LONG TERM SURVIVAL IN BREAST CANCER

A thesis submitted in fulfilment of the requirement for the degree of Master of Surgery of the University of London.

by

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March 1984

ABSTRACT

It has been estimated that 60% of the women who survive ten years after initial therapy for breast cancer will nevertheless die of recurrent disease, but previous reports suggest that the rate of dying is independent of tumour associated variables such as tumour size, stage and lymph node status (Mueller & Jeffries 1975, Langland et al 1979). The aim of this thesis was to investigate tumour, and host related factors that might influence long term survival (of ten years or more) in order to identify patients still at risk, and assess whether a biological basis exists for further therapy.

In the first section of the thesis in which tumour associated factors were studied, it was demonstrated that only tumour fixation and pathological lymph node status had a measurable impact on survival beyond ten years, and that this influence progressively diminished. There was no basis on which to found a prognostic index for long term survival.

In the second section of the thesis, the endocrine status of the host was investigated in a study of serum oestrogens, oestrogen binding, androgens, prolactin and thyroid function in a large cohort of long term survivors, comparing them to patients with early breast cancer and normal women, as controls. Although several significant differences in

endocrine status were observed, there was no substantial evidence that long term survival is influenced by the endocrine status of the host.

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ACKNOWLEDGEMENTS

The experimental studies described in this thesis were carried out at Charing Cross Hospital Medical School, and in the Department of Clinical Endocrinology, Imperial Cancer Research Fund. I am indebted to my sponsors, Mr. J. I. Burn and Dr. J. Bulbrook, and also Mr. T. Cooke, for their support, enthusiasm and patience, without which this study would not have been possible.

I am grateful to the many surgeons and radiotherapists who permitted me access to their patients at Charing Cross, Wembley and Croydon Hospitals, but in particular to Mr. J. L. Hayward who allowed me honorary Registrar status within the Breast Unit, Guy's Hospital, and access to patients and computerised records, as well as invaluable assistance in setting up the study.

My thanks are due also to the staff at Imperial Cancer Research Fund, notably Dennis Wang, John Moore and Brian Thomas for teaching me research methods and offering critical advice, to Jack Cusick for statistical assistance, and to Angela Mott for help with computing.

The thesis was typed with great patience by Wendy Wallace and Maggie Lane on a Philips word processor. I am indebted to Alison Grant for the artwork.

Lastly, and by no means least, I thank my wife, Teresa, who has endured my many crises and provided unfaltering support.

Part of the work was completed whilst I was in possession of a Governor's Scholarship from the Trustees of the Charing Cross Hospital Medical School.

ARRANGEMENT OF THE THESIS

This thesis is presented in two sections. Section one, which commences with a general introduction, is concerned with the tumour and its influence on long term survival in breast cancer. The relevant literature, experimental methods, results and discussion are incorporated within each chapter. Section two is concerned with the endocrine status of the host and long term survival. Within this section, each chapter is divided into two parts, the first of which contains a literature review, and the second, the results and discussion of the study. This section ends with the general conclusion integrating the entire thesis.

Details of patients and experimental methods are given in appendices to each chapter prior to a list of references that have been cited within the text.

CHAPTER 1

INTRODUCTION

1.1 Introduction

Breast cancer is the commonest malignancy of women in the United Kingdom, with an incidence rate that is still rising. The conventional management of early breast cancer has failed to show any dramatic improvement in recent decades, and it seems likely that no further improvement in survival rates will be attained until there is a greater understanding of the biology of the disease.

1.2 Survival in breast cancer

Approximately 50% of women with breast cancer die within ten years, but the majority of the remainder nevertheless still die of their disease, albeit twenty, or even thirty years later.

Survival in cancer is based upon the interrelationship of the biology of the tumour with that of the host. In breast cancer the factors associated with the tumour include its growth rate, the ability to metastasise, the production of biologically active substances, and the possession of steroid hormone receptors. The tumour associated factors have been extensively investigated in relation to short term survival, and are reflected in clinical and pathological staging systems, and prognostic indices.

In contrast, the biology of the host has been relatively little investigated. In theory the immunocompetence of the host is likely to influence survival, but in practice this has been difficult to demonstrate. Hager concludes a comprehensive review of immune defence in mammary cancer with 'much of the data remains weak and inconclusive' (Hager and Heppner 1981). There are many epidemiological observations to suggest that development of breast cancer is influenced by hormonal mechanisms, and survival in breast cancer may too, be influenced by the endocrine status of the host. Thirdly, Stoll has suggested that host defence may be modified by physical or psychological stress (Stoll 1976).

1.3 Aim of thesis

Although there are few studies on long term survival in breast cancer it has been proposed that tumour associated factors such as tumour size and stage are unrelated to survival beyond ten years (Langlands et al 1979). This implies that host defence may be relatively more important in long term survival, and therapy aimed at stimulating such defence, for example by endocrinological means, might result in worthwhile improvement in overall survival.

The aim of this thesis is to investigate the biology of long term survival in breast cancer both by a study of the biology of the tumour, and of that of the host.

1.4 The biology of the tumour

The thesis is composed of two sections. Section One contains a review of previous reports on the pattern of survival, and curability of breast cancer. A study is presented of the influence of tumour associated prognostic variables on survival beyond ten years, in a large cohort of breast cancer cases with extended clinical follow-up. A further tumour associated factor is investigated in a study of the relationship of tumour carcinoembryonic antigen to other prognostic variables, and to long term survival.

1.5 The biology of the host

In the second section of this thesis the biology of the host is investigated in a study of the hormonal status of a large cohort of long term survivors from breast cancer, comparing them with normal women, and with patients with early breast cancer, as controls.

Due to the lack of a satisfactory method for studying cell mediated immunity in a predominantly elderly population, many of whom have additionally received either radiotherapy or chemotherapy, no attempt has been made in this study to investigate immunocompetence in long term survival. It is recognised that it may well have an important role in host defence.

CHAPTER 2

SURVIVAL IN BREAST CANCER

2.1 Introduction

The pattern of survival in breast cancer is unique. Although late metastases are reported for many carcinomas, their frequency in breast cancer is such that the risk of dying of the disease, even 15 to 20 years following apparently successful treatment, is still twenty times that of the normal population (Langlands et al 1979).

In many diseases curability may be assessed by fixed term survival rates at, for example, 5 or 10 years, but it is apparent that for a disease with a force of mortality still being exerted at 20 years or more after diagnosis, fixed term survival rates are clearly inadequate.

This chapter discusses the methods of analysis that are available and the results of studies of long term survival and curability in breast cancer.

2.2 Statistical Methods

Traditionally, survival has been measured in terms of fixed interval mortality rates, generally at 5 or 10 years. However fixed interval mortality rates do not take into account incomplete data on patients still alive, or lost to follow-up, and nor can they be related to single variables, without control of the others. Fixed interval mortality rates are most appropriate when survival is short and when the majority of patients under study have died; they are least appropriate when survival is long, and in the presence of a high incidence of late metastases, as is the case in breast cancer.

In order to study the curability of breast cancer, or the impact of independent variables on survival, more complex methods of analysis are required.

A life table for survival is derived from a determination of the annual mortality rates during each year under study. Patients may be entered and observed for varying lengths of time, but at the end point of the study all patients are accounted for, whether alive, dead or lost to follow up. When calculating the annual mortality rate an adjustment is made for those alive or withdrawn during that period. From this table, may be calculated cumulative survival rates, and estimates of probability using the log-rank test (Peto et al 1977).

A log rank test involves assessing the number of observed deaths in each group, and comparing it with the extent of exposure to risk of death in that group. If there is no survival advantage between two groups the observed deaths will equal the exposure to risk of death. The log-rank test compares observed deaths and extent of exposure (often called expected deaths) for each group and a chi-squared test may then be performed to obtain a level of significance.

A life table is therefore an appropriate method for survival analysis in breast cancer, although several problems can arise:

1. Errors of death certification.

A survey of the causes of death in 14,000 cases revealed that 20% of the deaths were wrongly attributed to cancer. This survey related only to hospital deaths and the figure is likely to be larger still if G.P. certification were included. Information from post-mortems is rarely available.

2. Extended follow-up

Because of the pattern of survival in breast cancer, follow-up periods in excess of 20 years are required if the survival of early cancer is to be studied. Such follow up is costly in terms of administrative expense and the paucity in numbers of patients with long term survival leads

to difficulties in statistical interpretation, requiring that very large numbers of patients be entered into a study.

3. Advancing Age

The survivors tend to be predominantly elderly, and thus an allowance has to be made for death from other causes. This is generally done by means of age-adjusted life tables based on age related mortality data from the Registrar General.

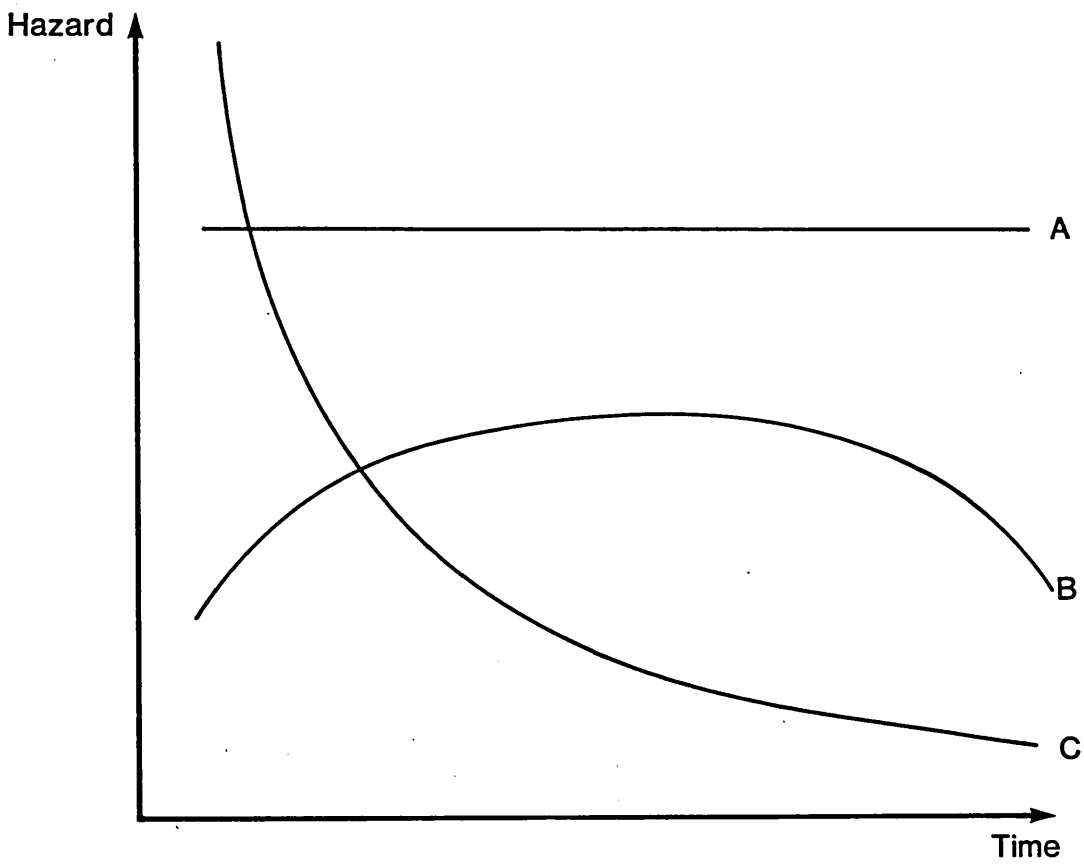
The pattern of survival may then be assessed from the life table by plotting cumulative probability of survival with time. If the distribution is exponential then a plot of log survival versus time will give a straight line. Alternatively if the distribution is log normal a plot of survival versus time on log probability paper will similarly yield a straight line. The problem with this method is the ease with which a straight line may be drawn irrespective of whether this is a correct interpretation of the data.

An alternative method of analysis is the hazard function, or force of mortality. This is the probability of a patient dying during a specified interval given that the patient has survived to the commencement of that interval. The interval is often taken to be one year and the hazard function may then be calculated as either an annual hazard rate, or as a cumulative hazard function.

The hazard function is able to distinguish reliably between different distributions. If survival is exponential then the force of mortality or hazard function is constant and is represented by a straight line whereas decreasing hazard represents a Weibull distribution for survival [see Fig 2.1].

Figure 2.1

Types of Hazard Function



	Hazard Function	Survival Distribution
A	Constant	Exponential
B	Parabolic	Log normal
C	Decreasing	Weibull

2.3 Survival in Breast Cancer

As early as 1926, Major Greenwood in "Natural Duration of Cancer" observed that the 'time specific risk of death' increased during the first three years following diagnosis and treatment, but declined slowly thereafter (Greenwood 1926). The time specific risk of death, or hazard function as it is now known, has been described in the previous section. Greenwood's observation that it changes with unit time was ignored for over 50 years.

Boag proposed a log normal distribution for survival based on his assumption that the interval between diagnosis and death from cancer was not modified by unsuccessful treatment (Boag 1949).

Berkson and Gage analysed a large series of breast cancer patients from the Mayo clinic observing that 50% of non-metastatic cases survived 15 years, whereas only 18% of cases with metastases did so. They suggested an exponential model of survival as it provided a good fit over 15 years (Berkson & Gage 1952). Haybittle disputed the assumptions made in these last two studies and proposed an extrapolated actuarial model that was fitted to the data on 704 patients diagnosed in the Cambridge area between 1947 and 1950 (Haybittle 1959, 1965). Haybittle noted that the model overestimated deaths in the first year, but underestimated them in the second year of follow up.

An exponential model implies that the force of mortality, or hazard function, remains constant with unit time, and thus if survival follows an exponential distribution it must be assumed that the risk of dying 20 years following treatment is still the same as it was at 5 years.

Mueller and Jeffries analysed the rate of dying and cause of death in a large retrospective study of 1513 patients from the Syracuse-Upstate Medical Cancer Registry (Mueller & Jeffries 1975). Ignoring the poor fit at the beginning and at the end of their 15 year survival curve, they computed a half life of 5.9 years and a constant annual 8% mortality rate, implying again, an exponential distribution.

A fifth study, by Myers, reached a similar conclusion, and it was not until some 50 years after Greenwood's initial observation concerning hazard functions, that it was again noted that the hazard function did not remain constant implying that survival could not follow an exponential pattern (Myers, Axtell & Zelen 1966, Blackwood et al 1977).

Survival distribution was analysed in one series of 10,752 patients and another of 656 patients, followed up for 8 and 18 years respectively. In both series the hazard function decreased with time following, approximately, a Weibull distribution [see Fig 2.1], indicating that the longer a patient survived the less chance she would have of dying from breast cancer.

Although making an important step forwards, the authors still did not recognise Greenwood's and Haybittle's observation that the hazard rate increases initially. A further, and very comprehensive, analysis of hazard function has been conducted by Gore on patients from the Western General Hospital, Edinburgh (Gore 1982). With a twenty year follow up on 3878 patients she has shown that the hazard function increases during the first 1 to 4 years from diagnosis due to early deaths from breast cancer. The peak hazard occurs earlier for Stage II & III disease than for stage I but by the tenth year they converge. The exponential and Weibull distributions provided a poor fit to the data, and simiarly a Cox Proportional Hazards model was inappropriate as the hazards were clearly not proportional. Instead she proposed a complex modification of the proportional hazards model that allowed for variation of the hazard function with time.

Having identified a statistical model that accurately reflects survival distribution in breast cancer it is possible to analyse the independent effect of individual tumour or host related factors on survival. Moreover one may also assess the curability of the disease.

2.4 Curability in breast cancer

Many attempts have been made to establish the absolute curability of cancer of the breast. The conclusions reached depend substantially upon the statistical model used and the authors definition of cure.

> "At no observed epoch from onset is the rate of mortality of the same order of magnitude as normal mortality"
>
> Greenwood 1928

Although based upon only 6 years follow up, Greenwood once more sums up precisely the problems of curability. Early studies were based mostly upon fixed interval mortality rates and although the fallacy of this has already been highlighted, useful observations can still be made.

Adair reported a study of 1458 breast cancer patients treated by radical mastectomy with an actuarial 30 year survival rate of 38% (Adair 1974). Only 4% of breast cancer deaths occurred in the third decade of follow-up and Adair assumed that three hundred of the 20 year survivors had lived a normal life span and could be considered cured. Interestingly, however, 21% of second cancers also occurred in the third decade of follow up, although the cumulative rate of metachronous tumours was 16.4%.

Adair's assessment of actuarial survival is seen to be

highly optimistic when a comparison is made with other studies [see Table 2.1].

Author	% Survival Rate 15 yrs	20 yrs	25 yrs	30 yrs
Berkson & Gage (1952)	50[1] 18[2]	-	-	-
Bond (1968)	-	-	17	-
Campos (1972)	74[1] 21[2]	-	-	-
Adair et al (1974)	-	42	-	38
Mueller & Jefferies (1975)	20	-	-	-
Brinkley & Haybittle (1977)	16.7	12.4	9.1	-
Hibberd et al (1983)	18	14	10	7.5
McBride et al (1983)	47[1] 9[2]	-	-	-

[1] metastatic
[2] non-metastatic

Table 2.1 Actuarial long term survival rates

The variation between these studies is almost certainly due to the criteria of selection, usually defined as being operable disease. The assessments of curability also differ but these depend, in large part, on the definition of the term cure.

Thus Adair describes 21% of his series to be cured at 30 years having lived a normal life-span. Mueller and Jeffries

however, assuming their exponential distribution of survival, calculate that 85% of all women with breast cancer will ultimately die of their disease.

An alternative concept of cure is that suggested initially by Greenwood, namely that the rate of mortality of treated cases should equal that of the normal population.

Easson and Russell studied 1812 cases of operable breast cancer over a 15 year follow-up period (Easson & Russell 1968). The observed mortality rate was only 1% above expected mortality but they concluded that a cured population had not been identified.

Their estimate of the ratio of observed: expected mortality agrees closely with other reported studies, although authors have drawn differing conclusions from their own results [see Table 2.2]

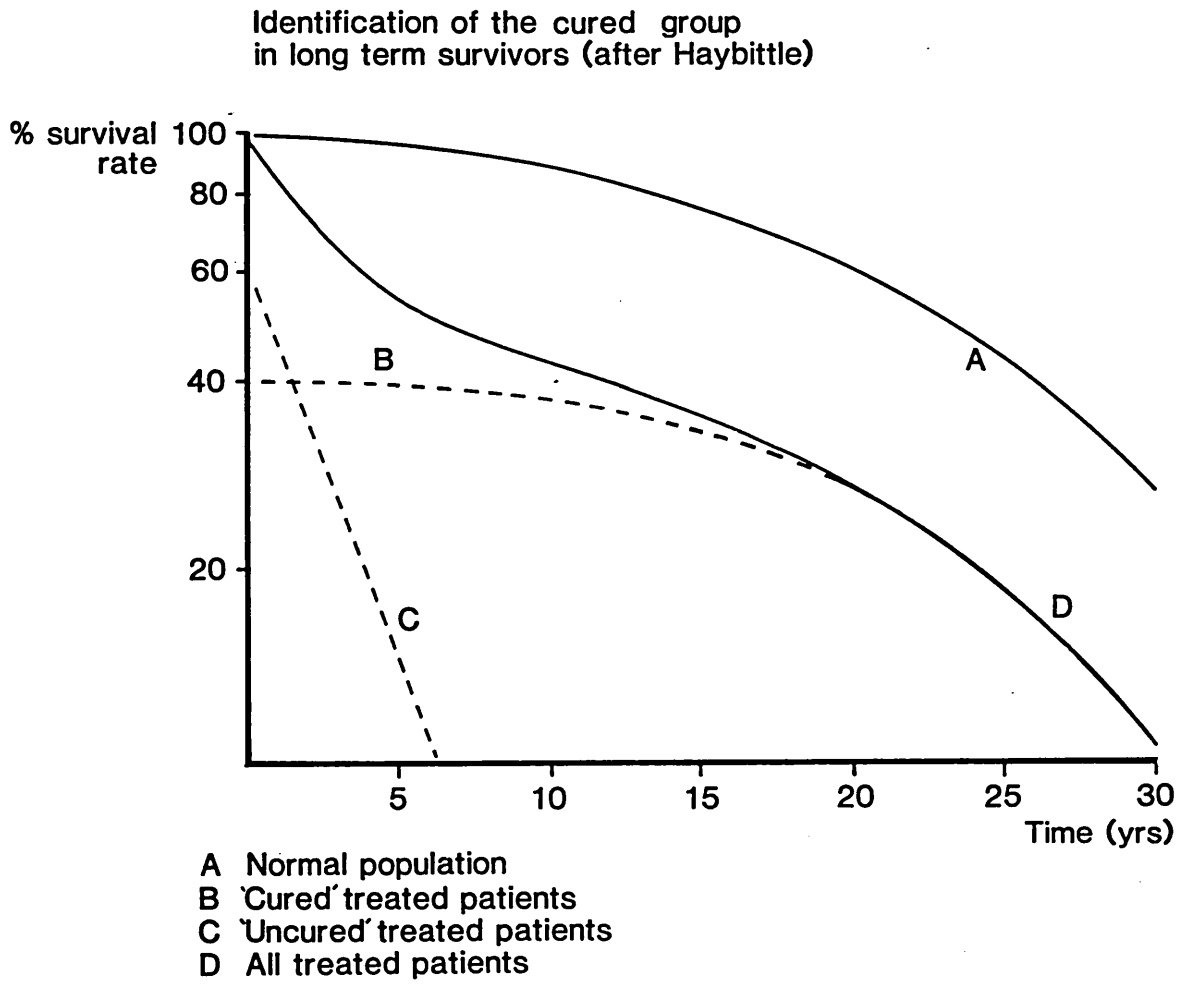
Author	Annual % excess mortality
Easson & Russel (1968)	1 at 15 years
Brinkley & Haybittle (1977)	1.1 at 20 years
Pocock et al (1982)	2 at 20 years
Hibberd et al (1983)	1 at 20 years

Table 2.2 Percentage excess observed: expected mortality from breast cancer

Brinkley and Haybittle in a series of papers concerning 704 women with operable disease demonstrate that 20 years or so following treatment, the observed mortality rate parallels that of a normal age-matched population (Brinkley & Haybittle 1977). They assume that the survival curve of the treated group is comprised of a cured group with the same mortality experience as the general population, and an uncured group [see Fig 2.2]. Extrapolation of the survival curve of treated patients from the point at which this curve parallels that of the normal population is assumed to give an indication of the percentage cured. However, the deaths from breast cancer 20 years or more following treatment are still twelve times that expected.

A 20 year retrospective study of 1982 women treated for localised disease related tumour size to the same criterion for 'cure' (Duncan and Kerr 1976). For women with tumours in excess of 4 cms in size, the 40% remaining alive at 10 years following treatment had normal life expectancy. Similarly the 50% of women remaining alive 13 years after treatment for a 3 cm tumour also had normal life expectancy and were considered cured. They predicted that for smaller tumours, up to an 80% cure rate could be achieved but that normal life expectancy might not be seen until beyond 20 years. However, an excess mortality from breast cancer of about 20 times that of the normal population was seen in all groups, but fell significantly with time.

Figure 2.2


Identification of the cured group
in long term survivors (after Haybittle)
% survival rate
100
80
60
40
20
A
B
C
D
5
10
15
20
25
30
Time (yrs)
A Normal population
B 'Cured' treated patients
C 'Uncured' treated patients
D All treated patients

In contrast, Langlands could demonstrate no effect of original stage of the disease on survival beyond ten years in a substantially larger retrospective study of 3878 cases of early disease. However excess mortality from breast cancer was still in evidence 20 years following treatment, with a twenty-fold excess of breast cancer deaths between 15 and 20 years (Langlands et al 1979). The authors conclude that cure can only be obtained when the observed mortality rate equals that expected, and that only pre-menopausal patients disease-free for at least 15 years could be considered potentially cured.

A somewhat different approach is taken by a study of curability in breast cancer patients from the M.D. Anderson Hospital (McBride et al 1983). The study is heavily weighted towards advanced disease with 50% of the patients having metastatic disease at presentation.

They define cure in three ways. Firstly, having demonstrated decreasing hazard function, they assume that cure is feasible, though undefined. Secondly, the authors define cure as death due to any cause other than breast cancer (irrespective however of time to death). This unusual definition yields a high percentage cure rate [see Table 2.3].

Stage	% cure
II	41
III	23
IV	2

Table 2.3 Percentage cure rate by stage (McBride et al 1983)

Thirdly, the study uses Berkson and Gage's exponential model to describe the distribution of survival of those patients not cured. They describe a proportion (P) that are cured, with the same mortality experience as the normal population, and another proportion (Q = 1-P) that experience the force of mortality due both to breast cancer and other causes of death. They estimated the parameters of the cancer mortality function and then used the method of maximum likelihood to estimate the proportion cured and the parameter of the exponential [see Table 2.4].

Stage	% curve	probability
II	12	N.S.
III	15	0.01
IV	5	0.001

Table 2.4 Curability of breast cancer (McBride et al 1983)

The reason why it is easier to demonstrate a statistically significant cure rate in advanced disease rather than in early disease, as might initially be expected, is because

probability is assessed on the difference in hazard functions between the normal population and the treated group. In early breast cancer the hazard rates approach so closely that the null hypothesis cannot be rejected. Alternatively, one could consider that a 'cure' in early breast cancer might add one year to expected life time, which is not significant, whereas 'cure' in advanced disease may add 5 years for stage III disease, or over 10 years for stage IV, which is highly significant.

In summary, the absolute curability of cancer is somewhat dependent upon definition. The assumption that breast cancer survival follows an exponential distribution with continuing constant mortality has been shown to be incorrect. If cure is defined as living to normal life expectancy then approximately 20% of all breast cancer cases are cured, or 30% of early breast cancer. However, a substantial proportion of these patients will die from their disease and probably only 15% of patients will live a normal life expectancy and die disease-free. The curability of advanced cancer is easier to prove statistically but is substantially less than 5%.

2.5 Conclusions

The natural history of treated breast cancer has been presented in some detail, with the continuing, although decreasing, force of mortality due to breast cancer present even thirty years beyond treatment. The conventional explanation is of the orderly progression of breast cancer with the risk of dissemination of metastases increasing with tumour age prior to treatment.

An alternative view is that of Baum who considers that all breast cancers should be considered as a systemic disease and that only a minority are non-metastasising although the metastasising potential varies according to the biological nature of the tumour (Baum 1976). This view is a variation on the theory of 'biological predetermination' which states that the natural history of a breast cancer is dependent upon the biological characteristics of the tumour and not upon its chronological age at presentation.

Survival in cancer is the result of the balance between the two opposing forces of the tumour and of the host. If long term survival is to be modified by treatment then it is important to assess whether it is dependent or independent of the biological characteristics of the tumour. One method for approaching this question is to assess the longer term effects of prognostic factors that are known to influence short term survival. This will be discussed in the following chapter.

An alternative explanation is that long term survival may be influenced by host related mechanisms and these will be examined in subsequent chapters.

CHAPTER 3

PROGNOSTIC FACTORS IN LONG TERM SURVIVAL

3.1 Introduction to literature review

3.1.1 Problems in statistical analysis

Prognostic factors are widely used in the management of patients with early breast cancer for the selection of therapy. Although the majority of studies have analysed the influence of individual prognostic factors, some attempts have been made to conduct multivariate analyses in order to examine the simultaneous effect of several variables.

Myers, in the third statistical analysis of data from the Yale - New Haven Medical Centre investigated four prognostic factors, pathological nodal status, nuclear grade, sinus histiocytosis and tumour size, in 375 patients (Myers et al 1966). The study concluded that there was no interaction between these variables and that the relative order of prognostic importance was nodal status, tumour size, sinus histiocytosis and nuclear grade. The study illustrates, however, some of the problems in analysis of prognostic factors.

Firstly, for the reasons outlined in the previous chapter, life table analysis must be used. Secondly, the patients should be unselected. In Myers' study over 400 patients (52%) were excluded because of lack of data. Thirdly, if there is interaction between prognostic variables a multivariate analysis must be performed, although in Myers'

study it was concluded that such interactions did not exist. Lastly, there is a problem in the method by which deaths are coded. One might assume that all deaths are breast cancer related, in which case death is taken as the endpoint regardless of cause. Alternatively it may be argued that prognostic factors only effect disease-related deaths, in which case a distinction must be made between deaths due to breast cancer, and intercurrent deaths due to other causes. It should be noted that intercurrent deaths will be highly age related, whereas disease-related deaths may not be.

3.1.2 Aim of the study

There have been very few studies on the influence of prognostic factors on long term survival in breast cancer, and only three major studies are reviewed, two based on patients treated in Edinburgh between 1943-1953 and 1954-1964, and one based upon patients treated at the Memorial Hospital, New York between 1940-1943 (Duncan and Kerr 1976, Langlands et al 1979, Langlands and Kerr 1979, Pocock et al 1982, Gore 1983, Adair et al 1974). These retrospective studies have been concerned mostly with clinical prognostic factors. In large retrospective studies of this nature there are few centres where accurate clinical data is available spanning twenty years or more; histological data is usually not available or is not of sufficient accuracy to warrant statistical analysis.

In the first part of this chapter these major studies are reviewed, together with several smaller such studies. In the second part, an analysis of the influence of clinical and pathological prognostic variables on the long term survival of 890 patients treated, and followed up by the Breast Clinic, Guy's Hospital, is presented.

3.2 Stage

The pattern of survival by clinical stage on the second series of Edinburgh patients has been reported (Pocock et al 1982). Of 3922 patients all but 85 were classified by Stage according to:

Stage I	Tumour <5 cm. diameter
Stage II	As stage I plus palpable lymph nodes
Stage III	Tumour >5 cm. or fixation, fixed nodes etc.
Stage IV	Metastatic disease.

No division between cause of death was made, but patients were censored at age 85 to prevent weighting by elderly deaths. Analysis of the data was by life table and hazard function. Of 3922 patients in the study, 1281 (33%) were alive at ten years, beyond which point there was no difference in survival noted between patients classified as Stage I or Stage II (chi^2 = 0.54, df 2). By 15 years from treatment there was no statistically significant difference in survival between Stages I to III with an average 4-5% mortality per annum continuing to 20 years. It was concluded from this study that stage has very little influence, if any, on survival beyond ten years.

In the other published studies of long term survival no independent analysis of the effect of stage is documented.

3.3 Axillary node status

Pathological lymph node status is the single most important prognostic indicator in short term survival and exceeds that of tumour size when both are considered together, as in a staging system (Myers et al 1966). In the Edinburgh patients, only clinical nodal status has been analysed (Gore 1983). Forty-one per cent of patients whom the clinician assumed to have non-palpable axillary nodes survived ten years, as opposed to 27% of patients with mobile nodes, and 7% of patients with fixed homolateral axillary nodes. When analysed by hazard function the estimated hazard for patients with no clinically involved nodes converged with that of patients with clinically palpable nodes, the conclusion being that clinical nodal status does not influence survival beyond ten years.

Although Adair, in his 30 year report on the survival of 1458 patients treated by radical mastectomy, graded patients according to the anatomical level of histological nodal involvement, no numerical data was published to permit analysis of this prognostic information over fixed time periods (Adair et al 1974).

In the Cambridge series it was noted that of 81 twenty year survivors at least 21 (26%) had positive axillary node histology at the time of treatment, but a separate analysis of histological nodal status was not recorded (Brinkley and Haybittle 1977).

Dawson studied the pathology of 107 women who had survived 25 years or more following radical mastectomy (Dawson et al 1982). These patients were matched to a control group who had died within a short time (median 3.4 years, range 0.9 to 9.9 years) of radical mastectomy. Although, not surprisingly the long term survivors had smaller tumours, less involved nodes, and were younger than the control group, 12% of the survivors had tumours larger than 5.0 cms, and 11% had four or more involved nodes. Whilst the study supported the importance of well-known prognostic factors it highlights the impossibility of predicting long term survival in individual patients.

3.4 Tumour size

The correlation between tumour size and survival is well documented and is second in prognostic importance only to nodal status in short term survival (Myers et al 1966, Fisher et al 1969). The influence of tumour size on long term survival was analysed in both cohorts of Edinburgh patients though with differing conclusions. In the first study survival data was analysed by tumour size above and below 3.0 cm, in 1.0 cm intervals (Duncan and Kerr 1976).

There was little difference in survival in tumours of 4.0 cm and above although tumours over 6.0 cm had marginally better prognosis than the remainder. For tumours of 3.0 cm and less, survival was inversely proportional to size, with 1.0 cm tumours having significantly better prognosis than 2.0 cm tumours. After ten years the age-corrected survival rates for tumours of 4 cm and larger were parallel to the time axis, indicating a normal life expectancy, uninfluenced by the tumour. For patients with 3 cm tumours, the curve became parallel at about 13 years, but in the smaller tumours it continued to fall, throughout the follow-up period, indicating a persistent influence on survival extending to twenty years.

In the second Edinburgh series the effect of tumour size was examined initially only for patients Stage I and II on the grounds that Stage III patients included those with other, and less favourable prognostic variables (Langlands et al 1979). There was no evidence of an effect of tumour

size on survival between ten and twenty years. A second study analysed the influence of tumour size on the entire cohort of patients (Gore 1983). Tumours of 5.0 cm and greater demonstrated a constant decreasing hazard function, whereas smaller tumours showed non-monotone hazard. Size was an important determinant of survival until 10 years with 53% of tumours 2.0 cm or less surviving, compared to 35% of tumours between 3.0 and 4.0 cm, and only 14% of tumours 5.0 cm and above, but beyond ten years, the hazard functions were constant implying no further influence on survival.

Adair analysed the impact of tumour size only on crude survival rate up to 30 years (Adair et al 1974). As in the overall survival rates presented in the last chapter, the survival analysed by tumour size is substantially better than in any other comparable series, suggesting that their definition of local disease is much more rigid [see Table 3.1].

Tumour size (cms.)	Crude Survival Rate (%) 10 years	20 years	30 years
2.0	74	62	58
2.0	58	49	48
3.0	42	33	28
4.0	37	30	27
5.0	25	21	20

Table 3.1 Survival in relation to tumour size (Adair et al 1974)

3.5 Fixation

Fixation to pectoral fascia or muscle within the diameter of the tumour is permitted within TNM Stage I and II, but fixation to the chest wall is TNM Stage III (UICC, 1980). In the analysis of the second Edinburgh series, all tumours with fixation, whether superficial or deep, were classified as Stage III (Langlands et al 1979). However, a separate analysis of the influence of fixation and ulceration on survival was subsequently conducted (Gore 1983). In this analysis, fixation was graded as none present, skin fixation, deep fixation to muscle/fascia, and deep fixation to chest wall. Deep fixation to muscle or fascia did not have an unduly poor prognosis, but fixation to the chest wall, although uncommon, was highly unfavourable to survival. Of interest was the hazard function curves for patients stratified according to whether fixation was present or not. These did not converge, even at 15 years, suggesting that fixation, even to skin, is a highly adverse prognostic factor exerting an influence on survival well into the second decade of follow-up. This contrasts somewhat with the earlier finding in the same data series that patients with fixation (classified as Stage III) had no difference in survival with patients without fixation (classified as Stage I or II) (Langlands et al 1979).

3.6 Parity

Nulliparity is associated with an increased risk of development of breast cancer compared to an early first birth or multiparity [see Chapter 6]. Parity has therefore been investigated as a prognostic factor in breast cancer survival in several studies (MacKay and Sellers 1965, Donnegan et al. 1978, Papatestas et al 1980, Juret 1981). None of these reports have studied the influence of parity on long term survival, and only five year life table analysis has been presented.

An analysis of 608 women with operable breast cancer revealed that parous women had a significantly higher 5 year cumulative disease-free survival rate (60%) than nulliparous women (46%, $p = 0.012$) (Papatestas et al 1980). The survival rates were evaluated within each tumour stage, and for premenopausal and postmenopausal women, and parous women had a significant survival advantage in each stratum. This finding contrasts to that of an earlier study in which no difference in survival was demonstrated between nulliparæ and multiparæ up to 4 children, but where multiparity of 5 children or more was associated with a poorer prognosis (MacKay and Sellers 1965).

The influence of parity on long term survival will be analysed in the current study.

3.7 Other clinical prognostic factors

3.7.1 Menopausal status

Two other important clinical prognostic factors remain, namely age and menopausal status. Because these two variables are highly interdependent they have caused much confusion in the past literature, and the majority of studies have employed inadequate statistical methods to analyse the independent effect of either variable, or have not allowed for the effect of peri-menopausal patients.

The influence of menopausal state on long term survival has been studied in the second Edinburgh series of patients (Pocock et al 1982). Actuarial survival analysis demonstrates premenopausal women to have significantly better prognosis than postmenopausal women, with peri-menopausal women (within 5 years of menopause) having intermediate survival. However this takes no account of the age difference between the groups and the associated intercurrent deaths from other causes which are highly age dependent. Age corrected life tables (using national age-specific death rates) demonstrate that premenopausal and postmenopausal patients have the same percentage survival. In an alternative analysis using relative survival (the ratio observed: expected deaths) the same conclusion is reached. However, menopausal patients have a significantly

poorer prognosis over the first five years, although this unfavourable prognostic effect declines thereafter, so that after 15 years of follow-up, there is no impact whatsoever of menopausal status on survival.

3.7.2 Age

The influence of age on survival is not clear. There are a great number of reports in the literature on the effect of age on 5 year survival and although many suggest that young women with breast cancer have a worse prognosis than other age groups there are as many that find no effect of age (Earley et al 1969, Kleinfeld et al 1963, Norris and Taylor 1970, Schwartz and Zeok 1976, Langlands and Kerr 1979).

