary breast cancer treated by mastectomy. Only four significant prognostic variables have been identified - tumour size, axillary lymph node status, histological grade and age group; all of which may be easily measured. It has been possible to correctly predict the fate of more than 70% of the women in the study population with regard to death or recurrence of breast cancer.

This type of prognostic information could be applied to the design and analysis of trials of treatment and may also enable clinicians to follow up patients more rationally.

Introduction to Part II

With a knowledge of several simple clinical and pathological characteristics it has been possible to identify individual women and groups of women who have a poor prognosis following mastectomy for primary breast cancer. Such discrimination will only be relevant if it is possible to influence the prognosis by appropriate treatment. At present, the prognosis does not seem to be appreciably modified by giving adjuvant systemic drug therapy. However, there may be significant benefits from this form of treatment which are obscured by giving adjuvant therapy empirically to all women in "high risk" groups, irrespective of the individual tumour drug sensitivity. Consequently some women will endure the unpleasant side effects of drugs unnecessarily whilst others will be denied effective treatment.

Prognosis therefore, may be improved if systemic treatment is given knowing each individual tumour's sensitivity to a variety of agents. If drug sensitivity could be identified by an in-vitro method this information could then be applied both in advanced cancer, and in selecting effective adjuvant treatment.

Previous experience with in vitro drug assays will be reviewed. The evolution of a drug sensitivity assay using short term organ culture is described. The application of this assay to a study of primary and advanced breast tumours will be presented and discussed.

PART II

IN VITRO DRUG SENSITIVITY ASSAY

Introduction

Although advanced breast cancer can rarely be cured (Fishcer, 1982) useful palliation may be achieved with endocrine manipulation, cytotoxic chemotherapy or radiotherapy used either individually or in combination. Endocrine manipulation usually takes the form of ovarian ablation in pre-menopausal women and the synthetic anti-oestrogen Tamoxifen in post-menopausal women. Overall response rates of the order of 30 percent can be expected from such treatment (McGuire, 1974). Oestrogen receptor (E.R.) status is acknowledged to be a sensitive predictor of response to hormone therapy in advanced breast cancer. Nevertheless, 40 percent of ER+ve tumours prove to be unresponsive and 5-10 percent of ER-ve tumours will respond to hormone therapy.

Response rates of up to 70 percent have been reported in women with advanced breast cancer treated with combination cytotoxic chemotherapy. Such responses are often short-lived, and at the expense of appreciable morbidity, which may even result in reduced survival (Cavalli, 1983). Lippmann (1978) has suggested that ER-ve tumours may be more responsive to cytotoxic chemotherapy but other workers have been unable to confirm this (Kiang, 1978). Sulkes (1979) reported a significant association between pre-treatment Thymidine Labelling Index and response to 5-fluouracil though this association is refuted by other studies (Wolberg, 1971).

At present, therefore, drug therapy is given to some extent, empirically. Drugs are selected on the basis of past experience of overall response rates to individual drugs or combination of drugs. Clearly this approach is inefficient, as it is known that many phenotypically identical tumours vary greatly in their response to treatment (Henderson, 1980).

CHAPTER I

History and Development of Drug Sensitivity Assays

In principle all in-vitro assays of drug sensitivity involve excising a tumour biopsy and maintaining the cells in tissue culture. The component cells are then exposed, either as tissue fragments or as cell suspensions, to a range of anti-cancer drugs. In vitro response is usually assessed by evaluating a facet of tumour metabolism or growth. Such crude approaches however, take no account of differences in host conversion and detoxification of the drug, tissue specific drug accumulation, and immuno-suppressive activity.

In 1957, Wright reported a clear association between clinical response to cytotoxic agents, and in-vitro response as judged by alterations in tumour biochemistry. Although several groups of workers, using different methods, have reported similar associations, others have reported negative results and alluded to the difficulties of technique and interpretations of such assays (Beeby, 1975). No single type of assay has been universally accepted, and the value of in-vitro sensitivity assays is still doubted by many clinicians (Lancet Editorial, 1982; Selby, 1983).

The techniques used to maintain tumour tissue in-vivo may be broadly classified into those using either cell culture or organ culture.

Cell culture involves disaggregating a tumour into its component cells and maintaining these cells in medium, either as a suspension or a single layer. Cell culture has the advantage of eliminating connective tissue elements. However such an in-vitro model is artificial and far removed from the in-vivo situation where tumour cells comprise only a small proportion of total tumour volume.

For organ culture the tumour is cut into fragments but not disaggregated. The structural integrity of the tumour is therefore intact. However fibroblasts may inhibit in-vivo growth and prevent the exposure of tumour cells to the drugs under evaluation.

The applications of these two types of tissue culture technique are described below.

(i) Cell Culture Techniques

Most tumour cells ultimately die or terminally differentiate. The tumour cells of importance are those capable of sustained replication and these have been defined as the stem or clonogenic cells. Stem cells constitute no more than 1% of the total cell population of many solid tumours. For this reason experiments to assess the behaviour of an entire tumour cell population may bear little or no relation to the behaviour of the subpopulation of clonogenic cells.

In 1955, Puck described an in-vitro colony assay using single cell suspensions of mammalian tumours dispersed in a semi-solid medium. Cells capable of sustained replication produced small colonies containing 50 or more progeny of the original cell. Clonogenic assays have since been widely used in the radiobiology and experimental chemotherapy of animal tumours. The results of in vitro assays in animal models have been shown to predict gross tumour response to chemotherapy (Elkind, 1977).

Salmon and Hamburger (1977) in Arizona have developed a clonogenic assay in which disaggregated tumour cells are pre-treated with chemotherapeutic agents and then cloned in agar. They have reported that the in-vitro inhibition of cell growth after treatment with a specific drug correlates with the clinical response of the same

patient treated with the same drug (Salmon, 1978). However many of these, and other studies have been performed on small groups of patients with diverse tumour types (Mann, 1982).

A pre-requisite of the clonogenic assay is the production of a single cell suspension with uncompromised cell viability. Cell suspensions can be produced with relative ease from leukaemias and myelomas, and even highly cellular solid tumours such as lymphomas and melanomas. Conversely many scirrhous breast carcinomas are extremely difficult to disaggregate due to the small number of tumour cells dispersed through a predominantly fibrous stroma.

The plating efficiency of a cell suspension is the total number of colonies expressed as a proportion of the total viable cell count. Plating efficiency is often less than 1 percent and although Courteney (1978) has described a method which provided qualitative and quantitative enhancement of colony formation, this has yet to be tested on human tumours.

A further theoretical problem is that of cytokinetic heterogeneity within individual tumours. The proportion of tumour cells in the mitotic phase - the growth fraction - is usually low. Thus within a given tumour, cells may be in the log or plateau phase and it is not known if these two kinetically different cell populations have the same plating efficiency.

Interpretation of the clonogenic assay requires the skill of an expert histopathologist and it may take several weeks before the in vitro result is available. Such a delay may be important if drug therapy is to be given on the basis of the in vitro result.

Clearly the performance of clonogenic assays requires facilities and expertise which would probably only be available in a specialist tissue culture laboratory. In addition, the assay may only be technically feasible on a small proportion of breast tumours.

Some workers have suggested that clonogenic assays could now be used routinely in predicting response to cancer chemotherapy, and for the in vitro testing of new anticancer drugs. Many feel that such optimism is both unfounded and premature (Von Hoff, 1983).

(ii) Organ culture techniques

Human breast cancer was first successfully maintained in short term organ culture by Cameron and Chambers in 1937. Longterm organ culture has proved more difficult due to the inhibition of epithelial growth by fibroblasts (Heuson, 1975).

In vitro response may be deduced by observing changes in cell morphology or by measuring the products of metabolism or suppression of isotope uptake by tumour protein. In 1973, Flax described a short term in vitro assay of hormone sensitivity in breast cancer. Response was assessed by observing histological changes and analysing pentose shunt activity histochemically. A correlation was found between in-vitro response and clinical response to specific hormone therapy. These findings were reproduced by Hobbs in 1974, but Masters (1976) could find no such association and concluded that the observed changes in histochemical activity merely reflected tumour heterogeneity.

More recently Lippman (1977) has used an in vitro assay to demonstrate consistent oestrogen stimulation and anti-oestrogen inhibition of dimethyl benzanthracene (DMBA) induced rat mammary carcinomas. This assay, which relates in-vitro response to the rate of RNA synthesis, has not been applied to the study of human tumours.

Cytotoxic agents exert their effects chiefly on multiplying cells - their biochemical target being nucleic acid synthesis. The mode of action of hormones and anti-hormones is not yet well defined, but it probably results in altered nuclear R.N.A. Polymerase B activity - which in turn influences the rate of synthesis of messenger R.N.A. precursors (Nicholson, 1976).

The in vitro effects of drugs on nucleic acid synthesis can be expressed quantitatively by measuring the incorporation of isotope labelled nucleic acid precursors (Masters, 1980; Poulson, 1982) or by analysing nuclear D.N.A. content (Engelholm, 1983). This latter

method has not been studied in human tumours and requires disaggregation and the use of an expensive Flow Cytometer.

Detection of labelled nucleic acid precursors can be made using auto-radiography (Wolberg, 1971) or by scintillation counting (Volm, 1979). Scintillation counting has been shown to be both quicker and more accurate (Tanigawa, 1982).

Conclusions

No single type of in-vitro drug sensitivity assay has been shown to accurately reflect the in-vivo response of a tumour to chemotherapeutic agents. Most recent literature has described the use of cell culture techniques. The results of these clonogenic assays have been encouraging when used on artificially-induced animal tumours and human cell lines. However, naturally occurring human solid tumours are usually heterogenous and most workers have reported difficulty in consistently producing isolated cell suspensions. Evaluation of in-vitro response involves expert histological examination for prolonged periods during which time the disaggregated cells are very susceptible to environmental changes.

The interpretation of in-vitro changes is crucial for any type of drug assay, but the preparation of organ cultures of solid tumours is technically easier and feasible in almost all cases. This may be of practical importance if in-vitro assays are to be tested clincially.

Although there has been little published on the use of organ cultures, in view of these practical considerations this type of assay was adopted for this study. The evolution of this drug sensitivity assay will be described in detail in Chapter 2.

CHAPTER 2

General Aims of the Study

The aim of the study was to devise and test a short term in-vitro drug assay using organ cultures of human breast cancer.

The method used involves incubating fragments of tumour with an isotope labelled nucleic acid precursor in a medium containing a fixed concentration of a drug. The uptake of this precursor by the tumour is then measured after homogenising the tumour fragments following a fixed period of incubation. The in-vitro effect of the drug is deduced by comparing the rates of isotope uptake by tumours incubated with and without the drug under evaluation.

Although the concept of this method appears simple there are many potential problems which must be considered. For example, the composition of the tissue culture medium, the drug concentrations and the isotopes may all influence the production and interpretation of in-vitro results.

These factors will be discussed individually in order to illustrate how the final method was developed. Where possible, preliminary experiments were performed to answer specific questions.

The results and interpretation of these initial studies will be presented in turn. This is then followed by a detailed description of the method finally used.

Evolution of the method

(i) The Medium

Components such as enzymes, cofactors and serum are added to tissue culture media in unphysiological doses. The selection of such additives is often arbitrary and there is little evidence that the complexity of a medium relates to its ability to carry out its desired function (Waymouth, 1974).

Serum has been thought to be essential for the growth and survival of mammalian cells in culture and to have a protective action on the tissue by acting as a physiological buffer and influencing adhesion to the growth surface (Mitchel, 1961). However, serum contains many hormones which may influence the in-vitro effects of oestrogens and anti-oestrogens which are to be studied.

Duncan (1981) has shown that explants of human breast cancer can be maintained satisfactorily in serum free tissue culture medium for up to 3 days without histological deterioration and similar observations have been made when studying the growth of MCF7 cells in culture (Barnes, 1979).

In view of these factors a preliminary study was performed to see if the use of serum-enriched tissue culture medium enhanced the uptake of isotope by tumour fragments maintained in organ culture.

Biopsy specimens of nine fresh breast carcinomas were incubated for 3 hours in medium with or without the addition of foetal calf serum (10percent). In all other respects the experimental method used was that described in detail on pp's 152 to 160.

The uptake of tritiated uridine was found to be significantly reduced in the presence of foetal calf serum ($p<0.01$ Wilcoxon paired sample test). The detailed results are presented in Appendix II.3(i).

This apparent suppression of isotope uptake is probably due to the non-specific binding of isotope to steroids in the serum. For this reason all subsequent experiments were performed using serum-free medium.

(ii) Drugs

A major problem inherent to all in-vitro drug assay systems concerns the concentration of drug to be used in the cultures. There is no readily applicable method of relating drug concentrations in an artificial in-vitro system to those found in-vivo. In 1962 Wright used drug levels which were known to inhibit growth of a cell culture line. Attempts have been made to relate in-vitro drug concentrations to clinically effective dosages - but such calculations assume a uniform plasma distribution and mode of action (Bickis, 1966). Most hormone and cytotoxic agents are complicated and have unique pharmacokinetics which are not clearly understood. Clinically drug absorption and distribution are influenced by dosage schedules, route of administration, extent of disease, and the nutritional and metabolic state of the patient.

Gilette (1968) produced a mathematical formula computing the amount of drug to be added to the medium for in-vitro experiments. However, this calculation requires measurement of drug concentration in extracellular fluid and in cells under conditions of equilibrium and is therefore not readily applicable to the study of human tumours.

The drugs studied using the in-vitro assay were Tamoxifen and Adriamycin.

Tamoxifen

This synthetic anti-oestrogen, the trans isomer of 1-(P-B dimethyl-aminoethyoxyphenyl) 1-2 diphenylbut-1-ene is widely used in the management of advanced breast cancer. Tamoxifen is most effective against ER+ve tumours and is therefore particularly useful in postmenopausal women, and has an acceptably low incidence of toxic side effects (Patterson, 1978).

The precise mode of action of Tamoxifen is not yet clear. It is thought to act primarily by blocking the association of oestrogens with binding proteins (Nicholson, 1980). At high concentrations Tamoxifen can prevent the binding of 3H oestradiol to oestrogen receptors from both human and rat mammary tumours. This simple concept however fails to explain the inhibitory action of Tamoxifen as opposed to the stimulatory effects of oestradiol which has many similar characteristics.

Tamoxifen has been shown to inhibit the activity of RNA polymerase and invoke a transition delay in the G1 phase of cell cycle (Osborne, 1983). Although the in-vitro effects of Tamoxifen have mostly been studied using mammary cell carcinoma lines it seems that antioestrogens may act by failing to stimulate the synthesis of growth regulatory products at a rate sufficient to support cell growth.

Relating the actions of Tamoxifen to serum and tissue concentrations is difficult. The principle metabolite of Tamoxifen found in serum is a monohydroxylated derivative - thought to be formed in the liver and undergo entero-hepatic recirculation. This metabolite occurs in the plasma of treated women at a lower concentration than that of the parent Tamoxifen. Paradoxically a further metabolite N-desmethyl Tamoxifen has been found in serum and tissue in concentrations in excess of that of Tamoxifen (Daniel, 1981). Although Tamoxifen accumulates in tissues during longterm treatment,

Adam (1981) found no correlation between either plasma or tissue concentrations of Tamoxifen and its metabolites, and subsequent clinical response to treatment.

Using a mammary carcinoma cell line, Sutherland (1981) clearly demonstrated a dose dependent growth inhibition and cytotoxicity, maximal at an in-vitro concentration of 10^{-6} molar.

Adriamycin

Danorubicin was first isolated from streptomyces caeruleorubidus in 1963 and Doxorubicin (Adriamycin) was subsequently recovered from a mutant strain. Adriamycin has been used in a wide range of human malignancies and is probably the single, most effective cytotoxic agent used in the management of advanced breast cancer (Carter, 1976). The cytotoxic action arises from binding with double stranded DNA which inhibits polymerase and nuclease activities (Momparler, 1976). Adriamycin is short acting and following bolus intravenous administration the plasma disappearance curve is triphasic with half lives of 12 mins, 3 hours, and 30 hours (Benjamin, 1977).

In-vitro, Adriamycin has been shown to inhibit incorporation of nucleic acid precursors into Hela cells (Kim, 1972). Krishnan (1976) has studied the in-vitro effects of Adriamycin on cell cycle kinetics of cultured human lymphoblasts and demonstrated a dose-related response with maximal cell death at an in-vitro drug concentration of 10 µg/ml. Volm (1979) found a clear correlation between the in-vitro inhibitory effects of Adriamycin at 10 µg/ml and clinical response to therapy in breast, lung and ovarian carcinomas.

The concentration of drug used in these experiments may be critical. Both of the agents studied have complicated pharmacokinetics. Although their in-vivo actions have been thoroughly investigated, it is not known if their in-vitro activity is in any way comparable. For these reasons the drug concentrations have been selected on the basis of previously reported in-vitro studies of breast cancer using the same drugs. The concentrations used in all assays were Tamoxifen 10^{-6}M and Adriamycin 10 µg/ml.

(iii) Isotopes

The in-vitro effects of drugs may be deduced by measuring changes in the uptake of nucleic acid precursors by tumour tissue. Thymidine is incorporated into DNA and uridine into RNA. The utpake can conveniently be measured by using isotope labelled precursors. Tritiated isotopes are used as they are readily available commercially and relatively inexpensive. The isotopes are safe to use being stable in aqueous solution and having a long half-life.

Although Tamoxifen and Adriamycin have different modes of action they both influence nucleic acid synthesis directly or indirectly. Adriamycin has been shown to exert its maximal in-vitro effect on RNA synthesis (Kaufmann, 1980) and Tamoxifen also predominantly effects RNA synthesis (Nicholson, 1980).

It would therefore seem logical to assess in-vitro response by measuring the uptake of tritiated uridine rather than thymidine. However in view of the complex and poorly-defined actions of the drugs under evaluation a preliminary experiment was performed to compare the uptake of tritiated uridine and thymidine under control conditions. Paired samples of 5 tumours were incubated in medium containing 25 μCi ^{3}H uridine or thymidine respectively. The experimental details are otherwise as described on pp's 152 to 160.

The uptake of isotope by the tumour fragments was significantly greater when uridine was used ($p<0.01$ Wilcoxon paired sample test). The results are presented in Appendix II.3 (iii).

In view of these findings all subsequent experiments were performed using tritiated uridine.

(iv) Oxygen

Both in-vivo and in-vitro tumour metabolism is influenced by tissue oxygenation. This may be of particular importance in organ culture where the delivery of a drug or isotope to the tumour cells may be impaired. In-vitro thymidine labelling studies have shown that the penetration of isotope is often confined to the surface of tumour fragments. Fabrikant (1969) found that the penetration of thymidine was enhanced by incubating the tumours under hyperbaric conditions.

However, it is known that, paradoxically, high concentrations of oxygen may exert a toxic effect on living tissues. In 1972, Richter demonstrated that in vitro growth was suppressed when cells were cultured under hyperbaric conditions.