There are relatively fewer reports on age as a determinant of long term survival. Mueller analysed 3558 women from the Syracuse Cancer Registry who had been followed up for 19 years after treatment for breast cancer (Mueller et al 1978). A life table analysis was performed on patients stratified by age (21-50 years, 51-70 years, and over 70 years) taking death due to breast cancer only as the end point and this concluded that the younger age group had a significantly better prognosis (50% mortality at 11.5 years) than the middle (50% mortality at 7.2 years) or older age group (50% mortality at 4.0 years). Unfortunately, in the studies in which younger patients are found to have a poorer

prognosis, it is in patients aged 40, or even 30 years and less. Because of the age distribution of breast cancer, the 2% incidence rate in women under 30 years would have entirely masked any impact of this age group in Mueller's study. In contrast, Langlands and Kerr found that women aged 40 to 44 years have enhanced prognosis but that women aged under 40 years, notably with Stage III disease, had a poorer prognosis (Langland and Kerr 1979). When the same data was analysed by hazard function, women under 34 years of age have a markedly poorer prognosis, whereas women aged 35-44 years do best. Elderly patients of 64 years and over have an intermediate prognosis (Gore 1983).

Laterality is the only other clinical prognostic factor to have been analysed in long term survival, and it does not appear to exert any influence (Gore 1983).

3.8 Late recurrence

There are many single case reports of late recurrence of breast cancer in the literature, and many of these have been summarised in a study on the chronicity of cancer (Morton and Morton 1953). These authors collected 55 cases of late recurrent breast cancer occurring between 10 and 50 years following primary treatment, of which 71% were local metastases, and 33% distant metastases. However, very little clinical or pathological data on these cases is published, the major feature of interest being that late recurrence had occurred.

In a later review, including many of the same cases, the average age at initial presentation was 43.1 years for those patients who subsequently manifested late recurrence, compared to an average mean age at initial presentation of 53 years (Herrmann 1972). Of interest, 14 of 19 cases (74%) were left sided lesions, but again, clinical and pathological data are sparse, allowing very little further interpretation.

3.9 Introduction to statistical study

A review of the literature on the influence of clinical prognostic factors on long term survival in breast cancer has suggested that very few, if any, of the variables which are important in short term survival still exert an effect on long term survival beyond ten years. However, many of the studies were based on a single cohort of patients treated in one centre, and the analyses were performed with death due to any cause taken as the end point. The statistical study presented in this chapter is on patients treated in the Breast Clinic, Guy's Hospital, London between the years 1952-1972 and followed up until June 1983. Deaths due to causes other than breast cancer were censored as free of disease at time of death and the analysis therefore refers only to deaths due to breast cancer. This permits examination of the impact of prognostic factors upon the natural history of breast cancer rather than upon survival. The disadvantage of the method is that deaths certified as being due to breast cancer may be inaccurate, and secondly, that deaths due to unknown causes(10.5% in this study) must be included. Separate hazard function plots were obtained for patients known to have died from breast cancer, and for those patients whose cause of death was unknown. Inclusion of the latter did not bias the result.

3.10 Patients

All breast cancer patients treated at the Breast Unit, Guy's Hospital, London are seen annually after the 5th year of follow-up. If the follow-up appointment is not kept, or if the patient is unable to attend for follow-up, the G.P. is contacted annually. In the event of the G.P. being unable to supply clinical information on a patient, the Registrar General, NHS Central Register, Southport, is requested to search and inform the Unit of the patient's status. The clinical and pathological data on all NHS patients treated in the Breast Unit since 1952 are stored on a mainframe computer at the Imperial Cancer Research Fund (ICRF).

3.10.1 Entry criteria

The computerised data on all patients treated between 1952-1972 was examined, and those patients surviving for ten years or more, disease-free from the time of primary treatment for breast carcinoma were entered into the study (n = 907). Seventeen patients were subsequently excluded because details of date of birth, date of operation or date of death were not available. The remaining 890 patients were entered into the study.

3.10.2 Clinical data

The clinical data was abstracted from the computerised record and checked by carrying out an analysis of variance

on each of the variables, and an analysis of correlation where appropriate. The data on patients aged 80 and above, and on those patients surviving twenty years or more, was checked by reference to the clinical notes to ensure accuracy. The clinical data abstracted from the computerised record is summarised in Table 3.2.

3.10.3 Treatment

The primary treatment of breast cancer in the patients in the study is summarised in Table 3.3. The principal treatment received was a modified radical mastectomy with axillary nodal clearance. The variation in surgical treatment did not differ substantially between the first decade of entry and the second, but a significantly greater proportion of patients received adjuvant cytotoxic chemotherapy in the second decade of entry.

Treatment	No. of patients (%)
Excision of tumour	142 (16)
Radical mastectomy	656 (74)
Simple mastectomy	86 (9.5)
Pre-op DXT	6 (0.5)
Post-op DXT	462 (52)
Cytotoxic chemotherapy	180 (20)
Ovarian ablation	0

Table 3.3 Treatment of long term survivors

1. Hospital number
2. Date of birth
3. Date of operation
4. Date first symptom
5. Breast involved: (Right, Left, Bilateral)
6. Site: (Upper lateral, Lower lateral, Upper medial, Lower medial, Upper, Lower, Lateral, Medial, Central, Diffuse)
7. Axillary gland involvement: (Clinical: involved/not involved; Pathological: involved/not involved)
8. Size 1 (mm)
9. Size 2 (mm)
10. Fixation (None, skin, deep, both)
11. Tumour TNM stage (T_o, T_1, T_2, T_3, T_4)
12. Nodal TNM stage (N_o, N_1, N_2, N_3)
13. Metastasis TNM stage (M_o, M_1)
14. Parity
15. Children breast fed
16. Menopausal state pre-treatment (Premenopausal, Postmenopausal, Oophorectomy)
17. Date of menopause (Year)
18. Treatment (Excision, Radical mastectomy, Simple mastectomy, DXT pre-op., DXT post-op., Cytotoxic chemotherapy, Oophorectomy)
19. Local metastasis (Month, Year)
20. Distant metastases (Month, Year)
21. Death (Day, Month, Year)
22. Cause of death
23. Date last follow-up (Month, Year)

Table 3.2 Clinical and pathological data on long term survivors

3.11 Methods

The influence of clinical prognostic factors on survival beyond ten years was estimated using computerised life table analysis. The patients were stratified by the following method:

3.10.1 Clinical stage

The primary tumour, regional lymph nodes and distant metastases were staged by the examining clinician according to the International Union Against Cancer pre-treatment clinical classification (UICC 1980).

Tumour:

- T_0 No evidence of primary tumour
- T_1 2.0 cms. with/without muscle fixation. Limited skin fixation.
- T_2 2.0-5.0 cms. with/without muscle fixation. Limited skin fixation.
- T_3 5.0 cms. with/without muscle fixation. Limited skin fixation.
- T_4 Any size and oedema, ulceration, peau d'orange, satellite nodules.

Nodes:

- N_0 Not palpable
- N_1 Palpable, not fixed
- N_2 Fixed homolateral axillary nodes
- N_3 Clavicular nodes palpable. Oedema of arm.

Metastasis M_0 None present

M_1 Present

3.11.2 Tumour size

The tumour size was measured in two dimensions at right angles, in millimetres. The maximum diameter was taken as the larger of the two dimensions.

3.11.3 Tumour fixation

The tumour was classified as:

no fixation present
skin fixation only present
deep fixation only present (to fascia, muscle or chest wall)
skin and deep fixation present.

For the purpose of statistical analysis deep fixation, and both skin and deep fixation were considered together.

4.11.4 Tumour site

The tumour was recorded as being in the right, left or both breasts, and by the following classification as to site:

0. upper lateral
1. lower lateral
2. upper medial
3. lower medial
4. upper
5. lower
6. lateral
7. medial
8. central
9. diffuse

The patients also were stratified horizontally (medial, central or lateral) and vertically (lower, central, or upper).

3.11.5 Recurrence and death

Recurrence was recorded as local (month, year) or distant (month, year). The date of death (day, month, year) was recorded and the cause of death coded as:

1. death due to breast cancer
2. death, unknown cause
3. death, cause other than breast cancer (specified).

The end point was taken as death due to breast cancer or cause unknown. Deaths due to other known causes were censored as free of breast cancer if no previous recurrence had been recorded. Patients were censored at age 85 in order to prevent undue weighting by age related intercurrent death, and inaccurate data.

3.11.6 Statistical analysis

The life table analyses were performed using two computer programs.

The 'Surv-c' program based on a log rank analysis estimated survival distribution observed over varying time periods and plotted Kaplan-Meier survival curves for each treatment

group. Where patients were stratified into groups the output gave a chi-squared analysis for trend, non-linearity, and homogeneity, from which two tailed p values were estimated, as well as a log rank test.

The 'BMDP' programme estimated the cumulative proportion surviving for each 25 month interval of follow-up from 10 to 30 years, with an estimated hazard function (and standard error), and density function (and standard error).

Further details on data management and statistics are given in Appendix 3.

3.12 Results

The 890 patients entered into the study were followed up for periods of between 11.5 years (138 months) and 30 years (360 months). The number of patients entered and withdrawn into each interval of follow-up is summarised in Table 3.4.

Interval (months)	Entered	Withdrawn	Dead
120 - 180	890	488	65
180 - 240	337	212	10
240 - 300	115	74	11
300 - 360	30	26	4

Table 3.4 Survival Analysis

The life table analysis of all patients, unstratified, is presented in Fig.3.1 and the log survival curve in Fig.3.2. Because of the relatively small number of deaths due to breast cancer, estimates of hazard function were found to be unhelpful in this series.

3.12.1 Clinical stage

Life table analysis was performed for 779 patients stratified by tumour TNM stage. The relative survival is

Figure 3.1

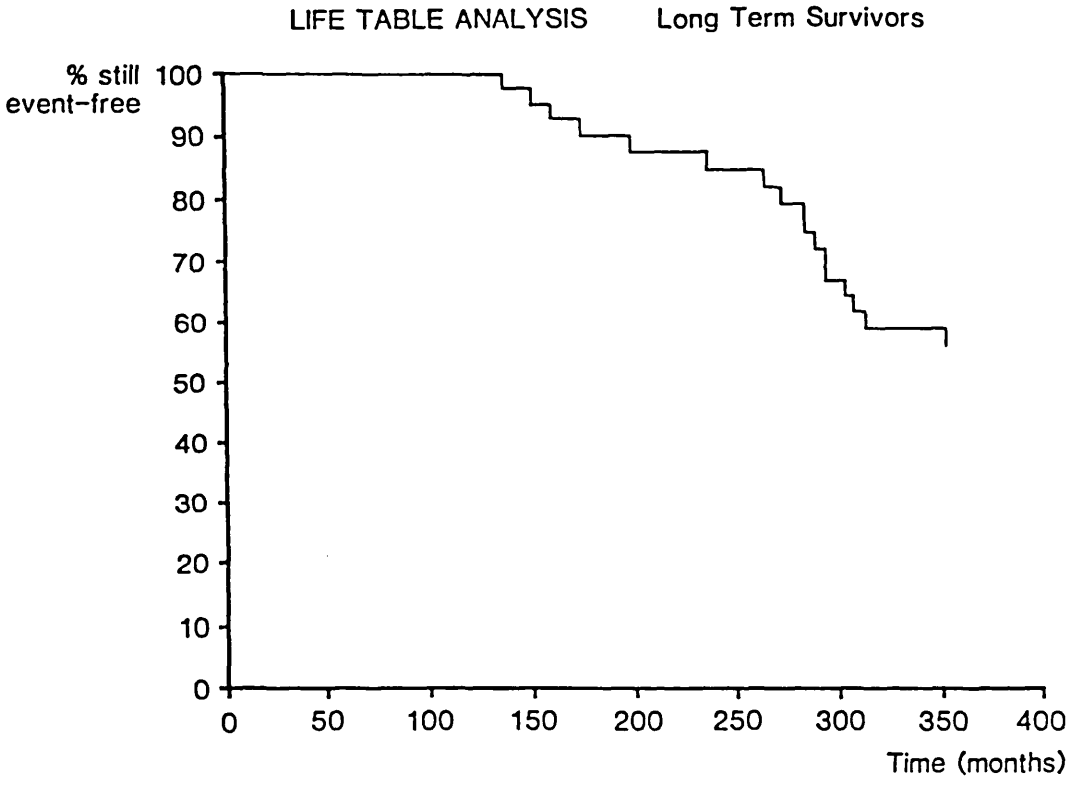


Figure 3.2

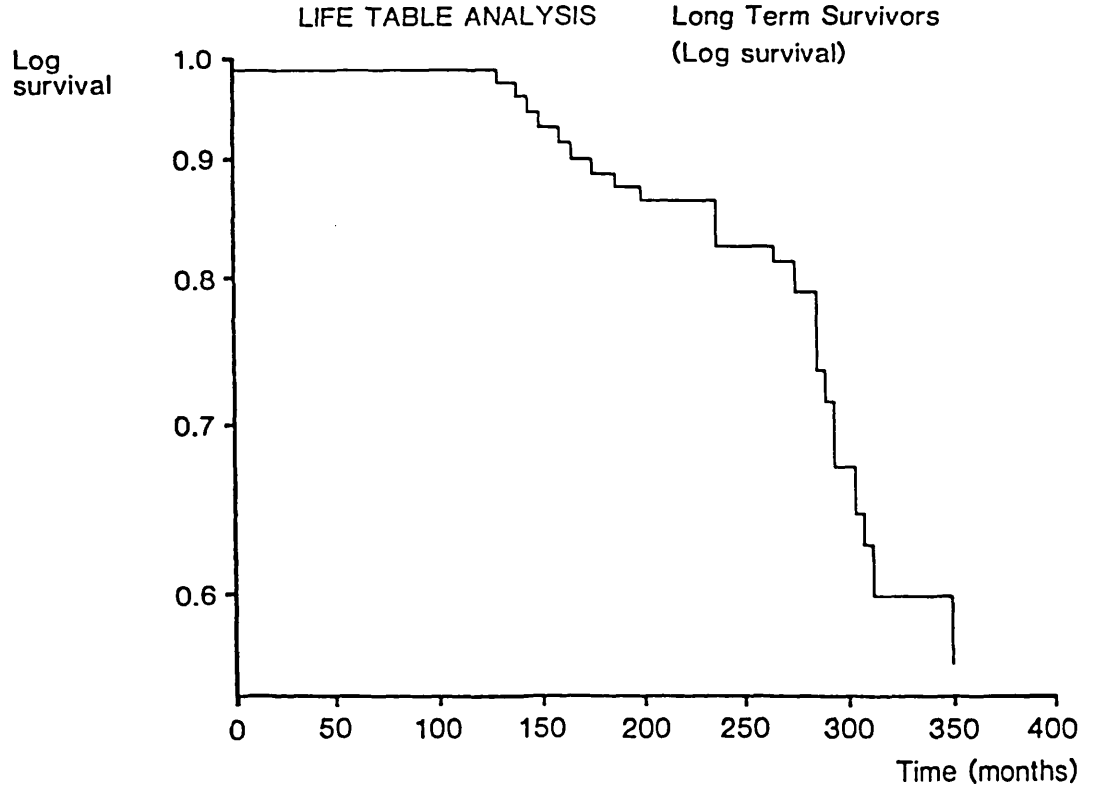
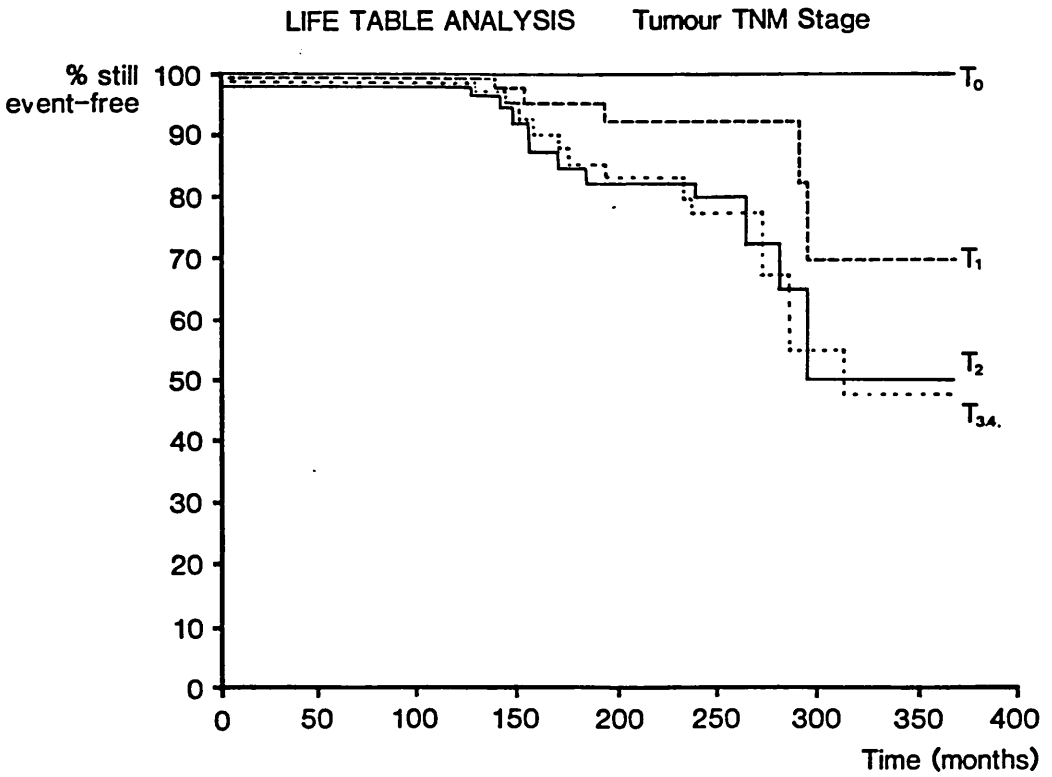


Figure 3.3


LIFE TABLE ANALYSIS
Tumour TNM Stage
% still event-free
100
90
80
70
60
50
40
30
20
10
0
0
50
100
150
200
250
300
350
400
Time (months)
T0
T1
T2
T3,4.

Stage	Patients (%)	Observed Events	Expected Events	Observed / Expected
T_0	30 (4)	0	2.71	0.00
T_1	189 (24)	9	19.58	0.46
T_2	443 (57)	50	40.09	1.25
T_3 T_4	117 (15)	17	13.63	1.25
Total	779	76	76	1.00

Table 3.5 Survival analysis: tumour TNM stage

A life table for patients stratified by tumour TNM stage is shown in Fig.3.3. A two-tailed p-value for positive trend estimated from a log rank test is 0.002.

chi^2 for: trend (1 df) = 8.89

non-linearity (2 df) = 2.86

hetero-geneity (3 df) = 11.75 p = 0.008

This demonstrates that there is a significant trend in decreasing survival with advancing tumour TNM stage even on patients who have survived disease free for an initial ten year period.

Patients were also stratified by nodal TNM status. The relative survival is shown in Table 3.6, and the life table analysis in Fig.3.4.

Stage	Patients (%)	Observed Events	Expected Events	Observed / Expected
N_0	561 (70)	45	54.28	0.83
N_1	222 (28)	30	23.78	1.26
N_2 N_3	16 (2)	5	1.94	2.58
Total	799	80	80	1.00

Table 3.6 Survival analysis: nodal TNM stage

Figure 3.4

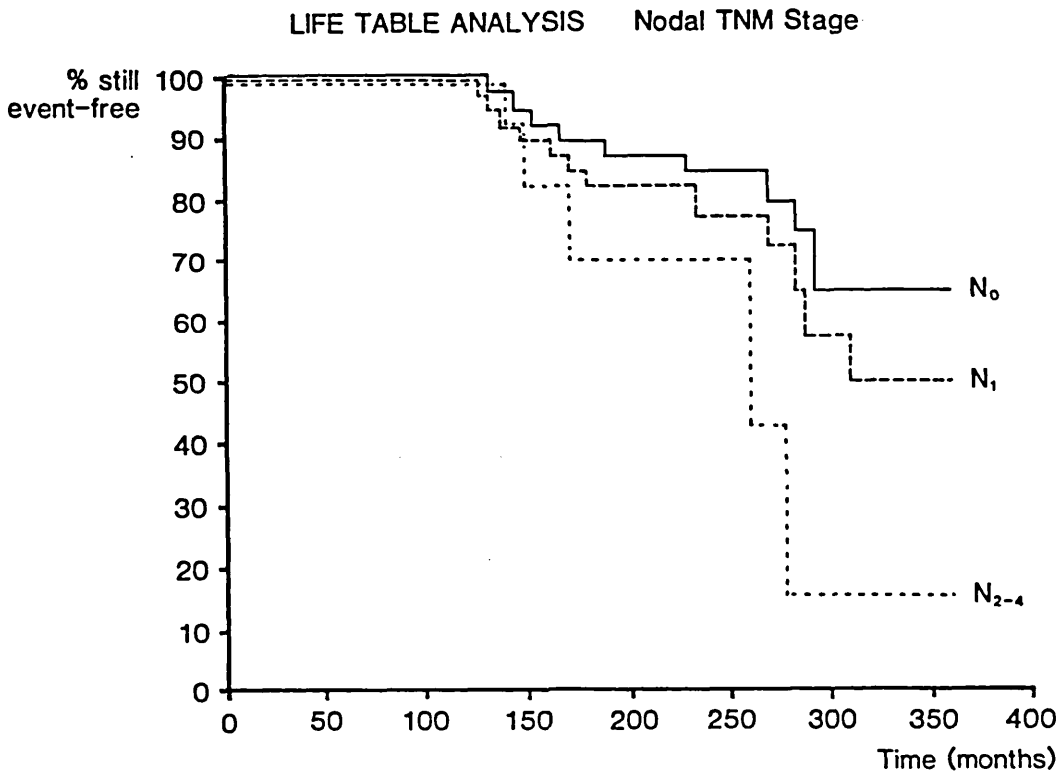
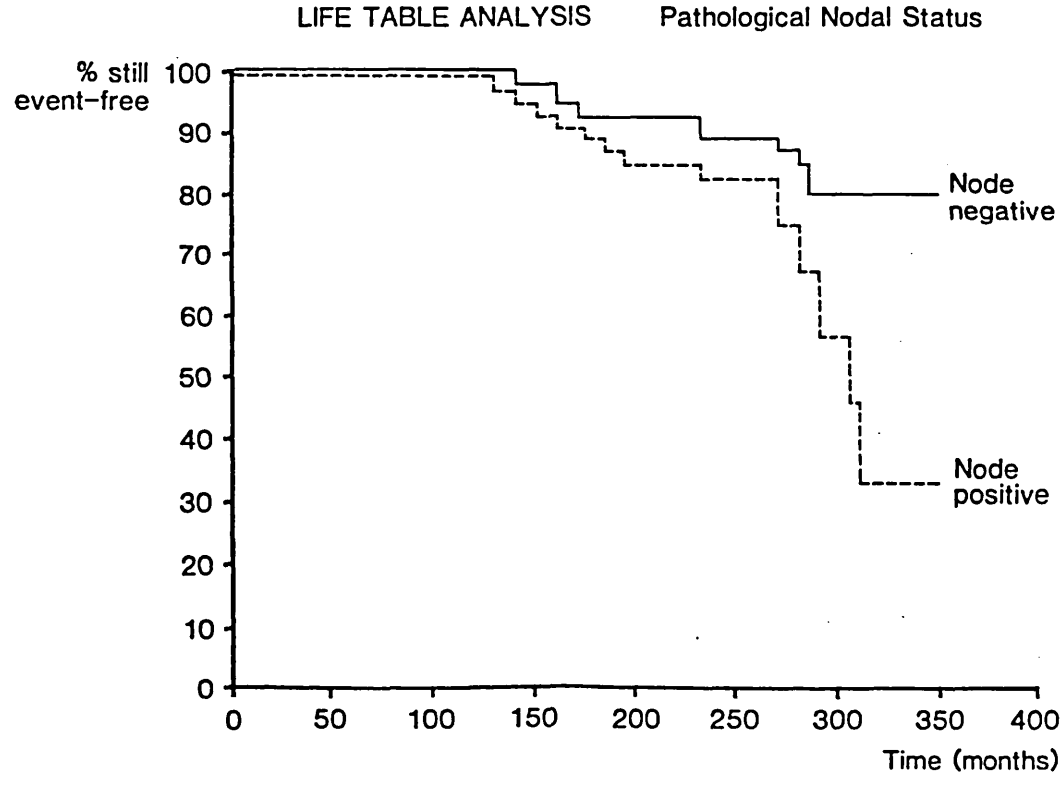


Figure 3.5



A two tailed p-value for positive trend from a log rank test gives p = 0.01.

chi^2 for: trend (1 df) = 6.99

non-linearity (1 df) = 1.12

heterogeneity (2 df) = 8.11 p = 0.01

Patients with clinically involved and fixed, axillary nodes have a worse long term prognosis than patients with no palpable axillary nodes. Whilst the chi squared value for trend is high it is of only moderate statistical significance, and examination of the life table analysis [Fig.3.4] shows N_0 and N_1 tumours to have approximately parallel survival curves.

3.12.2 Pathological nodal status

The pathological nodal status was known in 556 patients and these were stratified by node negative and node positive groups. The survival analysis on these patients is shown in Table 3.7, and the life table analysis in Fig.3.5.

Pathological nodal status	Patients (%)	Observed Events	Expected Events	Observed / Expected
Node negative	346 (62)	23	31.7	0.73
Node positive	210 (38)	28	19.3	1.45
Total	556	51	51.0	1.00

Table 3.7 Survival analysis: pathological nodal status

The two tailed p-value for positive trend from the log rank test gives p = 0.002, indicating that pathological nodal status has an important influence on survival

beyond ten years. The correlation between clinical and pathological nodal status for these patients is shown in Table 3.8. This demonstrates a false negative rate for clinical examination of 32% and a false positive rate of 47%.

Pathological status	Clinical status negative	positive	Total
negative	279	67	346
positive	134	76	210
Total	413	143	556

Table 3.8 Correlation of clinical and pathological node status

3.12.3 Tumour size

Tumour maximum diameter was estimated from the larger of the two dimensions estimated. A plot of cumulative proportion by tumour size (maximum diameter) is shown in Fig.3.6.
Mean tumour diameter (± S.D.) = 3.37 cms (± 1.9).
For the analysis of survival the patients were stratified according to maximum tumour diameter 2.0 cms , 2.0-4.9 cms and 5.0 cms , the tumour diameters equivalent to those in the UICC tumour TNM staging. The relative survival is shown in Table 3.9, and the life table analysis in Fig.3.7.

Tumour size (cms)	Patients (%)	Observed Events	Expected Events	Observed / Expected
2.0	215 (29)	13	21.05	0.62
2.0 - 4.9	386 (51)	43	37.80	1.14
5.0	149 (20)	20	17.15	1.17
Total	750	76	76	1.00

Table 3.9 Survival analysis: tumour size

Figure 3.6

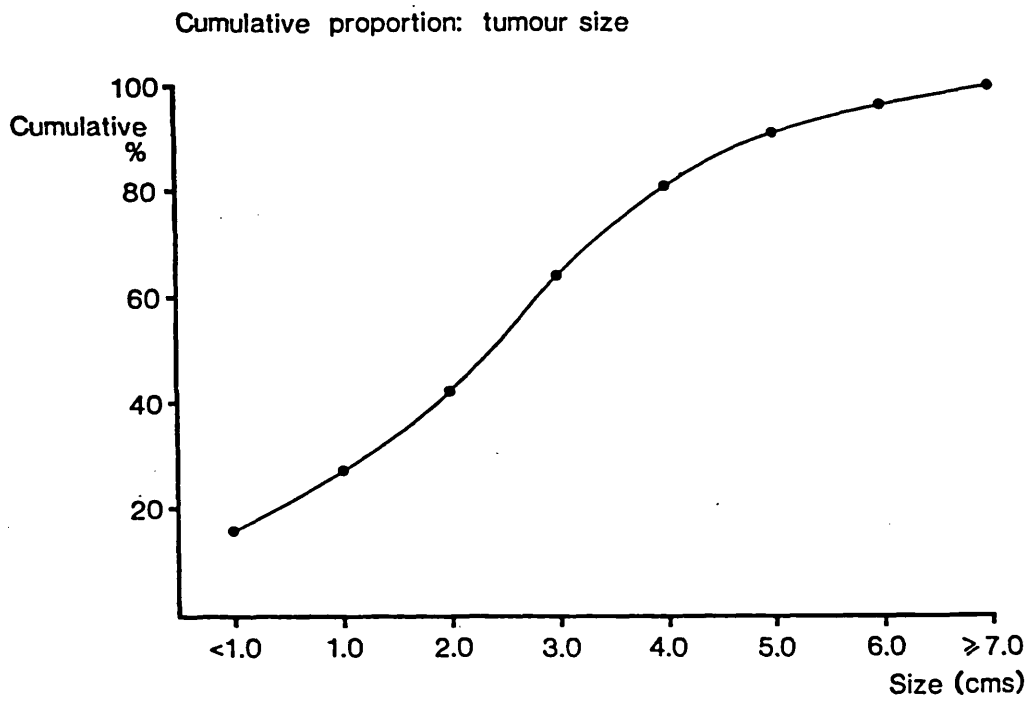
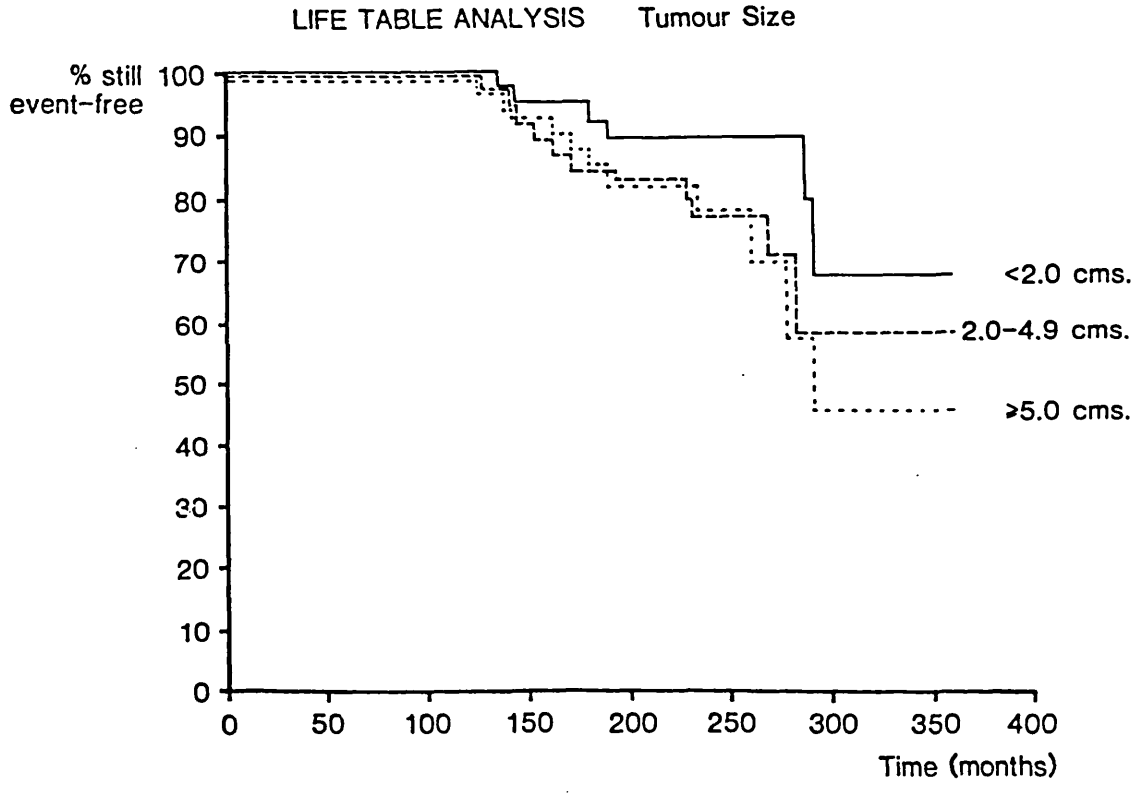


Figure 3.7



A two tailed p-value for positive trend estimated from a log rank test gives p = 0.07.

chi^2 for: trend (1 df) = 3.15

non-linearity (1 df) = 1.15

homogeneity (2 df) = 4.29 p = 0.1

Tumour size is inversely related to survival beyond ten years, but the trend does not reach statistical significance and it is concluded that tumour size is therefore not an important influence on long term survival.

3.12.4 Tumour fixation

The patients were stratified according to whether fixation was absent, tumour fixation to skin present, or tumour fixation to underlying fascia, muscle or chest wall present. The relative survival is shown in Table 3.10 and the life table analysis in Fig.3.8.

Fixation	Patients (%)	Observed Events	Expected Events	Observed / Expected
None	344 (44)	21	38.84	0.54
Skin	387 (50)	47	38.90	1.21
Deep	44 (6)	14	4.26	3.28
Total	775	82	82.0	1.00

Table 3.10 Survival analysis: tumour fixation

A two tailed p-value for positive trend estimated from the log rank test gives p = 0.00001.

Figure 3.8

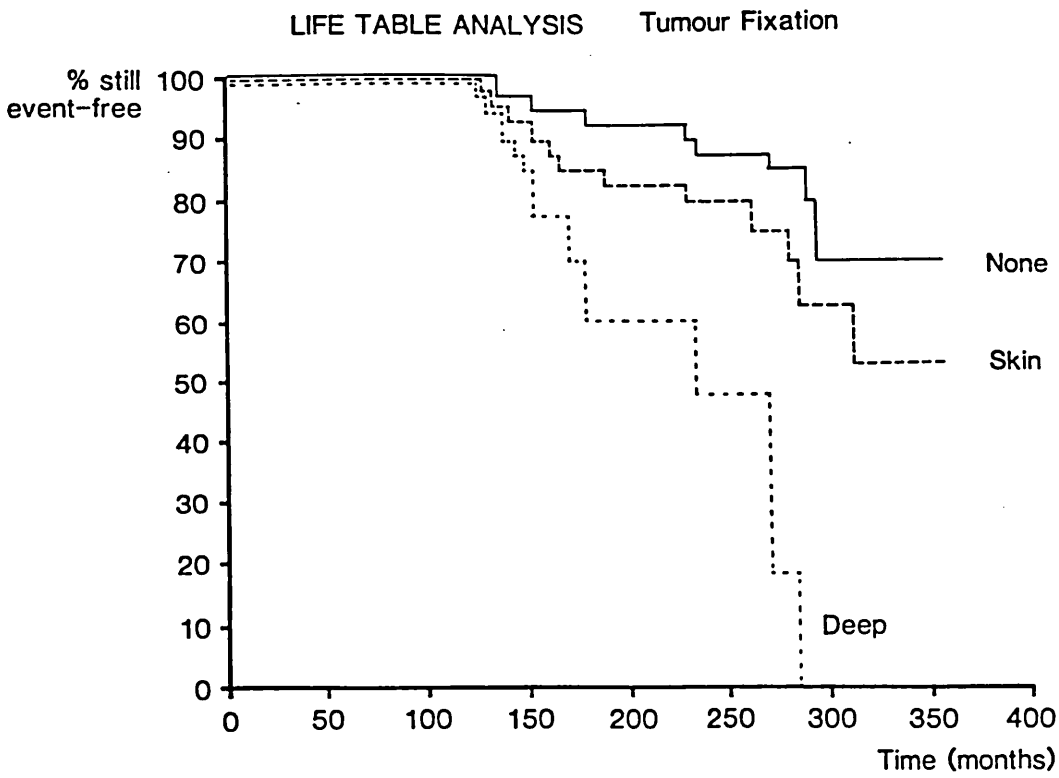
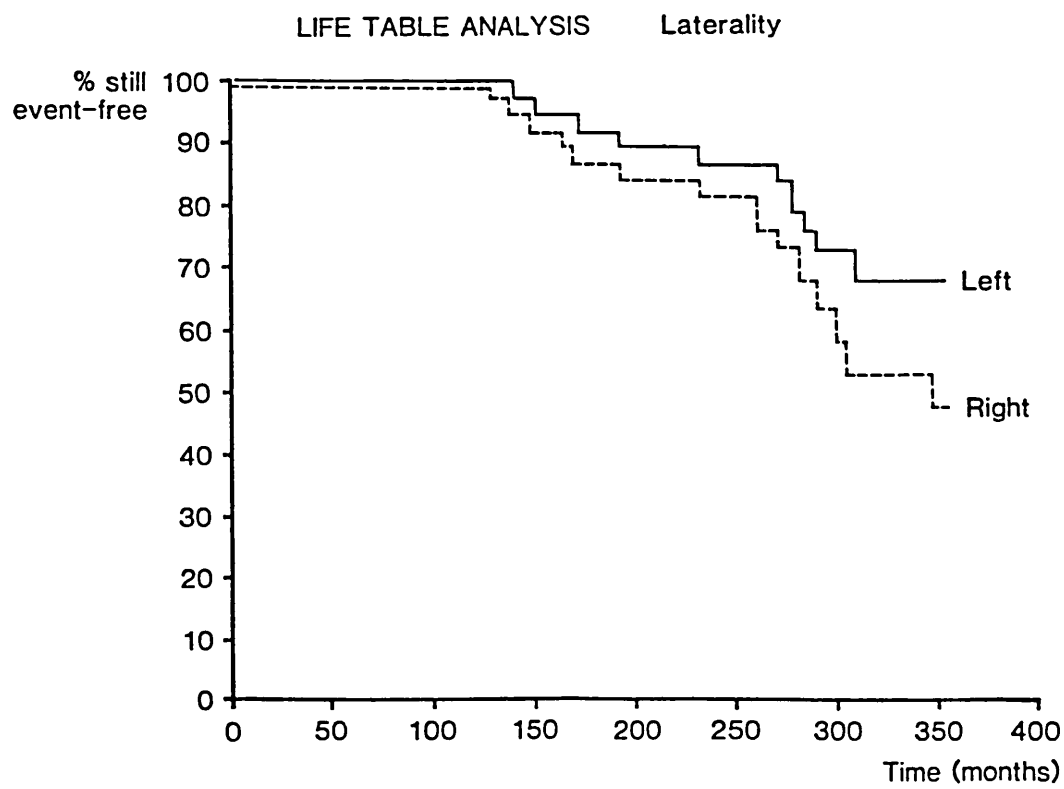


Figure 3.9



chi^2 for: trend (1 df) = 27.15

non-linearity (1 df) = 5.44

homogeneity (2 df) = 32.59 p = 0.00001.

This result indicates that tumour fixation is highly correlated to survival beyond ten years.

3.12.5 Tumour site

The patients were stratified by laterality, and by site, and a life table analysis performed for laterality, for site comparing medial to lateral tumour survival, and for site comparing upper and lower tumour survival.
Left sided tumours had marginally better overall survival than right sided tumours [see Table 3.11, and Figure 3.9].

Site	Patients (%)	Observed Events	Expected Events	Observed / Expected
Left	422 (48)	33	42.24	0.78
Right	454 (52)	55	45.76	1.20
Total	876	88	88	1.00

Table 3.11 Survival analysis: laterality

A two tailed p-value for positive trend estimated from a log rank test gives p = 0.02, which is only marginally significant. There was no significant difference in tumour size, stage, or fixation between right sided tumours and left sided tumours.

Survival analysis by site within the breast is shown in

Tables 3.12 and 3.13 and Figs.3.10 and 3.11.

Site	Patients (%)	Observed Events	Expected Events	Observed / Expected
Medial	215 (33)	15	20.63	0.73
Central	61 (10)	10	6.65	1.50
Lateral	373 (57)	40	37.73	1.06
Total	649	65	65	1.00

Table 3.12 Survival analysis: tumour site (horizontal)

The two tailed p-value for positive trend estimated from the log rank test gives a p-value which is not significant, although the life table shows central tumours to have a worse prognosis than either medial, or laterally placed tumours, which have parallel survival curves.

Site	Patients (%)	Observed Events	Expected Events	Observed / Expected
Lower	133 (19)	10	14.02	0.71
Central	61 (9)	10	7.03	1.42
Upper	513 (72)	52	50.95	1.02
Total	707	72	72	1.00

Table 3.13 Survival analysis: tumour site (vertical)

For the analysis of survival by vertical tumour site a two tailed p-value for positive trend estimated from the log rank test gave p not significant. The life table demonstrates no significant difference in survival between tumours placed in the lower half of the breast relative to the upper.

Figure 3.10

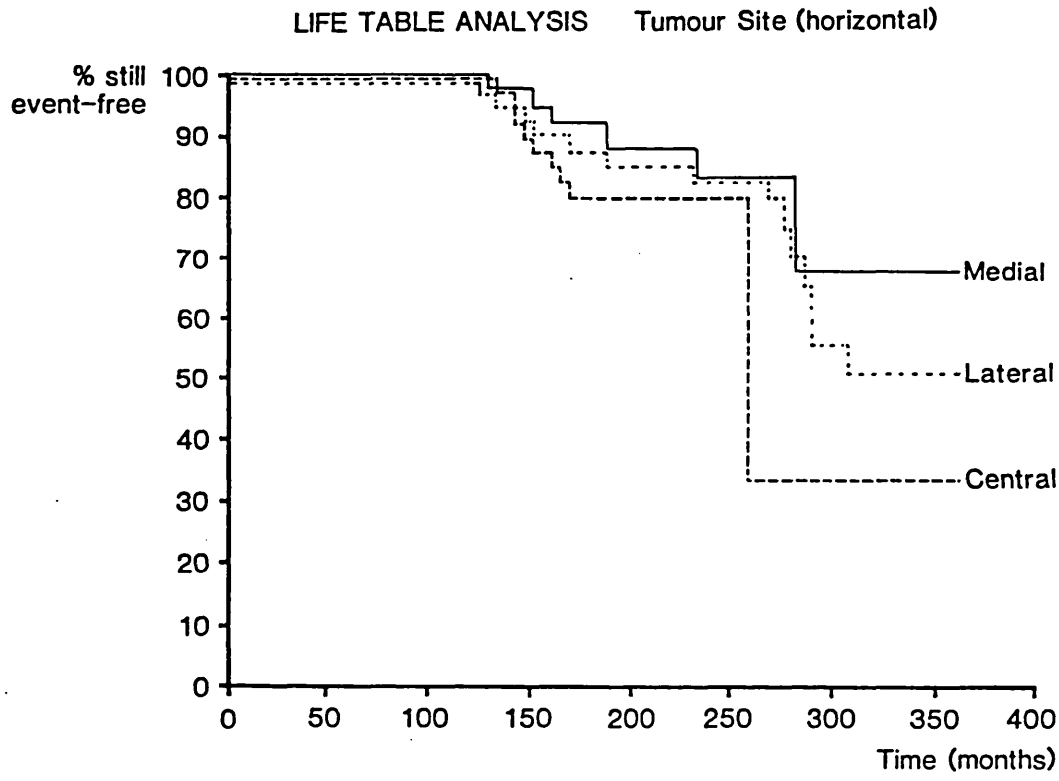
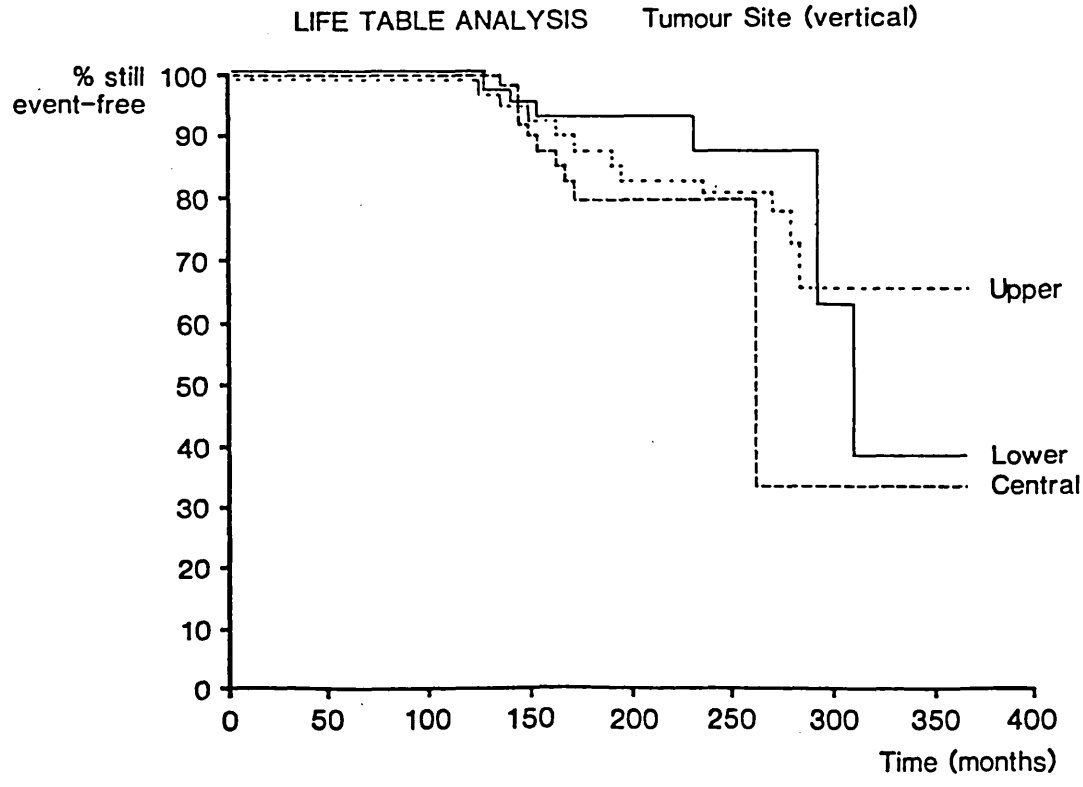


Figure 3.11



Tumour site and pathological nodal status were however interdependent related covariates [see Table 3.14].

Site	N_o	(%)	N_1 N_2 N_3	(%)		P
Medial	83	(67)	40	(33)	)	0.001
Lateral	170	(47)	188	(53)	)	
Lower	91	(64)	52	(36)	)	N.S.
Upper	138	(62)	84	(38)	)	

Estimate of p from chi^2 test.

Table 3.14 Analysis of pathological nodal status by tumour site

Tumour site was therefore analysed with respect to pathological nodal status. The survival analysis for patients stratified by nodal status (negative:positive) and site (horizontal) is shown in Tables 3.15 and 3.16, and the life table analysis in Figs.3.12 and 3.13.

Tumour Site	Patients (%)	Observed Events	Expected Events	Observed / Expected
Medial	166 (40)	7	12.79	0.55
Lateral	245 (60)	23	17.21	1.34
Total	411	30	30	1.00

Table 3.15 Survival analysis: node negative patients by tumour site (horizontal)

A two tailed p value for positive trend, estimated from the log rank test gives p = 0.03, for node negative patients analysed by horizontal tumour site.

Figure 3.12

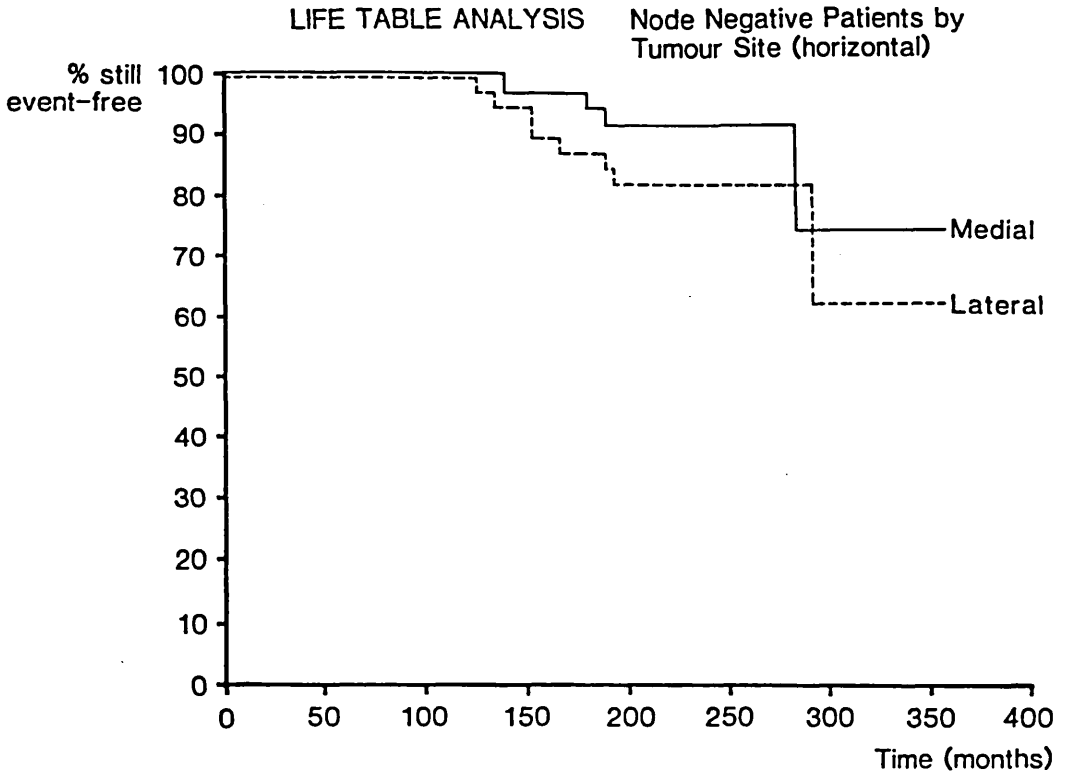
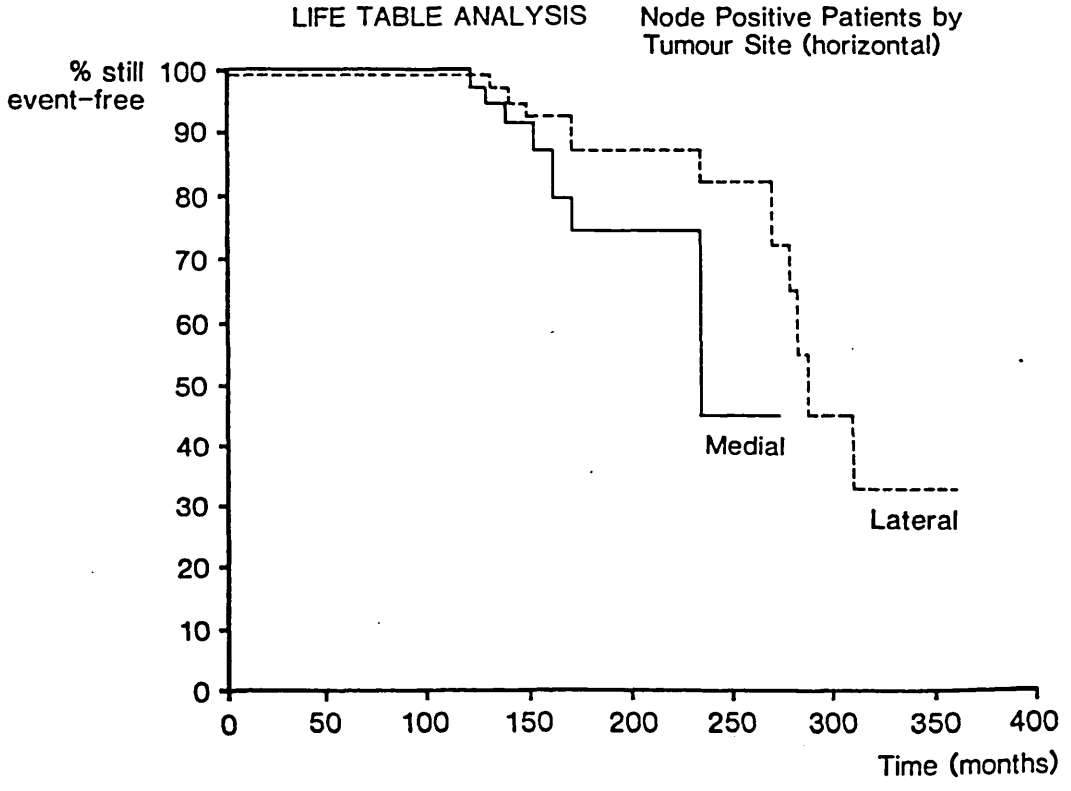


Figure 3.13



Tumour Site	Patients (%)	Observed Events	Expected Events	Observed / Expected
Medial	48 (29)	8	4.74	1.69
Lateral	116 (71)	16	19.26	0.83
Total	164	24	24	1.00

Table 3.16 Survival analysis: node positive patients by tumour site (horizontal)

For the node positive patients analysed by horizontal tumour site a two tailed p value for positive trend, estimated from the log rank test gives $p = 0.09$.

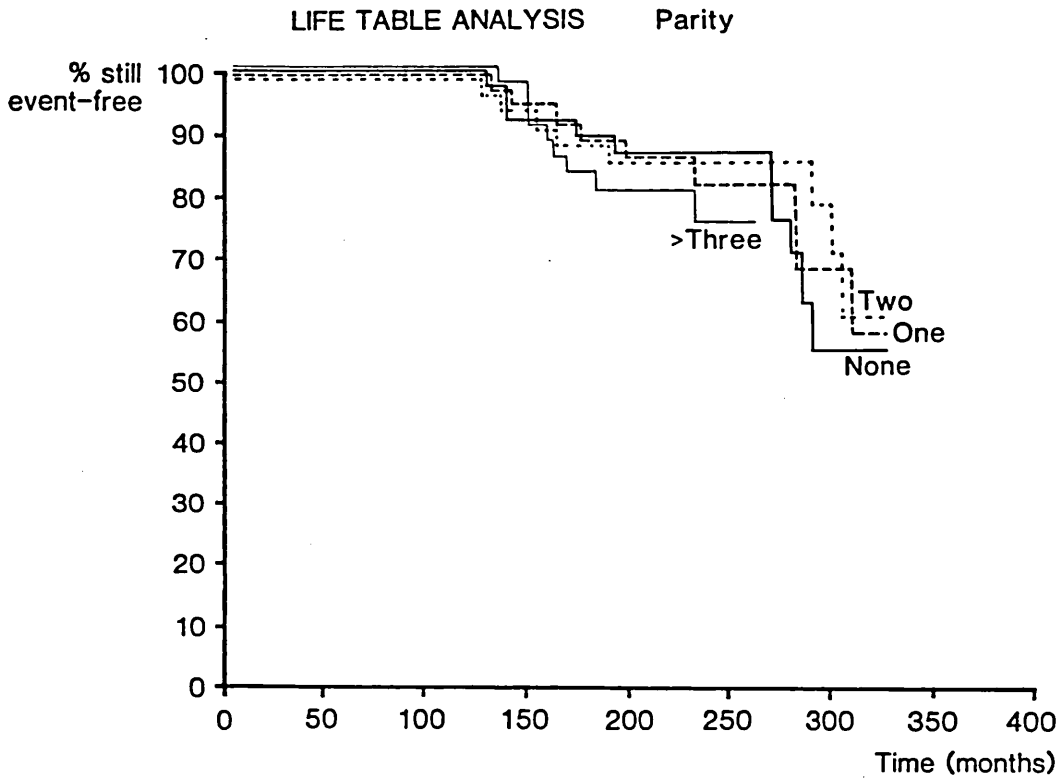
An overall regression analysis was performed for tumour site and nodal status and a two tailed test for trend estimated from the log rank test. This gave a non-significant p value, indicating no correlation between long term survival tumour and site stratified by nodal status, comparing medial and lateral tumours.

A similar analysis was performed for tumours stratified by pathological nodal status and tumour site, comparing upper tumours with tumours of the lower breast. Using the same statistical methods, there was no association between long term survival and tumour site.

3.12.6 Parity

A life table analysis was performed on 739 patients on whom data on parity was available. The patients were stratified

Figure 3.14


LIFE TABLE ANALYSIS
Parity
% still event-free
100
90
80
70
60
50
40
30
20
10
0
>Three
Two
One
None
0
50
100
150
200
250
300
350
400
Time (months)

probability estimated from the log rank test did not demonstrate a significant relationship, and it is concluded that long term survival is not related to parity.

3.12.7 Late recurrence

Of 890 patients in this study, 37 (4%) had a proven local or distant metastasis occurring ten years or more following treatment. Six patients had local recurrence only, 31 had distant recurrence only, and 7 patients had both local and distant recurrence. The mean age (± S.D.) of patients with late recurrence was 60.0 years (± 9.2).

The mean tumour diameter (± S.D.) was 4.3 cms. (± 2.2) compared to the mean diameter (± S.D.) of 3.37 cms. (± 1.9) of the total cohort. The difference is not statistically significant (Student's t test).

The primary tumours of the patients with late recurrences were left sided in 11 (32%) and right sided in 25 (68%) compared to 422 (48%) and 454 patients (52%) respectively in the total cohort, although this difference is not statistically significant (chi^2 = 3.63, p = N.S.).

Pathological nodal status was analysed in 25 of the patients with late recurrence, 10 (40%) having pathologically negative nodes, and 15 (60%) having pathologically involved nodes, compared to 346 (62%) and 210 patients (38%) respectively for the total cohort. This difference also

was not statistically significant (chi^2 = 1.10, p = N.S.). The patients with late recurrences were analysed with respect to tumour fixation and clinical TNM stage, and compared to the initial total cohort. The results are summarised in Tables 3.17 - 3.19.

Fixation	Patients (%) Late recurrence		Total cohort		p
None	12	(33)	344	(44)	N.S
Skin	20	(56)	387	(50)	N.S
Deep	4	(11)	44	(6)	N.S

p estimated from chi^2

Table 3.17 Late recurrence: tumour fixation

Tumour TNM stage	Patients (%) Late recurrence		Total cohort		p
T_o	0		30	(4)	N.S.
T_1	3	(9)	189	(24)	N.S.
T_2	22	(65)	443	(57)	N.S
T_3 T_4	9	(26)	117	(15)	N.S

p estimated from chi^2

Table 3.18 Late recurrence: tumour TNM stage

Nodal TNM stage	Patients (%) Late recurrence		Total cohort		p
N_o	23	(64)	561	(70)	N.S
N_1	11	(31)	222	(28)	N.S
N_2 N_3	2	(5)	16	(2)	N.S

p estimated from chi^2

Table 3.19 Late recurrence: nodal TNM stage

The patients with late recurrence were not significantly

different from the total cohort with respect to tumour fixation or clinical tumour stage.

For patients with distant recurrence the mean interval from appearance of distant metastasis to death (± S.D.) was 23.5 months (± 15.9, range 1 to 52 months) for patients with late recurrence. This is incomparison to a mean interval (± S.D.) of 18.0 months (± 15.8) between appearance of distant metastasis and death in patients followed up for ten years or less. (This latter cohort of patients are those used as breast cancer controls in the endocrinological study of survival in Section 2 of this thesis). The difference between the two cohorts in the mean interval between distant recurrence and death was significant ($p < 0.01$, Student's t test).

The mean time (± S.D.) from treatment of primary tumour to appearance of late distant metastasis was 13.3 years (± 4.0) and late local metastasis, 13.8 years (± 3.1).

The sites of distant metastases were:- opposite breast, 4 (9%); lung, 5 (11%); pleura, 8 (17%); bone, 11 (23%); skin, 6 (13%); liver, 3 (6%); and lymphatic, 10 (21%).

3.13 Conclusions

In this study, the end point of the life table analysis has been taken as death due to breast cancer. The study therefore investigates the influence of prognostic factors on the natural history of breast cancer, rather than overall survival. This may, at least in part, explain the differing conclusions from previous studies that have defined their end point as death from any cause.

3.13.1 Survival

The log survival curve [Fig.3.2] illustrates the continuing mortality from breast cancer between ten and thirty years from primary treatment. Following the restraints discussed in the previous chapter, it is incorrect to assume exponential survival, but deaths from breast cancer average 2.75% annually over the period analysed.

Although the incidence of patients receiving adjuvant chemotherapy increased during the second decade of entry to the study, there is no evidence that this can be expected to modify long term survival.

3.13.2 Tumour Stage

In an analysis of the Edinburgh series a previous study concluded that 'there was no evidence beyond 10 years of an effect on survival of the original stage of the disease'

(Langlands et al 1979). In contrast, this study shows tumour stage exerting an important influence extending beyond ten years [Fig.3.3]. The classification of tumour TNM stage is dependent upon the clinical estimation of tumour size and fixation, and so it is necessary to analyse these factors independently.

3.13.3 Tumour size

In agreement with previous studies, there was no significant correlation between tumour size and survival beyond ten years. In particular, the life table illustrates the close proximity of the survival curves for tumours 2.0 to 4.9 cm and those 5.0 cm and larger [see Fig.3.6]. Although lymph node status is related to tumour size, very large tumours (5.0 cm) have proportionately fewer involved nodes and may represent a cohort in which tumour size is a reflection of the ability of host resistance (Langlands et al 1979).

The absence of any impact on long term survival in the remainder of these patients would suggest that tumour size may be a reflection only of the chronology of the disease.

3.13.4 Tumour fixation

In contrast to tumour size, the presence of fixation is a highly unfavourable prognostic sign with an influence

extending well beyond the first decade [see Fig.3.8]. This study does not therefore support the findings of Langlands and co-workers that the influence of fixation has largely disappeared by this time, but is in agreement with the subsequent study, on the same data series, by Gore (Langlands et al 1979, Gore 1983).

The presence of tumour fixation, whether superficial or deep, is an important indicator, whether of tumour invasiveness or lack of host resistance, and is the most significant of the clinical prognostic factors studied. It appears to reflect accurately the subsequent tumour:host relationship, all patients with deep fixation dying of recurrent disease, albeit up to two decades later.

3.13.5 Axillary node status

Analysis of the clinical axillary node status in this series demonstrates, in agreement with a previous study, that long term survival is not influenced by clinical nodal status, with the exception of the presence of nodal fixation (Gore 1983). In view of the subsequent analysis of pathological nodal status, it seems that this reflects only the inaccuracy of clinical nodal staging.

Pathological nodal status, in contrast, exerts an important, and continuing impact on breast cancer survival [see Fig.3.5]. This lends credence to the hypothesis that regards regional lymph nodes as of more biological than

anatomical significance (Fisher et al 1980). Patients with positive axillary lymph nodes have a substantially greater risk of both local and distant metastases even in the third decade of follow-up, indicating that the positive node is a reflection of the tumour:host relationship and not a source in itself of distant metastases.

3.13.6 Tumour site

Long term survival was also analysed by tumour site. There was no difference in long term survival between medially and laterally placed tumours, nor between tumours in the upper or lower breast [see Figs.3.10 and 3.11]. Central tumours had a marginally worse prognosis but this was not statistically significant. Nodal status is related to tumour site, and it is recognised that approximately 40% of medial tumours have pathologically involved internal mammary nodes (Caceres 1967 and see Table 3.13). Life table analysis of patients by tumour site suggests that there is no difference in survival between axillary node negative, medial or lateral tumours. In the axillary node positive patients life table analysis shows a higher rate of recurrence in the medially placed tumours, although this did not reach statistical significance despite the fact that they are likely to have an increased incidence of involved internal mammary nodes. There was no difference in treatment protocol between the two groups.

3.13.7 Late recurrence

The overall conclusion to be drawn from the data on the small number of patients with late recurrence is the striking similarity between these patients and the control group in which clinical recurrence occurred on average, a decade earlier. This small study only emphasises further, the difficulties in predicting outcome.

CHAPTER 4

TUMOUR CARCINOEMBRYONIC ANTIGEN

4.1 Introduction

The preceeding chapter has demonstrated that the clinical prognostic factors governing short term survival in breast cancer have, with the exception of fixation, much less influence on long term survival, suggesting that they may reflect more the chronological age of the tumour than its biological growth characteristics. Some support for this interpretation comes from a study of the survival of 896 patients following the first appearance of distant metastases (Paterson et al 1982). In this study, clinical stage and pathological nodal status at presentation exerted no effect on survival following first distant metastasis, although the level of cytosol oestrogen receptor was highly significant. This suggests that both tumour fixation and oestrogen receptor content may be prognostic factors of a different order.

At the present time it is not possible to investigate the role of oestrogen receptor on long term survival, as prospective studies have barely reached ten years, and until the development of a satisfactory immunohistochemical method, retrospective analysis is not feasible. However tumour oncofoetal antigens have been related to survival in breast cancer, and appear to be independent of tumour size, clinical stage, histological type and histological grade (Shousha et al 1979, Wahren et al 1978, von Kleist et al 1982). Immunoperoxidase methods for carcinoembryonic antigen permit a retrospective study on the influence of

this oncofoetal antigen on long term survival.

4.1.1 Aim of study

The aim of this part of the study is to investigate the role of tumour carcinoembryonic antigen on long term survival in breast cancer, and its relationship to other known clinical and pathological prognostic factors. Conventional immunoperoxidase techniques will be used, but additionally a monoclonal anti-human CEA antibody will be used for the first time.

4.2 Carcinoembryonic antigen in breast cancer

Carcinoembryonic antigen (CEA), is normally produced, between the second and sixth month of intrauterine life, in the gut, liver and pancreas. It is a large glycoprotein of molecular weight around 200,000.

In adult life elevated levels of serum CEA may be associated with tumours of the alimentary tract, lung, ovary, bladder, thyroid and parotid gland, as well as in patients with breast cancer (Gold and Freedman 1965, Klockars et al 1980, Caselitz et al 1981). Although in colorectal cancer there is a good correlation between circulating serum CEA and tumour burden, this relationship is much poorer in patients with breast cancer, limiting its usefulness as a tumour marker (Swanson et al 1978, Steward et al 1974).

4.2.1 Serum CEA

The incidence of elevated levels of serum CEA in patients with breast cancer varies from 47% to 80% and is dependent upon age and smoking (Alexander et al 1976). However serum levels of CEA are very poorly correlated to the presence of tissue CEA as demonstrated by immunoperoxidase techniques, and the incidence of elevated levels of serum CEA in patients in whom CEA is present in tumour tissue, varies from 41% to 55% (Mansour et al 1980, Wahren et al 1978, von Kleist et al 1982).

Conversely, Miller has assayed CEA from the media in which breast cancer explants have been cultured, and in these circumstances 75% of tumours are found to secrete CEA, of which only 63% can be demonstrated to possess CEA by staining (Miller et al 1983). This would suggest that, although immunoperoxidase staining is more sensitive than serum assay, it may be less efficient in formalin fixed tissue.

4.2.2 Tissue CEA

CEA may be demonstrated histologically in between 60% and 80% of tumours (Steward et al 1974, Shousha et al 1978, Shousha et al 1979). However, there appears to be no correlation between tissue CEA and tumour stage, tumour size, histological type, histological grade, or cellularity (Wahren et al 1978, Shousha et al 1978, Shousha et al 1979, von Kleist et al 1982, Duffy et al 1983). The only dissenting study is that by Walker who did demonstrate significantly reduced immunoperoxidase detection of CEA in patients with poorly differentiated tumours (Walker 1980).

4.2.3 Tissue CEA and survival

A retrospective study of tissue CEA in 69 patients treated for early breast cancer and followed up for between six and thirteen years demonstrated a significant inverse relationship to survival (Shousha et al 1979) [See Table 4.1].

Patients		Survival 5 years	10 years
Unstratified	CEA +	29*	12
	CEA -	93*	67
No metastasis	CEA +	32*	9*
	CEA -	92*	80*

* p=0.001 estimated from chi^2 (1 df)

Table 4.1 Tumour CEA and survival (Shousha et al 1979)

The survival advantage of CEA - negative tumours was shown to be independent of the presence or absence of metastases although both this study, and a previous one had demonstrated a correlation between CEA positivity and presence of lymph node metastases (Shousha et al 1978). This finding was not supported in one other study in which there was no correlation of tumour CEA to lymph node status or 2 year survival, but there are no other reports in the literature concerning survival (Walker 1980).

Although oestrogen receptor content is known to be highly correlated with survival, tumour CEA content has been variously described as having a poor correlation to levels of oestrogen receptor, or none (Menendez-Botet et al 1976, Persijn and Korsten 1977, Silva et al 1982).
It therefore appears that a relationship may exist between histologically demonstrated CEA and prognosis that is either partly, or wholly independent of the major clinical and pathological prognostic variables.

4.3 Patients

Sixty-eight patients were investigated retrospectively in a study to assess the relationship of histologically demonstrated CEA in breast cancer and survival.

The patients were divided into two cohorts, short term survivors (n = 22) who had died of breast cancer between six months and five years following treatment, and long term survivors (n = 46) who had survived ten years or more disease-free from the date of primary treatment. In order to evaluate the relationship of CEA to survival independent of histological grade a larger proportion of long term survivors with moderate or poorly differentiated tumours was chosen. There were no other criteria of entry other than that formalin fixed histological material in paraffin blocks was available and that subsequent histological examination and haematoxylin and eosin stained slides demonstrated invasive breast cancer to be present.

The clinical stage of the tumour (Stage I-III, UICC 1980), and the pathological nodal status were obtained from patient records. Survival time was confirmed using the tumour registry, Charing Cross Hospital.

4.4 Methods

Five 4 µm. sections were cut from the stored formalin-fixed paraffin-wax-embedded blocks. The sections were de-waxed and the first section stained with haematoxylin and eosin. This was then examined microscopically and the histological type and histological grade noted. (Bloom and Richardson 1957).

Three sections from each patient were then stained using three separate methods for immuno histochemical localisation of CEA. The first section was stained using a peroxidase-antiperoxidase (PAP) technique in which the antigen is first reacted with a rabbit anti-human CEA, the primary antibody. A link antibody, swine anti-rabbit immunoglobulin, is then used to bind to the primary antibody, but since immunoglobulins have two binding sites, the other may be used to bind a peroxidase-antiperoxidase immune complex (PAP). Finally, the PAP complex can be localised using a substrate solution of diaminobenzidine tetrahydrochloride and (DAB) hydrogen peroxidase. DAB is oxidised in the presence of peroxidase to a brown deposit which can be visualised microscopically [See Fig.4.1].

The second method used was identical to the above PAP method except that the primary antibody was first absorbed with a perchloric acid treated extract of human spleen in an attempt to remove non-specific cross-reacting antigens (NCA) (Walker 1980).

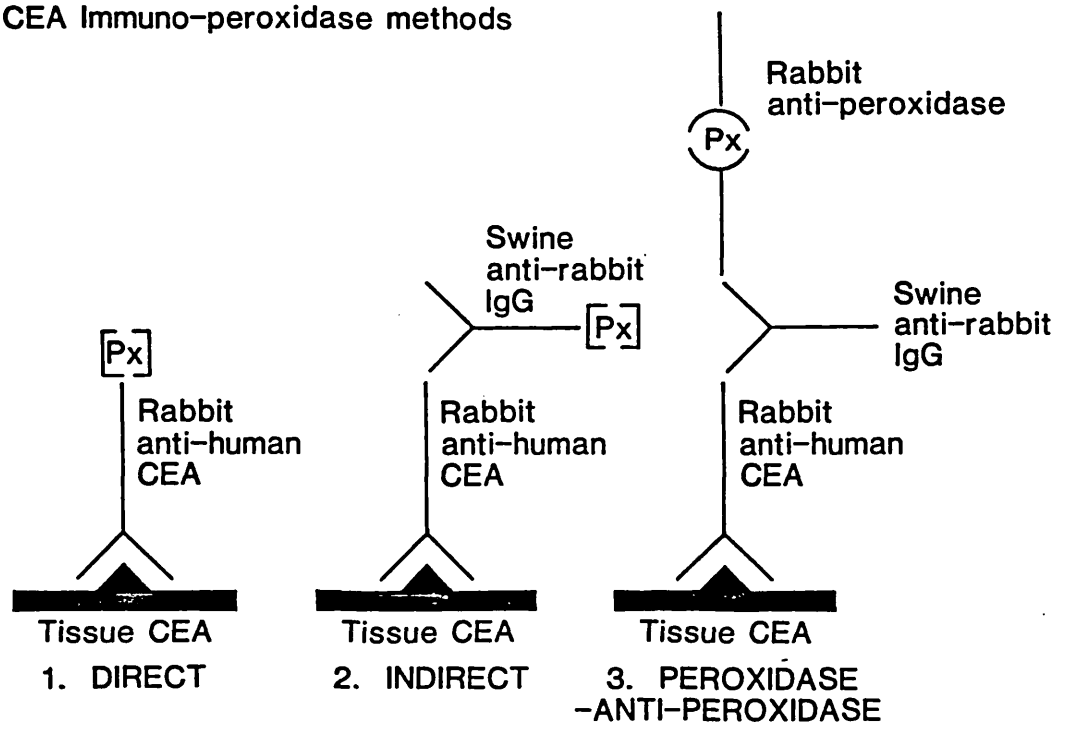
In the third method, a monoclonal mouse anti-human CEA antibody (H58) kindly provided by the Department of Medical Oncology, Charing Cross Hospital, was used to demonstrate CEA. An indirect immunoperoxidase method was used, utilising a rabbit anti-mouse immunoglobulin bound to peroxidase to localise the monoclonal antibody [see Fig.4.1].

4.4.1 PAP method

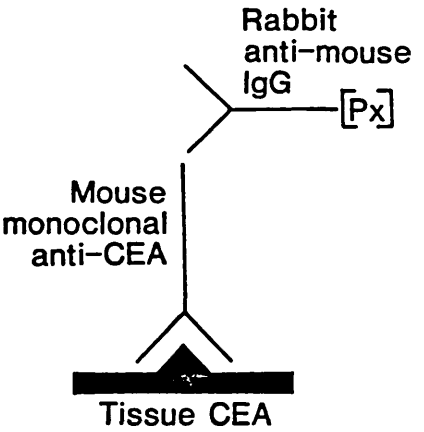
Paraffin sections of formalin fixed tissues were dewaxed in an Inhibisol/Xylene mixture (4:1) and brought to absolute alcohol. Endogenous peroxidase activity was blocked with a fresh solution of 3.0% Hydrogen Peroxide in absolute methanol for 30 minutes. The sections were then washed three times to remove excess peroxide. The sections were covered with a normal swine serum (NSS) diluted 1:10 with tris-saline buffer (pH 7.6) and left for 10 minutes before the excess NSS was removed. The sections were then treated with the primary antibody, rabbit anti-human CEA (diluted 1:60) (Dako-PATS) and left for 30 minutes at room temperature in a moist chamber. The sections were washed three times prior to addition of unlabelled swine anti-rabbit immunoglobulin diluted 1:50 and left for 30 minutes (Dako PATS, Z 196). The sections were again washed three times before the peroxidase anti-peroxidase complex (Dako-PATS, Z113) in dilution 1:50 was applied in a moist chamber for 30 minutes. After washing the peroxidase activity was

Figure 4.1

CEA Immuno-peroxidase methods



Monoclonal anti-CEA
immuno-peroxidase method



developed with a freshly prepared solution of 3.3' - diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide in tris-saline buffer (pH 7.6). The sections were counterstained with Coles' haematoxylin, dehydrated, cleared in xylene and mounted in DPX.