The influence of oxygen concentration on short term organ culture was assessed by performing control incubations under hyperbaric and normobaric conditions. Nine tumours were studied in this way and hyperbaric conditions were produced by injecting 10mls of a 95% O_2/5% CO_2 mixture via the vacutainer bung prior to each incubation. For each tumour a parallel incubation was performed at normal atmospheric pressure. The other experimental details are as described on pp's 152 to 160 and the results are presented in Appendix II 3 (iv)

The uptake of tritiated uridine was significantly greater under normobaric conditions ($p<0.05$ Wilcoxon paired sample test) and subsequent incubations were therefore performed at normal atmospheric pressure.

(v) Tumour heterogeneity

Tumour morphology has been shown to influence the penetration of isotopes using thymidine labelling techniques. Structural heterogeneity may also explain the differences in levels of steroid receptor proteins, and discrepancies in histological grading within tumours.

In an attempt to identify any possible sampling bias the uptake of tritiated uridine by tissue from the centre and periphery of tumours were compared.

In five consecutive tumours, samples were taken from the periphery and the centre of the tumour before preparing the tissue fragments as described on pp 152. These paired samples were then incubated under identical conditions and the uptake of tritiated uridine was measured for each sample.

There was no significant difference in the uptake of isotope by the centre or periphery of the tumour ($p<1>0.5$ Wilcoxon paired sample test). The detailed results are presented in Appendix II.3 (v).

Although this small study failed to demonstrate a significant sampling bias, all tissue fragments were randomised prior to incubation.

(vi) Tumour ultrastructure

The use of organ culture as an in-vitro model assumes that the metabolic activity of excised tissue is maintained throughout the incubation. In a recent study of breast tissue maintained in organ culture for 3 days there was clear histological evidence of areas of cell death which in some cases resulted in lysis of almost all cells (Duncan, 1981). The degree of necrosis appeared to be proportional to the duration of incubation and this would mitigate in favour of short term organ culture, incubating tissue for only a few hours.

The difficulty of relating the in-vitro activity of drugs to in-vivo response has been discussed previously. It is possible that drugs may act on organ cultures by producing necrosis as a surface effect rather than by suppressing nucleic acid synthesis.

In order to identify any gross changes in cellular architecture electron micrographs were prepared from tumours tested using the in-vitro drug assay. Examples of a tumour incubated under control conditions (Fig. II.1) and with Tamoxifen (Fig. II.2) and Adriamycin (Fig. II.3) are illustrated.

The specimen incubated under control conditions shows preservation of normal cell architecture. Incubation with Tamoxifen resulted in slight oedema and organelle disruption. Similar changes were observed following incubation with Adriamycin, but in addition drug particles were clearly demonstrated. It would appear, therefore, that short term incubation results in only minor changes in tumour ultrastructure.

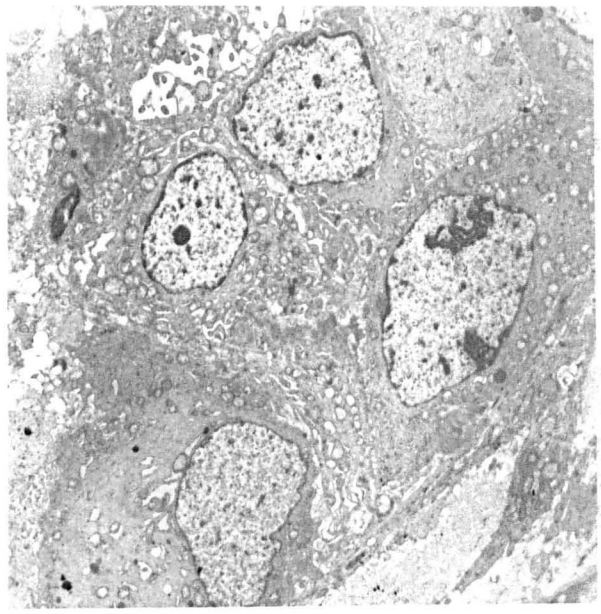


Fig. II.1
Electron micrograph x7K
Control incubation

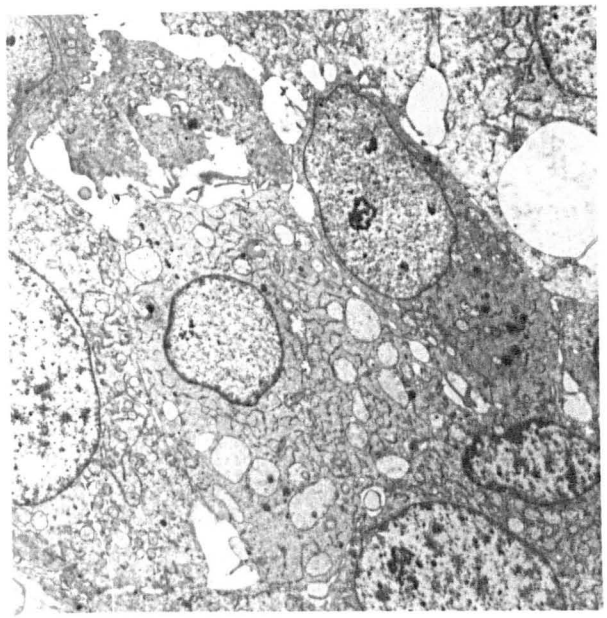


Fig. II.2
Tamoxifen incubation

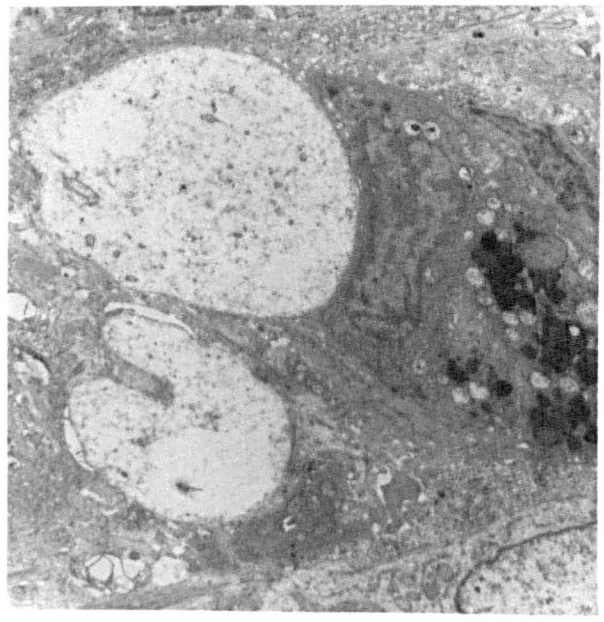


Fig. II.3
Adriamycin incubation

(vii) In-vitro response

When tissue is exposed to the action of a cytotoxic drug in-vitro, the response is not all or none. If the drug is effective it depresses metabolism to some degree, and difficulty then arises regarding the degree of metabolic inhibition that correlates with inhibition of tumour growth. This question has been studied mainly in animal model systems and to a lesser extent in human advanced breast cancer.

Dickson (1976) studied the in-vitro and in-vivo effects of cytotoxic agents on a rat sarcoma and found that the minimum curative dose of drugs was associated with as little as 25-30% inhibition of tumour glycolysis in culture and failure of the tumour to take on transplantation. Black and Speer (1954) found a positive correlation between the in-vitro effects of cytotoxic agents and their clinical efficacy when the drugs caused more than 30% inhibition of dehydrogenase activity after 1 hour incubation. Bickis (1966) examined the effects of drugs on respiration, glycolysis and precursor uptake and found that at least 50% suppression of isotope uptake into RNA, DNA or protein was necessary for reasonable expectation of a favourable clinical response. Recent studies of the in-vitro effects of oestradiol and adriamycin on RNA biosynthesis in breast cancer have suggested that as little as 15% suppression of uridine uptake is significant (Schlag, 1980).

In the light of these results it was decided that a 25% suppression of uridine uptake would be taken to indicate an in-vitro inhibition - although this figure is arbitrary and can only be considered meaningful when correlated with clinical response.

The Method

The method used in all drug assays is described in detail below and summarised diagramatically in fig. II.4 and illustrated in figs. II.5,6,7,8.

(a) A block of fresh breast tumour was sectioned into 2mm cubes using a sterile scalpel blade on a Petri dish. Cubes were randomised and approximately 10 cubes were placed in vacutainer tubes containing 5mls RPMI 1640 tissue culture medium. Tubes containing medium and tumour alone acted as controls, and in the remaining tubes the medium contained the drugs under evaluation in the following concentrations:-

Tamoxifen 10^{-6}M

Adriamycin 10 µg/ml

A minimum of 3 control and 3 drug treated tubes was used in each assay.

(b) Labelled vacutainer tubes were sealed with rubber bungs and secured in perspex clamps. 25 µCi tritiated uridine (vol. 0.25mls) was injected via the bung of each tube and all tubes were then incubated for 3 hours @ 37^{o}C in a shaking water bath.

(c) Following the incubation the contents of each tube were poured over a stainless steel grid and the tumour cubes were washed with 10mls phosphate buffered saline solution (P.B.S.). The cubes were then transferred to a universal container and homogenised for 1 minute in 2mls P.B.S. using a Polytron tissue homogeniser. A 400 µl aliquot of the homogenate was then decanted for a Lowry protein assay and the protein in the residual homogenate was precipitated with an equivalent volume (1.6ml) of 10% Trichlor acetic acid and stored at 4^{o}C for 2 hours to allow for stabilisation.

(d) This precipitate was reduced to a pellet by centrifugation @ 3000 r.p.m. for 10 mins. This pellet was washed and resuspended 3 times and on each occasion the supernatant was filtered and the residue retained. The protein pellet and filtered residue were dried and passed through a scintillation counter, having been placed in a glass vial containing 5mls Toluene liquid scintillation fluid.

(e) The scintillation count per mg tumour protein was then calculated for each tube and the mean values of the drug treated tubes compared with controls. The uptake of tritiated uridine was expressed as a precentage of the controls and more than 25% suppression of uridine uptake was taken to indicate in-vitro inhibition.

Fig. II.4 Drug assay - method

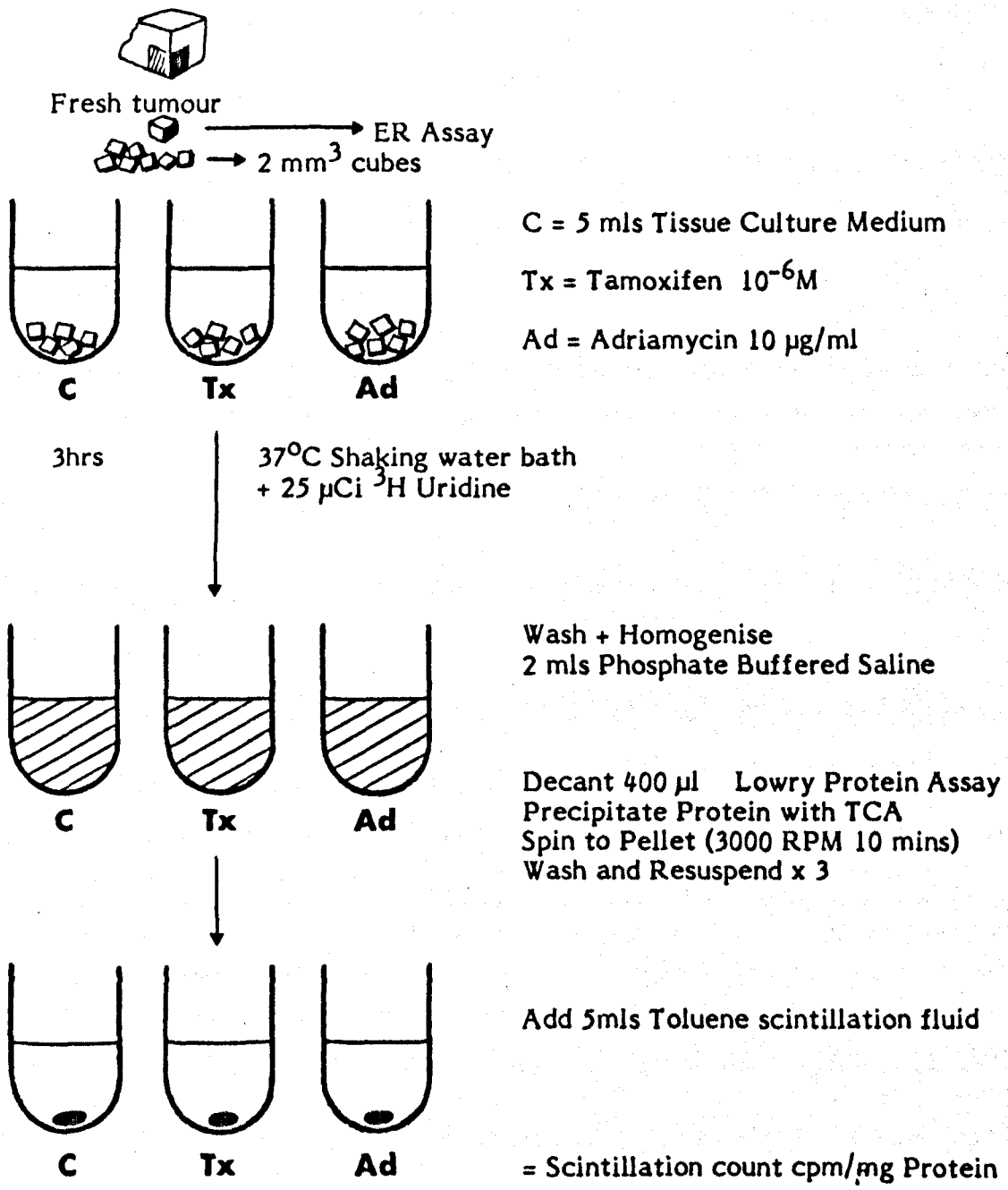
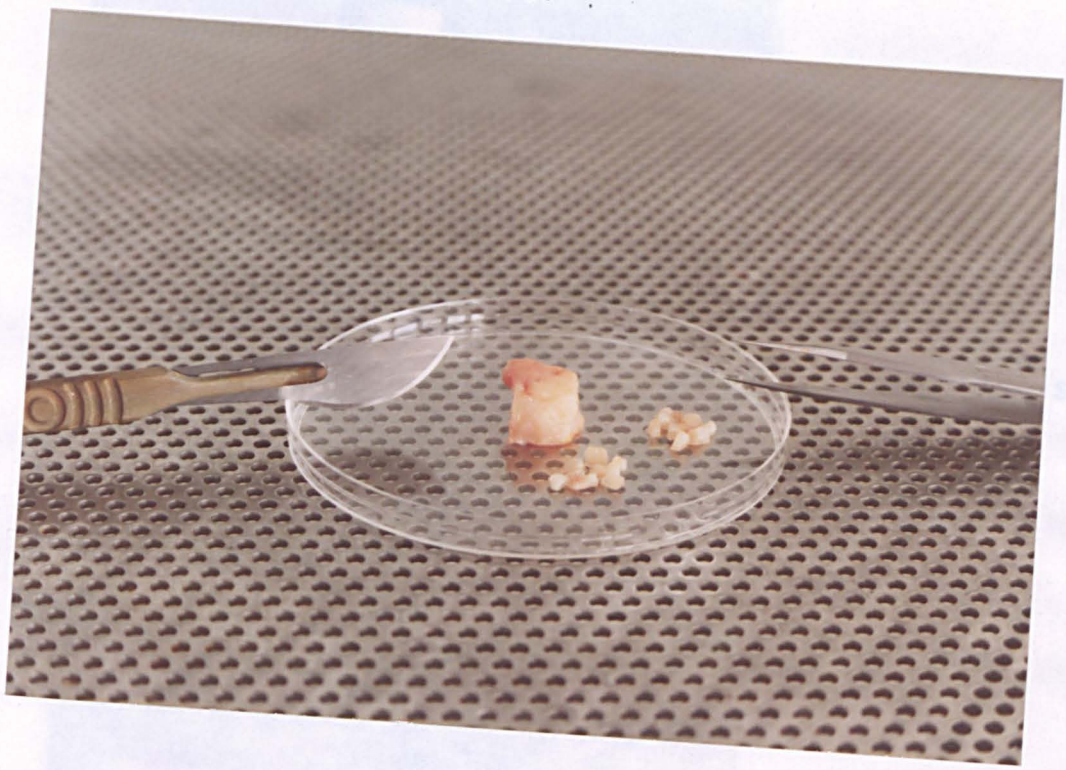


Fig. II.5 Tumour preparation



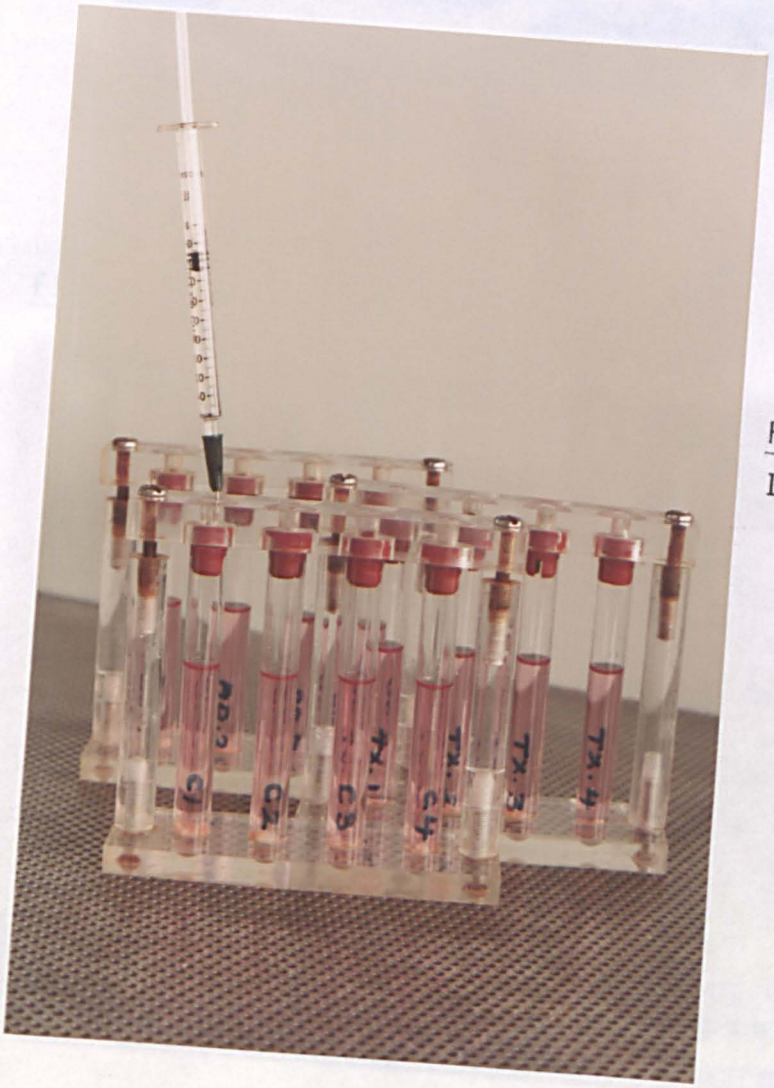
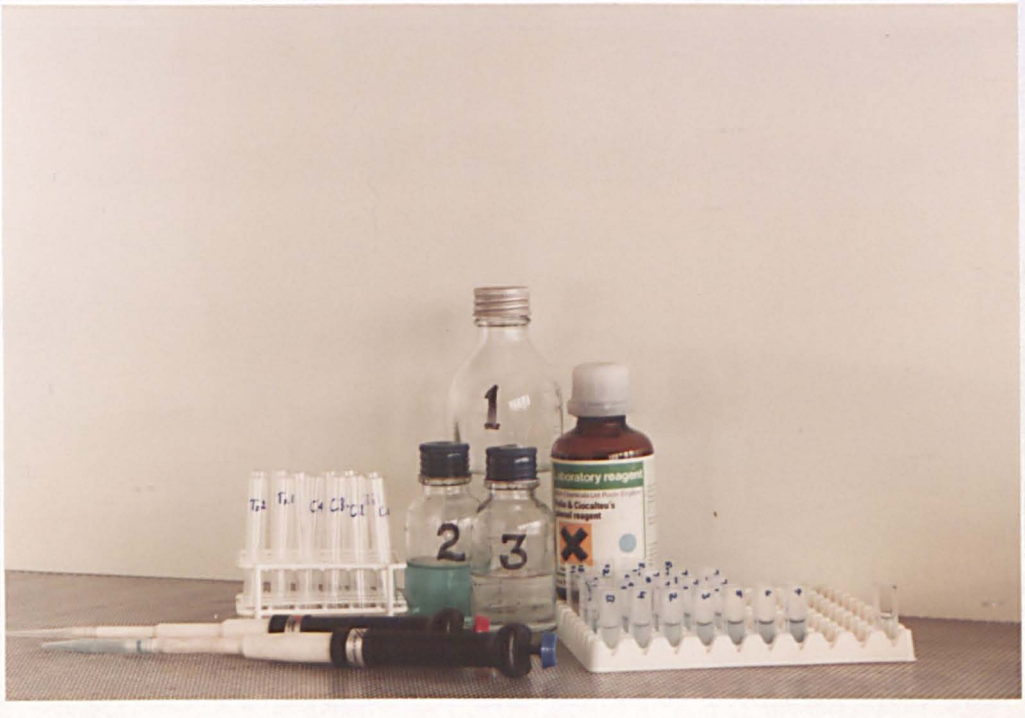


Fig. II.6
Incubation

Fig. II.7
Polytron Homogeniser

Fig. II.8 Protein Assay



A worked example:

The calculations for the drug assay of a single tumour (No. 1452) are presented below. The details of the Lowry protein assay and the equipment and reagents used appear in Appendix II.1 and 2.

No. 1452: From this tumour three control samples were prepared and compared with three samples incubated with Tamoxifen. Having followed steps (a) to (d) the total scintillation count was derived by adding the count of the protein pellet to that of the filtered residue for each sample.

Table II.1

		Counts Per Minute (CPM)				
		Protein Pellet	+	Filtered Residue	=	Total
Control	C_1	1533		1983		3521
	C_2	1489		1488		2977
	C_3	1542		1834		3376
Tamoxifen	T_1	1661		693		2354
	T_2	1184		1039		2223
	T_3	1915		1476		3391

For each sample the total scintillation count is corrected by dividing by the protein content. This is measured using the Lowry protein assay (Lowry, 1951) (Appendix II.2).

For each tumour a standard curve is first plotted using serial dilutions of a standard protein.

Table II.2

Fig. II.9

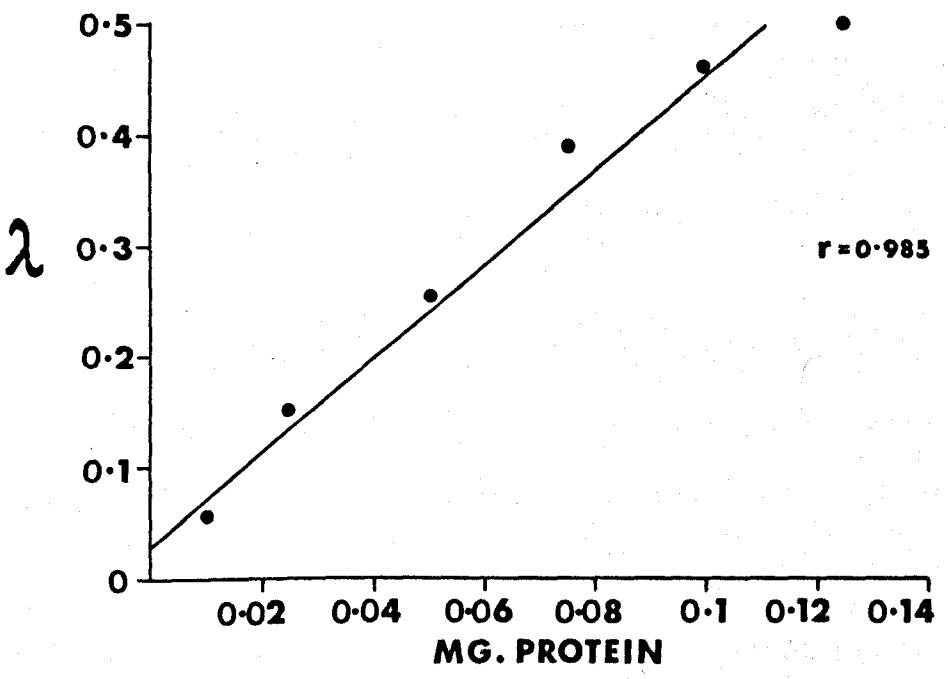
Mg Protein	Wavelength λ
0	0
0.01	0.055
0.025	0.150
0.05	0.255
0.075	0.390
0.1	0.460
0.125	0.500

The wavelength of each individual sample is measured by adding a 50 UL aliquot of homogenate to the indicator solution. From the standard curve the protein content is calculated. This figure is multiplied by 32 to produce the protein content of 1.6mls (being the volume of homogenate in which the protein was precipitated). Having divided the total scintillation count by this figure the corrected scintillation count is expressed as a mean $\pm$ 1 standard deviation.

Table II.3

Sample	λ	mgp	x32	cpm	cpm/mg	cpm/mg $\pm$ 1SD
C_1	0.445	0.090	2.88	3521	1223	
C_2	0.500	0.101	3.23	2977	921	1039 $\pm$ 160
C_3	0.535	0.108	3.46	3376	975	
Tx_1	0.490	0.099	3.17	2354	742	
Tx_2	0.470	0.095	3.04	2223	731	777 $\pm$ 71.0
Tx_3	0.610	0.123	3.94	3391	860	

Fig II 9
Tumour Nº1452



Expressing the uptake of tritiated uridine by the Tamoxifen incubated samples as a percentage of the uptake by the control samples (74.7%) this tumour would be judged to have been inhibited in-vitro by Tamoxifen.

CHAPTER 3

Aims of the Study

The in-vitro drug sensitivity assay was tested on a series of primary and advanced breast carcinomas.

The aims of this study were fourfold.

1. To determine the overall rates of in-vitro inhibition by Tamoxifen and Adriamycin.
2. To relate in-vitro inhibition to E.R. status.
3. To relate in-vitro inhibition by Tamoxifen to the clinical response of women with advanced breast cancer.
4. To relate in-vitro inhibition by Adriamycin to the clinical response of women with advanced breast cancer.

Patients

(i) General studies (1 and 2)

The patients studied presented to four hospitals within the Mersey Region from 24.6.81 to 15.8.82. A representative specimen of tumour was transported, on ice, to the Department of Surgery within one hour of operation.

Prior to the performance of a drug assay a 100g portion of the tumour was excised and stored in liquid nitrogen for estrogen receptor assay. These assays were carried out on batches of tumours at the Tenovus Institute, Cardiff, using the Dextran Coated Charcoal Technique (see Part I, Chapter 2, Appendix I.2).

Clinical information was later retrieved from the hospital case notes and recorded on a standardised proforma.

(ii) Clinical Studies (3 and 4)

A specific clinical study was performed to relate in-vitro inhibition to clinical response to Tamoxifen in advanced breast cancer (study 3). The patients in this part of the study were all treated by the Professorial Surgical Unit at the Royal Liverpool and Broadgreen Hospitals. All patients had either Stage III or Stage IV primary tumours or major local/regional recurrence of breast cancer.

Following admission to hospital all surface lesions were accurately measured in two planes and metastatic disease was documented by the appropriate radiological investigation. Treatment of local disease was initially by incisional biopsy followed by Tamoxifen 20mg b.d. orally. From the biopsy specimen the diagnosis was confirmed histologically, a drug-assay performed and estrogen receptor status measured.

The clinical response of the tumour to Tamoxifen was assessed by careful examination in the outpatient department at monthly intervals. At 3 months following operation, clinical response was classified using the U.I.C.C. criteria (Appendix II.4(a)). For the purpose of this study response includes both complete and partial response.

Clinical response, estrogen receptor status and in-vitro findings were not correlated until after the completion of the study.

A second clinical study (study 4) was performed on women with advanced disease who were treated with Adriamycin as part of a triple agent chemotherapy regime in monthly cycles:-

Vincristine 1mg	Bolus Intravenous
Adriamycin 80mg/metre surface area	Injection
Cyclophosphamide 200mg/m SA	Oral

Most patients were given this treatment following failure to respond to Tamoxifen and the details of all cases are presented in the Results Section. The drug assays were performed on biopsy specimens removed before cytotoxic therapy was given and the subsequent clinical response to Adriamycin was assessed using the U.I.C.C. criteria outlined previously.

Results (i) General Studies

(1) Overall inhibition rates

A total of 88 tumours was studied. Of these, 79 were incubated with Tamoxifen and 59 were also incubated with Adriamycin.

Table II.4

Total Assays	88
Tamoxifen Incubation	79
Adriamycin Incubation	59

The E.R. status of 54 of these tumours was also determined. Of these 31 (57%) were ER+ve and 23 (43%) were ER-ve. All 31 of the ER+ve tumours were incubated with Tamoxifen and 25 were also incubated with Adriamycin. Of the 23 ER-ve tumours all were incubated with Tamoxifen and 15 were in addition incubated with Adriamycin.

Table II.5 Drug assays in relation to ER status

		Incubation	
		Tamoxifen	Adriamycin
E.R. Status	+ve 31	31	25
	-ve 23	23	15
	Totals 54	54	40

When the total group of tumours was examined 28 of the 79 tumours incubated with Tamoxifen were inhibited in-vitro (35%) and 28 of the 59 tumours incubated with Adriamycin also showed inhibition (47%).

Table II.6 Rates of in-vitro inhibition

		Incubation	
		Tamoxifen	Adriamycin
In-vitro	Inhibition	28	28
	No Inhibition	51	31
	Total	79	59

The detailed results of all patients studied are presented in Appendix II.5.

(2) In-vitro inhibition in relation to E.R. status

Of the 31 ER+ve tumours incubated with Tamoxifen, 13 (42%) were inhibited. A similar proportion of the ER-ve tumours 9/23 (39%) was inhibited by Tamoxifen in-vitro. The difference in the inhibition rates of ER+ve and ER-ve tumours is not statistically significant (p=1.0 Fisher Exact Test)(Table II.7).

Table II.7 In vitro inhibition by Tamoxifen in relation to ER status

		ER Status	
		+ve	-ve
In Vitro	Inhibition	13	9
	No Inhibition	18	14
	Total	31	23

Adriamycin inhibited 8 of the 25 ER+ve tumours assayed (32%). Conversely of 15 ER-ve tumours assayed 9 (60%) were inhibited. Again, this difference is not statistically significant (p=0.160 Fisher Exact Test).

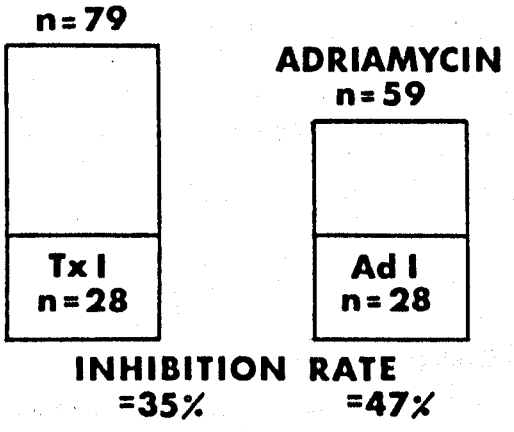
Table II.8 In-vitro inhibition by Adriamycin in relation to ER status

		ER status	
	Adriamycin	+ve	-ve
In-vitro	Inhibition	8	9
	No Inhibition	17	6
		25	15

The results of general studies (1 and 2) are summarised in Figs. II.10,11 and 12.

Study 1
OVERALL RATES OF IN-VITRO INHIBITION

Fig.10



Study 2
RELATIONSHIP of ER STATUS to IN-VITRO INHIBITION

Fig.11

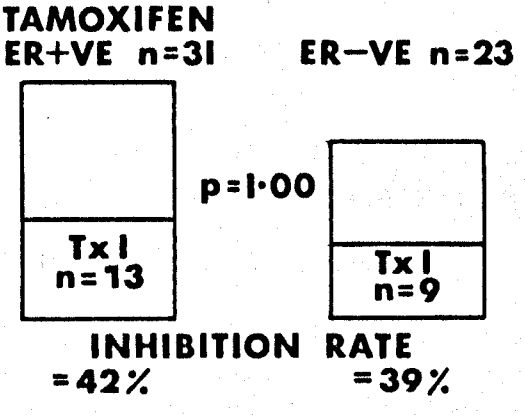
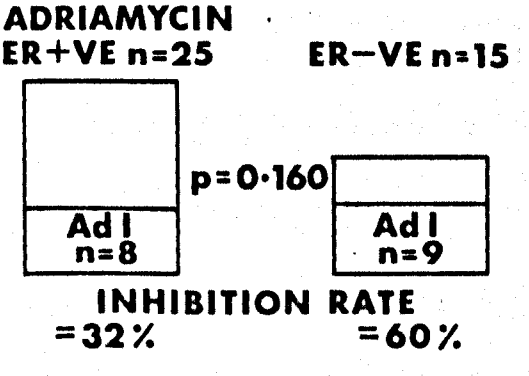


Fig.12



TxI = Tamoxifen Inhibition
AdI = Adriamycin Inhibition

(ii) Clinical Studies

(Study 3) Relationship of clinical response to in-vitro inhibition by Tamoxifen

A total of 27 women with advanced breast cancer was studied. The mean age of patients was 61 years with a range of 38 to 82 years. Only 2 of the patients studied were pre-menopausal. The mean follow up of the patients was from 2-27 months (mean 13 months) and there were 8 deaths during the period of study.

Looking at the 17 ER+ve tumours, 8 (47%) were inhibited in-vitro by Tamoxifen. Half of the 10 ER-ve tumours were inhibited by Tamoxifen.

Table II.9 In vitro inhibition in relation to ER status

		ER Status	
		+ve	-ve
Tamoxifen	Inhibition	8	5
	No Inhibition	9	5
	Total	17	10

The clinical details of all 27 patients are presented in Appendix II.5.

In-vitro inhibition and ER status were then examined in relation to clinical response to Tamoxifen. Of the 13 tumours responding clinically to Tamoxifen 9 were inhibited in-vitro. Conversely only 4 of the 14 tumours unresponsive clinically to Tamoxifen were inhibited in-vitro. The difference in response rates of inhibited and non-inhibited tumours failed to achieve statistical significance $p = 0.0824$ Fisher Exact Test (Table II.10).

Table II.10 In vitro inhibition in relation to clinical response

		Clinical	
		Response	No Response
In-vitro	Inhibition	9	4
	No Inhibition	4	10
	Total	13	14

As a predictor of response to Tamoxifen the in-vitro drug assay has a sensitivity of 69% and a specificity of 71%.

The clinical response of these 27 tumours were then considered in relation to ER status. Looking first at the 13 tumours which responded clinically to Tamoxifen, 9 of these (69%) were ER+ve. This was not significantly different from the proportion of ER+ve tumours 8/14 (57%) that did not respond to Tamoxifen (Table II.11).

Table II.11 Clinical response in relation to ER status

		Clinical Response	No Response
ER Status	+ve	9	8
	-ve	4	6
	Total	13	14

In this group study ER status and the in-vitro assay have an identical sensitivity (69%) in predicting response to Tamoxifen, though the specificity of ER status is lower at 43%.

These findings relating clinical response to in-vitro inhibition and ER status are summarised in Figs. II.13 and 14.

Looking specifically at the 8 ER+ve tumours that did not respond clinically to Tamoxifen only 2 of these tumours (25%) were inhibited in-vitro. Similarly of the 4 ER-ve tumours that did respond clinically to Tamoxifen only 1 of these tumours (25%) failed to show in-vitro inhibition.

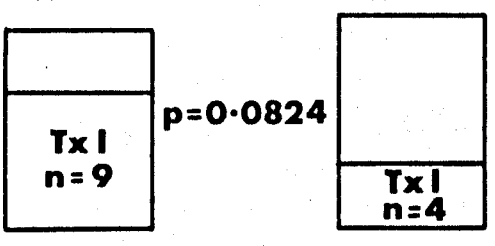
(4) Relationship of clinical response to in-vitro inhibition by Adriamycin

The clinical response of 7 women treated with Adriamycin were compared retrospectively with the in-vitro findings. Only 3 of these patients received Adriamycin as part of their initial management, the other 4 patients having first failed to respond to Tamoxifen. Only 2 of the 7 women were premenopausal. The patients were followed up for a mean of 19 months (range 5-36 months).

Study 3

RELATIONSHIP of CLINICAL RESPONSE to IN VITRO INHIBITION

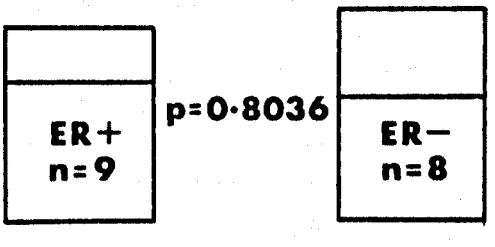
Fig 13



SENSITIVITY 69·2%
SPECIFICITY 71%
+ve PV 60%
−ve PV 67%

RELATIONSHIP of CLINICAL RESPONSE to ER STATUS

Fig 14



SENSITIVITY 69·2%
SPECIFICITY 43%
+ve PREDICTIVE VALUE 60%
−ve " " 67%

Of the 7 tumours, 4 (57%) responded clinically to Adriamycin, and 3 of these 4 responsive tumours were also inhibited by Adriamycin in-vitro (75%). None of the 3 tumours which failed to respond clinically to Adriamycin were inhibited in-vitro. This apparent difference in inhibition rates was not statistically significant as only small numbers of patients were involved ($p = 0.2296$ Fisher Exact Test).

Only 4 of the tumours were of known ER status, and all 4 were found to be ER+ve. Of these 4 only 1 was inhibited in-vitro by Adriamycin, and the same tumour also responded to Adriamycin.

The details of all 7 patients are presented in Appendix II.5.