4.4.2 Indirect method

For the monoclonal mouse anti-human CEA an indirect method was used. The sections were deparaffinised, treated with hydrogen peroxide and after washing, covered with normal rabbit serum diluted with TRIS-saline buffer (1:10). The sections were then treated with monoclonal antibody for one hour at room temperature in a moist chamber. They were then washed, treated with peroxidase conjugated rabbit anti-mouse immunoglobulin (DAKO, P161) diluted 1:30, and incubated for 30 minutes. Following washing, the sections were stained with 0.05% Diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide in tris-saline buffer (pH 7.6). The sections were counterstained and mounted as previously. Duplicate sections of some tumours were treated with trypsin before applying this indirect technique (Fox et al 1982).

4.4.2 Controls

1. Sections of colonic carcinoma known to be positive for CEA were simultaneously stained with each set of breast carcinoma sections, using each of the methods described (positive control).

2. Endogenous peroxidase control: sections were treated with Hydrogen Peroxide solution followed by DAB (negative control).

3. Primary antiserum omission control: the primary antiserum was omitted, but the sections were processed through all the remaining steps (negative control).

4. Non-immune serum control:

(a) PAP method - primary antibody was replaced by normal rabbit serum the protein concentration of which was equivalent to the primary antibody (negative control).

(b) Indirect method - monoclonal antibody replaced by non-immune mouse IgG at an equivalent concentration to the primary antibody (negative control).

4.4.3 CEA staining

The presence of CEA was noted by visualisation of dark brown staining in tumour cells whether within the cells (intracellular), on their surface (surface staining), or within the lumen of neoplastic cells (intraluminal).

The results were expressed in four grades dependent upon the density of staining:

Grade 0 - No staining present.

Grade 1 - Faint CEA staining, usually of only a small proportion of malignant cells.

Grade 2 - Moderately positive staining.

Grade 3 - Strongly positive staining present.

In many cases, positive staining was localised, with other unstained areas present. When the intensity of staining varied within the section, the CEA grade was based upon the highest grade present. Histiocytes, mast cells and areas of necrosis were also noted to stain positively but these did not influence assessment of CEA grade.
All sections were coded and analysed histologically without reference to other clinical or pathological data.

4.4.4 Statistical methods

The patients were stratified by survival into short and long term survivors. The patients were also analysed by stratification into pathological axillary node status, clinical stage, histological type, and histological grade.

Analysis was by a non-parametric statistical test. Tied values were assigned the average of their ranks, and the two tailed p value estimated from the standardised normal deviate (SND). This test gives a conservative value for probability as the variance has not been adjusted for tied values.

4.5 Results

4.5.1 CEA staining

Positive CEA staining was demonstrated as dark brown granules situated either within the cytoplasm of malignant cells, or occasionally on the cell surface or within ducts. CEA staining was also sometimes demonstrated in normal acini and areas of adenosis.

Positive CEA staining (Graded 1 to 3) was seen in 33 (49%) of breast carcinomas with the PAP absorbed technique and 46 (68%) of carcinomas with the non-absorbed PAP technique. With the monoclonal antibody however, only 6 patients (9%) had CEA positive staining, and in all cases these were patients who had Grade 3 staining by the alternative methods [See Figs. 4.2 - 4.9]. For this reason the subsequent analyses refer to CEA staining by the PAP techniques, using polyclonal antibody.

4.5.2 Age

The mean age (± S.D) of the 68 patients in the study was 52.4 years ± 15.7. The patients were analysed by grade of CEA staining (PAP absorbed and PAP non-absorbed techniques) against age. There was no correlation between grade of CEA staining and age (SND = 0.2, p = N.S.).

4.5.3 Histological type

The patients were stratified initially into short term survivors (mean survival ± S.D. = 3.15 years ± 2.4) and long term survivors (mean survival ± S.D. = 17.18 years ± 4.9). The tumour histological type within the two cohorts is shown in Table 4.2.

Histological type	Short term survivors	Long term survivors
Infiltrating duct	21	33
Lobular invasive	1	8
Medullary	0	1
Mixed (ductal & lobular)	0	4
Total	22	46

Table 4.2 Histological type

The grade of CEA staining was analysed against histological type. There was no correlation between grade of CEA staining and histological type (SND = 0.23, p = N.S.).

4.5.4 Survival

The grade of CEA staining (Grades 0,1,2,3) was analysed by survival cohort (short term, long term) for the PAP absorbed and PAP non-absorbed techniques, [see Table 4.3].

When all patients were analysed there was a significant difference in CEA staining between long term and short term survival (PAP (absorbed) method, SND = 2.35, p = 0.009; PAP (non absorbed) method, SND = 2.04, p = 0.02).

	CEA Grade							
	PN				PA			
Survival	0	1	2	3	0	1	2	3
short term	4	4	5	9	7	4	5	6
long term	18	9	8	11	28	5	9	4

PN PAP (nonabsorbed) method.
PA PAP (absorbed) method

Table 4.3 Tumour CEA grade and survival

When only infiltrating ductal carcinomas (n = 56) were analysed, there was also a significant difference (PAP (absorbed) method, SND = 2.81, p = 0.0025; PAP (non absorbed) method, SND = 2.25, p = 0.01) between the two cohorts. The cohort of long term survivors were further stratified into two subgroups, those patients with less than the median survival of the cohort (16 years) and those patients with greater than the median survival [see Table 4.4]. Analysis of CEA staining between these two groups demonstrated no significant difference (PAP (absorbed) method, SND = 0.2, p = N.S.; PAP (non-absorbed) method, SND = 0.25; p = N.S.).

Survival (yrs)	CEA Grade PN 0	1	2	3	PA 0	1	2	3
10-16	10	5	3	7	15	3	4	3
17-30	8	4	5	4	13	2	5	1

PN PAP (nonabsorbed) method
PA PAP (absorbed) method

Table 4.4 Tumour CEA grade and long term survival

4.5.5 Histological grade

CEA staining was analysed with the patients stratified by survival cohort and histological grade [see Table 4.5].

Histological grade	Short term survivors (%)		Long term survivors (%)	
I	0	(0)	3	(9)
II	7	(33)	16	(48)
III	14	(67)	14	(43)
Total	21		33	

Table 4.5 Histological grade

When all patients were analysed, there was no correlation between CEA staining and histological grade [see Table 4.6]. When moderately and poorly differentiated (histological grades II and III) tumours only were analysed, there was a statistically significant difference in CEA staining between the two survivor cohorts (PAP (absorbed) method, SND = 2.75, p = 0.003; PAP (non-absorbed) method, SND = 1.97, p = 0.02).

Histologial grade	CEA Grade PN 0	1	2	3	PA 0	1	2	3
I	3	0	0	0	2	1	0	0
II	6	4	5	8	12	4	3	4
III	7	8	6	7	14	3	7	4

Table 4.6 Tumour CEA grade and histological grade

4.5.6 Nodal status

The pathological nodal status was known for 49 patients, of whom 33 were long term survivors (node positive = 13, node negative = 20) and 16 were short term survivors (node positive = 10, node negative = 6).

The tumour CEA grade in these patients is summarised in Table 4.7. There was a statistically significant correlation between pathological nodal status and CEA staining (PAP (absorbed) method, SND = 23.2, p = 0.001; PAP (non-absorbed) method, SND = 23.3, p = 0.001) comparing tumours with no nodes involved, and those with one or more involved nodes.

Nodal status	CEA Grade PN 0	1	2	3	PA 0	1	2	3
negative	6	8	6	6	13	7	2	4
positive	5	3	5	10	8	4	6	5

Table 4.7 Tumour CEA grade and pathological nodal status

4.5.7 Tumour stage

The clinical tumour stage was known in 45 patients, and the tumour CEA grade in these patients is summarised in Table 4.8. There was no correlation between tumour stage and tumour CEA grade (PAP (absorbed) method, SND = 0.63, p = N.S.: PAP (non-absorbed) method, SND = 0.11, p = N.S.).

Tumour stage	CEA Grade PN 0	1	2	3	PA 0	1	2	3
I	1	2	2	1	3	1	2	0
II	6	6	4	11	11	8	2	6
III	3	3	4	4	6	1	4	3

Table 4.8 Tumour CEA grade and tumour stage

4.6 Conclusions

4.6.1 Survival

This study confirms that a strong inverse correlation exists between the presence of CEA in a breast tumour, as demonstrated by immunoperoxidase techniques, and survival. It does not appear however, at least within the limitations imposed by the number of patients in this study, that this influence on survival extends beyond 16 years after primary treatment.

4.6.2 Histological type and grade

Histological type is known to influence prognosis in breast cancer and this is reflected within this series by the significantly larger proportion of lobular carcinomas within the cohort of long term survivors. However, it is apparent that production of CEA is entirely independent of histological type, a finding supported by other studies. Histological grade is also an important prognostic variable, and previous studies have not been unanimous in finding CEA production to be independent. For this reason, this study was weighted to provide a cohort of long term survivors with moderate to poorly differentiated tumours. It is therefore of importance that comparison of CEA staining between short and long term survivors of equivalent histological grade, nevertheless provides a very significant difference.

4.6.3 Tumour stage

In agreement with an earlier study, a correlation was found between CEA production and pathological nodal status, although within the limitations of the number of cases involved this might represent a survival cohort effect. This finding supports the theory that the presence of oncofoetal antigens in adult tumours permits malignant cells to multiply with less restraint, and enables them to metastasise more readily (Stein et al 1978). Further evidence in favour of this theory comes from a study of multiple tissue biological markers, including CEA, in which the presence of four or more such markers was associated with lymph node metastasis (Walker 1982).

4.6.4 Immunoperoxidase methods

Previous studies have used both NCA absorbed and nonabsorbed methods and the differences in their findings attributed to non-specific binding. This study therefore compared both methods. The non-specific binding present in the non-absorbed method resulted in a higher overall percentage of CEA positive tumours (68%) than the absorbed method (49%), and also in a statistically less significant correlation with survival.

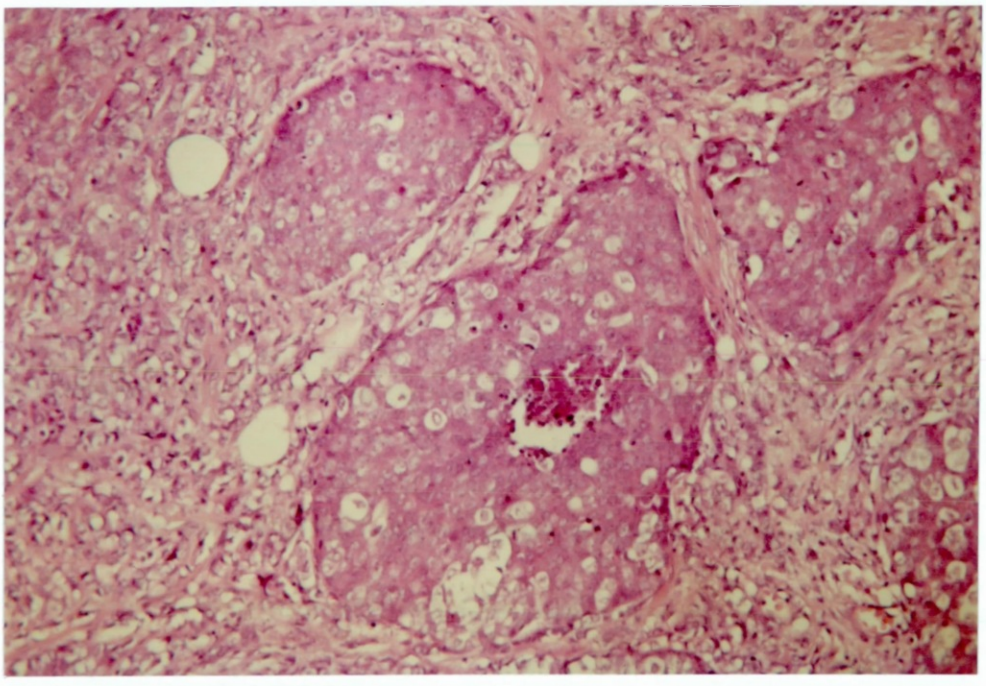
The monoclonal antibody failed to stain the majority of breast carcinomas when an indirect immunoperoxidase technique was used, even after trypsinization of the

sections before application of the monoclonal antibody, a technique known to enhance visualisation. The failure of the monoclonal antibody may be attributed either to its specificity, or possibly to the process of formalin fixation, although the same antibody successfully localised CEA in formalin processed sections of colonic tumours in the positive controls used in this study.

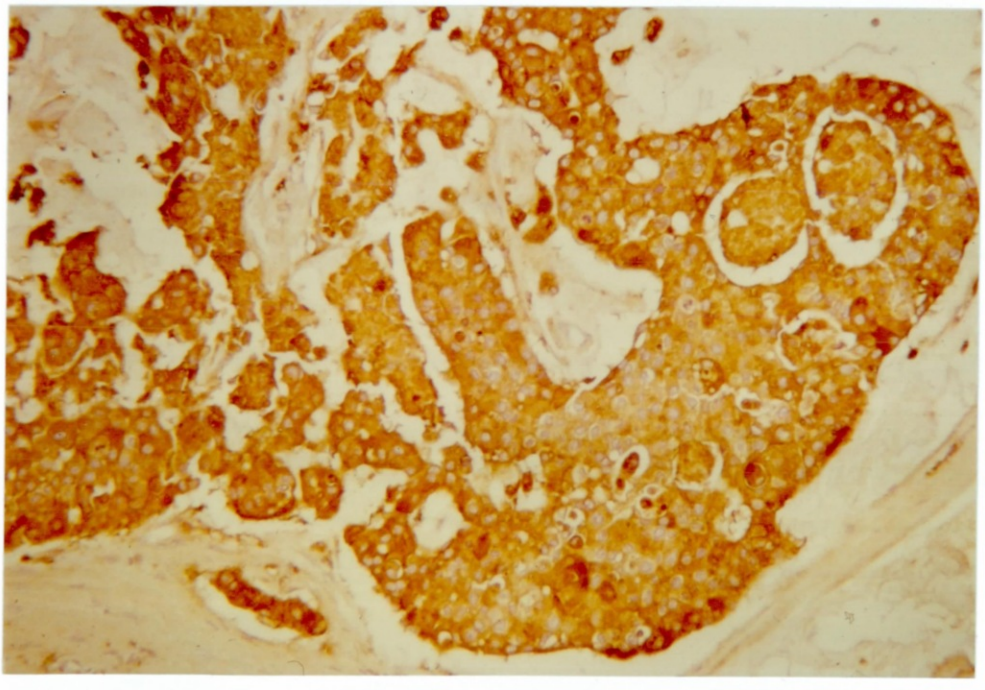
4.6.5 Summary

Tissue carcinoembryonic antigen has been demonstrated to be an important prognostic indicator in short term survival in breast cancer. As a biological variable it is independent of most other tumour prognostic variables with the exception of pathological nodal status, and possibly, of hormone receptors. As such, it appears to be an indicator of the metastasing ability of a tumour and is associated with poor prognosis even in the absence of pathologically involved axillary nodes. In view of the large literature on serum CEA, which, at least in breast cancer, has been found to be of limited prognostic value, the extreme paucity of studies on tissue CEA is surprising.

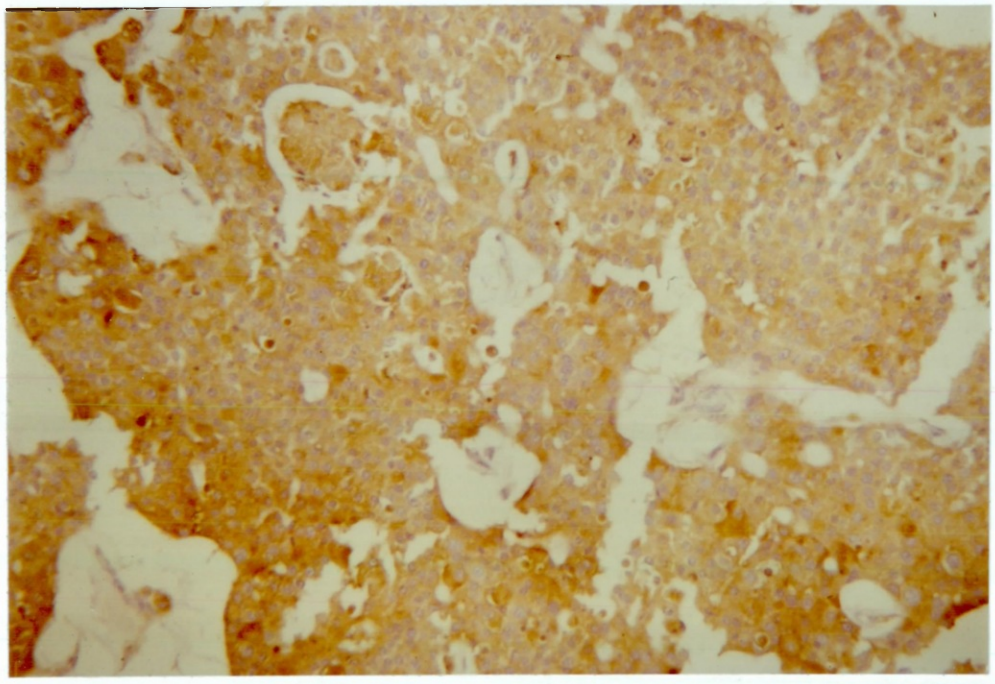
Figure 4.2



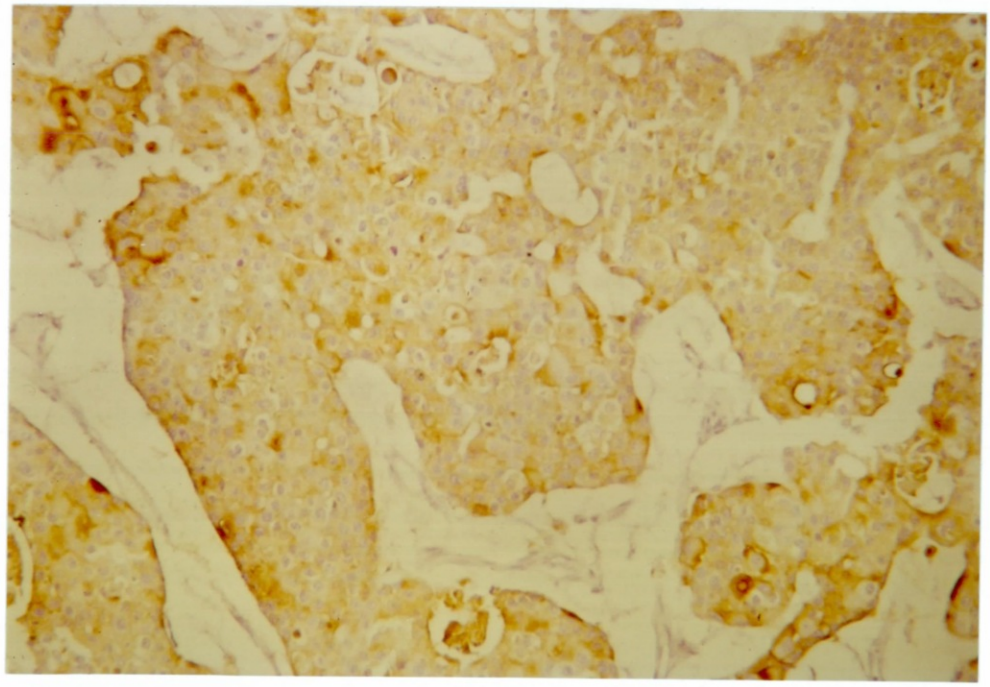
A. Haematoxylin & Eosin (x 60).



B. Dako-PAP (non-absorbed) anti-CEA [Grade II]. Scattered and intensely stained (brown) tumour cells are seen.

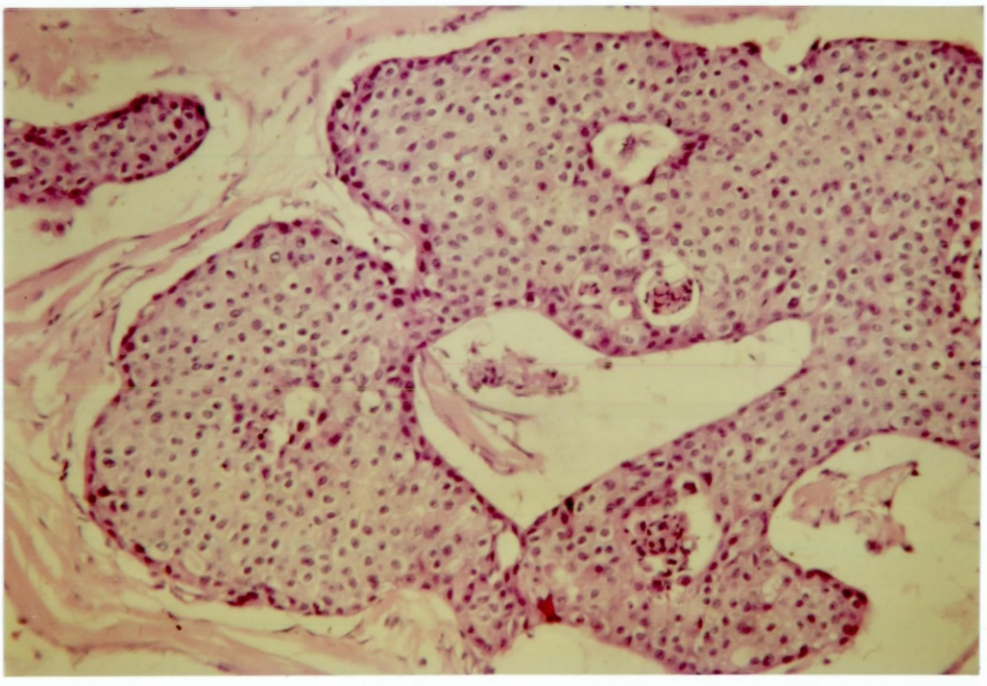


C. Dako-PAP (absorbed) anti-CEA [Grade I]
A few positively stained tumour cells.

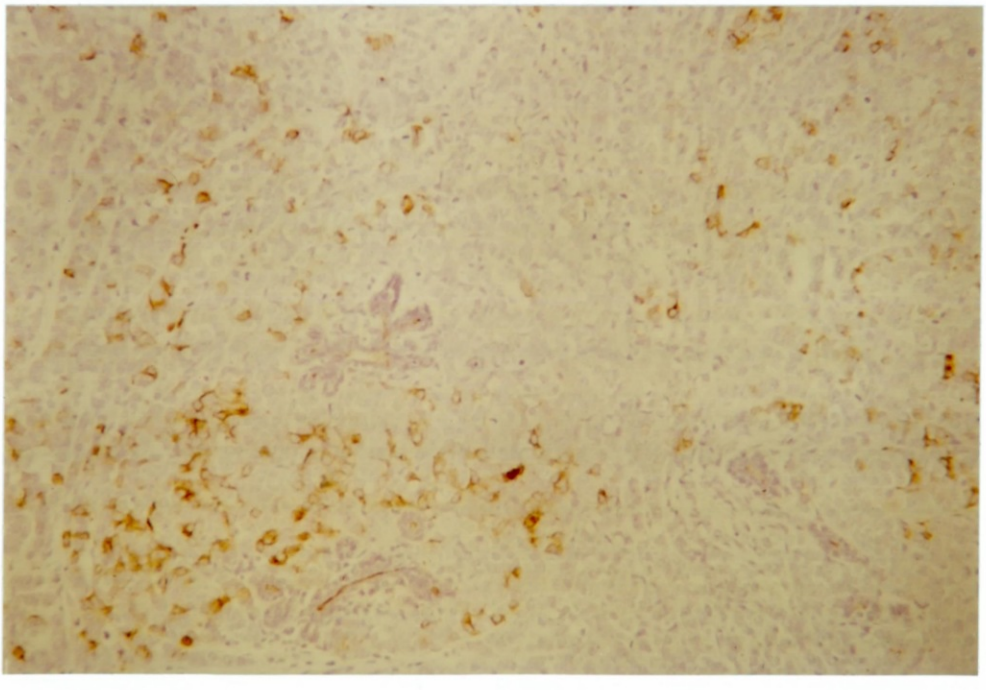


D. Monoclonal anti-CEA [Grade 0]
No brown staining is visualised

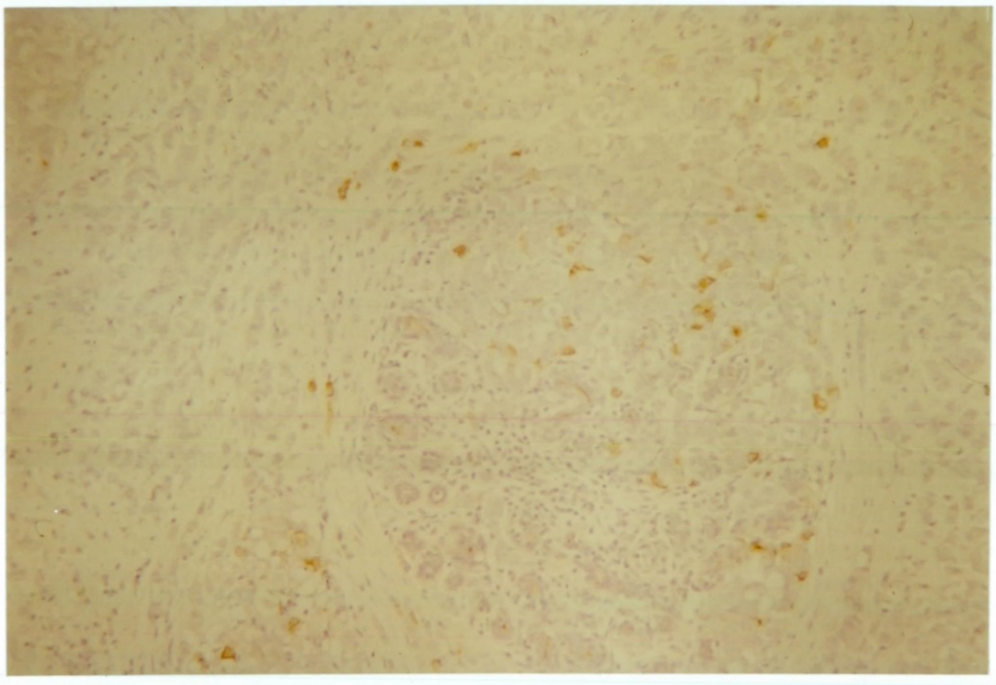
Figure 4.3



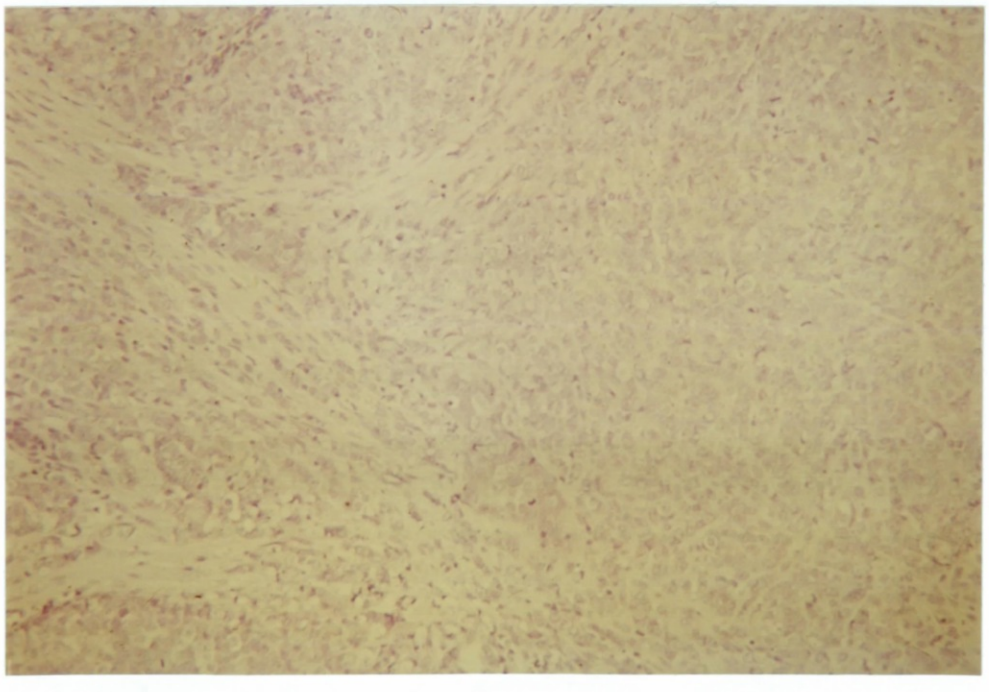
A. Haematoxylin and Eosin (x 60)



B. Dako-PAP (non-absorbed) anti-CEA [Grade III]
Intense brown staining of most tumour cells.



C. Dako-PAP (absorbed) anti-CEA [Grade III]
Staining is less intense but is still present in most tumour cells.



D. Monoclonal anti-CEA [Grade III]
One of the few sections that demonstrated positive staining.

S E C T I O N T W O

THE BIOLOGY OF THE HOST

CHAPTER 5

BREAST CANCER: HYPOTHESIS AND MODELS

5.1 Introduction

The second section of this thesis is devoted to the biology of the host, and in particular, the influence of host endocrine status on survival. Epidemiological and animal studies have suggested that several hormones may be involved in the development of mammary cancer, but despite this evidence, studies on urinary or plasma hormones in women at risk, or patients with early breast cancer have largely been inconclusive.

This section presents the results of a study analysing the endocrine status of a large cohort of long term survivors of treated breast cancer, comparing them with normal women, and patients with early breast cancer as controls. The justification for hormonal analysis on survivors of breast cancer is based largely on epidemiological evidence and studies of women at risk of breast cancer, or of patients with early disease that suggest that endocrine factors are important. This evidence is reviewed within each chapter of the section together with the smaller number of studies that have investigated endocrine mechanisms within the clinical course of the disease. In this chapter the major endocrine hypotheses are briefly reviewed in order to permit an understanding of the mathematical models subsequently proposed. These models relate epidemiological and serological evidence to cellular events and are therefore used in later chapters as a framework on which endocrine mechanisms can be discussed.

5.2 Endocrine hypotheses

Many endocrine hypotheses have been advanced, based mainly upon the weight of epidemiological evidence. The more important are presented briefly here, but are discussed in detail subsequently.

5.2.1 Oestrogens

Many of the epidemiological risk factors for breast cancer are associated with changes in oestrogen metabolism suggesting that they should be considered the primary carcinogens, or promoters, of mammary tumours.

Lemon, in the 'impeded oestrogen hypothesis', argued that oestriol, a less potent oestrogen than either oestradiol or oestrone, could modulate the effect of the more potent oestrogens by competitive binding of tumour receptor sites, but this hypothesis has been challenged by many subsequent studies (Lemon et al 1966).

Grattarola, noting that women with breast cancer may have abnormal endometrial biopsies in the luteal phase of the menstrual cycle proposed that excessive oestrogenic stimulation occurred due to inadequate progesterone secretion (Grattarola 1964). This theory was subsequently modified by Korenman who demonstrated that this effect occurred predominantly in a 'window' during puberty (Korenman 1980).

A fourth oestrogenic hypothesis was described by Siiteri based on epidemiological evidence in postmenopausal women that suggested that 'oestrone excess' stimulated mammary neoplasia (Siiteri et al 1974).

5.2.2 Progesterone

There is no direct evidence that progesterone is itself a carcinogen although it is required for normal cell growth and differentiation. On the contrary, the only direct evidence linking progesterone to mammary cancer is the inadequate corpus luteum function described by Korenman, in which progesterone secretion is reduced.

5.2.3 Androgens

Following a discovery by Bulbrook that measurement of urinary androgen metabolites could be used to predict the outcome of endocrine manipulation in women with breast cancer, several theories have been advanced that suggest that women with decreased secretion of adrenal androgens are at high risk of developing breast cancer (Bulbrook et al 1960).

5.2.4 Thyroid hormone

Since the observation, by Beatson in 1896, of regression in two breast cancers following administration of thyroid extract, there has been continuing debate as to the

influence of thyroid function on both the pathogenesis, and clinical course of breast cancer with much evidence, both in favour and against, such a relationship.

5.2.5 Prolactin

Prolactin, a pituitary hormone whose principal effect is on the initiation and maintenance of lactation, is a promoter of murine mammary cancer (Lyons 1958). There is however much less substantive evidence that it has a role in either human breast cancer development, or its subsequent clinical course.

5.3 Mathematical models of breast cancer

Experimental and epidemiological evidence that hormones may be responsible, at least in part, for the induction of breast cancer, suggests that this is a multistage process with a period of as long as 20 to 30 years between induction and subsequent clinical appearance of tumour.

Doll has described the value of relating such epidemiological evidence to cellular events by means of mathematical models (Doll 1978). These permit analysis of the relative importance of different cellular events in determining risk.

Early analysis of cancer mortality statistics lead to two similar hypotheses (Fisher and Holloman 1953, Nordling 1953). Both studies found that for the age group 25-74 years, the death rate increased proportionally to the sixth power of age, or more precisely:

$$\log \text{death rate} \propto \log \text{age}^6.$$

Nordling suggested that this relationship would be explained on the basis that seven successive mutations resulted in a cancer cell. However, this assumes that the rate of mutation remains constant, and this is not borne out by experimental evidence. Armitage and Doll developed Nordling's hypothesis further (Armitage and Doll 1954). They noted that whereas the log death rate v. log age is a straight line for many cancers including stomach, pancreas

and lung, this does not hold true for epithelial tumours in hormone dependent tissues such as breast or uterus [See Fig. 5.1].

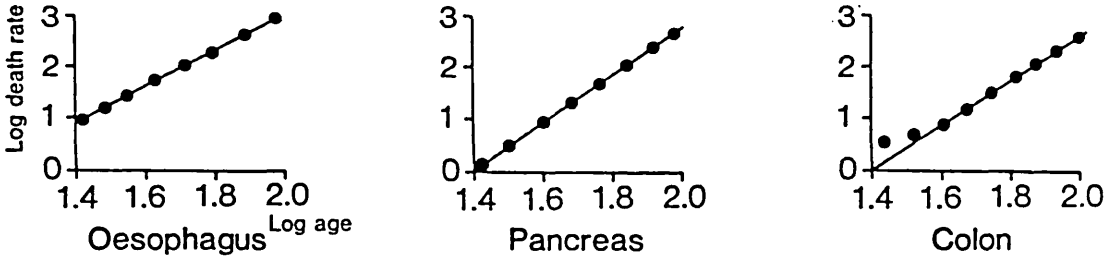
They suggested that the deficit in mortality with advancing age in these cancers might be due either to a reduction in the probability of one of the later stages of mutation occurring with advancing age, or conversely, that there was an increased chance of an initial stage of mutation occurring at an earlier age. They therefore developed a more complex mathematical model that allowed the impact of carcinogenic factors on mutation rates to vary with age.

Following the proposition by De Waard that breast cancer is two distinct diseases, one premenopausal, and the other postmenopausal, Manton and Stallard developed a multistage model that predicted fairly accurately age specific incidence rates (De Waard 1964, 1979, Manton and Stallard 1980). However, there are several grounds on which this model may be criticised. Firstly, the model, following de Waard's hypothesis, supposes that ovarian and extraovarian oestrogens have fundamentally differing actions on breast epithelium, and not only this, but that the former act through seven stages to produce cancer, whereas the latter act through only four. Although this may provide a fit for incidence data it is hardly a compelling explanation. Secondly, the model takes no account of the likely decrease in susceptability that might be expected to follow after the menopause when the breast epithelium commences to involute.

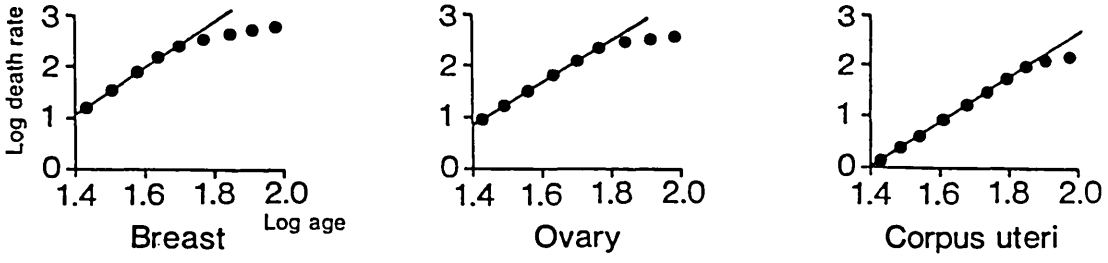
Figure 5.1

Log Death Rate: Log Age for Endocrine Dependant and Non-Dependant Tumours

Non-endocrine dependant tumours



Endocrine-dependant tumours



Thirdly, the model cannot account for the epidemiological data that suggest an increased risk associated with early menarche, and/or late first pregnancy.

For these, and other reasons, Moolgavkar has proposed a further modification of the multistage model (Moolgavkar 1978, 1979, Moolgavkar, Day and Stevens 1980).

This model will be discussed in some detail as it permits an explanation of how hormones might act on the various stages of a cancer from development of a transformed cell, through to the appearance, perhaps many decades later, of metastatic disease.

The description of the mathematical model is followed by that of an animal model which similarly provides evidence of the site and duration of action of hormones on the breast epithelium.

5.4 The Moolgavkar two stage model

The model proposed by Moolgavkar for cancers of hormone dependent tissues specifically takes into account the fact that such tissues grow rapidly at an early age and then the cells continue to turn over until the menopause whence they involute. There is good experimental evidence to suggest that susceptability to malignant change is associated with rapid cell turnover, and similarly, that the number of stages involved is less than the five to seven initially proposed (Cairns 1975).

The model makes several assumptions:

1. A tumour may arise from a single transformed cell following a 'hit'. (The nature of the transformation, whether mutation or not, does not in fact alter the mathematical concept).
2. Each susceptible cell within the breast is as likely to be transformed as any other similarly susceptible cell.
3. Variance from the mean time from transformation to tumour detection is small, and thus the latent period is treated as being constant. The target cell is unidentified, but likely to be a stem cell (Cairns 1975). It may normally be considered to either differentiate, or die, but in the proposed model a third possibility is that it may divide into two daughter cells, one normal, and one that has sustained 'a hit' and become an intermediate cell.

The possible fates of a target cell are shown in Fig 5.2. The cell may:

a) divide into two further normal cells.
b) Die or differentiate (either event removes it from the pool of susceptible cells).
c) divide into two cells, one normal and the other, having sustained the first 'hit', becoming an intermediate cell.

An intermediate cell may then in its turn:

a) divide into two daughter intermediate cells.
b) die.
c) divide into one intermediate cell and one transformed cell, having received a further hit. The transformed cell is malignant.

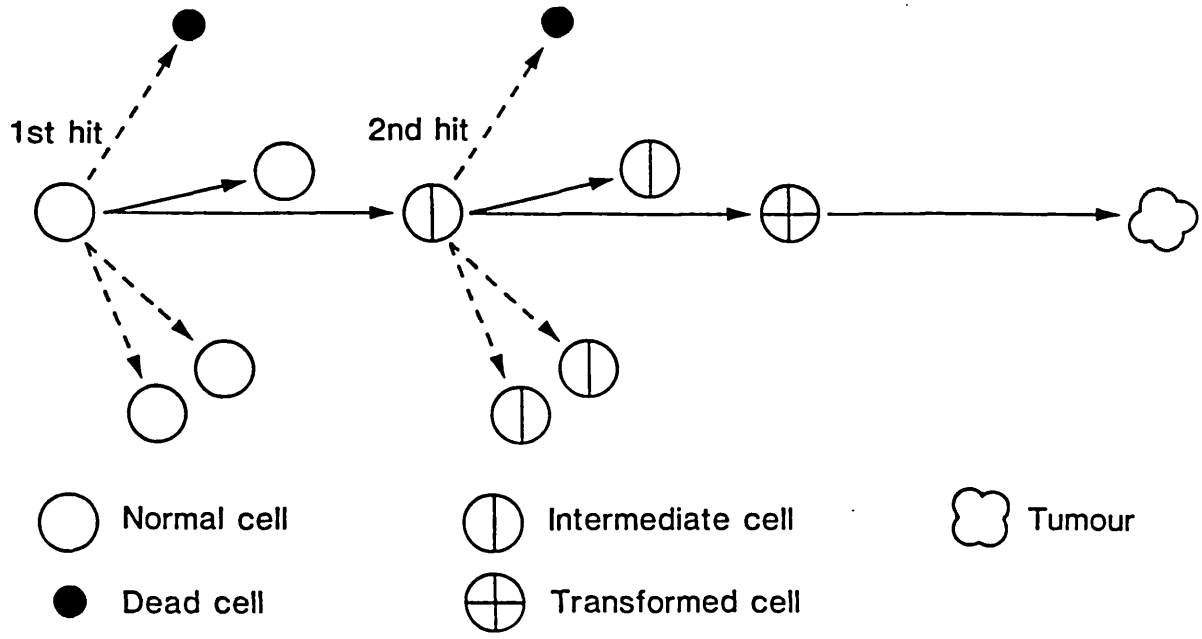
The growth of the tissue is determined by the relative rates of birth and death of cells, and is assumed to follow a logistic curve in this model. Although the 'hits' may be mutations, the exact cellular event is unimportant. The mutation rates of somatic cells are assumed to be of the order of 10^{-8} to 10^{-5}/cell/year/locus (Nei 1975).

How can this model be used to explain a hormonal basis of breast cancer induction and subsequent growth? Can known epidemiological facts also fit into the model?

The onset of menarche is associated with rapid breast growth and production of a large number of cells possibly susceptable to unopposed oestrogenic stimuli. This results

Figure 5.2

A two stage model for breast carcinogenesis (Moolgavkar, 1980)



in the first 'hit', and appearance of a population of intermediate cells. Early menarche increases the risk because of the appearance of such cells earlier in life. First pregnancy is known to be protective against breast cancer, the reduction in risk being proportional to the shortening of the 'menarche - first pregnancy' interval. This reduction in risk is life long, so that pregnancy must, in this model, reduce the number of susceptible cells, perhaps by favouring differentiation.

Following involution of breast epithelial cells at the menopause (whether artificial or natural) the pool of intermediate cells will gradually diminish and the rate of production of new such cells fall dramatically. The model therefore accounts for the protective effect of the early oophorectomy (which is life long) and for the drop in incidence following the menopause. Late menopause delays involution and is associated with increased risk.

The model supports Korenman's first window although the evidence for a second window is less convincing. Similarly, the fact that a small proportion of breast cancers are probably inherited can be accomodated by an extension of a hypothesis originally suggested by Knudson (Knudson et al 1975). Eighty percent of women with a presumed oncogene on chromosome 10 develop breast cancer by the age of 70. The hypothesis would suggest that these women have a substantailly higher initial population of intermediate cells and are thus more likely to develop breast cancer at

an early age. Epidemiological evidence supports the fact that familial breast cancer occurs earlier and is more frequently biateral (Wynder et al 1978). Whether familial breast cancer is associated directly with an increase in intermediate cells, or whether, as seems more probable, it is also due to a variety of other subtle hormonal imbalances is not proven.

An intermediate cell that becomes transformed will eventually appear as a clinical carcinoma, but the latent period is unknown. Tumour doubling times are assessed from the stage of clinical appearance (approximately 10^9 cells) to death, representing some ten doublings. From extrapolation one might infer that the time from one cell to 10^9 would be in the order of twenty to thirty doublings. However as tumour growth is almost certainly not exponential the latent period has been variously calculated at between 2.5 years to over 20. Certainly the evidence from young women exposed to radiation in Hiroshima is that the period between induration and clinical appearance of tumour is of the order of 20 to 30 years.

The Moolgavkar model thus provides an excellent framework in which to fit many of the known epidemiological facts concerning breast cancer.

5.5 Russo's animal model

Before considering in depth the multitude of hormonal data, both epidemiological and serological, that might support the Moolgavkar model, there is one animal model described that lends very considerable credence to the hypothesis (Russo et al 1981).

Although the development of human mammary cancer is influenced by many factors, it is probably reproductive history that is the most important. Mammary cancers in Sprague-Dawley rats induced by 7,12 dimethly-benz(a)anthracene (DMBA) are hormone dependent adenocarcinomas, histologically similar to human breast cancers and in which reproductive factors have a similar, though not identical impact to that in the human. The incidence of tumours is increased in nulliparous rats, but pregnancy stimulates tumour development unlike in the human.

The susceptibility of an individual cell also correlates with the rate of DNA synthesis (Brookes and Lawley 1964), the length of G_1, and S phases of the cell cycle, and cellular ability in DNA excision repair (Bertram and Heidelberger 1974).

5.6.1 Normal rat mammary development

The normal rat mammary gland is composed of a parenchymatous ducto-alveloar tree embedded in a connective tissue stroma.

At birth a single lactiferous duct is present which branches into some three to five secondary ducts which end in small terminal end buds [see Fig 5.3]. A rapid increase in secondary duct development occurs with a concomitant increase in terminal end buds. These latter then commence differentiation into small alveolar buds. At menarche further differentiation occurs, and the alveolar buds start forming clusters of 10-12 alveoli termed a lobule. This latter differentiation occurs focally, with a small increase in the lobule population occuring at each oestrus. Pregnancy induces a rapid differentiation of terminal end buds to alveolar ducts and subsequently lobules, but again, this is focal and results only in an overall increase in the lobule population. At the menopause (about 14 months in the rat) the gland involutes, terminal end ducts disappear, and lobules become smaller (Raynaud 1961). Oestrogen, progesterone, thyroid hormone, prolactin, growth hormone, insulin and gonadotrophin are all required for the normal development so described.

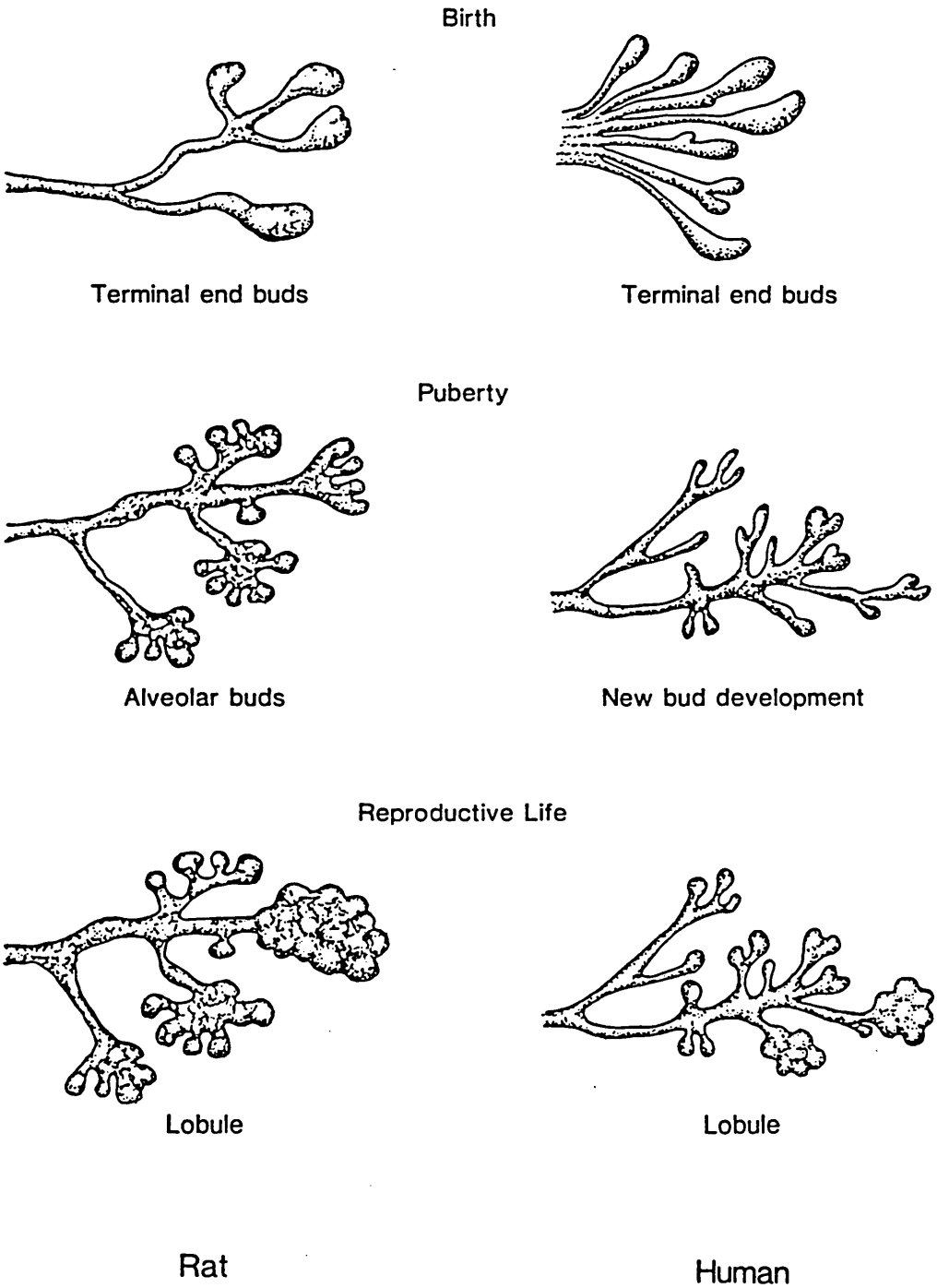
5.6.2 Micro-anatomy

The human mammary gland is composed of epithelial and myoepithelial cells. The former are divided into basal B-cells, clear A-cells, and basal clear-cells. It is thought that oestrogenic stimulation converts basal B-cells into the more differentiated A-cell.

The equivalent cells of the rat are described from their

Figure 5.3

Mammary Gland Development



morphological appearance as light, dark and intermediate cells. The dark cells are present in the largest number, with light cells being uncommon. The intermediate cells are morphologically similar to the basal B-cell of the human, and it is these cells that are responsive to oestrogenic stimulation, and which also are thought to be the target cell of carcinogens (Russo et al 1976).

5.6.3 The rat model

The pathogenesis of a DMBA induced rat mammary tumour depends on both the changes induced by the carcinogen, and the structure of the gland at the time of administration of the carcinogen. The effects of DMBA on cellular differentiation and proliferation, both in vivo, and in vitro, have been superbly described by Russo (Russo et al 1981).

Administration of DMBA to a young virgin rat at which the terminal end buds are actively differentiating results in the latter enlarging due to intraductal proliferation of the lining epithelium with increased mitotic activity. The intraduct proliferation increases in size and microtumours develop that are typical hormone dependent adenocarcinomas. The normal process of differentiation of terminal end buds to alveolar buds is halted, but those end buds that had already become alveolar buds do not form carcinomas, although occasionally benign adenomas or cysts are found. The more differentiated the mammary gland at the time of

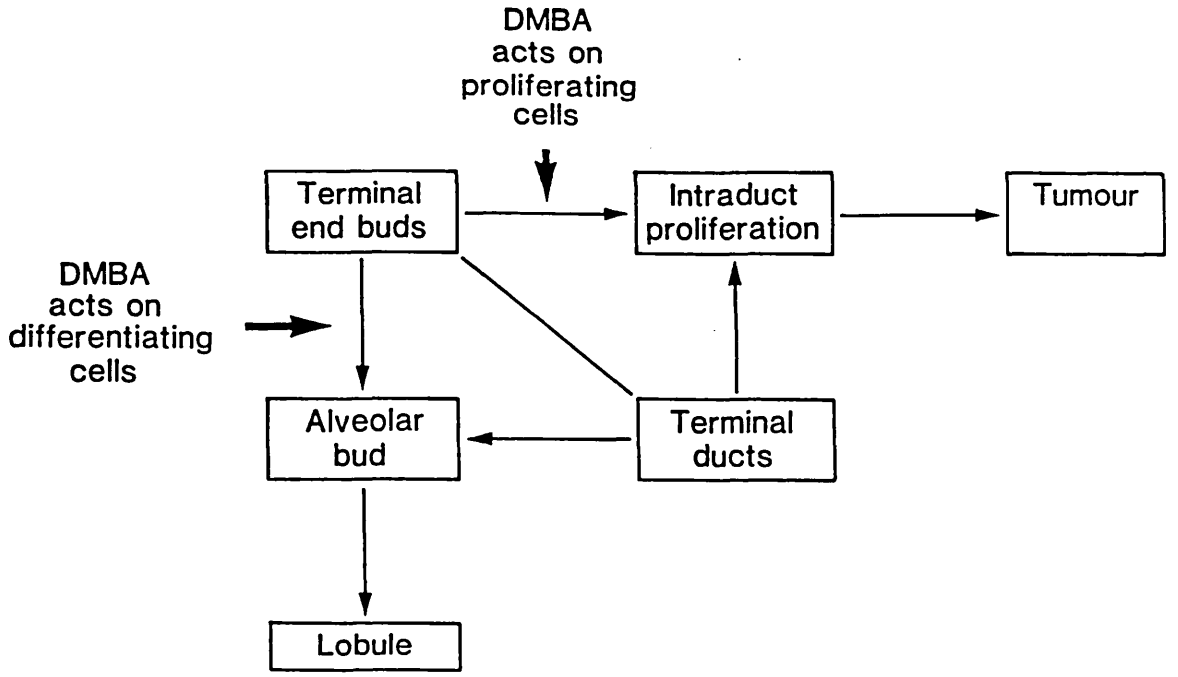
carcinogen administration the more benign, and the more organised is the lesion that develops [see Fig 5.4]. At a cellular level, the dark cells of terminal ducts and end buds decrease in number, with a concimitant increase in intermediate cells. No discernible changes occur in alveolar buds or the even more differentiated lobules. With the ductal hyperplasia intercellular spaces appear and may form a cribriform pattern of intraduct carcinoma and intermediate cells may account for up to 50% of the population within the end bud, this proportion increasing with the age of the tumour.

Russo has demonstrated that the incidence of tumour development is proportional to the density of terminal end buds ready to differentiate to alveolar buds, and to the DNA labelling index. The susceptibility of the mammary gland decreases significantly with age, presumably because of increasing differentiation, and fibroadenoma development becomes more common. Although this is not directly comparable to the human situation, it must be recalled that the time scale is greatly foreshortened in the rat.

Full term pregnancy renders the mammary gland less susceptible to DMBA carcinogenesis due to the reduced target pool of end and alveolar ducts. In the pregnant woman, both pituitary and placental prolactin, as well as oestrogen and progesterone act synergistically to promote breast growth and differentiation. Lactation confers no additional benefit in the rat model but does seem to exert a protective

Figure 5.4

Exposure to DMBA and Mammary Gland Differentiation



effect on prevention of subsequent benign lesions. This effect has not been observed in women.
Russo has also examined the cell kinetics of this model and noted that the growth fraction is highest in the least differentiated structures, and vice-versa (Russo et al 1977). Similarly, the least differentiated structures have short cell cycles whereas there is progressive lengthening of the G_1 phase in the more differentiated lobules.

In a further series of experiments Russo describes DMBA binding in the rat model, and notes that it is inversely related to the age of the animal and also to the degree of gland differentiation. Subsequently using normal human breast epithelial tissue in culture they were also able to correlate DMBA binding with differentiation, and in particular, the presence or absence of fully formed lobules. High levels of DMBA binding were observed in undifferentiated end buds and virginal lobules, but very low binding in breast tissue cultures with predominently well differentiated lobules (Tay and Russo 1981).

5.6.4 Human breast cancer

The conclusions to be drawn from Russo's observations may be applied almost directly to the human breast though the process of differentiation is slightly more complex [See Fig.5.3]. Lobule development begins at the menarche under oestrogenic stimulation and continues cyclically until pregnancy intervenes or until about the age of 35. However

the response is focal, and differs widely from individual to individual. Irregular or anovulatory cycles are associated with irregularities of lobule formation (Ingelby and Gerchon-Cohen 1960). Pregnancy causes true hypertrophy and by four months lobular development is maximal. However, there is still a highly significant number of primitive buds, and the process of differentiation remains focal. Further pregnancies cause further focal differentiation but some lobules do not respond to subsequent pregnancy or lactation and are termed 'virginal lobules'. These lobules, having no direct counterpart in the rat are considered to be potential targets for neoplastic transformation (Russo & Russo 1980).

Russo's findings in the rat model correlate well with the known data on human breast cancer and also with the mathematical model described by Moolgavkar.

Tumours originate in the terminal end buds of the rat or the corresponding terminal duct in the human. As the population of terminal ducts decrease with ovulatory menstrual cycles, or the onset of pregnancy, so the susceptibility to carcinogenesis decreases. Thus the effect of early first pregnancy or late menarche is explained, as is the increased incidence with nulliparity, and possibly also short or anovulatory menstrual cycles. The evidence from DMBA binding to the less well differentiated structures also supports this and suggests that the first 'hit' in the Moolgavkar model is acquired between the onset of puberty

and the age of 35. In the rat model the cell that is 'hit' is almost certainly the identically named intermediate cell of the Moolgavkar hypothesis. In the rat, a second 'hit' presumably occurs very shortly afterwards, but evidence from atomic bomb survivors would lead us to suspect that in the human the second 'hit' occurs much later, in order to partially account for the very long latent period (McGregor et al 1977, and Section 6.3).

CHAPTER 6

OESTROGENS

Part I

Literature review

6.1 Introduction

The first part of this chapter is devoted to a review of the literature concerned with the association of oestrogens with breast cancer. Following a brief outline of the physiology of oestrogens and their possible mode of action at a cellular level, the extensive epidemiological evidence is summarised. The majority of hormone studies of either women at increased risk of breast cancer, or in patients with breast cancer have utilised profiles of urinary metabolites, but the more recent plasma hormone studies are also reviewed. In the latter half of the review, the physiological, and possible pathological implications of oestrogen binding are discussed. Lastly, the evidence linking oestrogens to the development and clinical course of breast cancer is described on the basis of the Moolgavkar model presented in Chapter 5.

6.1.1 Structure and function

In the normal premenopausal, non-pregnant female, oestrogens are secreted principally by the theca interna cells of the ovary, with some production from the adrenal cortex, and peripheral aromatisation from androgens.

At least six natural oestrogens have been identified in the plasma of the human female, but only three are present in significant quantities, oestradiol, oestrone, and oestriol. Oestradiol and oestrone are secreted by the ovary, but

oestriol is derived from conversion principally in the liver [see Fig 6.1].

The oestrogenic potency of oestradiol is twelve times that of oestrone, and 80 times that of oestriol, and oestradiol is therefore considered to be the principal circulating oestrogen.

Oestrogens are conjugated by the liver to glucuronides and sulphates, which are then either excreted in the bile (20%) or in the urine (80%).

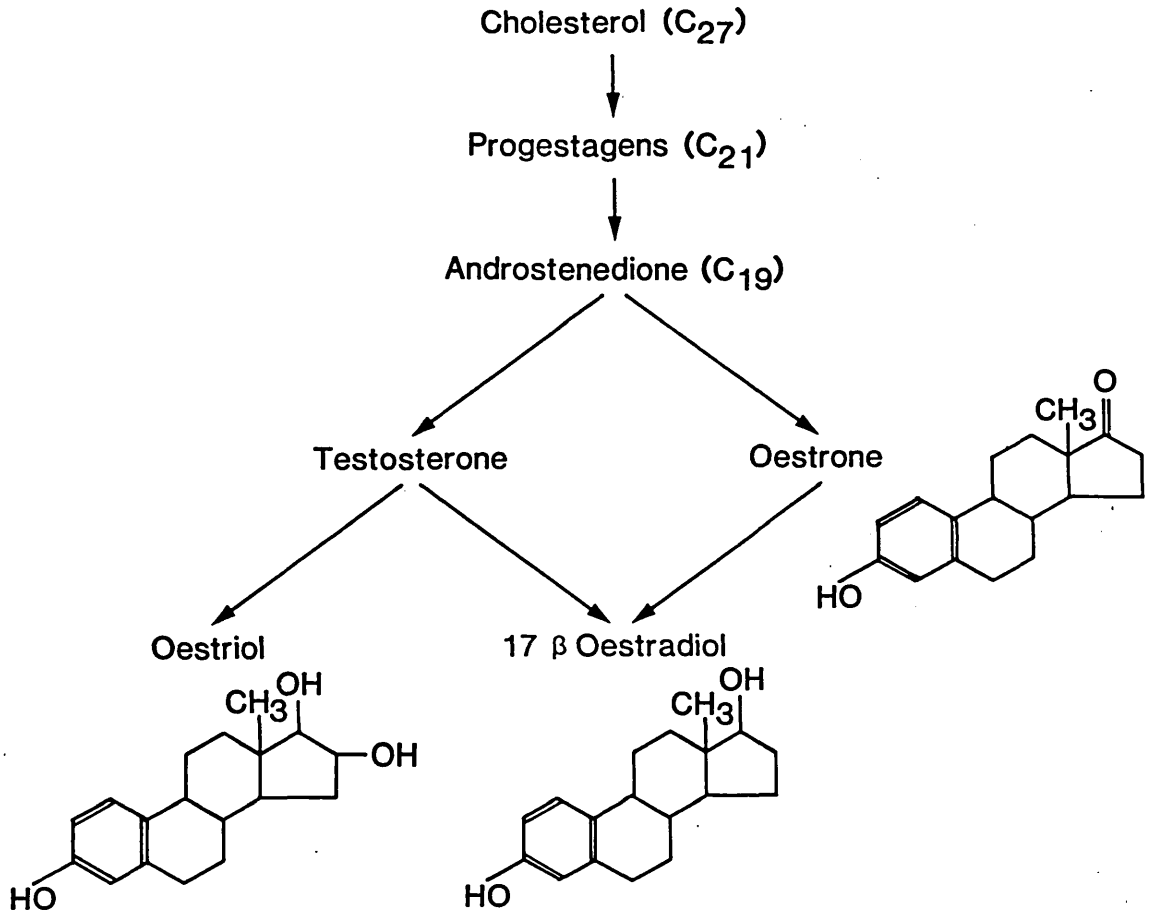
6.1.2 Postmenopausal oestrogens

The postmenopausal ovary continues to secrete significant amounts of androgenic hormones, but, during the climacteric, secretion of oestradiol falls dramatically, and after the menopause the contribution from the ovaries is thought to be unimportant (Adamopoulos et al 1971). Oestradiol in the normal postmenopausal female is probably derived partly from oestrone by reduction, and partly from testosterone by aromatisation. Oestrone is mainly derived from androstenedione by aromatisation in peripheral tissues.

Adipose tissue plays an important part in this conversion, and oestrone production from androstenedione correlates with weight (McDonald et al. 1978). Hyperthyroidism and cirrhosis are also associated with increased conversion of

Figure 6.1

Biosynthesis of oestrogens



androstenedione to oestrone (McDonald et al 1978).

Oestrone and oestradiol production may not remain constant in postmenopausal women. A study in which hormone profiles in postmenopausal women were measured noted that concentrations of androstenedione, oestrone, and oestradiol all fell to about 20% of their premenopausal values within a year following the menopause, but after five years androstenedione concentration rose, with a concomitant fall in testosterone. Over the next three decades the pattern reversed, with androstenedione increasing, and testosterone concentration falling. Oestrone and oestradiol concentration did not alter substantially in the first 10 years following the menopause, but there was a significant reduction in oestrone concentration with a concomitant increase in oestradiol, some 10 to 20 years later. The significance of this is unknown (Chakravarty et al 1976).

6.2 Cellular action of oestrogens

Oestrogens exert their action on very specific target tissues due to the presence of cellular receptors. The oestrogen molecule enters the cytoplasm of the target cell by diffusion and becomes bound to a cytoplasmic receptor causing a conformational change of the tertiary structure allowing translocation to the nucleus. Here it binds to a specific site on the chromatin, governed by the non-histone chromatin protein (NHCP), permitting gene activation with new RNA synthesis by a DNA-dependent RNA polymerase transcribing the DNA.

Human breast cancer tissue has been demonstrated to possess receptors for oestrogens, progesterone, cortisol and androgens. Approximately 75% of breast cancers in postmenopausal women are oestrogen receptor positive, and about 60% are progesterone receptor positive (McGuire 1978).

Progesterone appears to function as an anti-oestrogen and decreases the level of oestrogen receptor in the uterus, and possibly also the breast (Hsiah et al 1975). Progesterone can modulate tissue levels of oestradiol in premenopausal women which may be of considerable importance in cancer promotion. In postmenopausal women, progesterone levels are much lower, but it is possible that other steroids such as dehydroepiandrosterone (DHEA-S) may have a similar action (James and Reed 1981).

6.3 Oestrogens and epidemiological risk factors

A great many epidemiological studies have suggested an association between reproductive function, diet and obesity, and risk of subsequent breast cancer. The relative risk for the more important reproductive factors is shown in Table 6.1. Of the ovarian hormones the most likely candidates for carcinogens or promoters are the oestrogens, as they are carcinogenic in laboratory animals (Noble 1963).

6.3.1 Age at menarche

The majority of studies demonstrate that early age at menarche is associated with increased risk of subsequent breast cancer (see Kelsey 1979 for refs.). It is probable that this is a risk factor in its own right, but although Korenman initially proposed that this might be due to an increased number of early post-menarche anovulatory cycles leading to excessive oestrogenic stimulation of the breast due to inadequate luteal function, it has been shown more recently that the earlier the menarche, the sooner regular ovulatory cycles begin, and that ovulation is related to time from menarche, and not age at menarche (Sherman and Korenman 1974, Wallace et al 1978, Brown 1981).

Risk Factor	Odds Ratios	95% Confidence Limits	Statistical Significance of trend	
			No. Groups	P
Ever live birth (No:Yes)	1.4	1.1-2.0		
Ever pregnant (No:Yes)	1.3	1.0-1.7		
Age at first live birth (30: 20)	1.8	*	4	0.003
Age at menarche (12: 14)	1.4	*	5	0.02
Oophorectomy (Yes:No)	0.7	0.5-1.0		
Age at menopause (50: 40)	2.7	*	4	0.001
No. pregnancies with no live birth (4:0)	1.7	*	5	0.10

Odds ratios and 95% confidence limits for association between reproductive factors and breast cancer (Kelsey 1981).

(* For these variables there were monotonic increases or decreases of the odds ratios with increasing values of the risk factor.)

Table 6.1 Major epidemiological risk factors in breast cancer

6.3.2 Age at first child and parity

Early age at first birth is associated with decreased risk of breast cancer (Wynder 1960, McMahon and Cole 1970, Sartwell et al 1977, Brinton 1979, and others) but the protective effect is confined to full term pregnancies (Salber et al 1969). The earlier the first pregnancy, the lower the risk of cancer, and this protection appears to be lifelong. Interestingly it has also been shown that women aged more than 35 years at the time of first pregnancy have a greater risk than nulliparous women (MacMahon et al 1970).

Initial studies demonstrated that multiparity conferred no independent protection from that of age at first birth, but studies in Burma, Iceland and Sweden provide evidence that high parity may be a partially independent factor from that of age at first birth. (Thein-Hland and Thein-Manng-Myint 1978, Tulinius et al 1978, Adani et al 1978).

Breast feeding does not confer any protection independent of that of parity.

6.3.3 Natural and Artificial Menopause

Late menopause, like early menarche, is associated with increased risk (Wynder et al 1960, and others), and women with a menopause aged 55 or older have twice the risk of those with a menopause before age 45 (Trichopoulos et al

1972). There is no evidence to explain this finding, though it may be related to abnormal periods or endocrine function in late reproductive life. This, combined with the longer duration of menstrual cycles, might lead to further excess oestrogenic exposure.

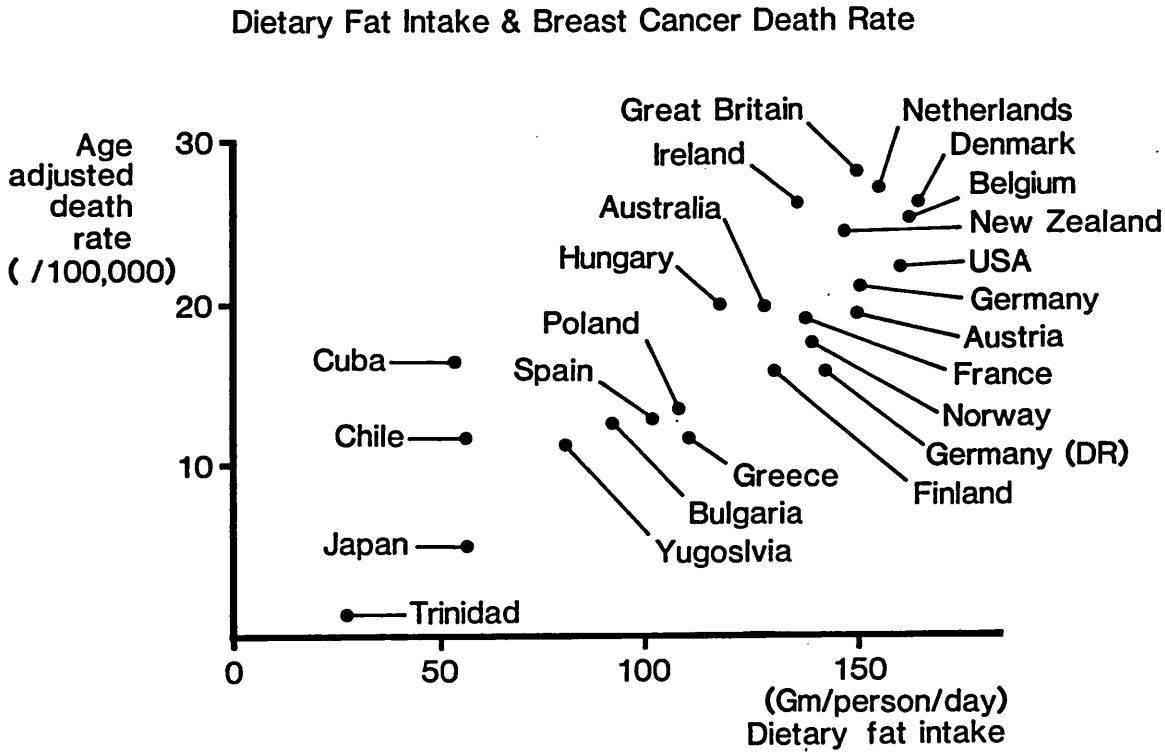
Women undergoing an artificial menopause acquire reduced risk, and the earlier the age at oophorectomy, the lower the risk (Feinleib 1968, Hirayama and Wynder 1962). An oophorectomy before the age of 35, reduces the risk of breast cancer by about 30% compared to a natural menopause at age 45. Interestingly, the protective effect of oophorectomy is life long (Trichopoulos et al 1972), further supporting the concept of oestrogenic action in the Moolgavkar model, and suggesting that endocrine intervention at an early age might lead to a substantial reduction in breast cancer incidence.

6.3.4 Dietary factors

The strong epidemiological evidence linking breast cancer risk to age at menarche and first child is unable to explain worldwide differences in incidence. The correlation between incidence and dietary fat intake is however compelling [see Fig. 6.2], and both Wynder and de Waard have been proponents of a nutritional aetiology in the pathogenesis of breast cancer.

A plateau in the age-specific incidence curve of breast

Figure 6.2


Dietary Fat Intake & Breast Cancer Death Rate
Age adjusted death rate (/100,000)
30
20
10
0
50
100
150
(Gm/person/day)
Dietary fat intake
Great Britain
Netherlands
Ireland
Denmark
Belgium
Australia
New Zealand
Hungary
USA
Germany
Poland
Austria
Cuba
Spain
France
Norway
Chile
Germany (DR)
Greece
Finland
Bulgaria
Japan
Yugoslvia
Trinidad

cancer occurs at about the age of 50; it was first described by Clemmesen and is now known as Clemmesen's hook (Clemmesen 1948). De Waard has proposed that this identifies two diseases in breast cancer, the former occurring in pre-menopausal women and due to unoppposed ovarian oestrogens, and the latter, a post-menopausal disease related to extra-ovarian oestrogen metabolism modified by nutritional factors (De Waard 1979).

Moolgavkar and others have argued that Clemmesen's hook is in fact a 'cohort effect' seen as a population changes from a low incidence curve such as that seen in Japanese women to a high incidence or Western type curve (Moolgavkar et al 1979). Nevertheless, there is good evidence to link nutritional factors to breast cancer development.

An early animal model developed by Tannenbaum demonstrated that caloric restriction tended to lower the number of spontaneous breast tumours in DMBA mice, and this was confirmed in a different model by Visscher (Tannenbaum 1942, Visscher et al 1942). High fat diets, and obesity, are also associated with increased tumour yield in animal models (Carroll 1975, Chan and Cohen 1974, Waxler et al 1979).

Dietary fat appears to effect predominently two hormones, prolactin and oestrogens. A high fat diet has been shown by several workers to be associated with elevated serum prolactin, although the evidence that this is a

carcinogenic co-factor is confusing (Hill and Wynder 1976, Carroll 1981). A vegetarian diet however is associated with increased urinary oestrogen excretion (Goldin et al 1981), decreased serum prolactin (Hill et al 1977), an earlier menopause, and increased SHBG (Armstrong 1979). In addition to the effect of dietary fat, certain dietary fibres may increase faecal oestrogen excretion by binding to bile salts (Goldin et al 1981).

6.3.5 Body weight

There is a positive association between ideal body weight and breast cancer, although for postmenopausal women this risk is substantially greater than for premenopausal women (Wynder 1960, De Waard 1975, MacMahon 1975). Two studies have even found a negative correlation between body weight and breast cancer risk in young women (Fasal 1975, Miller et al 1978).

Weight, weight/height ratios, height, and total body mass have all been studied as potential risk factors, but there is no clear indication as to which variable is associated with greatest risk, principally because of the variables being highly correlated with each other.

The metabolic consequences of obesity have been reviewed by Kirshner (Kirschner et al 1981). Several studies have demonstrated both increased production of the androgen, androstenedione, and its conversion to oestrone (see

Kirshner et al 1981). Additionally, the menarche has been reported to occur earlier in obese girls, and the menopause to occur later (Frish and Revelle 1970, Sherman et al 1979). This, together with the finding of an increased incidence of abnormal and anovulatory menstrual cycles in obese women, lends considerable weight to the hypothesis that obesity is associated with chronic exposure to excess oestrogenic activity. It is also quite compatible with Korenman's hypothesis on the importance of a 'window' [see Section 6.3.6].