Discussion

(i) The Method

This type of in-vitro drug assay is based on simple concepts. There are, however, many inherent difficulties both in the production and interpretation of results. There were practical problems associated with the use of breast carcinomas in organ culture. Scirrhous tumours were often difficult to homogenise, although the Polytron homogeniser (Appendix II.1) was much more effective than a simple teflon-tipped liquidiser used in initial experiments. Conversely fatty or mucoid tumours were easier to homogenise but the resultant homogenate was often viscid and impossible to aspirate accurately in micropipettes. The other technical aspects of the assay were relatively straightforward and it proved possible to perform the assay on almost all of the tumours studied.

The biological significance of this in-vitro model is uncertain. It would be naieve to anticipate that tumours maintained in organ culture proliferate significantly during a short 3 hour incubation. Nevertheless, changes in metabolism and cell cycle kinetics do occur during this time. These changes could be influenced by infection or necrosis of the tumour tissue but this was not evident in the preliminary electron micrographic studies.

The heterogeneity of breast tumours has been shown to influence the performance and interpretation of steroid receptor assays and histological grading. Tumour heterogeneity is no less a problem with the in-vitro drug assay. The intra- and inter-tumour variability of scintillation counts were considerable, although this variability

diminished as the number of control and drug-treated tubes increased. The number of tubes assayed was, in turn, limited by the size of the biopsy specimen. The sample of tumour was often very small after representative portions had been taken for histological evaluation and estrogen receptor assay.

(ii) The Results

1. The overall rate of in-vitro inhibition by Tamoxifen was 35%. This is similar to the response rates of women with advanced breast cancer given Tamoxifen empirically (Westerberg, 1978). The in-vitro inhibition rate with Adriamycin was 47%. Some workers have reported that clinical response rate to Adriamycin may be as high as 80% (Cooper, 1969); however, more detailed studies using objective criteria of response have found much lower clinical response rates of the order 30-50% (Carter, 1976).

2. Only 42% of ER+ve tumours were inhibited in-vitro by Tamoxifen. This is lower than the 60% of ER+ve tumours that would be expected to respond clinically to Tamoxifen (McGuire, 1978). Conversely the in-vitro inhibition rate of ER-ve tumours (30%) is much higher than would be expected from clinical studies.

The rate of in-vitro inhibition by Adriamycin was greater with ER-ve tumours (60%) than with ER+ve tumours (32%). This concurs with several clinical studies which have shown that ER-ve tumours are more responsive to cytotoxic chemotherapy than their ER+ve counterparts (Lippman, 1978).

3. Approximately 70% of the tumours that responded clinically to Tamoxifen were also inhibited in-vitro. Conversely only 30% of clinically non-responsive tumours were inhibited in-vitro. This observed difference in response rates was not statisticaly significant although this may be a reflection of the small number of patients studied.

When clinical response was examined in relation to ER status, 53% of ER+ve tumours responded to Tamoxifen. However, 40% of ER-ve tumours also responded clinically to Tamoxifen, a much higher response rate than one would have expected.

Similarly although 47% of ER+ve tumours were inhibited in-vitro by Tamoxifen, an even greater proportion (50%) of ER-ve tumours were also inhibited in-vitro.

These apparent anomalies could be due to either inaccuracies in the ER assay or misinterpetation of in-vitro inhibition and clinical response. Although the ER assays were all performed in the same laboratory using an identical technique, some of the tumours were stored in liquid nitrogen for up to 6 months before the assays were performed. There is now evidence that incorrect handling or prolonged storage of tumour tissue may lead to a decline in the levels of receptor protein (Braunsberg, 1975; Leake, 1981). For this reason some ER+ve tumours may have been falsely assayed as ER-ve.

As discussed in Chapter 2 the definition of in-vitro inhibition is arbitrary. Nevertheless, raising or lowering the threshold of inhibition to, for example, 50% or 10% of control values does not alter the overall results. Clinical response is also difficult to

assess in advanced breast cancer (Campbell, 1983). Although every effort was made to assess response accurately, it is well recognised that local and regional disease may regress whilst metastatic disease accelerates. Such paradoxical behaviour could obviously influence the interpretation of response.

If the drug assay and ER assay are compared as predictors of clinical response to Tamoxifen, both have a sensitivity of 69%. The specificity of the drug assay is higher (71%) than that of ER status (43%) in this study. Furthermore, the drug assay has a positive predictive value of 69% and a negative predictive value of 71%, comparing favourably with the positive and negative predictive value of ER status which were 53% and 60% respectively.

Finally the drug assay successfully identified 75% of the ER+ve tumours that did not respond clinically to Tamoxifen and 75% of ER-ve tumours that did respond to Tamoxifen.

4. At present cytotoxic therapy is not commonly used as initial treatment in breast cancer and consequently very few patients are included in this study. Nevertheless, of the 4 tumours that responded clinically to Adriamycin, 3 were also inhibited in-vitro. Conversely, none of the 3 clinically unresponsive tumours were inhibited by Adriamycin in-vitro.

Only 4 of the tumours were of known ER status, all were ER+ve. Of these only one tumour responded clinically to Adriamycin, and the same tumour was also inhibited in-vitro by Adriamycin. This finding would support the hypothesis that ER+ve tumours are less responsive to cytotoxic agents.

Conclusions

This in-vitro drug assay may be performed by a laboratory technician using relatively inexpensive reagents and equipment available in most large hospital laboratories. A major advantage of this assay is that each individual tumour acts as its own control. Although ER assay is probably no more demanding technically it does require constant quality control to validate the results of assays from each individual laboratory. For this reason it is now accepted that ER assays should only be performed by a limited number of specialist laboratories. Under these circumstances ER assays may become more expensive and available only for selected cases and specific research projects.

At present this drug assay necessitates an open biopsy and it would be desirable to adapt the technique to smaller quantities of tumour tissue. It has been possible to measure the ER content of fine needle aspirates of breast tumours both quantitatively (Silfversward, 1980) and qualitatively using immunoperoxidase staining (Pertshuk, 1978). The validity of these assays, however, has not been confirmed in comparison to more conventional techniques.

If in-vitro studies are to be used in the selection of systemic drug therapy it is important to produce results quickly and consistently. Using this assay a result is always available within 24 hours following biopsy. Although ER assays could theoretically be done in this time, in practice samples are stored, and transported to specialist laboratories and assayed in batches. Clonogenic assays are notoriously difficult to perform even with the expertise of a tissue culture laboratory. Interpretation requires accurate histological evaluation over a period of several weeks and as many breast carcinomas will not disaggregate it may be possible to test only a minority of tumours.

The overall rates of in-vitro inhibition by Tamoxifen and Adriamycin were found to be similar to expected clinical response rates using the same agents. The drug assay was as effective as ER assay in identifying Tamoxifen sensitive tumours. However when clinical response is considered in relation to the ER status there are discrepancies which cannot be explained satisfactorily. The results of the small study relating clinical and in-vitro response to Adriamycin are encouraging, but too few patients have been studied to draw any meaningful conclusions.

In summary this type of in-vitro assay has several practical advantages when compared to either Estrogen Receptor or clonogenic assays. The rates of in-vitro inhibition by Tamoxifen and Adriamycin are similar to the expected clincial response rates. Although the drug assay appears to be comparable to ER assay in predicting clinical response to Tamoxifen the results of the clinical studies are inconclusive. More extensive studies are required before this type of assay could be applied in the selection of drug treatment for breast carcinoma.

APPENDICES

Appendix I.1

Computer Code

Tumour Number 0001-0992

Surgical Unit 1 = Professorial Surgical Unit
2 = Royal Liverpool Hospital
3 = Broadgreen
4 = Walton and Fazakerley
5 = Whiston and St. Helens
6 = Clatterbridge
7 = Wallasey and Birkenhead
8 = Other

Age In years at the time of diagnosis

Menopausal status 0 = unknown
1 = premenopausal
2 = postmenopausal

Parity 0 = unknown
1 = parous
2 = nulliparous

Tumour size 1 = T_1
2 = T_2
3 = T_3

Axillary node status 0 = N? = unknown
1 = N_0
2 = N_1

Oestrogen Receptor 0 = unknown
1 = ER+ve
2 = ER-ve

Progesterone Receptor 0 = unknown
1 = PR+ve
2 = ER-ve

Histological Grade 0 = unknown
1 = I
2 = II
3 = III

Thymidine Labelling Index 00.0
99.9 = unknown

Operation Date 00 00 00

Date 1st recurrence 00 00 00 = not applicable

Site 1st recurrence 0 = N/A
1 = local
2 = nodal
3 = bone
4 = visceral
5 = > 1 site

Treatment 1st recurrence 0 = none or N/A
1 = DXT
2 = hormone
3 = cytotoxic
4 = combination

Date last follow up 00 00 00

Site of recurrence at last follow up 0-5

Treatment at last follow up 0-4

Date of death 00 00 00

Category
1 = alive and well
2 = alive with breast cancer
3 = died breast cancer
4 = died (other cause)

FILE; CANCDATA SPSS A LIVERPOOL 4341

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0005 2 77 2 2 3 1 2 0 0 99,9 061175 010679 3 0 010679 3 0 010679 3
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0194	1	77	2	0	3	1	2	0	0	99,9	210777	000000	0	0	040778	0	0	040778	4
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FILE: CANCDATA SPSS A LIVERPOOL 4341

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0198 8 53 2 2 2 1 2 0 1 99.9 270777 000000 0 0 270781 0 0 000000 1
0200 3 43 1 2 1 1 1 0 2 99.9 280777 000000 0 0 070979 0 0 000000 1
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0207 3 53 2 2 2 0 2 0 0 99.9 050877 010278 5 2 010680 5 4 010680 3
0208 7 62 2 0 2 1 1 0 3 99.9 050877 000000 0 0 050881 0 0 000000 1
0210 7 38 1 2 1 2 2 0 0 99.9 100877 000000 0 0 230481 0 0 000000 1
0211 8 63 2 2 2 0 2 0 1 99.9 150877 000000 0 0 010180 0 0 000000 1
0212 1 64 2 1 2 2 1 0 0 99.9 230877 220679 5 2 110382 5 0 110382 3
0213 4 62 2 2 2 1 1 0 1 99.9 020977 000000 0 0 050381 0 0 000000 1
0214 4 69 2 2 2 1 1 0 2 99.9 050977 000000 0 0 090181 0 0 000000 1
0217 3 47 1 2 2 1 2 0 0 99.9 140977 011078 2 4 010281 5 4 010281 3
0219 3 63 2 2 2 2 2 0 0 99.9 210977 040882 5 4 010283 5 4 000000 2
0220 3 52 2 2 2 1 2 0 0 99.9 300977 000000 0 0 140980 0 0 000000 1
0221 1 50 1 2 2 1 2 0 1 99.9 041077 000000 0 0 010782 0 0 000000 1
0222 4 76 2 0 3 2 2 0 1 99.9 051077 061178 5 0 061178 5 0 061178 3
0223 1 57 2 1 2 1 2 0 0 99.9 051077 060778 1 1 010681 1 0 000000 2
0224 4 50 1 2 2 2 2 0 2 99.9 071077 000000 0 0 040981 0 0 000000 1
0225 8 65 2 2 3 2 1 0 2 99.9 131077 000000 0 0 100679 0 0 100679 4
0226 5 42 1 2 1 2 2 0 0 99.9 141077 171080 5 0 170180 5 0 170180 3
0227 4 45 1 2 2 1 1 0 2 99.9 141077 000000 0 0 061279 0 0 000000 1
0229 4 51 2 1 3 2 1 0 1 99.9 171077 060781 5 2 041281 5 0 000000 2
0230 1 65 2 1 2 1 1 0 0 99.9 181077 000000 0 0 091281 0 0 000000 1
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0233 8 61 2 0 2 2 1 0 3 99.9 261077 010580 5 0 010580 5 0 010580 4
0234 1 48 1 2 2 0 2 0 0 99.9 271077 260778 5 2 121079 5 0 121079 3
0235 3 69 2 2 2 1 1 0 0 99.9 281077 000000 0 0 130781 0 0 000000 1
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0248 2 64 2 2 2 1 1 0 1 99.9 171177 000000 0 0 110981 0 0 000000 1
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0251 1 46 1 0 2 2 2 0 0 99.9 221177 180878 1 2 181184 1 2 000000 2
0252 8 57 0 0 2 1 2 0 0 99.9 221177 000000 0 0 010781 0 0 000000 1
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0255 4 60 2 2 1 0 2 0 2 99.9 291177 100580 2 2 010980 2 2 000000 2
0256 2 57 2 2 2 2 1 2 3 99.9 011177 010179 1 2 010782 5 4 010782 3
0257 8 57 0 0 2 1 1 0 2 99.9 301177 000000 0 0 011279 0 0 000000 1
0258 1 77 2 1 3 1 2 0 2 99.9 021277 000000 0 0 010481 0 0 000000 1
0259 3 49 1 2 2 2 2 0 0 99.9 021277 280778 5 0 011078 5 0 011078 3
0260 3 57 2 2 2 1 2 0 0 99.9 021277 000000 0 0 190681 0 0 000000 1
0261 2 47 0 2 3 2 2 0 2 99.9 071277 010180 5 0 010180 5 0 010180 3
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FILE; CANCDATA SPSS A LIVERPOOL 4341

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0266 2 84 2 0 2 1 1 0 3 99,9 040178 000000 0 0 010781 0 0 000000 1
0269 1 35 1 2 2 1 2 2 0 99,9 100178 030578 1 1 110879 5 4 110879 3
0270 1 57 0 0 1 2 1 0 0 99,9 170178 000000 0 0 170382 0 0 000000 1
0271 8 70 2 1 2 0 2 0 0 99,9 180178 000000 0 0 010680 0 0 000000 1
0272 2 48 2 2 3 2 2 0 2 99,9 180178 310878 5 0 310878 5 0 310878 3
0274 3 57 2 2 1 2 2 0 0 99,9 200178 000000 0 0 010181 0 0 000000 1
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0276 8 40 1 2 2 1 2 0 3 99,9 240178 000000 0 0 240181 0 0 000000 1
0277 8 64 2 0 3 2 2 0 3 99,9 240178 000000 0 0 240179 0 0 000000 1
0279 1 48 0 0 2 2 2 0 0 99,9 260178 261078 1 5 271080 5 4 271080 3
0281 8 69 2 0 3 2 1 0 2 99,9 270178 000000 0 0 220778 0 0 000000 1
0282 3 65 2 2 3 1 1 0 0 99,9 300178 000000 0 0 140581 0 0 000000 1
0283 3 46 1 0 1 1 1 0 0 99,9 300178 000000 0 0 180581 0 0 000000 1
0290 3 62 2 2 2 2 2 0 0 99,9 150278 190980 5 2 010581 5 0 010581 3
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0292 8 63 2 2 2 1 1 0 1 99,9 170278 000000 0 0 200578 0 0 000000 1
0294 8 58 2 2 3 2 2 0 3 99,9 210278 000000 0 0 030481 0 0 000000 1
0297 1 46 1 2 3 2 1 0 2 99,9 240278 260581 5 2 080383 5 4 000000 2
0298 5 47 2 2 2 2 1 0 0 99,9 270278 050279 5 0 050279 5 0 050279 3
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0302 1 35 1 2 2 1 2 0 0 99,9 090378 000000 0 0 130781 0 0 000000 1
0303 8 46 1 2 3 2 2 0 3 99,9 150378 090779 5 0 090779 5 0 090779 3
0304 2 55 2 2 3 1 2 0 2 99,9 150378 000000 0 0 010681 0 0 000000 1
0305 4 43 1 2 2 1 2 0 0 99,9 170378 000000 0 0 080581 0 0 000000 1
0306 8 36 1 2 2 2 2 0 2 99,9 170378 110879 5 0 011079 5 0 011079 3
0307 1 66 2 1 2 2 2 0 0 99,9 210378 000000 0 0 030781 0 0 000000 1
0310 1 36 1 1 1 2 1 0 0 99,9 280378 021178 1 2 170282 5 4 170282 3
0311 4 68 2 2 2 2 2 0 3 99,9 280378 010578 5 2 200680 5 2 200680 3
0313 3 58 2 1 3 0 2 0 0 99,9 210378 130878 5 2 131079 5 2 000000 2
0314 2 76 2 1 2 0 1 0 1 99,9 290378 000000 0 0 180180 0 0 000000 1
0318 2 86 2 0 2 2 1 0 3 99,9 310378 000000 0 0 040480 0 0 040480 4
0319 3 31 1 2 2 2 1 0 0 99,9 310378 260181 5 2 110381 5 2 000000 2
0322 3 57 2 2 2 1 2 0 0 99,9 070478 000000 0 0 070481 0 0 000000 1
0323 4 63 2 2 2 1 1 0 0 99,9 100478 000000 0 0 231179 0 0 000000 1
0324 6 48 2 1 2 1 1 0 2 99,9 110478 000000 0 0 110481 0 0 000000 1
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0327 1 47 1 2 2 0 2 0 3 99,9 140478 111078 1 0 140282 5 4 140282 3
0328 4 54 1 2 1 1 2 0 1 99,9 170478 000000 0 0 070581 0 0 000000 1
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0331 6 82 2 2 3 2 2 0 3 99,9 180478 000000 0 0 091081 0 0 000000 1
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0335 4 62 2 0 3 2 1 0 3 99,9 200478 000000 0 0 200481 0 0 000000 1
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0337 1 58 2 2 3 1 2 0 0 99,9 250478 000000 0 0 060982 0 0 000000 1
0338 3 37 1 2 3 2 1 0 0 99,9 250478 000000 0 0 010781 0 0 000000 1
0339 8 63 2 1 3 2 1 0 1 99,9 260478 000000 0 0 260481 0 0 000000 1
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0342 1 59 2 2 1 1 1 0 0 99,9 270478 000000 0 0 010681 0 0 000000 1
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0344 3 70 2 0 1 2 1 0 0 99,9 030578 030581 4 2 010681 5 0 010681 3
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FILE; CANCDATA SPSS A LIVERPOOL 4341