Further studies on the effects of obesity on peripheral oestrogen metabolism have also revealed what may be important metabolic changes. Fishman has shown that in obese women there is a significant increase in 16-hydroxylation of oestrone to oestriol, in preference to the 2-hydroxylation to 2-hydroxyoestrone [see Fig.6.3] (Fishman et al 1975). 2-hydroxyoestrone may also be of importance as an 'impeding oestrogen' as it has been shown to bind to cytosolic oestrogen receptors (Marticci and Fishman 1976).

6.3.6 Luteal inadequacy hypothesis

A further hypothesis has been proposed based upon epidemiological findings. It developed from a finding, originally reported by Grattarola, that a significant proportion of women with breast cancer had abnormal endometrial biopsies in the luteal phase of the menstrual

cycle. This indicated that the women concerned may have had anovulatory cycles due to inadequate circulating progesterone in the luteal phase (Grattarola 1964).

Unfortunately, biochemical data has failed to entirely support this hypothesis. Three separate studies all found normal levels of luteal phase serum progesterone in women with breast cancer (Swain et al 1974, England et al 1975, Malarkey et al 1977). In studies of women at risk of breast cancer, the results are more confusing. Pike reported elevated serum progesterone in women at risk, whereas Bulbrook reported lower luteal phase serum progesterone in proportion to increasing familial risk (Pike et al 1979, Bulbrook et al 1978).
The hypothesis is supported by the findings of Sommers' autopsy study, of an increased incidence of endometrial hyperplasia in women who have died of breast cancer (Sommers 1955, and see Section 9.2).

Korenman, in 1974, modified the luteal inadequacy hypothesis to that of the 'oestrogen window' (Korenman 1974). This suggests that oestrogenic stimulation of breast tissue, acts unopposed by progestagenic influences, during two vital periods. The first window, is the period between Tanner stage 'Breast-2', at around 8-10 years until the establishment of normal ovulatory periods. During this time cycles may be anovulatory or short, leading to inadequate luteal phases. The second window occurs during the latter years of reproductive life when cycles may again

become anovulatory.

The hypothesis is supported by evidence of breast cancer incidence in atomic bomb survivors, a study of whom showed that the highest rates of subsequent breast cancer occurred in girls who were 10-14 years old at the time of radiation exposure, or in women over 50 years. The induction period was about 20 years (Tokunaga et al 1979). The hypothesis is also supported by the epidemiological evidence that early menarche increases breast cancer risk, as this elongates the first 'window'. The hypothesis assumes that oestrogens are inducers or promoters of breast cancer. Although this is not proven, the evidence is summarised in Section 5. To the detractors of this hypothesis it is significant that the contraceptive pill has not been related to an increased incidence of breast cancer (Kelsey 1979, BMJ 1981).

In a further review of the hypothesis, one of the authors, Sherman, has acknowledged that nutritional factors may also play a role, increasing the oestrogenic environment over a much longer period, and modifying the effect of oestrogens on the subsequent course of any tumour (Sherman 1981).

6.4 Urinary and plasma oestrogens

Studies of urinary and plasma oestrogens can be generally classified into those concerned with potential differences in the hormonal profiles of women with breast cancer as compared to normal controls, studies of oestrogen profiles of women at risk of breast cancer, and lastly, studies of women with treated breast cancer and the association between recurrence and urinary oestrogens.

6.4.1 Profiles in women with breast cancer and at risk

Early studies measured either 'total urinary oestrogens' or the three major oestrogens (oestrone, 17β oestradiol and oestriol) by extraction, purification and colorimetric measurement of Kober's reaction. This method, pioneered by Brown, was used extensively with no clear trend emerging until publication of a study by Lemon in 1966 (Brown 1957). By calculating a ratio of 'the impeded non-carcinogenic oestrogens with the most carcinogenic oestrogens' he demonstrated that urinary oestriol excretion was more markedly reduced than oestrone or 17 β oestradiol, compared to normal controls. This was expressed as the urinary oestrogen excretion quotient derived from:

$$\frac{E_3 \text{ (µg/24 hours)}}{E_1 - E_2 \text{ (µg/24 hours)}} = \text{Equivalent (Lemon 1966)}$$

Abbrev.: E_1 Oestrone
E_2 Oestradiol
E_3 Oestriol

This quotient was subsequently formulated into an hypothesis by Cole and McMahon who proposed that the greater the amount of Oestriol, relative to Oestrone and Oestradiol, produced during the decade following menarche, the lower the risk of subsequent breast cancer. (Cole and MacMahon 1969). This explained the epidemiological finding of the protective effect of first pregnancy, as the third trimester of pregnancy is associated with an increase of oestriol relative to oestrone and oestradiol.

The hypothesis is supported by studies between populations at low risk compared to those at high risk. MacMahon and co-workers demonstrated higher quotient values in Asian women compared to N. American controls, and in Japanese women compared to Americans, with Hawaiian Japanese being intermediate (MacMahon et al 1971, 1974). Briggs also found the quotient to be higher in Asian or African women living in Zambia, compared to Europeans resident there (Briggs 1972). Bulbrook confirmed a higher urinary oestriol ratio in premenopausal Japanese women, as compared to British women, but could find no difference in plasma oestrogens (Bulbrook 1974).

Within population studies of urinary oestrogen profiles have not substantiated the Lemon quotient. Two studies found no significant difference in the oestriol ratio in postmenopausal women with cancer compared to normal controls (Schweppe et al 1969, Grouroos 1968). Arguelles did find an elevated oestriol ratio in premenopausal women with cancer, but the study lacks acceptable controls (Arguelles 1973).

More recently, using gas chromatography, all three urinary oestrogens have been found to be significantly higher in 35 post menopausal women with breast cancer than in 22 normal controls (Morreal et al 1979).

The hypotheses of Lemon, and of Cole and McMahon were based on evidence that oestrone and oestradiol were carcinogenic in experimental rodent tumours, but oestriol was not. It is now known however, that plasma levels of oestriol are very low compared to urinary levels, and that it does not effectively compete with oestradiol at receptor sites (Anderson et al 1975). Oestriol is also now known to be an oestrogen in its own right and can induce breast tumours in mice (Clarke et al 1977). Although Wotiz has countered these arguments it remains very difficult to draw satisfactory conclusions from the experimental data on urinary oestrogen excretion (Wotiz et al 1978).

6.4.2 Plasma Oestrogen studies

Studies of plasma oestrogens in breast cancer patients using radioimmunoassay methods have generally concentrated on 17 β oestradiol [see Table 6.1].

England assayed 17 β oestradiol daily throughout the menstrual cycle in 32 normal women, 31 women with benign breast disease, and 10 women with breast cancer. In women with benign breast cysts 17 β oestradiol was found to be significantly lower during the luteal phase. Although

Author	Hormone	Patients & Controls	Results
Wang & Swain (1974)	E_1, E_2	Pre and post N + BC	No significant difference
England et al (1974)	E_2	Pre and post N + BC + BBD	Pre BC : small elevation of Oestradiol in follicular and luteal phases
Hill et al (1976)	E_1, E_2	Post N + BC	No significant difference BC:
McFayden et al (1976)	E_2	Post N + BC	Oestradiol but no significant difference
Malarkey et al (1977)	E_2	Pre N + BC	No significant difference
Jones et al (1977)	E_2	Post N + BC	No significant difference
Adami et al (1979)	E_1	Post N + BC	BC:sig
Moore et al (1982)	E_2	Pre and post N + BC	Elevated E_2 and free E_2 in BC

Abbreviations:
Post - postmenopausal
Pre - premenopausal
BC - breast cancer
BBD - benign breast disease
N - normal controls
E_1 - oestrone
E_2 - oestradiol

Table 6.2 Plasma Oestrogen in breast cancer

there was similarly a small elevation in oestradiol in the follicular and luteal phases of premenopausal women with breast cancer there was no significant difference either in these, or the post menopausal women (England et al 1974).

Adami studied a wide hormonal spectrum in a much larger population. Oestrone was the only oestrogen measured, but this was found to be significantly higher in the breast cancer patients than their postmenopausal matched controls. There was no correlation between oestrone and weight, but oestrone did correlate to raised levels of both Androstenedione and Testosterone. The significance of these findings is difficult to assess (Adami 1979).

Despite the considerable amount of epidemiological evidence suggesting the ovarian function plays an important role in the pathogenesis of breast cancer, there is up to this point no clear or convincing differences in measurable ovarian hormones between women with breast cancer and normal controls.

An alternative approach has therefore been the study of oestrogen metabolism. Oestradiol is firstly oxidised to oestrone and thence by one of two alternative hydroxylations at the C-2 or 16 α position.

Numerous studies have examined urinary oestrogen metabolites but these have not produced any conclusive findings (Dao 1979, Hellman et al 1971). A more recent

study has used a radiometric method for the measurement of the oxidative metabolites 'in vivo' (Schneider et al 1982). This demonstrates that in breast cancer patients there is a 50%, and highly significant increase in 16 α hydroxylation in women with breast cancer. The importance of this finding is that the 16 α metabolite is a potent oestrogen compared to the C-2 metabolite that exhibits virtually no oestrogenic effects. If this alteration in oestrogen metabolism can also be demonstrated in women at risk it may represent an important endocrine parameter.

6.4.3 Plasma free oestradiol

Recently a simple method has been designed for the measurement of free oestradiol in serum; that is, the fraction of oestradiol that is not bound to either sex hormone binding globulin (SHBG) or albumin. In a preliminary study using this method free oestradiol was shown to be considerably elevated in patients with breast cancer when compared to normal controls (Siiteri et al 1981).

In a further study of 42 premenopausal and 38 postmenopausal women with breast cancer these initial observations were confirmed and it was noted that the patients had normal levels of SHBG despite the elevated free oestradiol (Moore et al 1982).

These results indicate that women with breast cancer may

indeed be subjected to elevated circulating levels of oestradiol. The significance of this and the relationship of ovarian hormones with SHBG are discussed further, later in this chapter.

6.4.4 Oestrogens and familial high risk.

Several studies have used daughters of breast cancer patients as examples of high risk and compared them to age matched daughters of normal women. Henderson assayed oestrone and 17 β Oestradiol in the follicular and luteal phases (defined as Day 6 and Day 22) and reported that 17 β Oestradiol was raised in the luteal phase of patient's daughters (Henderson 1975).

In a very similar study this finding could not be replicated either on urinary or plasma assays (Pike 1977). Fishman compared two age matched groups of young women, one at high risk, and the other with normal risk, measuring all three major oestrogens every other day thoughout the cycle. Surprisingly all three oestrogens were lower in the high risk group, but the difference was not statistically valid (Fishman 1978).

Bulbrook and co-workers analysed both urinary and plasma hormones in 1485 women divided into 5 risk categories. Although they found a significant link between risk and reduced progesterone in the luteal phase, there was no substantial correlation with plasma Oestradiol, although in

the younger premenopausal women plasma oestradiol was significantly raised with increased risk (Bulbrook et al 1978).

These findings were compatible with Korenman's theory of unopposed oestrogen action in the luteal phase, but it is impossible to draw any further firm conclusions from the available data on serum oestrogens to this point.

6.5 Oestrogens and recurrence: clinical evidence

Survival in breast cancer is highly correlated with the presence, or absence, of Oestrogen Receptors, and this appears to be independent of other prognostic factors such as lymph node status, age, menopausal status, and size of tumour (Hahnel 1981). Tumours possessing oestrogen receptors are endocrine dependent and have a significant survival advantage over tumours in which they are absent. The latter also have a significantly higher thymidine labelling indexes, indicating higher cellular proliferation rates (Cooke et al 1980).

Response to endocrine deprivation therapy (whether by castration, adrenalectomy, hypophysectomy, high dosage oestrogen, anti-oestrogen, or aminoglutethimide) is similarly correlated with the level of oestrogen receptor in the tumour (Hahnel 1981).

Although it is known that some tumours are oestrogen dependent, extremely small amounts of circulating oestrogens, such a those present after hypophysectomy, may be sufficient to promote tumour growth. Pearson has demonstrated a further regression in response to Tamoxifen following response to hypophysectomy (Pearson 1978).

Clinical evidence that oestrogens may modify the course of breast cancer comes from the observation that early artificial menopause may reduce the subsequent incidence of

relapse (MacKay and Sellar 1965). This parallels the life-long reduction in breast cancer risk confered by early artificial menopause [see section 6.3].

6.5.1 Plasma oestrogens and recurrence

Oestrone is the principal circulating oestrogen in the post menopausal woman and as it is principally derived from the adrenal androgen, androstenedione, there is no significant fall following oophorectomy. However oophorectomy in a premenopausal woman reduces plasma oestrogen and oestrone significantly. Corticosteroid therapy has been used to further suppress oestrogen production by its action on adrenal steroidogenesis, but androstenedione is suppressed much less readily than cortisol.

Early measurements of urinary oestrogens following a variety of endocrine deprivation therapies, noted significant depression but still detectable levels of oestrogens that could not be related to subsequent recurrence [see Table 6.2].

It has been suggested that response to chemotherapy may be due, at least in part, to chemotherapy induced endocrine ablation. Whilst this has not yet been fully evaluated the amenorrhoea associated with chemotherapy is due to a direct cytotoxic action on the ovaries, and is associated with reduced plasma oestradiol and oestrone.

Author	Therapy	Assay
Bulbrook et al (1958)	Oophorectomy & Adrenalectomy	reduced urinary oestrogens (no correlation with recurrence)
Jull et al (1963)	Oophorectomy	reduced urinary oestrogens (no correlation with recurrence)
Kodama et al (1977)	Chemotherapy induced amenorrhoea	reduced urinary oestrogens
Rose & Davis (1977)	Chemotherapy induced amenorrhoea	reduced plasma oestradiol & oestrone
Borkowski et al (1977)	Dexamethasone	reduced plasma oestradiol & oestrone
Santen et al (1977)	Aminoglutethimide	reduced plasma oestradiol & oestrone
Barone et al (1979)	Testololactone	reduced plasma oestrone

Table 6.3 Oestrogen deprivation therapy

More recent data on circulating oestrogens and response to endocrine ablation has come from the use of aminoglutethimide in postmenopausal patients with advanced breast cancer. Aminoglutethimide reduces oestrogen production by blocking the synthesis pathway between cholesterol and pregnenolone, and also by inhibiting androstenedione aromatisation to oestrone. Both surgical adrenalectomy and aminoglutethimide produce a substantial fall in circulating oestrogens, and the difference does not appear to be significant (Santen et al 1981). The separate effect of aminoglutethimide or androgen production may be significant but two authors have failed to correlate the remaining circulating oestrogens to subsequent recurrence (Santen et al 1977, Newsome et al 1978).

Since aminoglutethimide or corticosteroid therapy only reduces oestrogen production by some 60% and it is known that very small amounts of oestrogen can maintain tumour growth, it may be necessary to produce a far more substantial reduction before a correlation between circulating levels and response is seen.

6.6 Sex Hormone Binding Globulin - physiological role

Sex hormone binding globulin (SHBG), first demonstrated by Mercier in 1966, binds reversibly with both Testosterone and Oestradiol (Mercier et al 1966). Testosterone binds with high affinity to SHBG and with low affinity to albumin. Oestradiol however binds much less avidly to SHBG, but its binding to albumin, also with low affinity, is still of importance, due to its large capacity (Siitteri 1981).

It has been demonstrated in an 'in vitro' study that a change in the concentration of SHBG produced a relatively large alteration in levels of unbound testosterone, with a smaller alteration in unbound oestradiol, due to the relative affinity constants (Burke and Anderson 1972). It is known that oestrogens stimulate, and androgens inhibit SHBG production and this may therefore provide a mechanism for control of androgen - oestrogen balance. Anderson postulated that a rise in oestrogen production will thus indirectly cause a reduction in unbound androgens, and conversely, a rise in androgen production might cause a rise in the unbound testosterone fraction (Anderson 1974).

At least one study has disputed this, finding no correlation between oestradiol and SHBG, but this study can be criticised because the serum was diluted and this in

itself alters the affinity constants of binding proteins (Vigersky et al 1979, Westphal 1971). Using an ultrafiltration dialysis technique at 37°C on undiluted serum, other studies have confirmed a significant inverse relationship (Nisker et al 1980, Moore et al 1982).

Thyroid hormones are potent stimulators of SHBG and very high levels of SHBG are found in thyrotoxicosis (Anderson 1974). It has also been reported that there is an inverse relationship between body weight and serum SHBG binding capacity although it is not known whether this is hormonally mediated (DeMoor and Joossens 1970). However this immediately opens up several pathways in which SHBG may play a role in breast cancer pathogenesis.

6.7 SHBG - breast cancer

There are at least three mechanisms by which SHBG might be implicated in the pathogenesis of breast cancer.

Obesity has often been cited as a promoting factor in breast cancer [see section 6.3], and can affect the availability of free oestrogen in three ways. Firstly, there is increased peripheral conversion of androstenedione to oestrone, although this is seen predominantly in grossly obese postmenopausal women (Siiteri et al 1976). Secondly, androstenedione synthesis is stimulated by cortisol, increased adrenal secretion of which is associated with obesity (Migeon et al 1973). Thirdly, it has been shown that serum SHBG binding capacity is inversely related to body weight (Nisker et al 1980).

All these mechanisms increase the availability of free oestrogens and may act over prolonged periods.

Hypothyroidism has also been implicated as a risk factor [see section 9.2] and although this may be controversial, thyroid hormones exert an important and direct effect on oestrogen availability. Depressed levels of thyroid hormones are associated with decreased serum SHBG and thus increased free oestradiol (Anderson 1974).

Thirdly, Siiteri, and subsequently Moore, have shown that women with primary breast cancer have elevated levels of

free oestradiol, though with normal levels of SHBG binding capacity (Siiteri 1981, Moore et al 1981). This raises the possibility that such patients may have abnormal binding.

6.8 SHBG - prognosis

Murayama proposed that an alteration in SHBG binding capacity might be responsible for oestrogen receptor synthesis, and investigated the relationship between serum SHBG and hormone dependence (Murayama et al 1979). A positive correlation was reported between raised serum SHBG, and cytosol oestrogen receptor levels and subsequent endocrine responsiveness. However other groups have failed to repeat this finding albeit with differing methodology, and it thus remains uncertain whether such a relationship exists (Harris et al 1981, Bowen et al 1980, Mason et al 1981).

6.9 Conclusion

Progress towards understanding the role of oestrogens in breast cancer has been slow. In particular, measurement of serum and urinary oestrogens has generally been unable to explain the more obvious trends arising from epidemiological data. Nevertheless it is possible to make certain predictions concerning the mode of action of oestrogens using the Moolgavkar model.

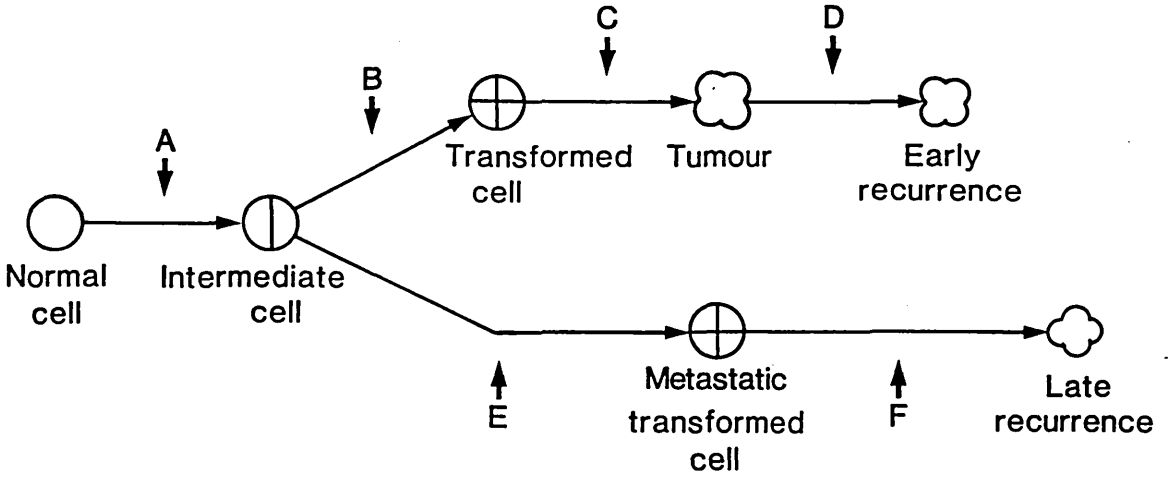
Excess oestrogenic influence, whether due to abnormal luteal phases, anovulatory cycles, increased 'menarche - first pregnancy' interval, or obesity act on the normal cell (A), and probably to a lesser extent the intermediate cell (B), increasing the probability of a 'first hit' [see Fig.6.3].

Early oophorectomy reduces risk, as does early first pregnancy. Although their mode of action is dissimilar, the former reducing circulating oestrogens, and the latter increasing cell differentiation, their impact is likely to be greatest on the intermediate cell population (B).

The presence of a transformed cell implies inevitable subsequent tumour development even though the time interval to clinical apperance may be as long as a further ten to twenty years. Women 'at risk' are highly likely to posess transformed cells and may have low oestriol ratios or possible abnormalities in oestrogen metabolism (C).

Figure 6.3

The Modified Moolgavkar Model



Approximately 30% of breast tumours are oestrogen dependent and endocrine deprivation can modify the recurrence pattern. Late recurrence (twenty or thirty years later) can not be explained on the basis of metastasising tumour cells unless it is assumed that they are capable of remaining in a resting phase for this period. Alternatively, it could be proposed that intermediate cells metastasise, and, continuing to be acted upon by the hormonal environment of the host, subsequently transform (E), resulting ultimately in clinical late recurrence.

Part II

Endocrine study of long term survival

6.10 Introduction

The second part of the chapter is concerned with the study of hormonal influences in long term survival following treatment of breast cancer in a group of patients who survived ten years or more disease-free following primary treatment. The hormone profiles of the long term survivors are compared with those of patients with breast cancer pre-treatment, and a cohort of normal women.

6.10.1 Hormone assays

The hormone assays discussed in detail in this chapter are total oestradiol, free, or non-protein bound oestradiol, and sex hormone binding globulin. Although other hormones studied will be alluded to (androgens, prolactin, and thyroid function) full details of these will be found in the appropriate chapters.

The analysis of free oestradiol was performed using a newly described method, ultrafiltration dialysis (Hammond et al 1980). In this procedure samples are incubated with [^{3}H] steroid and [^{14}C] glucose and duplicate aliquots are subjected to centrifugal ultrafiltration through a dialysis membrane at 37°C. The percentage free steroid is estimated by comparing the [^{3}H] : [^{14}C] ratio of the ultrafiltrate compared to the serum retained by the dialysis membrane. It is assumed that [^{14}C] glucose is able to cross the membrane freely in both directions. The results using

this method are highly reproducible.

Total oestradiol estimation was performed using a conventional radio-immunoassay technique with extraction of the steroid with ether, purification by Lipidex chromatography and labelling with an antibody to oestradiol.

The sex hormone globulin binding capacity was measured by the method described by Iqbal (Iqbal and Johnson 1977). In this method the serum sample is preincubated with a trace amount of labelled 5 α dihydrotestosterone (52-DHT) and added to a two tier column prepared in a pasteur pipette. The upper section of the column is Cibachrome Blue linked to Sepharose 4B, and the lower section contains Sephadex LH20. The two tiers are supported by a fibre glass paper disc. As the serum passes through the upper tier, albumin and albumin-bound 5 α DHT are immobilised by the Cibachrome Blue. SHBG-bound and non-bound steroid pass through to the sephadex layer where the non-protein-bound fraction is retarded. SHBG-bound steroid is collected in a scintillation vial and the radioactivity counted. The mass of DHT bound is calculated from the specific activity of the steroid added.

Full details of these methods are given in Appendix 6.

6.10.2 Results

The presentation of the results commences with a comparison of the long term survivor cohort under study (n = 161) with the much larger cohort of long term survivors (n = 890) analysed in Chapter 3, to determine that the patients in this study are a representative group.

In this, and subsequent chapters, the remainder of the results section is formatted as follows.

An analysis of quality control is presented with calculation of the within-batch and inter-batch coefficient of variation (C.V.). The hormone values obtained for the normal postmenopausal women in this study are compared to those of other authors.

The results of the hormone values for the long term survivors and the control groups are analysed by linear regression on the dependent physiological factors of age, height and weight. Because of the inter-dependence of weight upon height, the hormone values are also correlated against Quetelet's Index.

$$\text{Quetelet's Index} = \frac{\text{Weight (Kg)}}{\text{Height (m)}^2}$$

This allows a more accurate correlation to be made to inter-dependent variables (Khosla and Lowe 1967). The hormone profile of the survivors is then contrasted to those of the control groups using either Student's t test

for parametric data, or the Mann-Whitney test for non-parametric data. Where log transformation of serum hormone values produced a normal distribution, a Student's t test was performed on log transformed data. A linear regression is then performed for the hormonal data on both the long term survivors, and the breast cancer control group against tumour pathological stage and grade (TNM stage, size, histological grade, and fixation). Correlation of oestrogen and oestrogen binding with other hormones assayed is expressed as Pearson's correlation coefficient with a two tailed t test. Finally a life table analysis is presented for the hormonal data on the breast cancer control group using clinical data on these patients supplied by the Breast Unit, Guy's Hospital. This permits an analysis of the influence of hormonal status over the first decade of follow-up on the early breast cancer control group.

Further details of statistical analysis are presented in Appendix 3.

6.11 Patients

Serum oestrogens and oestrogen binding were analysed in long term survivors and two control groups, women with early breast cancer, and normal women.

6.11.1. Long term survivors

n = 161

mean age ± S.D. 66.8 yrs ± 9.0

mean survival ± S.D. 16.5 yrs ± 10.5

Source:

The long term survivors were patients being followed up usually annually, having had treatment for breast cancer at least 10 or more years previously, in the Departments of Radiotherapy or Surgery, Charing Cross Hospital, or the Breast Clinic, Guy's Hospital. All patients were seen by myself and permission was requested for participation in the study following an explanation of the protocol.

Entry criteria:

All patients entered into the study had survived ten years or more disease-free from the time of initial treatment and were clinically, and where possible mammographically, free of disease at time of entry. All long term survivors were post-menopausal or had received a surgical or radiation induced ovarian ablation at least five years prior to entry into the study.

Treatment:

All patients had received treatment for breast cancer whether by surgery, radiotherapy or chemotherapy, or any combination of these. Full details of treatment are presented in Appendix 6. Hormonal therapy at any time from initial diagnosis precluded entry into the study.

1. Hospital Number
2. Date of birth
3. Date of treatment
4. Date, first symptoms
5. Breast involved (Right, Left, Bilateral)
6. Site (upper lateral, lower lateral, upper medial, lower medial, upper, lower lateral, medial, central, diffuse)
7. Axillary gland involvement:
   clinical (pos., neg., ?)
   pathological (pos., neg., ?)
8. Size 1 (cms)
9. Size 2 (cms)
10. Fixation (none, skin, deep, both)
11. TNM Stage T (0,1,2,3,4)
12. N (0,1,2,3,4)
13. M (0,1) [As defined by U.I.C.C., 1980]
14. Parity
15. Children breast fed
16. Menopausal state at entry (Pre-, Post-, Hysterectomy, Oophorectomy)
17. Year of menopause
18. Treatment (Radical mastectomy
    Simple mastectomy
    Local excision
    DXT pre-op
    DXT post-op
    DXT only
    Ovarian ablation
    Chemotherapy)
19. Weight (Kg)
20. Height (m)

Appendix

19. Date, first metastasis
20. Date, first distant metastasis
21. Date of death
22. Cause of death (breast cancer
    not known
    other cause)

Table 6.4 Clinical and pathological data

Clinical and Pathological data:

The patients were examined clinically, and where appropriate mammographically, at the time of entry into the study. Where the clinical and pathological data summarised in Table 6.5 was not available from records, the patient was questioned for further details. Data on height and weight was not available for all long term survivors, but the remaining clinical and pathological data is summarised in Appendix 6.

6.11.2 Breast cancer control group

n = 93

age ± S.D. 65.5 yrs ± 6.8

Source:

Early breast cancer controls were chosen from a large cohort of 759 patients who had operable disease treated at the Breast Unit, Guy's Hospital, between 1974 - 1980.

Entry criteria:

Any post-menopausal patient with operable disease who did not subsequently receive hormonal therapy was eligible for admission into the study within the breast cancer control group.

Treatment:

The patients were treated by surgery, radiotherapy, chemotherapy, or any combination of these modalities. The treatment received is summarised in Table 6.5

Treatment	Number of patients
Radical mastectomy	320
Simple mastectomy	54
Excision or wedge biopsy	90
DXT only	0
DXT pre operative	0
DXT post operative	293
Cytotoxic chemotherapy	114
Oophorectomy	0

Table 6.5 Treatment: breast cancer control group

Clinical and Pathological data:

The clinical and pathological data on these patients was obtained from computerised records held by Imperial Cancer Research Fund (ICRF) at Guy's Hospital. The data is summarised in Table 6.4, and presented in Appendix 6.

6.11.3 Normal women control group

n=97

mean age ± S.D. 65.1 ± 7.1

Source:

The normal controls were obtained from a large cohort of entrants to the screening programme being carried out by the ICRF on the Island of Guernsey. In this programme which commenced in 1978, all women on the island were offered clinical and mammographic screening for breast cancer. At the time of screening detailed personal and family histories were taken, as well as blood and urine samples.

Entry criteria:

Only post menopausal women clinically and mammographically free of breast cancer and with no history of hormone therapy were used as controls in this group, and the controls were matched by age and weight to the long term survivors, and breast cancer control group.

Clinical data:

The data used in this study were age, height, weight, menopausal status and hormone therapy.

6.12 Results

6.12.1 Comparison of long term survivors: control groups

In order to ensure that the 161 long term survivors in the hormone study were a representative group of survivors, the clinical and pathological data were compared to similar data on ten, and twenty year survivors obtained from the large cohort of survivors analysed in the statistical study in Chapter 3. A summary of this data is presented in Table 6.6. It may be seen from this table that the 161 long term survivors under study are a representative group.

Height and weight data for the breast cancer control group (n=89) and normal women control group (n=62) were compared. Breast cancer patients were taller, and had less deviation from ideal body weight when compared to normal women [see Table 6.7]. There was insufficient data for a comparison with the long term survivor cohort.

Median	Breast cancer controls	Normal controls	P
Height (m)	160	157	0.0005
Weight (Kg)	64.5	64.5	N.S.
% deviation ideal body wt.	103.2	112.0	0.01

Mann Whitney test

Table 6.7 Control groups: height, weight and ideal body weight

		Survivors 10-20 yrs (%)	Survivors 20 yrs (%)	Long term survivors (%)
Number		638	69	161
Mean survival		14.7 yrs	24.5 yrs	16.5 yrs
Age at survival		66.9 yrs ± 11.3	71.8 yrs ± 9.42	66.8 yrs ± 9.0
Fixation:	None	256 (46)	30 (52)	48 (56)
	Skin	280 (50)	25 (44)	34 (40)
	Deep	4 (1)	1 (2)	1 (1)
	Both	18 (3)	1 (2)	3 (3)
T	1	147 (27)	15 (28)	31 (21)
	2	315 (59)	28 (53)	44 (56)
	3	65 (12)	9 (17)	15 (11)
	4	9 (2)	1 (2)	2 (2)
N	0	404 (71)	43 (72)	99 (70)
	1	160 (28)	16 (27)	38 (30)
	2	8 (1)	1 (1)	
M	0	571 (99)	69 (100)	161 (100)
	1	4 (1)		
Parity	0	194 (30)	26 (38)	26 (27)
	1	142 (22)	19 (28)	21 (22)
	2	190 (30)	19 (28)	27 (30)
	3	64 (10)	3 (4)	10 (11)
	4	48 (8)	2 (2)	9 (10)
Grade	I	68 (49)		18 (69)
	II	52 (38)		6 (23)
	III	18 (13)		2 (8)

Survivors (10 - 20 years and 20 years) from statistical study, Chapter 3.
Long term survivors, from endocrine study, Section two.

Table 6.6 Long term survival: comparative data (Statistical study: Endocrine study)

6.12.2 Serum Total Oestradiol (Total E_2)

1. Quality control

Analysis of intra-assay variation was carried out using duplicate serum samples. Patient sera with results outside two standard deviations from the mean, or with duplicate results varying by more than 25% were repeated. Duplicate samples from a Quality Control pool were included in each assay batch in order to calculate inter-assay variation. The within-batch coefficient of variation was 10.5% and the inter-batch coefficient of variation 15.3%.

2. Normal values

The values obtained from serum total E_2 in the normal women control group (mean ± S.D., 26.5 pmols/l ± 11.5) are within the ranges quoted by other authors [see Table 6.8].

Authors	n	Mean (pmols/l)	± S.D.
Baird & Guevara (1969)	6	47.7	± 7.3
Nagai & Longcope (1971)	13	16.5	
Korenman et al (1974)	12	40.3	
Vermeulen et al (1976)	20	73.0	± 3.67
Moore (1984)	34	47.7	± 29.4

Table 6.8 Total E_2 in normal postmenopausal women

3. Serum total E_2: age, height, weight.

Serum total E_2 is not significantly correlated to age but

there is a slight upward trend in hormone values in the eighth and ninth decades seen in the breast cancer control group [see Fig 6.4].

Serum total E_2 is significantly correlated to weight in both the normal women and patients with early breast cancer [see Table 6.9, and Fig.6.5]. Multiple linear regression analysis was performed on serum total E_2 and % ideal body weight for the normal women, and breast cancer control groups, and the slopes and intercepts of regression lines compared by analysis of covariance. There was no statistical difference in the slopes, but there was a statistically significant elevation of the regression slope for breast cancer controls compared to normal women ($F = 23.5$, $p < 0.001$). This demonstrates that at any given body weight, patients with breast cancer have approximately 25% more circulating serum E_2.

	Breast cancer (n = 89)		Normal women (n = 62)	
	r	p	r	p
Weight	0.218	0.05	0.278	0.05
Height	-0.216	0.05	-0.100	N.S.
% deviation ideal body weight	0.296	0.01	0.358	0.001
Quetelet's index	0.323	0.01	0.339	0.01

r Pearson's correlation coefficient
p two tailed t test
% deviation ideal body weight (Kg) = height (cm) - 100

$$Q \text{ index} = \frac{\text{weight (Kg)}}{\text{height (m)}^2}$$

Table 6.9 Correlation: Serum total E_2: height and weight

Figure 6.4

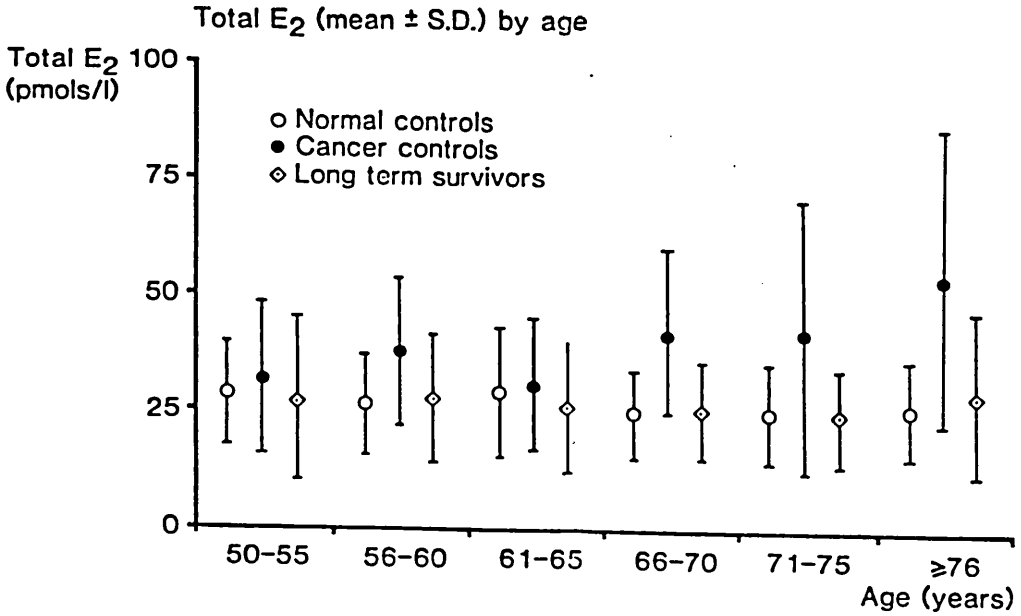
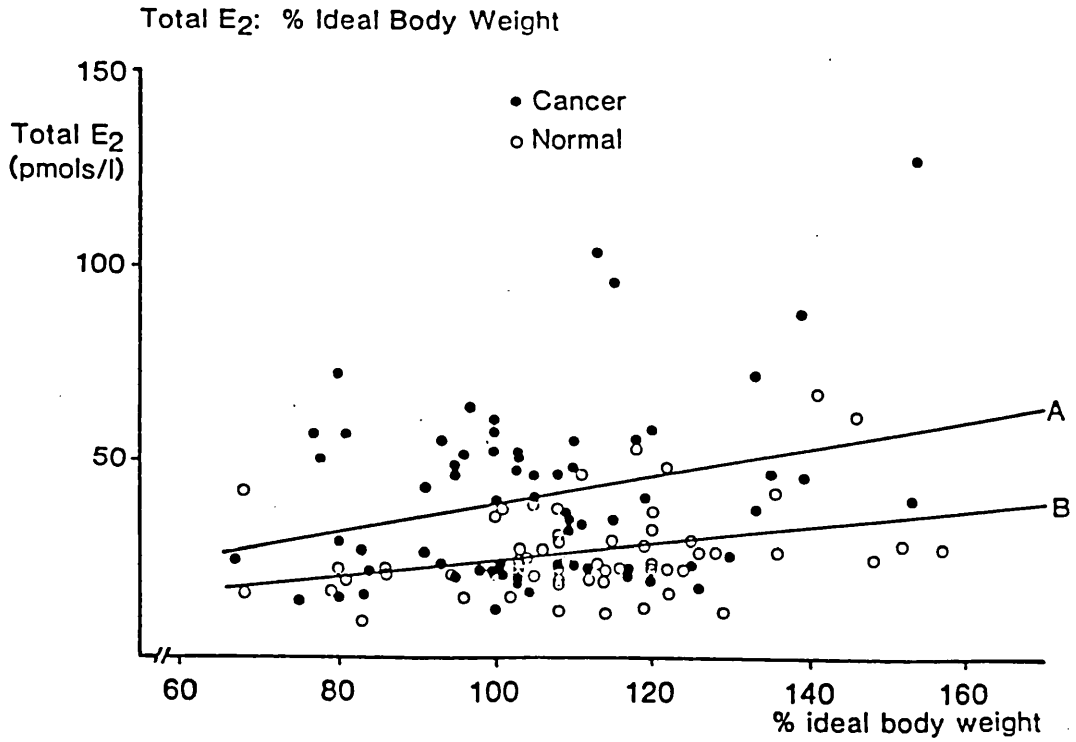


Figure 6.5



A, Cancer $y = 0.368x + 1.65$
B, Normal $y = 0.210x + 3.00$

4. Serum total E_2: long term survivors: control groups

The values for serum total E_2 were not normally distributed so serum total E_2 in the long term survivor cohort was compared to that of the control groups by students' t test on log transformed data. Serum total E_2 was significantly higher ($p < 0.00001$) in the breast cancer control group compared to either the long term survivors or normal postmenopausal women [see Table 6.10, and Fig. 6.6].

Cohort	n	mean (pmol/l) ± S.D.
Long term survivors	145	1.45 ± 1.32
Early breast cancer	77	1.61 ± 1.42
Normal women	63	1.42 ± 1.05

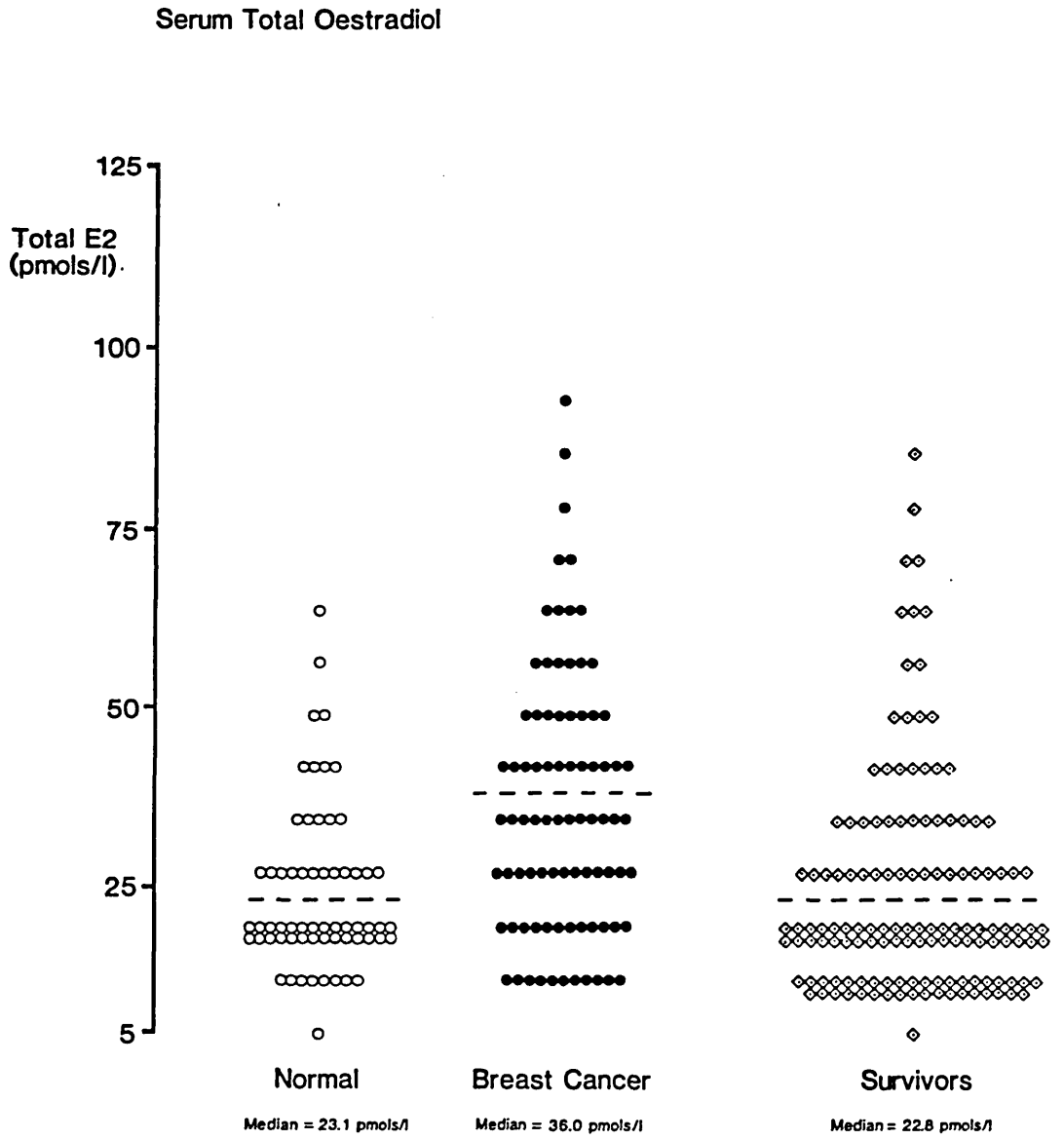
Table 6.10 Log serum total E_2: long term survivors and controls

There was no significant difference in serum total E_2 between the long term survivors and normal postmenopausal women.

5. Serum total E_2: pathological stage.

A linear regression analysis was performed on serum total E_2 against tumour and nodal TNM status (UICC stage), fixation, size, and histological grade for both long term survivors and women with breast cancer. No significant correlations were obtained.

Figure 6.6


Serum Total Oestradiol
125
Total E2
(pmols/l)
100
75
50
25
5
Normal
Breast Cancer
Survivors
Median = 23.1 pmols/l
Median = 36.0 pmols/l
Median = 22.8 pmols/l

6. Serum total E_2: other hormone assays.

There was no significant correlation between serum total E_2 and any of the other hormones assayed (free E_2, DHEA-S, Adiol., DHEA, prolactin or free T_4). The correlation with sex hormone binding globulin is presented in section 6.12.4.

7. Life table analysis: serum total E_2.

A life table analysis was performed on serum total E_2 in the women with early breast cancer using clinical recurrence data provided by Guy's Hospital [see Fig 6.7]. The patients were stratified into two subgroups, above and below pre-treatment median serum total E_2 (36.0 pmol/l). There was no significant difference in survival between the subgroups (log rank test).

Figure 6.7

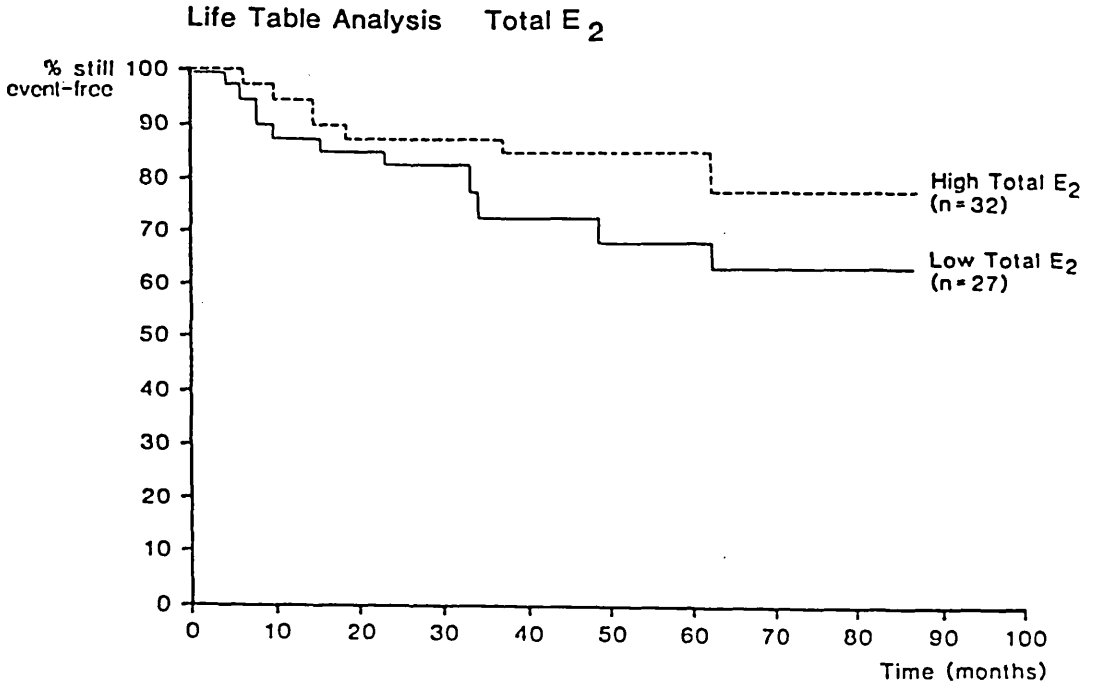
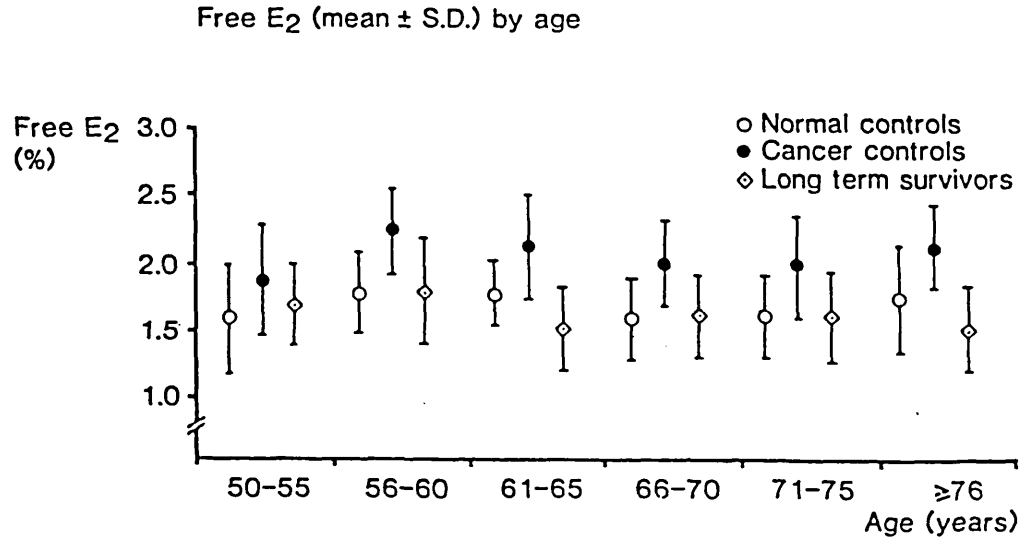


Figure 6.8



6.12.3 Serum % free oestradiol (free E_2)

1. Quality control

Intra-assay variation was calculated from the results of duplicate serum samples, and inter-assay variation from Quality Control sample duplicates. Patient sera with results outside two standard deviations from the mean, or with duplicate results varying by more than 15% were repeated. The within-batch coefficient of variation was 7% and the inter-batch coefficient of variation was 10%.

2. Normal values

The values obtained for serum free E_2 in normal post menopausal women in this study (mean % ± S.D., 1.68 ± 0.32) were in agreement with those obtained by other studies [see Table 6.11].

Authors	Mean (%)
Chopra et al (1973)	1.47
Fisher et al (1974)	1.49
Radar et al (1976)	1.39
Wu et al (1976)	2.21
Moore et al (1982)	1.72

Table 6.11 % Free serum oestradiol in normal post menopausal women

3. Serum % free E_2: age, height, weight.

Serum % free E_2 is not significantly correlated to age, but in similarity to serum total E_2, an upward trend in hormone values is seen in the eighth and ninth decades in the normal women, and in those with early breast cancer. This trend is not seen in the long term survivors [see Fig 6.8].

Unlike serum total E_2, there is no significant correlation of % free E_2 with either weight, height, % ideal body weight, or Quetelet's Index, in any of the three cohorts studied.

4. Serum % free E_2:- long term survivors: control groups

Serum % free E_2 in the long term survivors was compared to that in the control groups using students' t test. There was a highly significant elevation ($p < 0.001$) in the serum % free E_2 in women with early breast cancer, compared to the long term survivors, or normal postmenopausal women [see Table 6.12, and Fig. 6.9]. There was no significant difference however, between the long term survivors and normal postmenopausal women.

Cohort	n	mean (%) ± S.D.
Long term survivors	155	1.70 ± 0.41
Early breast cancer	89	2.09 ± 0.36
Normal women	97	1.76 ± 0.39

Table 6.12 Serum % free E_2 in long term survivors and controls

Figure 6.9

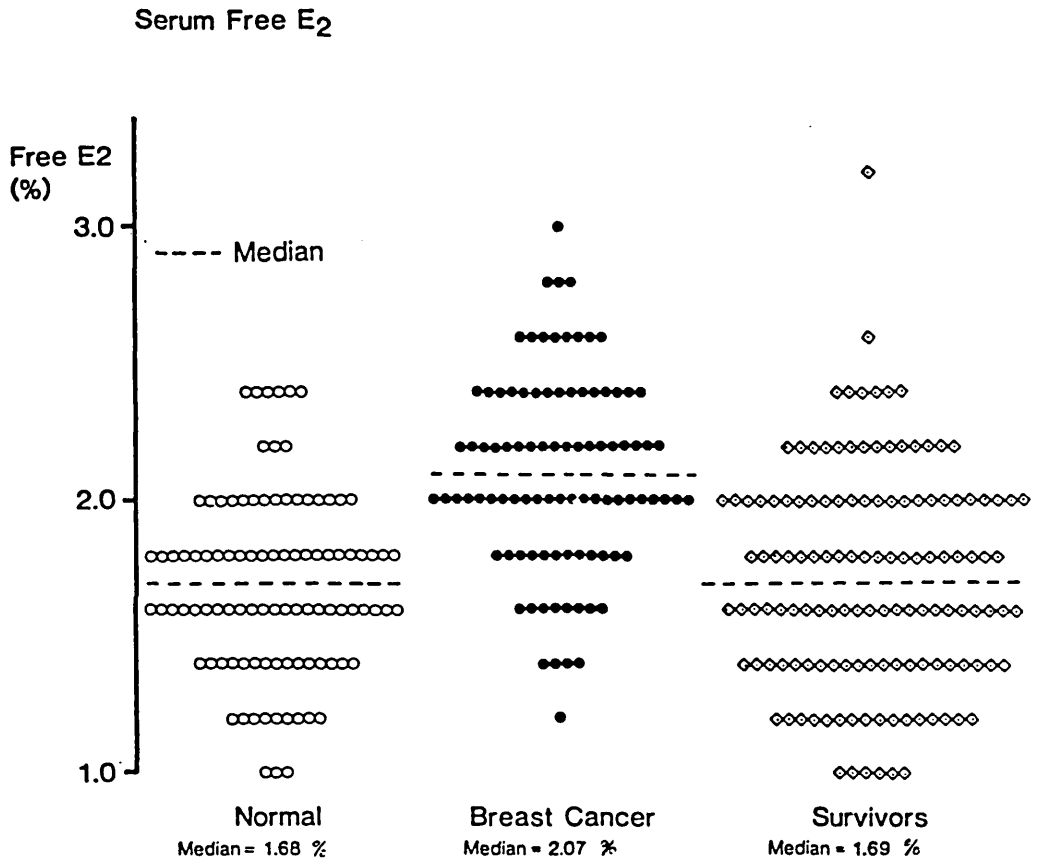
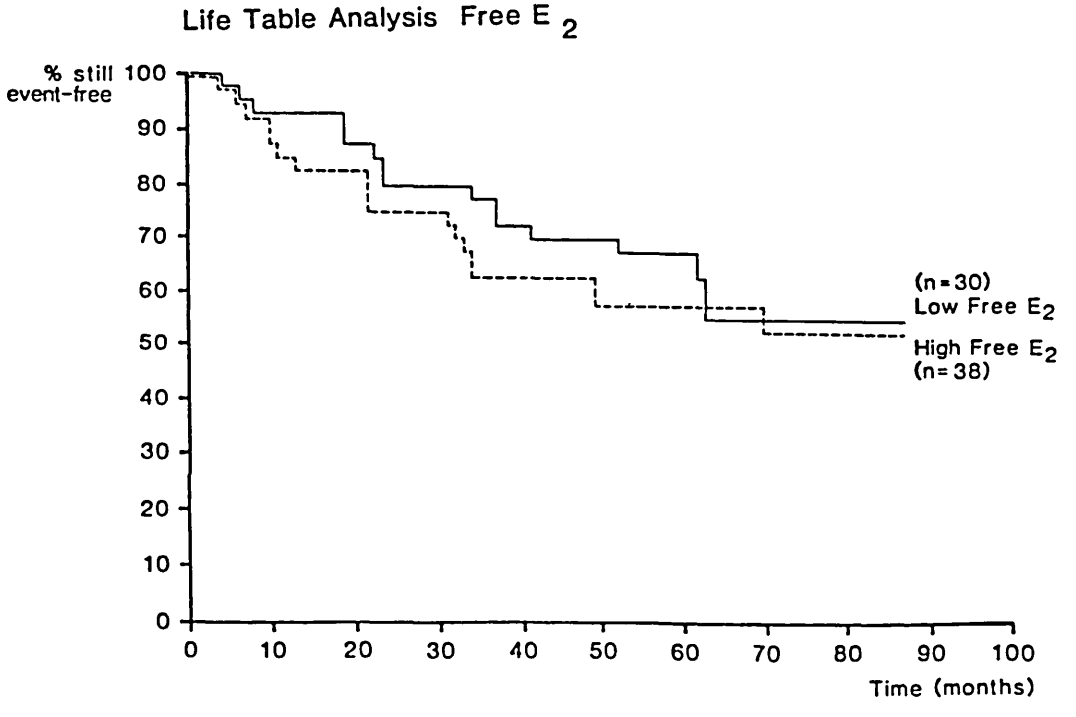


Figure 6.10



5. Serum % free E_2: pathological stage

A linear regression analysis was performed on serum % free E_2 against tumour and nodal TNM status (UICC stage), fixation, size, and histological grade for both long term survivors and women with early breast cancer. No statistically significant relationship was observed.

6. Serum % free E_2: other hormone assays.

There was no significant correlation between serum % free E_2 and other hormones assayed (total E_2, DHEA-S, Adiol, DHEA, prolactin, and free T_4). The correlation with sex hormone binding globulin binding capacity is presented in Section 6.12.4.

7. Life table analysis: serum % free E_2.

A life table analysis was performed on serum % free E_2 in the women with early breast cancer using clinical recurrence data from Guy's Hospital [see Fig. 6.10]. The patients were stratified into two subgroups, above and below median pre-treatment serum % free E_2 (2.07%). There was no statistically significant difference in survival between the two subgroups (log rank test).

6.12.4 Sex hormone binding globulin binding capacity (SHBG binding capacity)

1. Quality control

Inter-batch variation was calculated from the results of duplicate serum samples, and inter-batch variation from Quality Control pool samples. Patient samples with results outside two standard deviations from the mean, or with duplicate results varying by more than 10% were repeated. The within-batch coefficient of variation was 4.5% and the inter-batch coefficient was 6.0%.

2. Normal values

The SHBG binding capacity in normal postmenopausal women in this study (median 71.0 nmols/l, range 20-175) was in agreement with other published series [see Table 6.13].

Author	median (nmols/l)	range
Iqbal and Johnson (1977)	51.0	32-80
Rosner (1972)	54.0	26-136
Hammond et al (1980)	51.0	
Moore et al (1982)	53.3	26-136

Table 6.13 SHBG binding capacity in normal post menopausal women

3. SHBG binding capacity: age, height, weight.

A linear regression analysis of SHBG binding capacity with age demonstrated no significant correlation in any of the three cohorts studied.

SHBG binding capacity in the normal postmenopausal women in this study was significantly inversely correlated to weight ($r=-0.520$, $n=43$, $p < 0.001$) [see Fig 6.11]. This correlation was not statistically significant in women with early breast cancer.

4. SHBG binding capacity:- long term survivors: control groups.

SHBG binding capacity was not normally distributed and was therefore compared in long term survivors and controls by students' t test on log transformed data. The SHBG binding capacity of women with early breast cancer was significantly depressed compared to long term survivors ($p < 0.001$) and normal postmenopausal women ($p < 0.002$) [see Table 6.14 and Fig. 6.12]. The SHBG binding capacity of long term survivors was elevated when compared to normal postmenopausal women ($p < 0.02$).

Figure 6.11

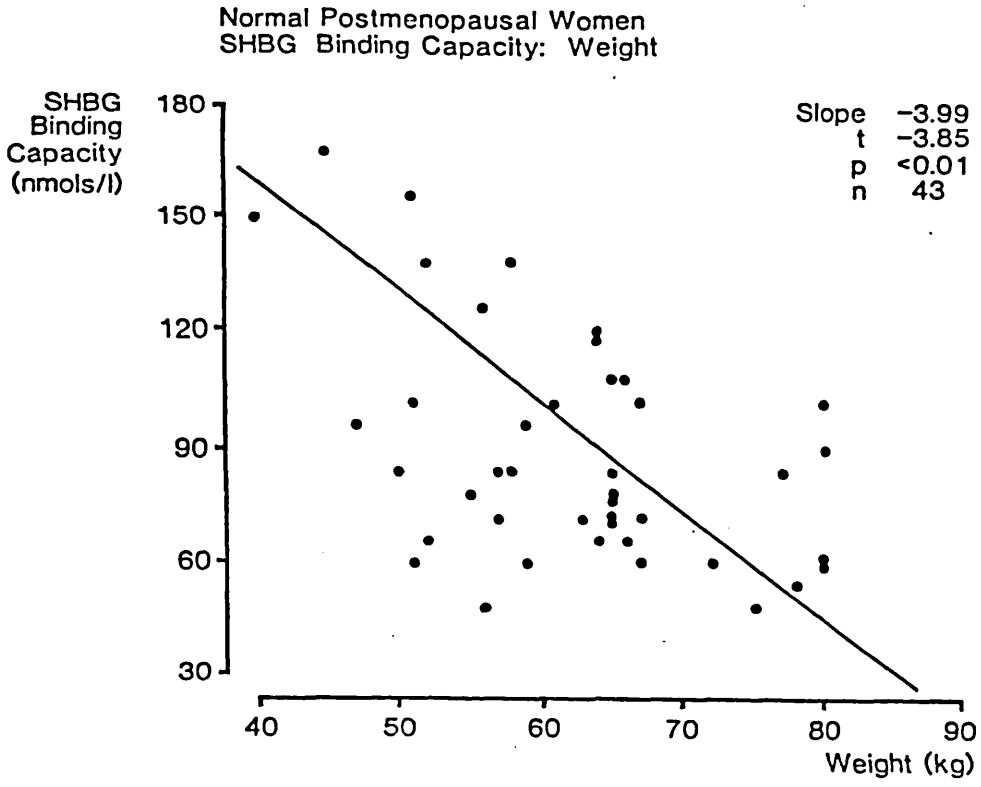
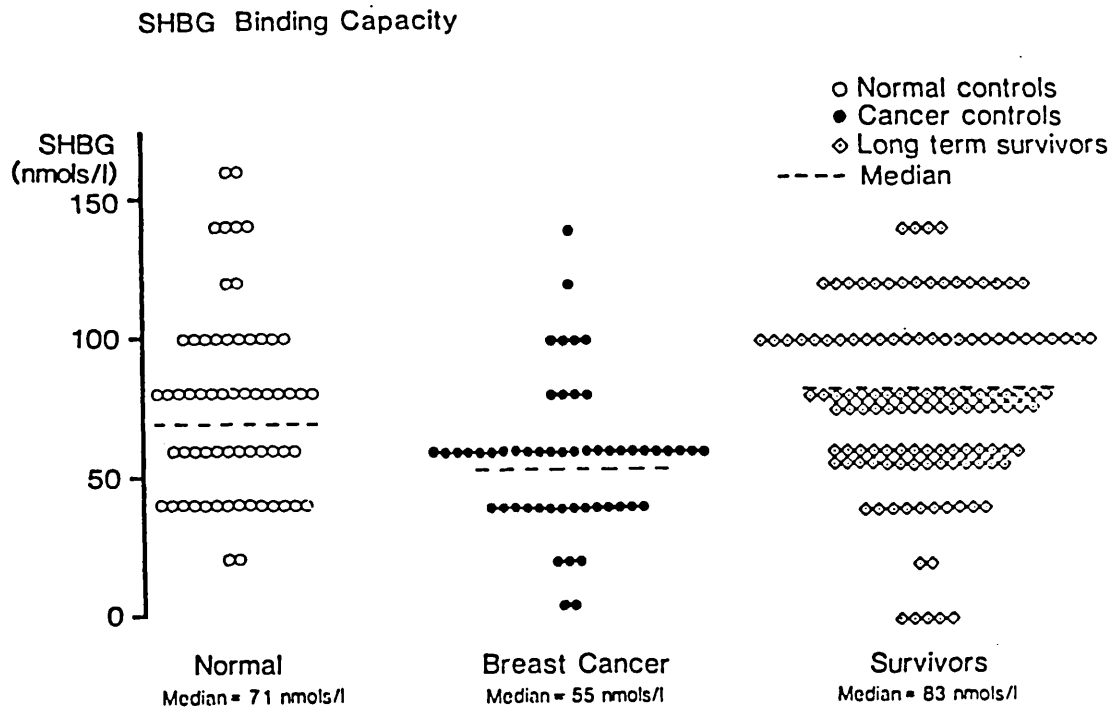


Figure 6.12



Cohort	n	mean (nmols/l) ± S.D.
Long term survivors	148	1.93 ± 1.48
Early breast cancer	47	1.79 ± 1.37
Normal women	70	1.88 ± 1.51

Table 6.14 Log SHBG binding capacity in long term survivors and controls

There was a highly significant inverse relationship between SHBG binding capacity and serum % free E_2 in each of the cohorts under study [see Figs. 6.13 to 6.15]. There was also a correlation between SHBG binding capacity and serum total E_2, although this was only statistically significant in normal postmenopausal women [see Table 6.15].

Correlation	Long term survivors		Early breast cancer		Normal Women	
	r	p	r	p	r	p
SHBG:% free E_2	-0.595	0.001	-0.337	0.001	-0.519	0.001
SHBG: total E_2	-0.184	N.S.	-0.085	N.S.	-0.399	0.001

r = Pearson's correlation coefficient
p = two tailed t test

Table 6.15 Correlation: SHBG binding capacity: serum % free E_2

Figure 6.13

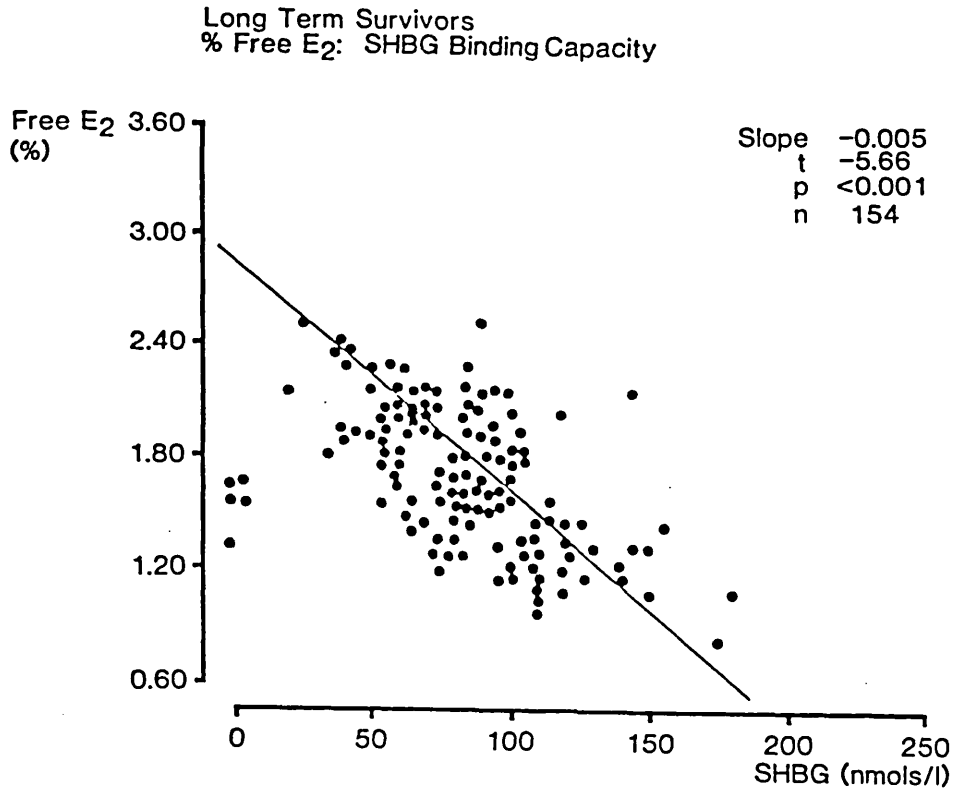


Figure 6.14

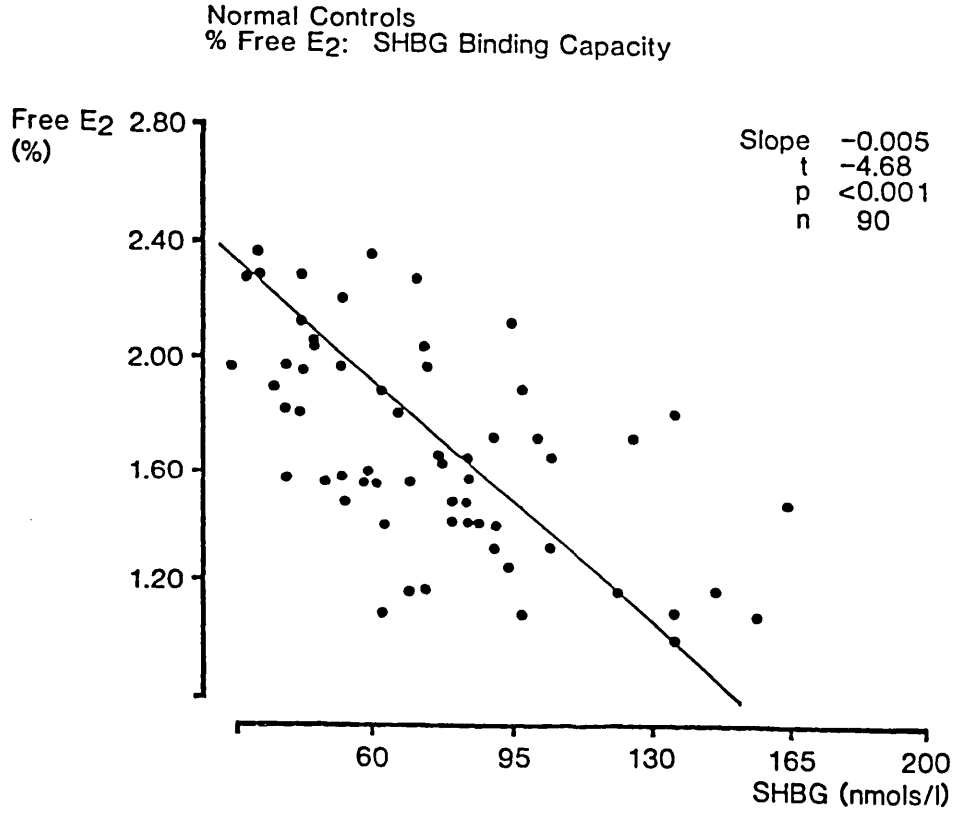
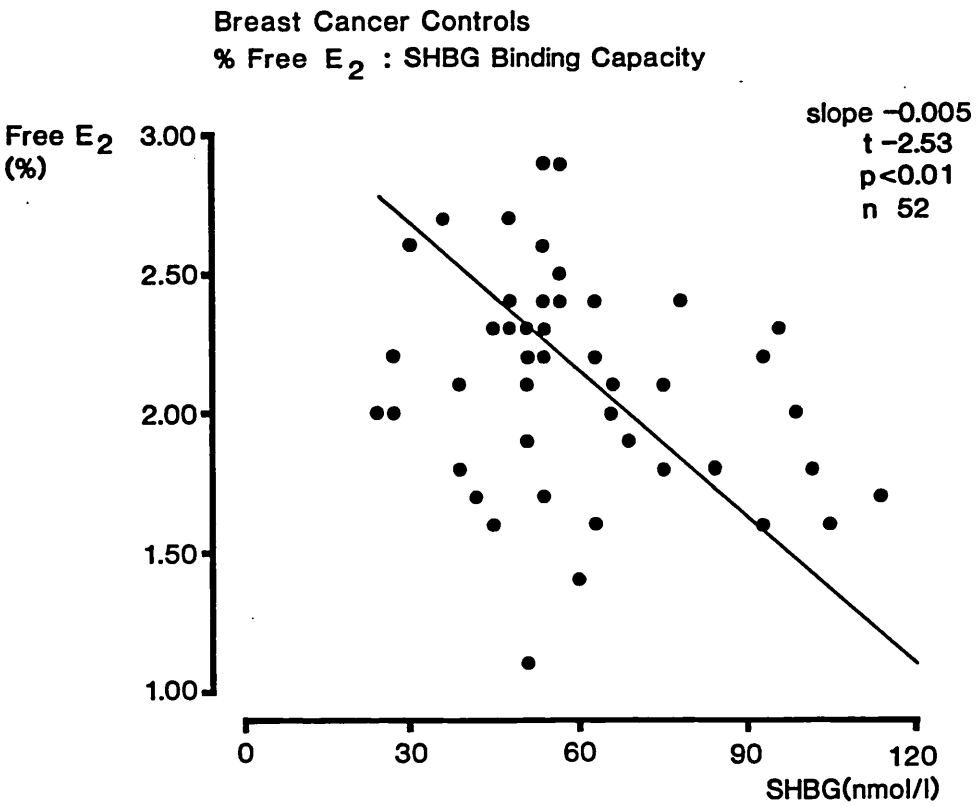


Figure 6.15


Breast Cancer Controls
% Free E_2 : SHBG Binding Capacity
slope −0.005
t −2.53
p<0.01
n 52
Free E_2 (%)
3.00
2.50
2.00
1.50
1.00
0
30
60
90
120
SHBG(nmol/l)

5. SHBG binding capacity: pathological stage.

There was no significant relationship between SHBG binding capacity and tumour or nodal TNM stage, fixation, size, or histological grade, by linear regression analysis on these variables.

6. SHBG binding capacity: hormone assays.

There was no correlation between SHBG binding capacity and any other hormone assays (DHEA-S, Adiol, DHEA, prolactin, or free T_4).

6.13 Conclusions

6.13.1 Normal ranges

The values for serum oestrogens and oestrogen binding in this study are within the ranges quoted by other authors. It must be recognised that a variety of methods have been used previously, some of which were less accurate than the current methods.

6.13.2 Age

The slight increase in serum total and % free oestradiol occurring some 20 years following the menopause noted in this study parallels a corresponding fall in serum androstenedione over the same period noted by Chakravarty, and may be due to increased peripheral conversion of androgens to oestradiol (Chakravarty et al 1976) [see Chapter 7]. It is possible that this late surge in circulating oestrogen could affect late recurrence but analysis of the survival data presented in Chapter 3 does not demonstrate any abnormal pattern of recurrence occurring in the eighth or ninth decade in support of this.

6.13.3 Oestrogens and oestrogen binding

The results demonstrate that both total and non-protein-bound oestradiol are significantly elevated in postmenopausal women with breast cancer, and that this is

associated with a corresponding reduction in SHBG binding capacity. Although two previous studies have shown elevation of serum free E_2 in breast cancer patients, neither demonstrated a significant corresponding decrease in SHBG binding capacity (Nisker et al 1980, Moore 1982). In a more recent, and unpublished study, non-protein-bound E_2 and SHBG binding capacity were measured in 12 women between 6 and 48 months prior to the appearance of clinical breast cancer (Moore 1984). When a comparison was made with matched normal controls, the cases were found to have significantly elevated levels of free E_2, and significantly decreased levels of SHBG binding capacity.

Following the results of the current study it seems probable that the increased availability of E_2 in women with breast cancer is due to an alteration in binding capacity which may preceed cancer development.

The patients with breast cancer were also found to have elevated levels of total E_2, but although it has been suggested that some breast tumours posess aromatase activity and can actively synthesise oestrogens, it is questionable both whether this occurs in a significant proportion of tumours, or is of physiological importance (Dao 1979, Siiteri et al 1976). If aromatase activity is important then it is unlikely to correlate to tumour size, stage, or fixation, as the increase in serum oestrogens has been demonstrated in this study to have no correlation with these factors.