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0350 4 61 2 2 1 2 2 0 1 99,9 080578 000000 0 0 120681 0 0 000000 1
0351 5 50 1 1 2 0 1 0 0 99,9 110478 000000 0 0 061279 0 0 000000 1
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0353 5 46 1 1 3 0 1 0 0 99,9 050578 000000 0 0 250579 0 0 000000 1
0354 4 43 1 1 3 2 2 2 1 99,9 110578 200381 3 1 200581 3 0 000000 2
0355 8 55 2 2 2 2 2 2 1 99,9 120578 000000 0 0 120581 0 0 000000 1
0356 8 53 0 0 2 1 1 2 3 99,9 170578 000000 0 0 170681 0 0 000000 1
0357 3 44 2 2 2 0 1 2 0 99,9 190578 260379 3 2 200979 3 0 200979 3
0358 5 65 2 2 2 0 1 2 0 99,9 190578 000000 0 0 080579 0 0 000000 1
0359 1 86 2 2 3 0 1 1 1 99,9 230578 020279 2 2 020580 2 0 000000 2
0361 1 70 2 1 3 1 1 2 2 05,1 230578 000000 0 0 010381 0 0 000000 1
0362 4 55 2 2 2 2 1 2 1 99,9 230578 000000 0 0 140581 0 0 000000 1
0363 4 49 1 2 3 0 1 1 3 04,6 240578 080782 4 2 080782 4 2 000000 2
0364 8 52 1 2 3 2 2 2 2 10,1 240578 250778 4 1 071079 3 0 071079 3
0365 7 68 2 2 2 2 1 2 1 01,7 250578 181078 3 2 110879 3 0 110879 3
0366 8 73 2 2 2 1 1 1 0 99,9 250578 000000 0 0 260381 0 0 000000 1
0367 5 77 2 2 3 0 1 1 0 99,9 260578 000000 0 0 190380 0 0 000000 1
0368 2 40 1 1 1 1 2 2 2 99,9 310578 000000 0 0 080782 0 0 000000 1
0369 6 54 2 2 1 1 1 1 1 99,9 010678 000000 0 0 290481 0 0 000000 1
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0371 4 49 2 2 2 0 2 2 0 99,9 060678 000000 0 0 280580 0 0 000000 1
0372 7 73 2 2 3 1 1 1 0 01,4 070678 000000 0 0 170281 0 0 000000 1
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0374 2 51 1 2 2 0 2 2 3 99,9 140678 030882 4 4 010283 3 4 000000 2
0375 3 32 1 2 2 0 2 2 0 99,9 130678 000000 0 0 200280 0 0 000000 1
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0377 4 62 2 2 3 2 2 2 3 99,9 150678 010979 3 2 140679 3 4 140679 3
0378 8 68 2 1 2 0 2 2 2 99,9 140678 000000 0 0 121278 0 0 000000 1
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0382 8 60 2 2 1 1 1 2 0 99,9 210678 000000 0 0 120480 0 0 000000 1
0384 2 49 1 1 2 2 1 2 3 99,9 220678 000000 0 0 120581 0 0 000000 1
0385 8 63 2 0 2 0 1 2 0 07,6 230678 000000 0 0 031279 0 0 000000 1
0386 3 64 2 2 2 1 1 2 0 99,9 300678 000000 0 0 010782 0 0 000000 1
0387 1 69 2 2 2 2 2 2 0 99,9 040778 130379 3 0 130379 3 0 130379 3
0388 2 43 1 0 3 2 2 2 3 07,2 050778 071178 2 3 140779 3 0 140779 3
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0399 3 44 0 0 2 1 1 0 0 99,9 250778 000000 0 0 010781 0 0 000000 1
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0401 4 58 2 2 2 0 2 0 0 99,9 270778 000000 0 0 101279 0 0 000000 1
0403 6 76 2 1 3 1 2 0 3 09,1 010878 200680 4 0 200680 4 0 200680 3
0404 1 55 2 2 3 2 1 0 0 99,9 010878 030681 1 0 021282 3 2 000000 2
0406 8 71 2 1 2 1 2 0 0 99,9 080878 000000 0 0 210181 0 0 000000 1
0407 3 69 2 2 3 2 1 0 0 99,9 150878 000000 0 0 280380 0 0 000000 1
0408 3 61 2 1 2 2 1 0 0 99,9 150878 000000 0 0 060881 0 0 000000 1
0410 2 39 1 0 3 1 2 0 0 99,9 160878 000000 0 0 120681 0 0 000000 1
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FILE; CANCDATA SPSS A LIVERPOOL 4341

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0415 4 47 1 0 1 0 2 0 0 99,9 300878 000000 0 0 130580 0 0 000000 1
0416 8 72 2 0 3 2 1 0 3 03,5 010978 210581 2 2 080183 2 2 000000 2
0419 1 58 2 1 2 2 1 0 0 99,9 220878 000000 0 0 030881 0 0 000000 1
0421 3 58 1 2 2 2 1 0 2 01,1 060978 000000 0 0 190281 0 0 000000 1
0422 4 39 1 2 3 2 2 0 3 07,2 060978 160879 5 4 260780 5 4 260780 3
0423 8 63 2 2 2 1 1 0 0 02,4 080978 010285 4 0 010283 4 0 000000 2
0424 1 59 2 2 2 2 1 0 0 99,9 120978 000000 0 0 111082 0 0 000000 1
0425 1 57 2 2 3 1 1 0 2 13,0 120978 241080 5 0 241080 5 0 241080 3
0427 8 62 2 1 2 1 1 0 0 99,9 130978 000000 0 0 011281 0 0 000000 1
0428 8 43 1 2 2 1 1 0 0 03,5 140978 230481 4 4 231282 4 4 231282 3
0430 8 40 1 2 2 1 1 0 0 99,9 150978 000000 0 0 170981 0 0 000000 1
0431 8 76 2 2 2 2 1 0 0 99,9 180978 000000 0 0 010481 0 0 000000 1
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0433 6 50 2 2 2 2 1 0 1 99,9 180978 000000 0 0 180981 0 0 000000 1
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0435 5 60 2 0 2 1 1 0 3 99,9 150978 140980 2 1 011281 2 0 000000 2
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0438 8 56 0 0 2 2 1 0 0 99,9 260978 000000 0 0 070881 0 0 000000 1
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0444 5 37 1 2 1 0 1 0 0 99,9 041078 000000 0 0 250280 0 0 000000 1
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0446 4 46 1 2 2 2 2 2 0 11,8 051078 210279 5 0 210279 5 0 210279 3
0448 4 56 2 2 1 0 1 0 0 02,1 061078 000000 0 0 030180 0 0 000000 1
0449 8 68 2 2 3 2 2 0 0 99,9 061078 000000 0 0 011081 0 0 000000 1
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0453 3 44 2 2 2 0 2 0 0 99,9 111078 000000 0 0 090680 0 0 000000 1
0455 3 66 2 2 2 1 1 0 0 99,9 161078 000000 0 0 011181 0 0 000000 1
0456 1 63 2 2 2 2 2 0 3 05,0 061075 171078 2 3 100679 5 4 100679 3
0457 5 61 2 1 2 2 1 0 2 99,9 171078 000000 0 0 011081 0 0 000000 1
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0461 2 56 2 1 2 1 1 0 2 02,5 261078 000000 0 0 270381 0 0 000000 1
0463 3 58 2 1 2 0 1 0 0 99,9 301078 000000 0 0 210380 0 0 000000 1
0464 3 36 1 2 2 2 1 2 0 01,7 311078 150879 5 2 151280 5 2 151280 3
0465 1 59 2 1 2 2 2 0 1 03,4 311078 000000 0 0 171282 0 0 000000 1
0466 1 45 0 0 1 0 1 2 0 99,9 311078 000000 0 0 240980 0 0 000000 1
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0470 1 79 2 0 2 1 1 1 2 99,9 031178 010981 1 0 041180 1 0 000000 2
0471 2 56 0 0 2 1 1 1 1 10,2 061178 000000 0 0 011081 0 0 000000 1
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0475 1 80 2 2 2 2 2 0 2 99,9 101178 000000 0 0 010580 0 0 000000 1
0477 1 80 2 2 1 0 1 2 0 99,9 151178 000000 0 0 120283 0 0 000000 1
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0479 4 71 2 2 3 0 1 2 0 11,7 221178 000000 0 0 100480 0 0 000000 1
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0482 1 44 1 0 2 1 2 0 0 99,9 200778 200779 1 1 010283 1 2 000000 2
0484 1 81 2 0 2 0 2 2 0 99,9 011278 091079 5 0 240980 5 0 000000 2
0485 4 54 2 2 3 0 2 2 0 99,9 061278 000000 0 0 030480 0 0 000000 1
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FILE; CANCDATA SPSS A LIVERPOOL 4341

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0490 4 47 1 1 1 0 1 2 0 99,9 131278 000000 0 0 300480 0 0 000000 1
0491 1 65 2 2 3 2 1 1 1 02,1 141278 000000 0 0 111082 0 0 000000 1
0492 1 70 2 0 1 1 1 2 0 99,9 141278 260582 1 0 080183 1 0 000000 2
0493 4 51 2 1 3 0 1 2 0 03,6 151278 000000 0 0 220181 0 0 000000 1
0494 1 72 2 2 3 1 1 2 0 99,9 191278 000000 0 0 111082 0 0 000000 1
0495 3 69 2 0 2 1 2 2 2 09,2 201278 220581 2 0 220581 2 0 220581 3
0498 1 60 2 2 2 2 2 2 1 99,9 050179 000000 0 0 050181 0 0 000000 1
0500 1 54 2 2 2 2 1 1 3 01,2 090179 041180 1 2 161181 2 4 161181 3
0501 4 42 1 2 2 2 1 2 1 04,2 110179 000000 0 0 010981 0 0 000000 1
0503 1 72 2 2 2 1 1 2 3 99,9 160179 000000 0 0 111082 0 0 000000 1
0504 4 62 2 2 2 2 2 2 2 12,2 230179 000000 0 0 220181 0 0 000000 1
0505 1 57 2 2 2 2 1 1 1 01,2 230179 000000 0 0 010181 0 0 000000 1
0506 7 44 1 0 2 1 2 2 0 10,6 240179 150480 4 2 070681 2 0 070681 3
0507 1 78 2 0 2 0 1 1 1 02,0 250179 000000 0 0 120581 0 0 000000 1
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0511 2 62 2 0 2 1 1 1 0 01,2 080279 000000 0 0 080281 0 0 000000 1
0513 3 69 2 2 2 1 1 2 0 99,9 090279 000000 0 0 090281 0 0 000000 1
0515 3 68 2 2 2 1 1 2 1 03,2 130279 000000 0 0 010381 0 0 000000 1
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0517 1 64 2 2 2 1 2 2 2 11,2 200279 000000 0 0 010381 0 0 000000 1
0518 1 36 0 0 2 2 2 2 0 12,2 011076 110777 2 2 101180 2 4 101180 3
0519 2 41 1 2 2 2 1 2 2 05,2 220279 240580 2 2 010780 2 2 010780 3
0520 1 58 0 0 2 2 2 2 3 11,7 010877 011277 1 1 010979 2 4 010979 3
0521 6 55 2 2 2 1 1 2 1 05,9 220279 000000 0 0 131081 0 0 000000 1
0522 4 70 2 0 2 1 1 2 2 06,2 230279 000000 0 0 230281 0 0 000000 1
0524 2 57 2 2 2 2 2 2 3 99,9 270279 060680 2 4 090281 2 2 000000 2
0527 1 49 1 2 2 2 1 2 1 00,8 060379 000000 0 0 111082 0 0 000000 1
0528 4 65 2 2 2 2 2 2 0 09,7 060379 000000 0 0 020681 0 0 000000 1
0529 2 55 2 0 2 2 1 2 3 06,1 080379 000000 0 0 030881 0 0 000000 1
0530 1 55 0 0 3 0 1 2 1 04,7 130379 000000 0 0 010881 0 0 000000 1
0531 1 37 1 2 1 1 1 1 2 99,9 130379 100180 4 3 030380 4 3 030380 3
0533 3 74 2 0 2 0 1 1 0 00,9 160379 000000 0 0 100481 0 0 000000 1
0534 4 47 1 0 3 1 1 1 0 99,9 160379 180780 2 2 070881 2 2 000000 2
0535 4 50 1 2 2 1 2 2 3 12,9 210379 000000 0 0 090681 0 0 000000 1
0536 1 61 2 1 2 1 1 2 3 05,1 220379 000000 0 0 110581 0 0 000000 1
0538 1 44 1 0 3 2 1 2 3 05,2 270379 250582 2 4 200482 2 4 200482 3
0540 4 35 1 2 2 2 2 2 3 10,2 290379 200880 2 4 220181 2 4 220181 3
0541 3 89 2 0 3 2 1 2 1 04,7 300379 000000 0 0 080681 0 0 000000 1
0542 4 51 2 2 2 1 1 2 3 06,2 300379 000000 0 0 220181 0 0 000000 1
0543 4 56 0 0 2 0 1 2 0 05,2 050479 000000 0 0 150481 0 0 000000 1
0544 4 70 2 0 2 2 2 2 0 15,1 060379 000000 0 0 190381 0 0 000000 1
0545 4 46 1 2 3 2 1 2 0 06,4 060479 000000 0 0 010183 0 0 000000 1
0546 1 58 2 0 2 1 1 0 2 99,9 100479 000000 0 0 100481 0 0 000000 1
0547 1 69 2 2 2 1 1 0 0 99,9 100479 000000 0 0 111082 0 0 000000 1
0548 6 47 1 0 2 2 2 0 0 99,9 100479 090480 1 0 090480 1 0 000000 2
0550 1 72 2 0 2 1 1 0 0 99,9 100479 000000 0 0 240681 0 0 000000 1
0552 4 72 2 0 2 1 2 0 0 99,9 170479 000000 0 0 170481 0 0 000000 1
0553 1 40 1 2 2 1 1 0 0 99,9 170479 000000 0 0 080881 0 0 000000 1
0554 7 51 0 0 3 2 2 0 3 99,9 200479 121079 4 2 120380 4 2 000000 2
0555 3 52 2 0 1 0 1 0 0 99,9 200479 000000 0 0 200481 0 0 000000 1
0556 3 74 2 2 2 0 2 0 0 99,9 230479 000000 0 0 040880 0 0 000000 1
0559 6 45 0 0 2 1 0 0 0 99,9 240479 000000 0 0 240481 0 0 000000 1
```

FILE: CANCDATA SPSS A LIVERPOOL 4541

```
0061  4 61 2 0 2 1 2 2 3 99,9 270479 000000 0 0 070581 0 0 000000 1
0065  6 37 1 2 2 2 2 2 3 99,9 020579 131279 1 1 010980 5 4 010980 3
0066  2 54 2 0 2 2 1 0 3 99,9 030579 010881 2 2 120881 2 2 000000 2
0068  1 62 2 2 2 2 1 2 1 99,9 080579 010882 4 3 101282 4 2 000000 2
0069  1 54 2 2 2 2 1 1 0 99,9 080579 000000 0 0 121182 0 0 000000 1
0070  6 63 2 0 3 2 2 1 3 99,9 080579 000000 0 0 010581 0 0 000000 1
0071  6 70 2 0 2 2 1 0 2 99,9 090579 000000 0 0 240981 0 0 000000 1
0073  1 53 2 0 2 2 2 1 0 99,9 110579 000000 0 0 110581 0 0 000000 1
0074  2 58 2 0 2 0 1 2 0 99,9 150579 000000 0 0 121280 0 0 000000 1
0075  1 64 2 0 2 0 2 2 0 99,9 100579 000000 0 0 031279 0 0 000000 1
0076  1 55 2 0 2 2 1 2 0 99,9 100579 080582 2 3 081282 2 3 000000 2
0077  1 73 2 2 2 1 1 2 2 99,9 220579 000000 0 0 150481 0 0 000000 1
0078  1 71 2 0 3 2 2 2 0 02,0 220579 101080 1 5 170682 5 4 000000 2
0079  1 60 2 0 2 2 1 0 0 99,9 220579 000000 0 0 291081 0 0 000000 1
0080  7 53 0 0 2 2 2 0 0 99,9 230579 280679 4 0 280679 4 0 210779 3
0081  7 57 2 2 2 2 2 2 3 99,9 250579 010481 5 0 010481 5 0 010481 3
0082  6 75 2 0 2 1 1 2 0 99,9 250579 000000 0 0 051181 0 0 000000 1
0084  1 67 2 2 2 2 2 2 0 99,9 140679 300681 2 3 301081 2 0 000000 2
0086  7 63 2 0 1 1 2 2 1 99,9 150679 000000 0 0 180781 0 0 000000 1
0087  1 60 2 0 2 1 1 2 2 99,9 150679 000000 0 0 140681 0 0 000000 1
0089  1 55 0 0 2 2 2 2 2 01,2 190674 190679 1 0 171281 1 0 000000 2
0091  6 56 2 0 2 2 2 2 1 99,9 190679 110180 1 0 030480 1 0 030480 3
0092  7 76 2 0 2 2 1 2 0 99,9 190679 000000 0 0 230781 0 0 000000 1
0093  2 53 2 0 2 1 2 2 2 99,9 210679 101281 1 2 210581 1 2 000000 2
0094  6 69 2 0 2 1 2 2 3 99,9 220679 000000 0 0 010180 0 0 000000 1
0095  6 46 1 2 2 1 1 2 0 99,9 010878 210679 2 2 230180 2 2 000000 2
0096  1 42 0 2 2 1 2 2 2 01,0 260679 000000 0 0 091282 0 0 000000 1
0097  4 75 2 0 2 0 2 2 0 99,9 260679 000000 0 0 290280 0 0 000000 1
0098  1 42 1 1 1 1 2 1 1 03,1 270679 000000 0 0 130583 0 0 000000 1
0099  6 75 2 0 2 2 1 1 3 99,9 270679 000000 0 0 131279 0 0 000000 1
0600  4 75 2 0 2 1 1 1 1 01,5 290779 040781 5 0 040781 5 0 040781 3
0602  1 59 2 2 2 0 1 0 0 99,9 290875 150879 5 4 180881 5 4 000000 2
0603  1 46 1 0 1 0 1 2 3 99,9 290679 000000 0 0 010881 0 0 000000 1
0604  1 64 2 0 2 2 1 2 1 00,6 030779 000000 0 0 200182 0 0 000000 1
0605  3 57 0 0 2 2 1 1 2 00,8 020479 020480 5 2 270682 5 2 270682 3
0607  6 39 1 0 2 2 2 2 3 99,9 030779 000000 0 0 130182 0 0 000000 1
0609  7 44 1 2 2 1 1 2 3 99,9 030779 041079 2 2 230481 0 2 000000 2
0612  4 42 1 0 3 0 2 2 2 01,3 040779 220481 5 1 220481 5 1 000000 2
0614  4 69 2 0 2 1 1 2 1 99,9 060779 000000 0 0 200881 0 0 000000 1
0615  1 67 2 2 2 1 1 2 3 01,5 100779 230282 5 2 120882 5 2 000000 1
0617  1 59 2 0 2 1 2 2 2 00,9 100779 000000 0 0 250382 0 0 000000 1
0318  1 69 2 0 2 2 1 2 0 05,9 100779 011080 4 0 011180 4 0 011180 3
0619  7 63 2 0 2 1 1 2 0 99,9 100779 000000 0 0 180681 0 0 000000 1
0622  1 71 2 0 2 0 1 1 0 99,9 170779 070182 5 4 070182 5 4 000000 2
0623  4 35 0 0 2 0 2 1 0 99,9 200779 000000 0 0 230581 0 0 000000 1
0624  2 43 1 2 3 2 1 1 2 99,9 250779 090780 2 1 220481 2 0 000000 2
0625  5 64 2 2 2 1 1 2 0 99,9 300779 270481 1 1 270481 1 1 000000 2
0626  2 70 2 0 2 0 1 2 0 99,9 310779 000000 0 0 040782 0 0 000000 1
0627  2 60 2 2 2 0 1 2 0 99,9 020879 000000 0 0 180481 0 0 000000 1
0628  5 33 1 0 2 1 1 2 0 99,9 140879 000000 0 0 160681 0 0 000000 1
0630  6 48 0 0 2 1 1 2 0 99,9 140879 050680 5 4 280682 5 0 000000 2
0631  6 55 2 2 2 1 2 2 1 99,9 070879 120681 1 0 190881 1 0 000000 2
0632  6 74 2 2 2 0 2 2 0 99,9 150879 000000 0 0 290580 0 0 000000 1
0633  4 50 0 0 2 1 1 1 3 99,9 140879 000000 0 0 100481 0 0 000000 1
0635  4 42 1 0 2 1 1 1 1 99,9 140879 000000 0 0 180381 0 0 000000 1
```

FILE; CANCDATA SPSS A LIVERPOOL 4341

```
0036 1 47 2 2 2 1 1 2 2 01,2 280879 030182 1 1 030183 1 0 000000 2
0037 3 59 2 2 2 2 2 2 2 02,0 280879 000000 0 0 010281 0 0 000000 1
0038 5 67 2 2 2 1 1 2 0 99,9 300879 000000 0 0 131081 0 0 000000 1
0041 7 64 2 0 1 1 1 2 0 05,0 040979 000000 0 0 030780 0 0 000000 1
0042 1 52 2 2 3 2 1 2 1 03,1 040979 010281 3 3 010281 3 3 010281 3
0047 4 48 0 0 2 2 1 2 2 05,1 050979 000000 0 0 280280 0 0 000000 1
0049 1 66 2 2 2 0 2 2 0 99,9 060979 231080 1 0 150781 1 0 000000 2
0050 6 68 2 2 2 2 1 1 1 03,2 130979 000000 0 0 030280 0 0 000000 1
0051 6 52 2 1 2 2 2 2 2 00,3 130979 000000 0 0 010581 0 0 000000 1
0052 3 54 1 2 2 0 1 0 0 99,9 130979 000000 0 0 180381 0 0 000000 1
0053 3 66 2 2 2 2 1 1 0 00,8 140979 000000 0 0 300381 0 0 000000 1
0055 4 45 1 0 3 2 1 2 1 99,9 180979 000000 0 0 090582 0 0 000000 1
0056 4 69 2 2 3 2 2 2 1 99,9 140979 000000 0 0 310881 0 0 000000 1
0058 7 49 1 2 2 2 2 2 2 00,7 250979 020181 3 0 020181 3 0 020181 3
0059 4 74 2 0 2 2 1 2 1 99,9 250979 000000 0 0 020681 0 0 000000 1
0060 7 48 1 0 2 2 2 2 1 05,4 260979 000000 0 0 120581 0 0 000000 1
0063 6 55 0 0 2 1 1 2 0 00,9 091079 000000 0 0 200281 0 0 000000 1
0064 2 55 2 1 2 2 1 2 1 02,0 101079 010480 4 0 010480 4 0 010480 3
0065 2 57 1 0 2 1 2 2 0 03,0 111079 000000 0 0 011081 0 0 000000 1
0068 1 53 2 2 2 1 1 1 0 05,1 181079 000000 0 0 210781 0 0 000000 1
0069 1 40 1 2 2 1 2 1 0 99,9 191079 000000 0 0 270481 0 0 000000 1
0071 4 58 2 2 3 2 1 2 0 00,3 231079 170180 2 1 091180 3 4 090180 3
0073 1 50 2 2 2 1 1 1 3 99,9 231079 000000 0 0 140182 0 0 000000 1
0074 1 41 1 2 2 0 2 2 3 19,1 301079 010380 2 4 120682 2 0 000000 2
0075 1 44 1 2 1 1 2 2 3 99,9 301079 000000 0 0 270582 0 0 000000 1
0076 6 68 2 0 2 1 1 1 1 99,9 301079 000000 0 0 190681 0 0 000000 1
0077 6 69 2 2 2 0 2 2 1 01,7 311079 000000 0 0 011082 0 0 000000 1
0078 6 53 0 0 2 2 2 2 3 14,8 081179 110580 4 3 140580 4 0 140580 3
0079 1 51 1 2 2 2 1 1 3 08,1 081179 010982 3 2 010383 3 0 010383 3
0080 2 76 2 1 3 2 2 2 3 08,2 121179 000000 0 0 010481 0 0 000000 1
0081 1 38 1 2 3 2 2 2 2 07,4 131179 010880 3 4 110382 3 4 110382 3
0082 1 47 1 2 2 2 1 2 3 06,3 131179 000000 0 0 171281 0 0 000000 1
0083 2 47 1 2 2 2 2 2 2 06,3 151179 000000 0 0 030281 0 0 000000 1
0084 1 79 2 2 2 1 2 2 3 99,9 191179 000000 0 0 010482 0 0 000000 1
0085 1 56 2 2 1 1 1 2 1 99,9 191179 000000 0 0 120882 0 0 000000 1
0087 7 58 0 0 2 1 1 1 1 99,9 211179 000000 0 0 101181 0 0 000000 1
0088 7 68 2 0 2 0 1 2 0 99,9 211179 000000 0 0 200580 0 0 000000 1
0089 4 48 1 2 2 0 1 2 0 99,9 211179 000000 0 0 250382 0 0 000000 1
0092 6 53 2 2 2 1 1 2 1 09,0 271179 000000 0 0 040281 0 0 000000 1
0093 1 46 1 0 2 1 2 2 2 01,3 271179 030681 4 4 201281 4 4 201281 3
0094 1 75 2 2 2 0 1 2 2 00,9 041279 000000 0 0 081282 0 0 000000 1
0095 1 46 1 2 2 1 1 2 0 09,3 051279 000000 0 0 100681 0 0 000000 1
0096 1 61 2 2 3 2 2 2 0 99,9 041279 050881 3 4 050881 3 4 000000 2
0097 1 30 1 2 2 1 2 1 0 15,0 041279 000000 0 0 010482 0 0 000000 1
0099 6 40 0 0 2 1 2 2 2 99,9 041279 161280 1 1 010283 1 0 000000 2
0701 1 75 2 2 2 0 1 2 3 02,8 111279 000000 0 0 060982 0 0 000000 1
0702 3 55 2 0 1 2 2 2 0 99,9 011279 010181 4 3 050881 3 0 000000 2
0703 3 60 2 0 2 0 2 2 0 99,9 101279 000000 0 0 011281 0 0 000000 1
0704 3 71 2 0 2 1 2 2 0 99,9 141279 140281 3 0 140281 3 0 140281 3
0705 3 61 2 0 2 1 1 2 0 99,9 181279 000000 0 0 080681 0 0 000000 1
0706 6 46 1 0 2 1 1 2 0 99,9 181279 160480 4 0 160480 4 0 160480 3
0707 4 74 2 1 2 0 1 2 0 99,9 161179 000000 0 0 290580 0 0 000000 1
0710 4 54 2 2 2 1 1 2 2 99,9 211279 000000 0 0 161080 0 0 000000 1
0711 6 45 1 2 2 2 1 1 3 99,9 030180 000000 0 0 011081 0 0 000000 1
0712 1 58 2 1 2 2 2 2 3 00,9 080180 000000 0 0 190882 0 0 000000 1
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FILE: CANCDATA SPSS A LIVERPOOL 4541

0713	1	66	2	2	2	2	1	2	0	99,9	080180	260582	2	0	260582	2	0	000000	2
0718	2	63	2	2	2	0	1	2	1	01,2	150180	000000	0	0	010481	0	0	000000	1
0720	1	64	2	0	1	0	2	2	0	99,9	150180	000000	0	0	011282	0	0	000000	1
0721	2	54	2	2	3	2	1	2	0	99,9	150180	100480	3	4	010382	3	0	010382	3
0723	1	58	2	2	3	1	1	2	0	01,1	170180	240980	1	1	011080	1	0	000000	2
0724	4	45	2	0	2	2	1	2	1	03,0	210180	000000	0	0	090181	0	0	000000	1
0725	4	50	2	2	2	1	2	1	0	99,9	281279	000000	0	0	020781	0	0	000000	1
0726	2	62	2	2	2	1	2	2	1	01,8	210180	000000	0	0	030681	0	0	000000	1
0727	1	60	2	0	2	1	1	2	1	01,9	220180	000000	0	0	080183	0	0	000000	1
0728	4	69	2	2	3	2	1	2	2	01,0	230180	000000	0	0	040382	0	0	000000	1
0729	5	46	0	0	2	0	1	2	0	99,9	250180	000000	0	0	250182	0	0	000000	1
0730	7	52	2	2	2	1	1	2	0	00,9	240180	000000	0	0	120881	0	0	000000	1
0731	1	43	1	2	2	0	2	2	0	99,9	240180	060782	3	0	060782	3	0	060782	3
0733	2	52	1	2	3	1	1	2	0	02,0	290180	220481	2	1	010782	3	0	000000	2
0734	1	41	1	2	3	2	1	2	0	00,3	290180	120381	1	1	030683	1	0	000000	2
0735	6	51	0	0	2	2	2	3	0	99,9	010280	000000	0	0	110381	0	0	000000	1
0736	6	72	2	1	2	0	1	2	0	99,9	240180	000000	0	0	240183	0	0	000000	1
0737	1	68	2	2	3	1	1	2	0	99,9	310180	000000	0	0	120683	0	0	000000	1
0739	5	57	1	2	2	2	2	2	0	01,9	050280	000000	0	0	050681	0	0	000000	1
0740	1	59	2	2	2	1	1	2	1	99,9	050280	000000	0	0	101082	0	0	000000	1
0741	7	52	1	2	2	1	1	2	1	05,8	060280	011480	1	1	100281	0	0	000000	2
0742	4	67	2	2	2	2	2	1	2	99,9	070280	000000	0	0	161181	0	0	000000	1
0743	4	50	1	2	2	2	2	2	1	01,8	080280	000000	0	0	010282	0	0	000000	1
0744	7	69	2	2	2	1	2	2	0	99,9	070280	000000	0	0	131081	0	0	000000	1
0745	3	67	2	0	2	1	1	2	0	99,9	080280	000000	0	0	110881	0	0	000000	1
0746	5	53	2	2	3	2	2	2	0	99,9	120280	050880	2	1	250281	2	0	000000	2
0749	1	51	1	2	1	1	2	2	0	99,9	120280	000000	0	0	111082	0	0	000000	1
0750	1	46	1	0	2	0	2	1	3	05,2	120280	250183	2	1	250183	2	1	000000	2
0751	1	74	2	1	3	1	1	2	0	99,9	120280	000000	0	0	151282	0	0	000000	1
0754	1	55	0	0	2	0	1	2	0	99,9	140280	120580	2	1	200682	0	0	000000	2
0756	6	31	1	2	2	2	1	2	3	99,9	110280	000000	0	0	131081	0	0	000000	1
0760	3	53	0	0	2	1	1	1	0	99,9	190280	000000	0	0	120182	0	0	000000	1
0762	5	33	1	1	2	0	1	1	0	01,2	220280	000000	0	0	270481	0	0	000000	1
0763	6	40	0	0	2	1	2	2	0	02,4	250280	311280	3	2	010381	3	2	010381	3
0767	5	69	2	0	2	1	2	1	0	99,9	300180	000000	0	0	090281	0	0	000000	1
0768	4	67	2	2	2	0	2	2	0	00,9	290280	000000	0	0	010281	0	0	000000	1
0772	1	48	1	2	3	2	1	2	3	02,6	040380	140781	4	0	070881	4	0	070881	3
0775	1	64	2	2	2	2	2	2	0	99,9	110380	230481	1	1	160981	1	1	000000	2
0777	1	71	2	2	2	2	1	2	2	03,8	110380	000000	0	0	010482	0	0	000000	1
0779	6	66	2	0	2	0	1	2	2	02,5	140380	000000	0	0	020982	0	0	000000	1
0780	7	44	1	0	2	1	1	1	1	99,9	140380	000000	0	0	130481	0	0	000000	1
0781	1	56	2	2	2	2	1	1	2	06,0	180380	000000	0	0	110382	0	0	000000	1
0782	3	69	2	1	2	2	2	2	0	99,9	240380	150880	2	2	050181	2	2	000000	2
0784	1	57	2	2	2	2	1	2	2	99,9	250380	000000	0	0	120882	0	0	000000	1
0785	5	62	2	2	3	0	1	2	0	02,9	260380	000000	0	0	160382	0	0	000000	1
0786	6	39	1	1	3	1	2	2	2	01,9	010480	000000	0	0	180381	0	0	000000	1
0789	1	66	2	1	2	2	1	2	1	99,9	010480	000000	0	0	010482	0	0	000000	1
0790	2	35	1	0	2	2	1	2	3	99,9	020480	000000	0	0	300381	0	0	000000	1
0791	2	55	0	0	1	0	2	2	2	01,9	010480	000000	0	0	100482	0	0	000000	1
0792	4	52	2	0	2	0	2	2	1	04,5	100480	230481	1	2	010681	1	0	000000	2
0793	3	59	2	0	2	2	2	2	0	99,9	220480	000000	0	0	050281	0	0	000000	1
0794	1	75	2	1	2	2	1	2	2	11,4	220480	000000	0	0	160981	0	0	000000	1
0796	3	43	0	0	1	1	1	2	0	09,9	250480	000000	0	0	230381	0	0	000000	1
0799	1	39	1	1	2	1	2	2	3	02,1	290480	000000	0	0	051181	0	0	000000	1
0800	6	72	2	0	3	2	1	1	0	99,9	230480	010581	3	2	310581	3	2	310581	3

FILE: CANCDATA SPSS A LIVERPOOL 4341

0805	1	38	1	1	2	0	2	2	3	99,9	090580	021081	5	3	080282	5	3	000000	2
0808	5	49	1	2	2	0	1	1	2	05,6	190580	000000	0	0	270561	0	0	000000	1
0809	5	51	0	0	2	0	2	2	0	01,1	130680	000000	0	0	160381	0	0	000000	1
0812	4	69	2	0	1	1	1	2	2	99,9	140380	000000	0	0	030382	0	0	000000	1
0813	4	39	2	2	2	1	2	2	2	99,9	220480	000000	0	0	291081	0	0	000000	1
0814	1	64	2	0	2	1	1	1	0	02,9	270580	000000	0	0	280182	0	0	000000	1
0815	1	67	2	0	2	1	1	2	1	99,9	270580	000000	0	0	170382	0	0	000000	1
0818	1	43	1	2	2	2	2	2	0	99,9	290580	000000	0	0	050682	0	0	000000	1
0819	1	43	1	2	2	1	2	1	0	03,6	290580	000000	0	0	060482	0	0	000000	1
0825	3	65	2	1	2	1	2	2	3	99,9	050680	000000	0	0	040681	0	0	000000	1
0828	7	49	1	2	2	1	2	2	0	01,0	110680	000000	0	0	030981	0	0	000000	1
0835	1	41	2	2	2	1	1	2	3	99,9	170680	000000	0	0	181182	0	0	000000	1
0836	1	43	1	2	2	2	2	2	2	00,7	170680	000000	0	0	020981	0	0	000000	1
0839	5	61	2	0	2	0	1	2	0	02,5	180680	000000	0	0	150582	0	0	000000	1
0842	1	30	1	2	2	1	2	2	0	01,0	240680	000000	0	0	010483	0	0	000000	1
0843	1	43	1	2	2	2	2	2	1	03,2	240680	000000	0	0	160682	0	0	000000	1
0846	1	59	2	1	2	1	1	2	2	00,5	010780	000000	0	0	080782	0	0	000000	1
0849	4	69	2	0	2	1	2	2	1	01,2	020780	000000	0	0	190381	0	0	000000	1
0850	1	52	2	2	2	2	2	2	2	07,0	010780	221080	2	0	111181	5	4	000000	2
0851	5	44	1	2	2	0	1	2	0	99,9	030780	060581	2	1	060582	2	0	000000	2
0853	2	31	1	2	2	1	2	2	2	99,9	110780	000000	0	0	190881	0	0	000000	1
0855	6	65	2	0	2	2	2	2	0	99,9	030780	010980	5	3	290581	5	3	290581	3
0858	2	67	2	0	3	0	2	2	0	00,5	180780	000000	0	0	010381	0	0	000000	1
0860	4	43	1	2	2	2	2	2	2	08,1	220780	000000	0	0	010881	0	0	000000	1
0861	2	44	1	1	2	1	2	2	2	99,9	220780	000000	0	0	010183	0	0	000000	1
0862	1	57	2	2	2	1	2	2	2	07,5	220780	000000	0	0	080482	0	0	000000	1
0863	1	61	2	1	3	2	2	1	1	08,7	290780	000000	0	0	160682	0	0	000000	1
0864	4	38	1	0	2	2	2	2	2	11,1	310780	060981	2	4	660182	2	4	000000	2
0865	1	32	1	0	1	1	2	2	1	02,8	010880	030581	1	0	200582	1	0	000000	2
0867	1	66	2	2	1	1	2	2	2	99,9	050880	000000	0	0	081081	0	0	000000	1
0869	1	69	2	2	2	1	2	2	1	99,9	050880	000000	0	0	031282	0	0	000000	1
0873	1	40	1	2	3	2	1	2	0	99,9	120880	120581	1	0	270482	1	0	000000	2
0874	2	65	2	0	2	2	2	2	2	01,6	130880	000000	0	0	050382	0	0	000000	1
0876	4	67	2	0	2	2	1	2	3	99,9	280780	000000	0	0	170381	0	0	000000	1
0877	1	56	2	2	2	1	1	2	2	00,7	020980	000000	0	0	250283	0	0	000000	1
0878	2	48	1	2	2	2	2	2	3	01,0	020980	000000	0	0	130482	0	0	000000	1
0882	1	70	2	2	2	1	1	2	2	13,4	170980	000000	0	0	120382	0	0	000000	1
0885	2	59	2	1	2	2	0	0	0	04,0	250980	010181	4	2	180383	4	2	000000	2
0886	7	67	2	0	2	2	2	2	1	03,6	260980	000000	0	0	010481	0	0	000000	1
0887	1	66	2	2	2	1	2	2	0	99,9	280880	000000	0	0	090981	0	0	000000	1
0889	1	60	2	0	2	1	1	1	2	99,9	040980	000000	0	0	090182	0	0	000000	1
0890	1	55	2	0	3	0	2	2	0	99,9	180980	260581	1	1	260382	1	1	000000	2
0892	1	53	2	2	3	2	1	2	0	08,0	021080	000000	0	0	230182	0	0	000000	1
0896	6	47	1	1	2	1	1	2	2	99,9	230980	230781	1	2	230782	1	2	000000	2
0898	6	67	2	0	3	1	2	2	2	01,5	081080	000000	0	0	270381	0	0	000000	1
0901	1	53	2	2	3	1	1	2	3	99,9	071080	051181	1	1	030183	5	0	030183	3
0902	1	57	2	2	2	1	1	2	0	99,9	101080	000000	0	0	240981	0	0	000000	1
0904	1	75	2	2	2	0	1	2	0	09,2	141080	000000	0	0	141081	0	0	000000	1
0906	1	49	1	2	2	2	2	1	3	00,5	151080	000000	0	0	030683	0	0	000000	1
0907	1	59	2	0	2	2	2	2	0	99,9	141080	000000	0	0	300183	0	0	000000	1
0910	1	46	2	2	1	0	1	2	1	00,5	070178	211080	1	1	111082	1	0	000000	2
0911	1	62	2	1	3	2	1	2	2	99,9	211080	270782	5	4	061283	5	4	061283	3
0914	1	64	2	2	2	1	0	0	0	99,9	281080	000000	0	0	170282	0	0	000000	1
0915	1	49	0	0	2	1	2	2	2	99,9	281080	000000	0	0	301282	0	0	000000	1
0928	1	59	2	2	2	0	1	2	0	01,8	111180	000000	0	0	111182	0	0	000000	1

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0952 1 60 2 0 3 2 1 2 2 03,4 161280 000000 0 0 161282 0 0 000000 1
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0958 1 52 2 1 3 2 0 0 2 08,2 060181 051101 5 3 010382 5 3 010382 3
0959 1 50 1 2 3 1 2 1 0 03,7 060181 000000 0 0 120882 0 0 000000 1
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Appendix I.2

Steroid Receptor Assays

A tumour tissue cytosol was prepared and high speed supernatant incubated with 10 concentrations of H^3 oestradiol-17B ranging from 200-5000 pmol/l for 16 hours @ 4°C. Similar incubations were carried out in the absence of cytosol to determine the inefficiency of separating free from bound label using charcoal. Non-specific binding was estimated at each concentration of oestrogen used in the incubation, from binding data obtained by addition of a 100 fold excess of diethylstilboestrol to the medium at three selected oestradiol concentrations. Estimates were calculated from a linear regression analysis of non-specific binding and oestradiol 17B concentration.

The "true" specific binding and concentration of available table were calculated using a computer programme, and the cytosol receptor level was derived from this data. The affinity equilibrium constant can be expressed by the Law of Mass Action.

$$\text{Constant} = Ka = \frac{[\text{oestrogen-receptor complex}]}{[\text{oestrogen}] \times [\text{receptor}]}$$

The receptor value is considered valid if this constant Ka is less than 10 litres/mol. The accepted "cut off" value of 5 fmol of specific binding per mgm of cytosol protein ensures that the probability of detection is adequate for the assay method.

PR

Progesterone receptor assays were performed using a similar technique substituting $_3H$ progesterone for $_3H$ oestradiol.

Appendix I.3

T.L.I.

Equipment and Reagents

Vacutainer Tubes

Medium RPMI 1640 T.C.M.

Subbed Slides 0.05% chrome alum.

Photographic Emulsions - AR10 stripping film (shelf life 6 months @ 4°C)

Dark Room Wratten No. 2 safelight filter
18-21°C
Rel. humidity 60-65%

Developer Kodak D19

Fixation Kodak Rapid Fix

Thymidine $_3$H Thymidine. Amersham International
1 mCi/ml Specific Activity 44C/mmol

Appendix I.3

Reproducibility Study

S.H. = The Author

R.C. = 2nd Observer (Mr. Roger Croton)

I = first count

II = second count

$$\text{Proportion} = \frac{\text{NO. OF LABELLED NUCLEI}}{\text{TOTAL NO. TUMOUR NUCLEI}}$$

T.L.I. = Thymidine Labelling Index (%)

	S.H.^{I}		R.C.		S.H.^{II}		% Observer Error	
Tumour No.	T.L.I.	Proportion	T.L.I.	Proportion	T.L.I.	Proportion	Inter	Intra
958	8.2	$^{164}/_{2000}$	4.07	$^{141}/_{2000}$	3.5	$^{70}/_{2000}$	33.6	40.2
1102	3.5	$^{70}/_{2000}$	6.3	$^{126}/_{2000}$	4.5	$^{90}/_{2000}$	16.7	12.5
1244	20.7	$^{414}/_{2000}$	16.7	$^{401}/_{2400}$	13.4	$^{269}/_{2000}$	10.8	21.2
1252	6.8	$^{136}/_{2000}$	7.05	$^{141}/_{2000}$	8.3	$^{166}/_{2000}$	8.14	9.93
1277	2.8	$^{56}/_{2000}$	3.0	$^{60}/_{2000}$	3.7	$^{63}/_{1700}$	10.5	13.9
1282	3.7	$^{74}/_{2000}$	1.9	$^{38}/_{2000}$	4.17	$^{50}/_{1200}$	37.4	5.93
1313	2.4	$^{48}/_{2000}$	4.85	$^{97}/_{2000}$	2.95	$^{59}/_{2000}$	24.4	10.3
1169	5.6	$^{112}/_{2000}$	5.6	$^{117}/_{2100}$	4.7	$^{97}/_{2000}$	6.92	7.18
1231	6.9	$^{138}/_{2000}$	7.75	$^{155}/_{2000}$	6.9	$^{138}/_{2000}$	5.80	0
1248	8.7	$^{174}/_{2000}$	3.42	$^{72}/_{2100}$	4.1	$^{84}/_{2000}$	8.75	34.9
1278	11.9	$^{238}/_{2000}$	7.19	$^{140}/_{2100}$	8.25	$^{165}/_{2000}$	10.6	18.1
1272	0.6	$^{12}/_{2000}$	1.4	$^{28}/_{2000}$	1.2	$^{6}/_{500}$	7.69	33.3
1305	4.8	$^{96}/_{2000}$	7.6	$^{152}/_{2000}$	5.05	$^{101}/_{2000}$	20.2	2.53
1310	3.5	$^{70}/_{2000}$	4.5	$^{90}/_{2000}$	3.7	$^{74}/_{2000}$	9.76	2.78
1319	8.7	$^{174}/_{2000}$	9.5	$^{180}/_{2000}$	8.2	$^{164}/_{2000}$	4.65	2.96
1333	0	$^{0}/_{2000}$	0	$^{9}/_{2000}$	0	$^{0}/_{2000}$	0	0
1168	3.8	$^{76}/_{2000}$	6.75	$^{135}/_{2000}$	6.4	$^{128}/_{2000}$	2.66	25.5
1210	0	$^{0}/_{2000}$	0	$^{0}/_{2000}$	0	$^{0}/_{2000}$	0	0
1218	5.8	$^{116}/_{2000}$	9.3	$^{186}/_{2000}$	6.95	$^{139}/_{2000}$	23.6	9.02
1219	7.96	$^{199}/_{2500}$	9.95	$^{199}/_{2000}$	8.04	$^{201}/_{2500}$	10.6	0.5

Mean Inter observer error = 12.6 S.D. 16.7 SE 3.71

Mean Intra observer error = 12.6% S.D. 23.4 SE 5.25

Appendix II.1

Vacutainers — Silicone coated. 100 x 13mm. Becton Dickinson, Heylan 38240, France.

Tissue Culture Medium — RPMI 1640 + 1% L. Glutamine < ampicillin 400 ng/ml, streptomycin 200 Ug/ml. Flow Laboratories Ltd.

Tamoxifen — I.C.I. Ltd., Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire.

Adriamycin — Farmitalia Carlo Erba.

Perspex Clamps — Bioengineering Department, Royal Liverpool Hospital

Uridine — ^{3}H Uridine Specific Activity 45 Ci/mmol. Max. disintegration @ $2^{o}C$ = 6% per month. Amersham International PLC.

Homogeniser — Polytron P.T.10 Kinematics CM 6010 Kriends, Switzerland.

Filter Paper — Millipore AP40 glass fibre. Millipore S.A. Holsheim, France.

Centrifuge — Beckman TJ6.

Scintillation fluid — Scintran. Toluene scintillation fluid. B.D.H. Chemicals Ltd., Poole, Dorset.

Scintillation Counter — Pye Unicam SP1800

Appendix II.2
Lowry Protein Assay (Lowry 1951)

(1) Solution A = 1ml 2% sodium potassium tartrate
+ 1ml aqueous copper sulphate solution.
+ 50ml 2% anhydrous sodium carbonate in 0.1 molar sodium hydroxide.

(2) Solution B = 50% aqueous folic indicator

(3) Solution C = Standard protein = 1mg/ml. Bovine serum albumin in phosphate buffered saline.

Using serial protein dilutions a standard protein curve is calibrated

Tube	1	2	3	4	5	6	7
H_2O ml	0.25	0.24	0.225	0.20	0.175	0.15	0.125
CµL	-	10	25	50	75	100	125
A ml	2.5	2.5	2.5	2.5	2.5	2.5	2.5
B ml	0.25	0.25	0.25	0.25	0.25	0.25	0.25

Each sample vortexed after addition of indicator - leave for 1 hour.

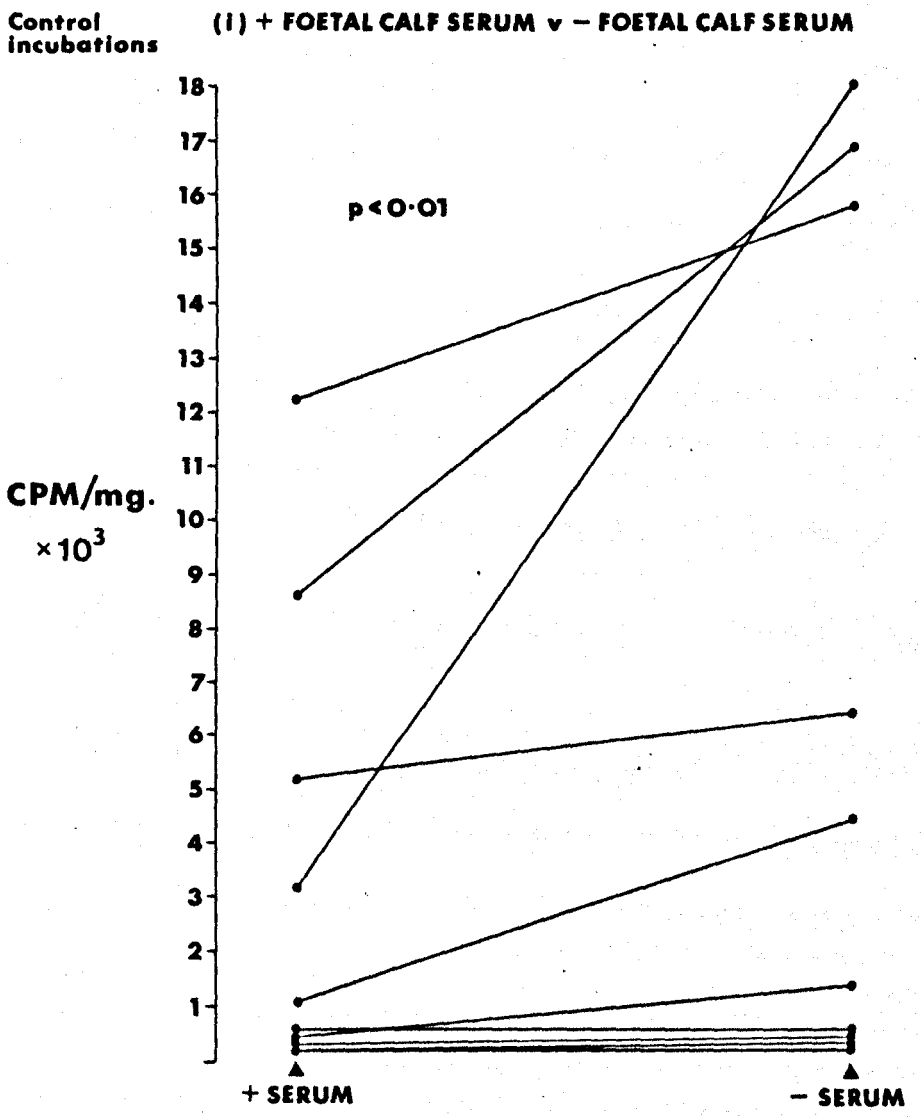
λ of each sample produced from spectrophotometer (using blanks to zero machine).

Appendix II.3

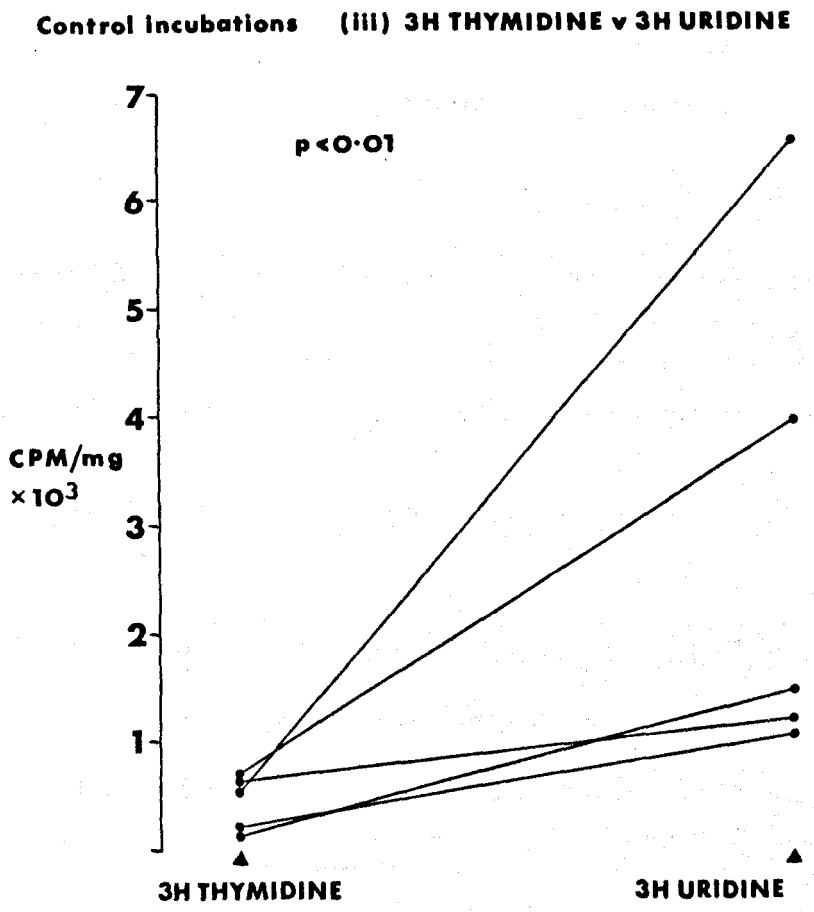
		cpm/mg ± S.D.	
(iii)	Tumour	^{3}H Thymidine	^{3}H Uridine
	C1	235 ± 31.9	1017 ± 230
	C2	702 ± 80.0	4467 ± 1929
	C3	643 ± 386	1222 ± 257
	1467	567 ± 384	6549 ± 908
	1472	184 ± 35.7	1494 ± 916
		+ Calf Serum	- Calf Serum
(i)			
	1386	1073 ± 393	4431 ± 414
	1387	3087 ± 144	23669 ± 6308
	1390	5127 ± 2438	6474 ± 3201
	1391	430 ± 61.2	1434 ± 182
	1398	211 ± 108	395 ± 6.4
	1482	531 ± 241	626 ± 154
	1483	12197 ± 2031	15765 ± 4597
	1484	360 ± 106	457 ± 18.0
	1485	8610 ± 2745	16921 ± 5360
	1400	378 ± 19.8	395 ± 6.36
		Hyperbaric	Non Hyperbaric
(iv)	256	269 ± 61.1	1297 ± 323
	M.S.	609 ± 122	893 ± 128
	1379	3861 ± 1420	1477 ± 173
	1445	1934 ± 242	5088 ± 2743
	1449	464 ± 151	617 ± 127
	1454	936 ± 333	2709 ± 1172
	1455	2915 ± 1147	10624 ± 6872
	J.R.	777 ± 71.5	1039 ± 160
	M.B.	890 ± 26.9	1388 ± 485

(v)

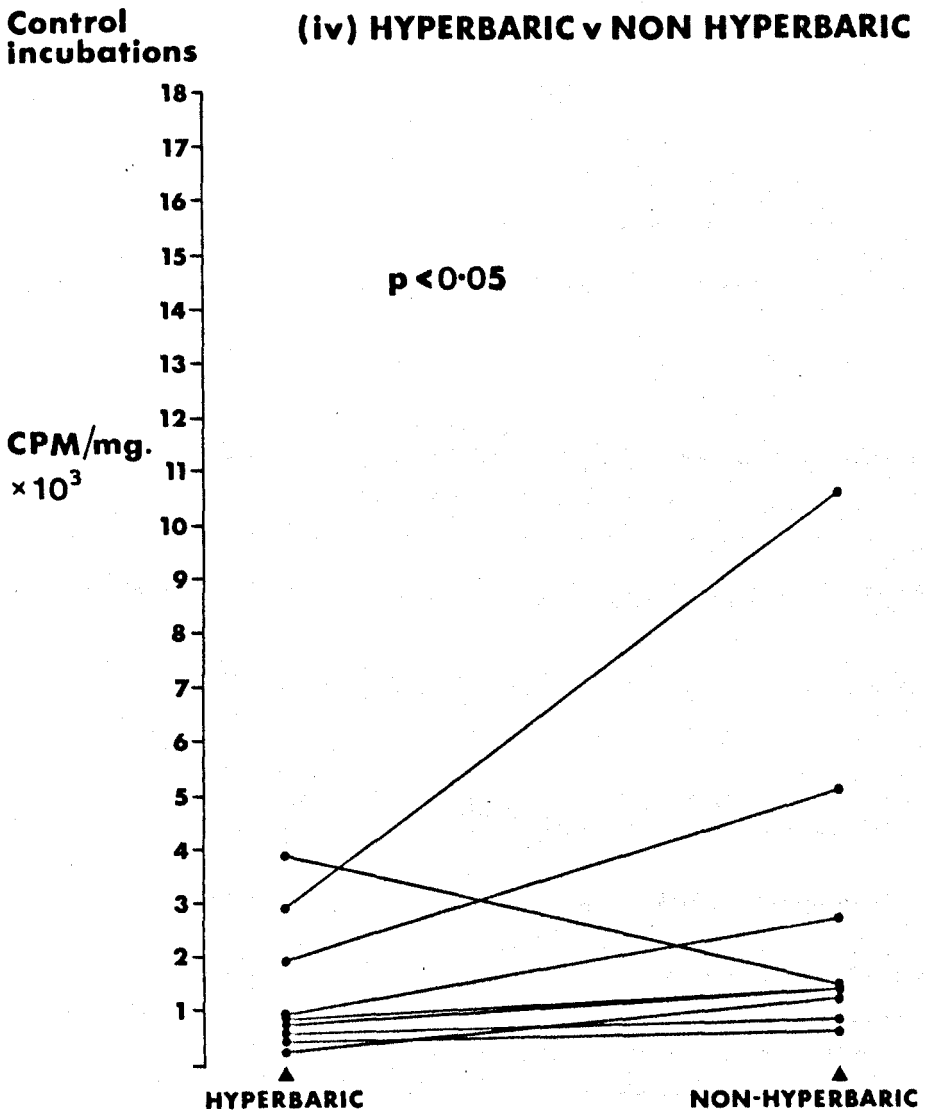
Tumour	Centre	Periphery
1462	110 ± 8.25	101 ± 31.6
1463	2882 ± 2441	2601 ± 311
1465	12606 ± 797	11380 ± 2394
1514	3490 ± 434	3606 ± 605
1518	1080 ± 15.4	1485 ± 50.8



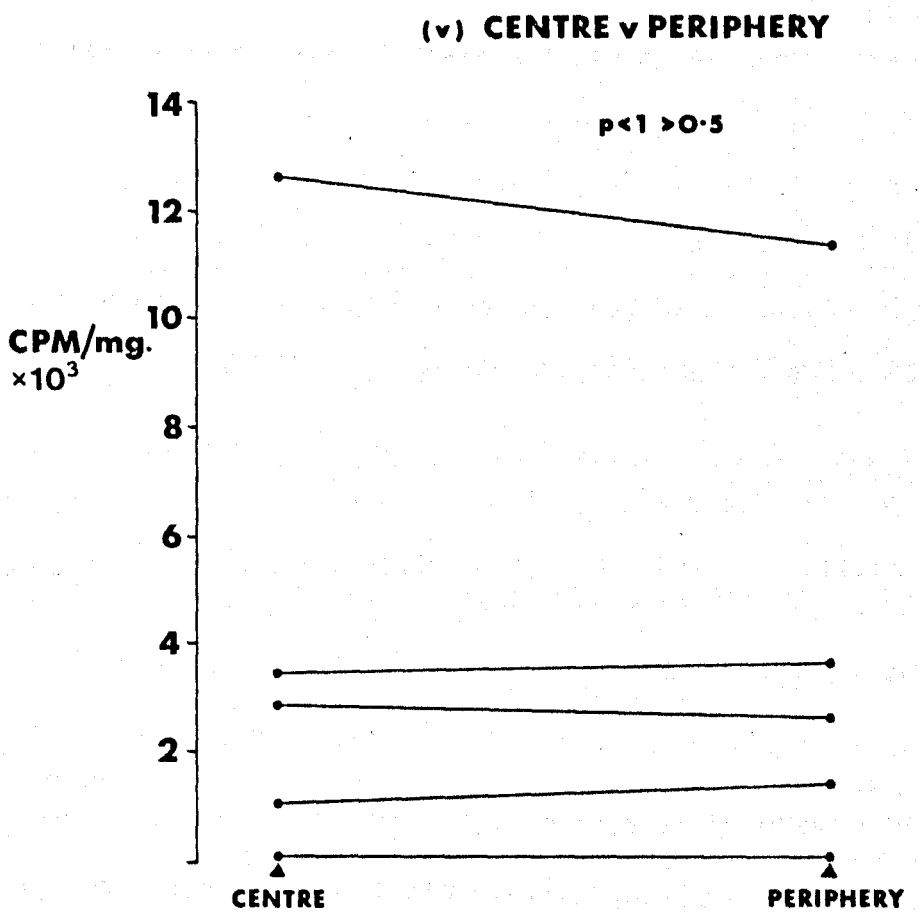
Appendix II.3



Appendix II.3



Appendix II.3



Appendix II.3

Appendix II.4

Interpretation of Results

(a) Clinical Response

Standard criteria must be used to objectively assess response to therapy in advanced breast cancer. Subjective information with regard to symptoms and morbidity may be of paramount importance when assessing whether a treatment is worthwhile - but this subjective response is influenced by the reaction of the patient to the treatment and has not been considered in this particular study when relating the in vitro to clinical findings.

Assessment criteria vary - the UICC criteria (Hayward, 1977) require a remission interval of only 4 weeks, whereas the British Breast Group (1974) recommend 6 months. In a recent study (Campbell, 1983), 61 women with advanced breast cancer were assessed for response at both 2 months and 6 months. Of 11 responders at 6 months, all had been considered responders at 2 months. However 8 women thought to have responded at 2 months had relapsed by 6 months.

In this study I have used the UICC criteria of response maintained for at least 3 months. Response includes both complete and partial response as defined.

Complete Response

Disappearance of all known disease. In the case of lytic bone metastases these must have been shown radiologically to have calcified.

Partial Response

> 50 percent decrease in measurable lesion and objective improvement in evaluable, but non-measurable lesions. No new lesions.

Treatment failure

Adjudged by unchanged lesion or progressive disease.

(b) In-vitro inhibition

In Vitro Results	I =	Inhibition = > 25% suppression of uridine uptake
	NI =	No inhibition = <25% suppression of uridine uptake
Clinical Response (UICC)	R =	Complete or partial response
	CR =	Complete response
	PR =	Partial response
	NR =	No response
	† =	Death

RESULTS *Studies 1 and 2. Overall rates of In Vitro Inhibition and ER status*

Study Number	Stage	ER	cpm/mg ± S.D. Control	Tamoxifen Tx	Adriamycin Ad	% Control Tx	% Control Ad	Result Tx	Result Ad
976	$T_4N_1M_0$	+ve	9487 ± 955	6136 ± 722	6205 ± 326	65	65	I	I
145	Local recurrence	+ve	2620 ± 362	2384 ± 262		91		NI	
787	$T_4N?M_0$	-ve	1211 ± 323	444 ± 276		37		I	
810	$T_3N_0M_1$	+ve	1625 ± 82	1511 ± 96	406 ± 70	93	25	NI	I
821	$T_4N?M_0$	+ve	2358 ± 620	871 ± 84	540 ± 12	40	25	I	I
870	$T_4N_1M_0$	+ve	745.4 ± 72	1821 ± 92		244		NI	
881	$T_4N?M_0$	-ve	2756 ± 64	2122 ± 112		77		NI	
910	Local recurrence	+ve	5924 ± 102	3504 ± 272		59		I	
1041	Lymph node recurrence	-ve	868 ± 252	201 ± 27.6		23		I	
1044	T_4	-ve	2982 ± 313	2171 ± 161		73		I	
1056	Local recurrence	+ve	313 ± 278	328 ± 342	49.0 ± 13	105	16	NI	I
1070	$T_2N?M_0$	-ve	3454 ± 203	3824 ± 380		111		NI	
1075	$T_2N_1M_0$	?	1091 ± 522	1460 ± 374		134		NI	
1083	$T_4N?M_0$	+ve	604 ± 108	655 ± 81.4		108		NI	
1102	$T_3N?M_0$	+ve	1141 ± 4260	7477 ± 3812		65		I	
1104	$T_3N_1M_0$	-ve	443 ± 143	296 ± 24.6		67		I	
1106	$T_2N?M_0$	-ve	5466 ± 3421	1369 ± 819	1493 ± 1079	25	27	I	I
1116	$T_3N_1M_0$	-ve	911 ± 378	780 ± 131	635 ± 154	86	70	NI	I
1137	$T_2N_0M_0$	-ve	237 ± 228	510 ± 402		215		NI	
1139	$T_3N_1M_0$	-ve	1156 ± 999	781 ± 410	960 ± 196	67	83	I	NI

Study Number	Stage	ER	cpm/mg ± S.D. Control	Tamoxifen Tx	Adriamycin Ad	Control Tx	Control Ad	Result Tx	Result Ad
1141	$T_2N_0M_0$	-ve	154 ± 9.31	155 ± 93.1		101		NI	
1145	$T_4N?M_0$	+ve	1111 ± 309	634 ± 438	1891 ± 341	57	170	I	NI
1146	$T_4N?M_0$	-ve	352 ± 98.0	353 ± 24.0	241 ± 98.7	100	68	NI	I
1152	Lymph node recurrence	+ve	462 ± 111	332 ± 177	596 ± 251	72	129	I	NI
1168	$T_4N_1M_1$	+ve	325 ± 216	545 ± 97.8	239 ± 239	168	73	NI	I
1169	$T_4N?M_0$	+ve	375 ± 158	268 ± 125	276 ± 105	71	74	I	I
1200	$T_2N_0M_0$	+ve	475 ± 9.14	44.7 ± 8.27	42.9 ± 9.26	94	90	NI	NI
1210	Local recurrence	?	25.5 ± 3.61	27.7 ± 6.55	29.3 ± 6.60	109	115	NI	NI
1211	$T_3N_1M_0$	+ve	35.1 ± 8.51	47.4 ± 7.21	20.4 ± 6.39	135	58	NI	I
1215	$T_4N_1M_0$	+ve	127 ± 10.5	90 ± 13.4	124 ± 57.9	71	98	I	NI
1216	Lymph node recurrence	-ve	93.0 ± 19.8	153 ± 12.8	168 ± 23.4	164	181	NI	NI
1218	$T_4N_1M_0$	+ve	190 ± 80.4	122.5 ± 6.90	147 ± 48.7	64	77	I	NI
1229	$T_3N_1M_0$	+ve	338 ± 205	323 ± 55.2	379 ± 27.5	96	112	NI	NI
1231	Local recurrence	+ve	242 ± 19.6	422 ± 156	242 ± 16.6	174	100	NI	NI
1244	$T_4N_1M_0$	+ve	577 ± 333	322 ± 34.9	1066 ± 21.4	56	185	I	NI
1248	$T_2N_0M_0$	+ve	117 ± 16.3	149 ± 13.0	166 ± 17.1	127	142	NI	NI
1251	$T_4N_1M_0$	+ve	423 ± 311	244 ± 7.0	595 ± 23.3	58	140	I	NI
1252	$T_2N?M_0$	?	1177 ± 99.8	928 ± 272	2611 ± 667	79	221	NI	NI
1261	Nodal recurrence	-ve	1501 ± 202	394 ± 18.2	1057 ± 158	26	70	I	I
1272	$T_3N?M_0$	?	1981 ± 738	1323 ± 553	1138 ± 82.7	67	57	I	I
1275	$T_4N?M_0$	-ve	812 ± 163	988 ± 126	1076 ± 185	121	132	NI	NI

Study Number	Stage	ER	cpm/mg ± S.D. Control	Tamoxifen Tx	Adriamycin Ad	Control Tx	Control Ad	Result Tx	Result Ad
1278	Local Recurrence	+ve	155 ± 8.25	297 ± 178	293 ± 23.8	192	189	NI	NI
1282	$T_2N_0M_0$	+ve	179 ± 17.8	164 ± 14.6	188 ± 14.6	92	105	NI	NI
1305	$T_3N?M_0$	-ve	1831 ± 226	1854 ± 349	954 ± 225	101	52	NI	I
1310	$T_2N_0M_0$	+ve	2476 ± 194	3153 ± 1738	3900	127	157	NI	NI
1312	$T_3N_1M_0$	+ve	3493 ± 944	2319 ± 68.1	1962 ± 57.0	66	56	I	I
1323	Nodal Recurrence	?	1665 ± 259	854 ± 177		51		I	
1333	Local Recurrence	+ve	1151 ± 17.0	1111 ± 47.1	1207 ± 5.87	96	105	NI	NI
1335	$T_4N_1M_1$	-ve	3107 ± 183	3620 ± 2130	2521 ± 346	116	81	NI	NI
1336	Local Recurrence	-ve	2004 ± 188	530 ± 79.9	1327 ± 698	26	66	I	I
1337	$T_2N_0M_0$	?	823 ± 300	499 ± 5.48	599 ± 184	61	73	I	I
1339	$T_2N?M_0$	-ve	822 ± 263	1114 ± 134	1411 ± 211	135	172	NI	NI
1343	$T_2N_0M_0$	-ve	628 ± 104	535 ± 72.9	593 ± 33.9	85	94	NI	NI
1344	Local Recurrence	+ve	153 ± 60.4	219 ± 128		143		NI	
1347	$T_4N_0M_0$	+ve	2118 ± 1660	377 ± 64.4	1283 ± 222	18	61	I	I
1352	$T_2N?M_0$	-ve	617 ± 27.9	624 ± 237	664 ± 312	101	62	NI	I
1353	$T_4N?M_0$	+ve	161 ± 39.8	152 ± 29.9	168 ± 60.9	94	104	NI	NI
1364	Local Recurrence	-ve	472 ± 57.3	273 ± 20.4	318 ± 63.0	58	67	I	I
1366	$T_4N_1M_0$	?	1384 ± 455	978 ± 185	1281 ± 379	71	89	I	NI
1367	$T_3N?M_0$	+ve	146 ± 59.1	187 ± 82.6	277 ± 15.9	128	155	NI	NI
1368	$T_2N_0M_0$	-ve	19592 ± 2158	25314 ± 3026	6112 ± 67.3	129	31	NI	I
1375	$T_4N?M_0$	?	4274 ± 682	7292	15251 ± 1093	170	356	NI	NI
1376	$T_3N_0M_0$	?	6272 ± 1435	3667 ± 1271	3231 ± 2259	58	51	I	I
1377	$T_2N_0M_0$	?	536 ± 15.2	1178 ± 539	822 ± 88.0	220	153	NI	NI

Study Number	Stage	ER	cpm/mg ± S.D. Control	Tamoxifen Tx	Adriamycin Ad	Control Tx	Control Ad	Result Tx	Result Ad
1380	Local Recurrence	?	514 ± 28.0	433 ± 29.2		84		NI	
1381	$T_2N_0M_0$	?	4677 ± 3536	5982 ± 578	2707 ± 313	126	58	NI	I
1382	$T_2N?M_0$	?	468 ± 111	722 ± 319	434 ± 127	154	93	NI	NI
1386	$T_3N?M_1$	?	4431 ± 414	5979 ± 5866	5374 ± 3425	135	121	NI	NI
1387	$T_4N?M_0$	?	23669 ± 6308	30374 ± 8339	14645 ± 2616	128	62	NI	I
1389	$T_4N_0M_0$	?	1359 ± 738	916 ± 430	922 ± 16.6	67	68	I	I
1390	$T_3N_0M_0$	?	6474 ± 3201	5632 ± 1638		87		NI	
1391	Local Recurrence	?	1434 ± 182	1536 ± 272	824 ± 120	107	57	NI	I
1392	$T_4N_1M_0$	?	3510 ± 1342	4150 ± 1491	3337 ± 260	118	95	NI	NI
1405	$T_4N?M_0$	?	5263 ± 1964	4947 ± 247		94		NI	
1416	$T_4N?M_0$	?	1188 ± 154	1234 ± 449	773 ± 158	104	65	NI	I
1418	$T_2N_1M_0$	?	2755 ± 799	3807 ± 1132	1506 ± 108	138	55	NI	I
1435	Nodal Recurrence	?	285 ± 103	243 ± 45.9	219 ± 14.8	85	77	NI	NI
1440	$T_4N?M_1$	?	120 ± 10.6	92 ± 3.4	70 ± 10.0	77	58	NI	I

Study 3 *Relationship of In Vitro Inhibition to Clinical Response to Tamoxifen*

Study Number	Stage	MP Status	ER	In Vitro	Clinical		Follow up
976	$T_4N_1M_0$	Post	+	I	R	PR $^{18}/_{12}$. Local progression and pleural effusion	$^{24}/_{12}$
145	Local Recurrence	Post	+	NI	NR	Disease static $^{12}/_{12}$ progression	$^{27}/_{12}$
787	$T_4N?M_0$	Post	-	I	R	PR	$^{14}/_{12}$
810	$T_3N_1M_1$	Post	+	NI	NR	NR $^{2}/_{12}$	† $^{2}/_{12}$
821	$T_4N_0M_0$	Post	+	I	R	PR $^{16}/_{12}$ multiple bone 2^os	† $^{16}/_{12}$
870	$T_4N_0M_0$	Post	+	NI	NR	Tumour unchanged	$^{26}/_{12}$
881	$T_4N_1M_0$	Post	-	NI	NR	NR Bone and liver 2^os	† $^{4}/_{12}$
910	Local Recurrence	Post	+	I	NR	Disease progression	$^{24}/_{12}$
1041	Nodal Recurrence	Post	-	I	R	CR	$^{15}/_{12}$
1044	$T_4N?M_1$	Post	-	I	NR	Progression of bone and lung 2^os	$^{4}/_{12}$
1083	$T_4N?M_1$	Post	+	NI	R	Local response. Bone 2^os unchanged	$^{18}/_{12}$
1145	$T_4N?M_0$	Post	+	I	R	PR	† $^{9}/_{12}$
1146	$T_4N?M_0$	Post	-	NI	R	PR	† $^{9}/_{12}$
1152	Nodal Recurrence	Post	+	I	NR	No change $^{9}/_{12}$. Progression	$^{17}/_{12}$
1168	$T_4N?M_1$	Post	+	NI	R	PR local disease. Bone 2^os unchanged	$^{12}/_{12}$

Study Number	Stage	MP Status	ER	In Vitro	Clinical		Follow up
1169	$T_4N?M_0$	Post	+	I	R	PR $^{6}/_{12}$. Local progression + bone 2^{o}s	$\dagger^{20}/_{12}$
1216	Nodal Recurrence	Pre	-	NI	NR	NR liver, lung, bone 2^{o}s	$\dagger^{18}/_{12}$
1218	$T_4N_1M_0$	Post	+	I	R	PR $^{4}/_{12}$. Progression. Bone 2^{o}s	$^{17}/_{12}$
1231	Local Recurrence	Post	+	NI	NR	Progressive local disease. Lung 2^{o}s	$^{10}/_{12}$
1275	$T_4N?M_0$	Post	-	NI	NR	Local progression	$^{5}/_{12}$
1282	$T_4N_0M_0$	Post	+	NI	R	PR	$^{13}/_{12}$
1323	Local Recurrence	Post	+	I	R	PR	$^{6}/_{12}$
1333	Nodal recurrence + bone 2^{o}s	Post	+	NI	NR	Bone 2^{o}s	$^{7}/_{12}$
1336	Local recurrence	Pre	-	I	R	CR	$^{11}/_{12}$
1353	$T_4N?M_0$	Post	+	NI	NR	NR	$^{3}/_{12}$
1364	$T_4N?M_0$	Post	-	I	NR	Progression	$^{12}/_{12}$
1375	$T_4N?M_0$	Post	-	NI	NR	NR	$^{12}/_{12}$

Study 4 *Relationship of In Vitro Inhibition to Clinical Response to Adriamycin*

Study Number	Stage	Meno-pause	ER	In Vitro	Clinical	
822	$T_4N_0M_0$	Pre	?	I	R	NR Tx $^{3}/_{12}$: V.A.C. $^{9}/_{12}$. PR $^{12}/_{12}$ †carcinomatosis 3 years.
1056	Local Recurrence	Post	+ve	I	R	V.A.C. $^{6}/_{12}$ PR $^{18}/_{12}$ † Pulmonary mets. 3 years.
1251	$T_4N_1M_0$	Post	+ve	NI	NR	V.A.C. NR Local progression + Pulmonary mets. † $^{17}/_{12}$
1278	Local Recurrence	Pre	+ve	NI	NR	V.A.C. $^{5}/_{12}$ NR Bone 2^{o}s † 2 years
1353	$T_4N?M_0$	Post	+ve	NI	NR	Tx $^{3}/_{12}$ NR V.A.C. $^{5}/_{12}$ NR † $^{5}/_{12}$ carcinomatosis
1375	$T_4N?M_0$	Post	?	NI	R	Tx $^{3}/_{12}$ NR V.A.C. $^{6}/_{12}$ PR Toilet mastectomy A + W $^{12}/_{12}$
1386	$T_3N?M_1$	?	?	~~4~~ I	R	Tx $^{3}/_{12}$ NR $^{6}/_{12}$ PR ~~d~~ Bone pain. Sclerosis bone 2^{o}s.

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