The reduced SHBG binding seen in the breast cancer control group is also significant in that the factors associated with decreased SHBG binding capacity, namely obesity, hyperandrogenism, and hypothyroidism, have all been linked to breast cancer pathogenesis (Anderson 1974). The possible roles of androgenic steroids and thyroid hormones are discussed in subsequent chapters.

6.13.4 SHBG binding capacity and weight

SHBG binding capacity is inversely proportional to weight in both normal women and the breast cancer control group. However the weight, and percentage deviation from ideal body weight are not significantly different between normal women and the early breast cancer patients studied, so weight is therefore an unlikely explanation of the reduced SHBG binding capacity in this latter group.

Obesity may nevertheless be a significant factor in women 'at risk' as circulating oestrogens are elevated in these women both by increased conversion from androstenedione, and the reduced SHBG binding capacity.

6.13.5 Oestrogens and recurrence

There was no evidence from this study that either short term, or long term recurrence was influenced by changes in circulating oestrogens. However, the finding of increased levels of SHBG binding capacity in long term survivors is

of interest as it could support the finding of a significant correlation between E_2 receptor status and SHBG binding capacity (Murayama et al 1980). The clinical relevance of this observation is discussed in the general conclusion.

6.13.6 Summary

The Moolgavkar model was used earlier to explain the possible action of oestrogens in the pathogenesis of breast cancer. A modification of the model was proposed in which hormonal action continued to influence the clinical course of the disease, but the only evidence in support of this, from a study of oestrogens, is the elevated SHBG binding capacity in long term survivors. These patients continue to be followed up annually, and additionally, are 'tagged' at the NHS Central Registry, Southport, so that deaths will continue to be reported. It is anticipated that recurrences in these patients will occur at an approximate 3.0% annual rate (from the date in Chapter 3), allowing further statistical analysis to be carried out.

CHAPTER 7

ANDROGENS

Part I

Literature Review

7.1 Introduction

The role of androgens in breast cancer first came to prominence when it was observed that response to endocrine manipulation could be predicted from the measurement of two urinary androgen metabolites (Allen et al 1957). Since that time interest has focussed principally on the role of androgens in the pathogenesis of breast cancer, and the clinical course of the disease.

In this chapter the literature on androgens and breast cancer is first briefly reviewed, and then in the second part, in a study designed to evaluate the role of androgens in breast cancer survival, hormone profiles of long term survivors are contrasted with those of control groups.

7.1.1 Principal androgens

The principal circulating androgens in man are testosterone (T), dihydrotestosterone (DHT), androstenediol (Adiol.), dehydroepiandrosterone (DHEA), and its sulphate (DHEA-S). The most potent androgen, by far, is testosterone. In the male, androgens are responsible for the development and function of the reproductive system. In the female their action is less well defined, and they have a less important role in the development and function of the reproductive system, although androgens have an anabolic effect on protein metabolism in both sexes.

7.1.2 Biosynthesis

Androgens are synthesised in the female in the zona reticularis of the adrenal cortex and in the stromal cells of the ovaries. Androstenedione is the principal ovarian androgen and it may be synthesised by either the $\Delta 4$ or the $\Delta 5$ pathway [see Fig. 7.1]. Testosterone is also synthesised by the ovary in inverse proportion to androstenedione secretion. Following the menopause, androstenedione synthesis falls, and testosterone synthesis increases, with the ovaries probably contributing about 30% of the former, and 50% of the latter (Vermeulen 1976). Androstenedione is of importance as it is aromatised in peripheral tissues, notably fat, to oestrone and thence to oestradiol, the conversion rate correlating to body weight.

The adrenal cortex secretes principally DHEA and DHEA-S. Although androstenedione is secreted in small amounts the majority probably arises from peripheral conversion of DHEA (Poortman et al 1977).

7.1.3 Transport and cellular action.

Circulating androgens in the blood are bound principally to albumin, with high capacity, but low affinity. Dihydrotestosterone and testosterone are however also bound with high affinity, to sex hormone binding globulin. There is good evidence that, like other hormones, only a very small unbound fraction of the androgens is biologically

Figure 7.1

Biosynthesis of Major Androgens

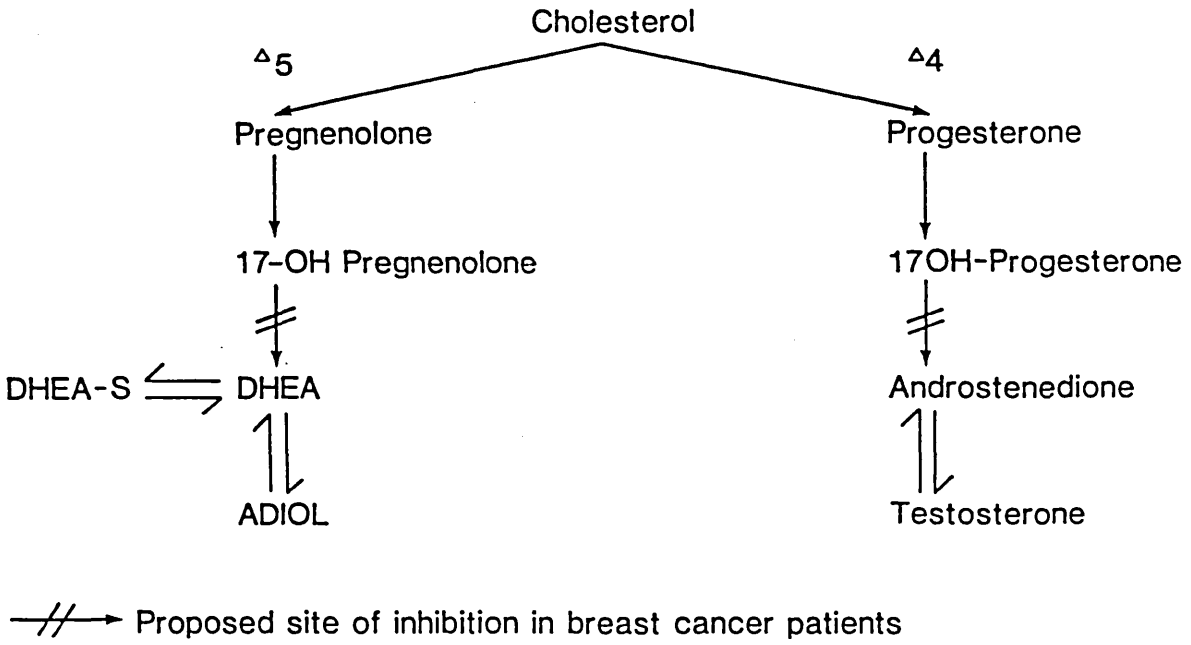
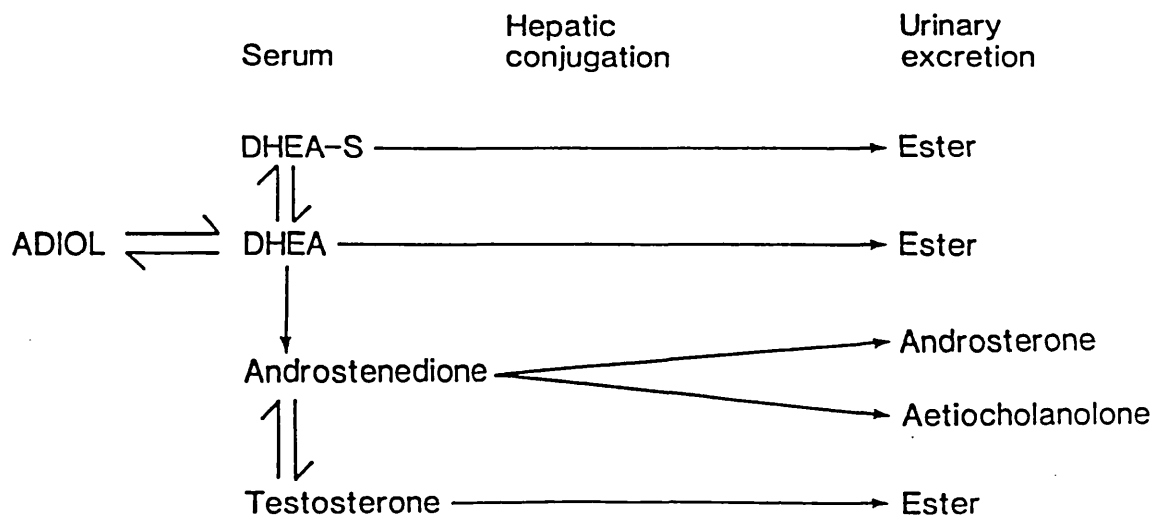


Figure 7.2

Metabolism of Androgens



inactive, and approximately 1% of circulating testosterone in women is in the free state.

Androgen responsive tissues, including the breast, possess receptors to testosterone. The hormone binds to the receptor activating it, and allowing translocation to the nucleus where the receptor complex binds to chromatin and controls genetic transcription and RNA, and subsequently protein, synthesis. It is probable that other androgens such as Adiol. and DHEA bind to the testosterone receptor rather than specific receptors, for in the testicular feminisation syndrome, the testosterone receptor is absent, but normal circulating levels of DHEA do not cause virilisation. Androgens may be able to bind to oestrogen receptors, and this factor, which may be of considerable importance in considering their role in breast cancer, will be discussed at greater length later in this chapter.

7.1.4 Metabolism

Androgens are principally excreted in the urine. Androgen sulphates are secreted by the renal tubule whereas androgens conjugated by glucuronidase in the liver are probably filtered through the glomerulus although a small proportion of unconjugated androgens may be conjugated with glucuronic acid in the kidney itself. The major urinary metabolites are glucuronosides or sulphate esters of androsterone and aetiocholanolone via either 17-hydroxylation or 17-oxidation pathways from androstenedione [see Fig. 7.2].

Approximately 10% of conjugated androgens are excreted in the bile about 50% of which is then reabsorbed in the gut.

7.2 The discriminant function

Following the observation by Allen that response to endocrine manipulation could be predicted by the ratio of the urinary 11-deoxy-17-ketosteroid metabolites to 11-oxy-17-ketosteroids, Bulbrook and Hayward commenced a long series of studies on androgens in breast disease (Allen et al 1957, Bulbrook 1974).

Measuring the three major urinary metabolites, aetiocholanolone, androsterone and DHEA, they formulated a discriminant function:

80-80 x 17-OH corticosteroids (mg/24hrs) + aetiocholanolone (µg/24hrs)

that gave a positive number to patients more likely to benefit from endocrine ablation, and a negative one to those less likely to. The reliability was only reasonable, 35% of positive discriminants responding compared to 16% of negative discriminants. There followed a series of studies in which modifications were made to the original function in an attempt to improve accuracy (Juret et al 1964, Kumaoka 1968, Wilson et al 1967, Miller et al 1967, Fotherby et al 1970, Sarfarty and Tallis 1970).

The conclusion from these studies is basically that women with advanced breast cancer having a low excretion of androgen metabolites respond poorly to endocrine ablation,

and have shorter mean survival. This finding led to the study of androgens in the preclinical stage of the disease.

7.3 Urinary and plasma androgens in women 'at risk'

Studies of women at increased risk of breast cancer have used both within-population studies of women at familial risk, and inter-population studies between geographical areas of high as opposed to low incidence of breast cancer.

7.3.1 Familial risk

A large prospective study of 5000 women on the island of Guernsey was set up in 1961 with all women being offered screening for breast cancer. In 27 women who subsequently developed breast cancer at a mean of 44 months later, analysis of urinary androgen metabolites demonstrated low excretion of aetiocholanolone and androsterone. This low excretion of urinary androgens was associated with a 5-fold increase in risk of developing breast cancer (Bulbrook et al 1971).

In a subsequent study by the same authors, plasma DHEA-S and androsterone-S was assayed as well as the urinary metabolites, in 87 daughters and 39 sisters of women with breast cancer, and compared to normal controls. Significantly lower plasma androgens, as well as urinary metabolites were seen in sisters of patients, but not in daughters. The difference was greater in younger (age 30-40 years) than in older women. It was concluded that familial risk may in part, at least, be due to inherited patterns of androgen metabolism (Wang et al 1975) [see Table 7.1].

Author	Assay	Conclusions
Wang et al (1975)	DHEA-S	Significantly lower in sisters of patients, but no significant difference in daughters.
	Androsterone-S	Ditto
	Urinary aetiocholanolone	Ditto
Wang et al (1979)	DHEA-S	Significantly lower in high risk premenopausal women
	Androstenediol	Ditto
	DHEA	Significantly lower in high risk postmenopausal women
	Androstenedione	No correlation with risk.
Boffard et al (1981)	Androstenedione	No significant difference in daughters of patients
	DHEA-S	Ditto
Secreto et al (1983)	Testosterone	No significant difference
	Androstanediol	No significant difference

Table 7.1 Plasma androgens in women at high familial risk

In a third study, plasma DHEA, DHEA-S, and androstenedione were assayed 5 years following urinary assay in the same women. Again, lower plasma androgens wee correlated with high risk, and also with the urinary excretion pattern of 5 years previously, indicating that the changes were not transient (Wang et al 1979) [see Table 7.1].

7.3.2 Geographical risk

With the evidence that subnormal androgen status is important in Caucasian women, a comparative study was conducted between normal British and Japanese women, who have a lower incidence of breast cancer (Wang et al 1976). Plasma DHEA-S and the principal urinary androgen metabolites were lower in Japanese women when matched for age and menopausal status. This was almost certainly due to their lower weight and does not necessarily imply that low androgenic status is not a risk factor in Japanese women, but no direct comparison can be made between the two populations.

7.4 Urinary plasma and androgens in women with breast cancer

Initial studies on androgens in patients with breast cancer were principally of the major urinary metabolites aetiocholanolone and androsterone. As might be anticipated from the previous studies of women 'at risk', the majority of authors found reduced levels of one, or both, of these metabolites in patients with early breast cancer, compared to normal women (Bulbrook et al 1964, Stern et al 1964, Bacigalupo and Lingk 1968, Gutierrez and Williams 1968).

With advancing methodology it became possible to measure the principal circulating androgens (DHEA, DHEA-S and Androstenedione), and these were found to correlate to their urinary metabolites (Deshpande et al 1965). The conclusions of some of the many studies on plasma androgens are summarised in Table 7.2. Rather surprisingly, in view of the general concordance on urinary androgens, there is no complete agreement on plasma androgens, though in the majority of studies, reduced levels of DHEA, and DHEA-S were observed in breast cancer patients.

Author	Assay	Conclusions
Brownsey et al (1972)	DHEA-S	Significantly reduced in advanced breast cancer. Normal in early breast cancer.
Wang et al (1974)	DHEA-S) Androsterone)	Reduced in early B.C. (not significant) Reduced post-mastectomy
Thomas et al (1976)	DHEA	Significantly reduced in early breast cancer.
McFayden et al (1976)	Testosterone	No significant difference. (6 pts. only)
Rose et al (1977)	DHEA-S	Significantly reduced in early breast cancer
	Androstenedione	No significant reduction in early/advanced B.C. No reduction post-mastectomy.
Wang et al (1977)	Androstenedione	Normal in early B.C.; significantly reduced in advanced B.C. Reduced post-mastectomy. Correlates to plasma DHEA-S.
Adams et al (1979)	Testosterone	Significantly elevated in early B.C.
Skinner et al (1980)	Hydroxy-DHEA	Significantly reduced in early B.C. Reduced post-mastectomy (advanced B.C. only)
Zumoff et al (1981)	DHEA-S DHEA	11 pts. only. Reduced premenopausal, elevated post-menopausal early B.C.
England et al (1981)	DHEA-S	No significant difference early B.C./normals/benign breast disease.

B.C. - breast cancer

Table 7.2 Plasma androgens in women with breast cancer

7.5 Urinary and plasma androgens, and breast cancer recurrence

7.5.1. Urinary androgens

Early studies using a discriminant function suggested that the prognosis of women with advanced cancer was poorer in those having low urinary androgen excretion. More recent studies on survival following mastectomy for early breast cancer have given a more equivocal result, but there is general agreement that low androgen excretion is associated with a poorer prognosis [see Table 7.3].

In the most recent of these studies 218 women with early breast cancer were followed up for a maximum of 60 months retrospectively (Thomas et al 1982). Recurrence was highly correlated with androsterone excretion ($p < 0.005$) even when patients were stratified by stage, or by histological grade (Grade III, $p < 0.025$) indicating that androgen excretion may be an independent prognostic factor. As patients in this retrospective study were biased towards those with recurrence, no attempt was made to analyse the predictive value of androgen excretion for prospective clinical analysis.

7.5.2 Serum androgens

The data on serum androgens and clinical recurrence is sparse. It has been shown that serum levels of DHEA-S,

Author	Low urinary androgens and clinical recurrence
Bulbrook et al (1962)	More rapid recurrence
Bulbrook et al (1964)	More rapid recurrence
Stern et al (1964)	More rapid recurrence
Hayward et al (1968)	More rapid recurrence
Miller et al (1975)	No significant difference
Prescott et al (1978)	No significant difference
Segaloff et al (1980)	More rapid recurrence (post-menopausal patients only)
Thomas et al (1982)	More rapid recurrence (pre-menopausal patients post-menopausal patients)

Table 7.3 Low urinary androgens and breast cancer recurrence

DHEA, and Adiol, correlate significantly with urinary androsterone and aetiocholanolone secretion (Wang et al 1979). From the evidence of urinary androgen studies it might be anticipated that early recurrence would be associated with low serum androgens. In an unpublished study, Moore could demonstrate no overall significant relationship between serum Adiol. or serum DHEA and recurrence rates after mastectomy, but in stage II patients elevated pre-treatment serum Adiol. was associated with significantly increased recurrence rates, as was elevated pre-treatment serum DHEA (Moore 1984).

Serum androgens have also been studied in patients with advanced cancer treated by Aminoglutethimide (Santen 1981). Aminoglutethimide is an enzyme inhibitor blocking aromatisation and hydroxylation of Δ^5 steroids. It results in the suppression of urinary and serum oestradiol and oestrone. Its actions on androgens are complex, but serum androstenedione is elevated, testosterone and DHT remain unaltered, and DHEA-S is markedly suppressed [see Figs. 7.1 and 7.2]. This contrasts with surgical adrenalectomy where, although oestrogen suppression is identical, serum and urinary androgens are uniformly depressed. In clinical studies it has been shown that mean levels of serum DHEA-S and androstenedione fall to a greater extent in objective responders than in women with disease progression (Santen 1981, 1982). The evidence from urinary and serum androgens is therefore conflicting.

7.6 Androgens and breast cancer

7.6.1 Adrenal biosynthesis

The preceding studies on urinary and plasma androgens have demonstrated significant associations between androgen metabolism and breast cancer but have not indicated its biological basis. The finding, in breast cancer patients, of subnormal plasma levels of DHEA and DHEA-S, which are synthesised predominantly in the adrenal, suggests that the biological defect may be at this level. To this end, Deshpande conducted a series of studies using radiolabelled precursors and examining the labelling of the androgens synthesised. His conclusions would indicate that if a primary adrenal defect is responsible it is at the level of the C_{21} side-chain cleavage from 17-OH pregnenolone and 17-OH progesterone to form DHEA and androstenedione respectively (Deshpande et al 1967) [see Fig 7.1].

This does not explain of course, how low circulating levels of androgens can be associated with increased risk, or modify the clinical course of breast cancer.

7.6.2 Androgens and breast development

There is no adequate evidence to define the role of androgens in the development of the normal human breast. It has generally been assumed that in this situation androgens are merely oestrogen antagonists and therefore have no role

to play, but this concept may be incorrect for several reasons. Firstly, it has been shown that androgens can stimulate breast growth in several animal models including the rat, mouse, guinea pig, and rhesus monkey (Wang 1979). Secondly, Adiol. and some other androgens have been shown to have true oestrogenic actions on for example uterine growth and vaginal epithelium (Huggins 1954).

It has also been shown that DHEA-S (and to a lesser extent, DHEA and Adiol.), reduces conversion of oestradiol to oestrone by competitive inhibition of 17β hydroxydehydrogenase, thus increasing exposure of target organs to oestrogenic stimuli (Bonner et al 1983).
A balance between oestrogenic and androgenic stimuli may therefore be required for normal human breast maturation.

7.6.3 Androgens and oestrogen receptors

In contrast, however, Adiol. has been demonstrated to displace oestradiol from oestrogen receptors, even at normal physiological concentrations (Poortman et al 1975). This would suggest that the role of androgens as oestrogen antagonists may be of importance, as low androgen concentrations in the plasma, perhaps from a familial enzyme defect, would permit greater oestrogenic stimulation on endocrine target organs leading to increased risk in breast cancer development. Of relevance also in this respect is the finding of Abul-Hajj that urinary androgen metabolite excretion in breast cancer patients is correlated to

possession of tumour oestrogen receptor, providing a possible link between androgens and the known survival advantage of oestrogen receptor possession (Abul-Hajj 1977).

7.6.4 Androgen receptors

Circulating androgens do not only bind to oestrogen receptors, for it has already been noted that endocrine target organs including the breast, possess specific androgen receptors. However, the role of androgen receptors in breast cancer is less well understood. Some experimental mammary tumours are androgen responsive, as is the human MCF 7 cell line, and in postmenopausal women response rates to androgen therapy vary between 10% and 38% (Henderson and Cannellos 1980). In two clinical trials in which response rates to hormone therapy have been equated to oestrogen and androgen receptor concentration, it appears that an equal response is obtained in tumours possessing either receptor (50-55%), but that an increased response (67%) can be anticipated when both are present (Wagner and Jungblut 1976, Engelsman et al 1977).

7.6.5 Breast cancer steroidogenesis

The above concepts are further complicated by the fact that not only are androgens converted in peripheral organs such as fat, skin and muscle to oestrogens, but also that some breast tumours can synthesise both androgens and oestrogens.

Most malignant human breast neoplasms possess aromatases that are capable of androgen interconversion and some can convert testosterone to oestradiol (Miller et al 1973). However, the conversion rates are very low, of the order of 0.1% to 0.5% and it is not clear how an androgen, or an oestrogen, synthesised in tumour cell mitochondria can then influence a tumour that may not possess a cytoplasmic endocrine receptor.

Human breast cancers also possess sulphurating enzymes, sulphuration being one of the major metabolic processes occurring before androgens are excreted in the urine. Dao and Libby reported a correlation between steroid sulphurating activity and response to endocrine ablation, in which case a correlation between sulphokinases and oestrogen receptors might be anticipated but there is no general agreement on this (Dao and Libby 1969, 1972, Leung et al 1973, Braunsberg et al 1974).

7.6.6 Transformation of carcinogens

A further link between androgens and cancer pathogenesis has been suggested by Schwarz, who demonstrated that DHEA can protect rat liver epithelial-like cells from aflatoxin-B induced carcinogenesis and also hamster embryonic fibroblasts from carcinogenesis induced by both aflatoxin and DMBA (Schwarz and Perantoni 1975). Again, however, this does not shed any further light on androgen mechanisms in human mammary cancer pathogenesis.

7.7 Conclusion

It has been seen in the previous chapter that the modified Moolgavkar model proposed earlier in the thesis could provide a reasonable fit with known epidemiological and hormonal data on oestrogens. There is substantially less weight of evidence concerning androgens, and it is probable that their action on breast cancer pathogenesis and clinical recurrence is indirect, rather than direct.

Low androgen secretion, perhaps occurring during early puberty and which may be an inherited metabolic defect, could cause an augmented oestrogenic influence during the first crucial decade of breast growth and maturation, delaying epithelial maturation and increasing risk of intermediate cell production [See Fig. 7.11 (A), (B)].

The low androgen secretion noted in women at risk, and up to 9 years before development of a clinical carcinoma is unlikely to be due to androgens synthesised by what would, at that point, be a micro-tumour, and is therefore more likely to be associated with increased transformation (C), again by an indirect mechanism.

Finally, androgens almost certainly do modify the clinical course of breast cancer (D) whether by continuing to exert an endocrinological action on receptors in micrometases and the subsequent appearance of early recurrence, or possibly because low androgen secretion is associated with an as yet

undefined tumour characteristic that itself is of prognostic significance.

In the second part of this chapter a further analysis of the actions of androgens in the clinical course of breast cancer is presented following the results of a study on long-term survivors.

Part II

Endocrine study of long term survival

7.8 Introduction

The second part of this chapter describes the results from the assays of androgenic hormones in the long term survivors and control groups. Dehydroepiandrosterone sulphate is the principal circulating post-menopausal androgen and the hormone most often assayed in previous studies. This was the only androgen assayed in the long term survivors. In order to widen the scope of the study into androgenic mechanisms in survival, a further group of patients, with very poor prognosis but survival equivalent to that of the long term survivors, was also studied, using both pre-treatment serum, and serum obtained at the time of survival.

The following abbreviations are used:

Dehydroepiandrosterone Sulphate - DHEA-S
Dehydroepiandrosterone - DHEA
Androstenedione - Adiol.

7.8.1 Patients

Serum androgens were assayed in long term survivors, women with early breast cancer, and in normal women, as in the preceding chapter, but in addition, a further group of poor prognosis survivors was also studied. Limited conclusions can be drawn from a study of long term survivors when comparison can be made only to matched control groups of normal women or women with breast cancer. Further

conclusions could be drawn if pre-treatment serum or hormone analysis were available for long term survivors for comparative analysis, but, because of the long duration of survival this was not possible. Therefore a group of poor prognosis survivors was defined in which, although duration of survival was significantly shorter than comparable survivors with better prognosis, the cumulative proportion surviving was equivalent to that of the cohort of long term survivors. The patients with poor prognosis were those with either histological grade III tumours or 4 or more pathological lymph nodes involved. The cumulative proportion surviving was matched between these groups and the long term survivors by life table analysis.

The poor prognosis survivors were chosen from the larger cohort of women with breast cancer in whom pre-treatment serum or hormone assays were available, thus allowing comparison of hormone profiles to be made with the control groups, both pre-treatment, and at the time of survival, when further blood sampling for hormone assay was carried out.

7.8.2 Methods

DHEA-S was assayed by the radio-immunoassay method developed at Imperial Cancer Research Fund by Wang (Wang et al 1979). Tritiated DHEA-S is added to the plasma sample which is then extracted to remove non-conjugated steroids and lipids. The DHEA-S is then hydrolysed and extracted. The antibody used

was raised against D7 (o-carboxymethyl)-immuno-bovine serum albumin raised in rabbit.

The methods used for Adiol. and DHEA were similar except that the steroids were first extracted by Lipidex 5000 chromatography (Moore 1979). Lipidex is a highly lipophilic sephadex derivative with a high separation capacity. The gel was contained in a 12 cm glass column, and a hexane: chloroform (97:3) mixture used as the eluting solvent. The radioimmunoassay method was the same as that for DHEA-S except that tritiated DHEA or Adiol. and the appropriate antibody were used.

Serum DHEA-S was assayed in the long term survivors, poor prognosis survivors and a proportion of the breast cancer and normal women control groups. Serum DHEA-S in the remainder of the control groups was performed by the Department of Clinical Endocrinology, Imperial Cancer Research Fund using methods identical to those described. Duplicates of controls and samples, as well as quality control samples were analysed to ensure accuracy.

Serum DHEA and serum Adiol. were analysed only in the cohort of poor prognosis survivors. Hormone assays for the control groups were performed by ICRF using methods identical to those described. Quality control and duplicate samples were assayed to ensure accuracy.

Full details of the assays are given in Appendix 7.

7.8.3 Results

The results of serum DHEA-S in the long term survivors, poor prognosis survivors (both pretreatment, and at time of survival) and the control groups were analysed as follows:

1. Quality control (coefficient of variation estimated for within-batch and inter-batch).
2. Normal range (comparison to previous studies).
3. Linear regression on age, height, weight and percentage ideal weight.
4. Comparison of:-
   long term survivors: age matched controls
   poor prognosis survivors (pretreatment): breast cancer controls
   poor prognosis survivors (at survival): normal controls
   (by multiple linear regression on age and estimation of variance of slope and elevation).
5. Linear regression on tumour pathological stage and grade.
6. Correlation of plasma DHEA-S and other hormones.
7. Life table analysis of pretreatment plasma DHEA-S in the breast cancer control group using clinical recurrence data provided by ICRF and Guy's Hospital.
   (Two tailed estimate of probability from a log rank test, and chi^2 estimation of trend, with patients stratified into two cohorts, above and below median serum hormone level).

The results of serum DHEA and serum Adiol. in the poor prognosis survivors and control groups were analysed following a similar format.

7.9 Patients

Four groups of patients were studied:

7.9.1 Long term survivors

n = 155

mean ± S.D 66.8 yrs ± 9.1

mean survival ± S.D 15.6 yrs ± 10.5

One hundred and fifty-five postmenopausal long term survivors were studied who had remained disease free for ten years or more from the time of treatment of breast cancer, and who were clinically free of disease at the time of entry into the study. None had received prior hormonal therapy. Full details of these patients are given in Section 6.11, and Appendix 6.

7.9.2 Breast cancer control group

n = 325

mean age ± S.D 62.4 yrs ± 11.0

Age matched postmenopausal patients were chosen from a large cohort of women with early breast cancer in whom either pre-treatment serum was available or serum hormone values were known. None had received adjuvant hormone therapy. Full details of clinical and pathological data on these patients is given in Chapter 6 and Appendix 6.

7.9.3 Normal women control group

n = 349

mean age ± S.D. = 58.9 yrs ± 4.9

Age matched postmenopausal women were chosen as controls from a cohort of 349 normal women from the Guernsey screening project who were clinically and mammographically free of breast cancer and in whom androgen hormone assays had recently been performed. None of these women had received hormone therapy. Full details of this cohort appear in Section 6.11 and Appendix 6.

7.9.4 Poor prognosis survivors

n = 321

mean age ± S.D. = 61.5 yrs ± 8.2

mean survival ± S.D. = 58.6 mths ± 11.0

A life table analysis was performed of the patients in the breast cancer control group stratified for pathological nodal status (no nodes, 1-3 nodes, 4 or more nodes involved) and histological tumour grade (Grades I to III, Bloom and Richardson 1955). Patients with 4 or more involved nodes, or tumours histologically grade III have very poor prognosis. A small cohort of postmenopausal patients, representing the uppermost 10th percentile of survivors in these two groups was chosen (n = 54). Of these patients,

32 were available for follow-up examination and blood sampling during the required time interval. Sixteen patients had histologically grade III tumours (median survival 68 months range 44 - 81 months), and 16 patients had 4 or more pathologically involved nodes (mean ± S.D. = 8.4 nodes ± 4.5, median survival = 48 months, range 39 - 69 months).

Comparison, by life table analysis, of the survival of patients in the breast cancer control group with four or more pathologically involved nodes alive at 30 months, or with histological grade III tumours alive at 68 months, demonstrates that there is an equivalent proportion surviving to unstratified long term survivors at 15.5 years [See Figs. 7.3 and 7.4].

This small group of poor prognosis survivors entered into the study had pre-treatment serum available for hormone analysis. None had received prior hormone therapy or adjuvant chemotherapy and all were clinically free of disease at time of entry into the study. Full clinical and pathological details are in Appendix 7.

Figure7.3

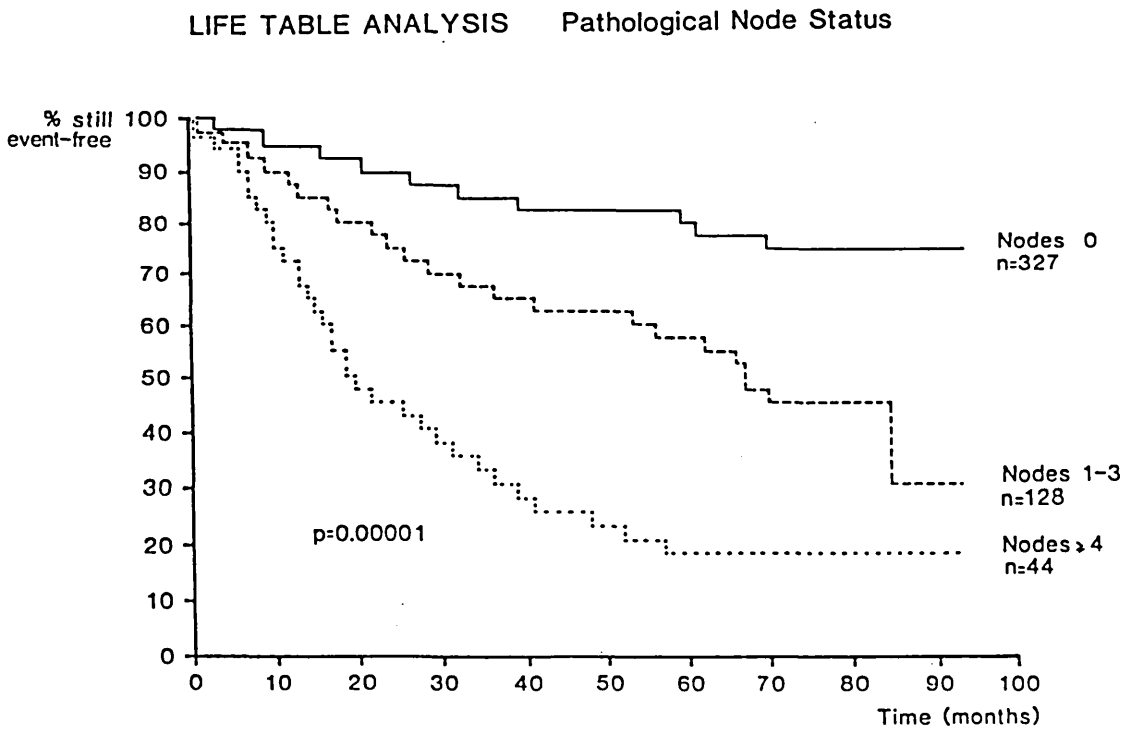
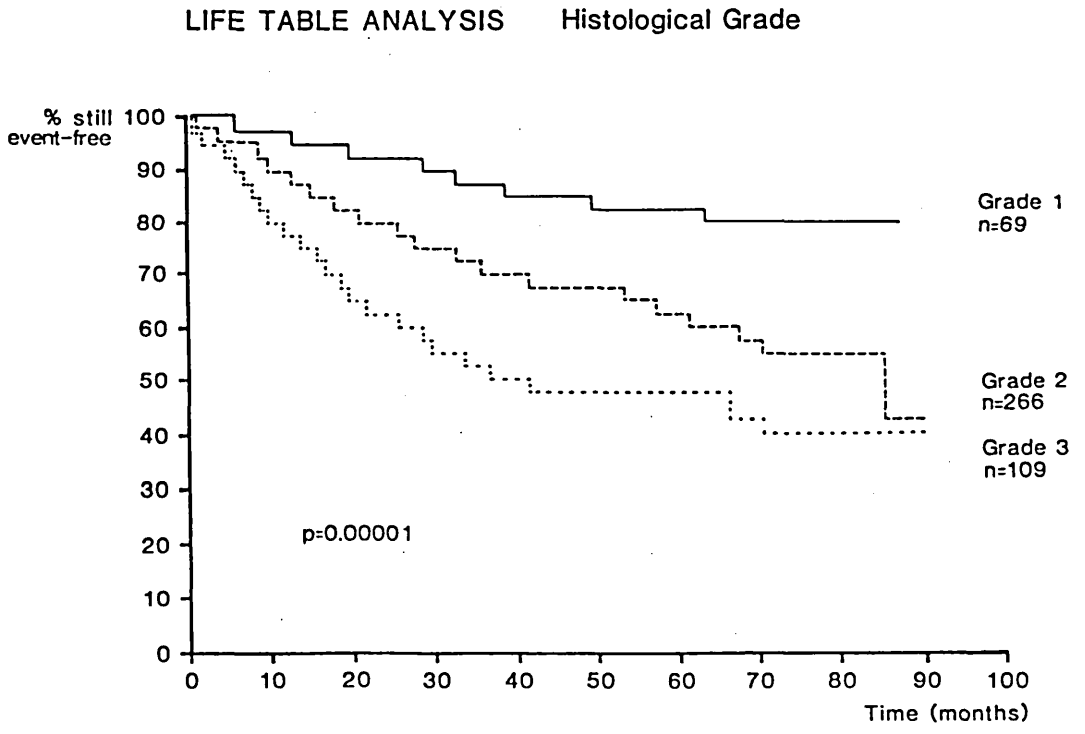


Figure 7.4



7.10 Results

7.10.1 Serum DHEA-S

1. Quality Control

Duplicate assays and quality controls were included in each batch of DHEA-S assays. The inter-batch coefficient of variation was 14%, and the within batch coefficient of variation was 10.7%.

2. Normal Values

Serum DHEA-S values in the normal women control group in this study (mean serum DHEA-S ± S.D. = 744.7 ng/ml ± 416) agreed closely with results of serum DHEA-S in postmenopausal women in other studies [See Table 7.4].

Author	serum DHEA-S mean ± S.D. (ng/ml)
Wang et al (1976)	650 ± 370
Meldrum et al (1981)	590

Table 7.4 Serum DHEA-S in normal postmenopausal women

3. Serum DHEA-S:- age, height, weight

There was no linear correlation between serum DHEA-S and weight, height, ideal body weight or Quetelet's Index.

Serum DHEA-S is significantly inversely correlated with age in all groups (long term survivors, $r = -0.368$, $p < 0.001$; poor prognosis survivors, $r = -0.438$, $p < 0.05$; breast cancer control group, $r = -0.338$, $p < 0.001$; normal post-menopausal women, $r = -0.322$, $p < 0.001$). Further analysis is therefore by multiple linear regression on age.

4. Serum DHEA-S:- long term survivors: controls

A linear regression of serum DHEA-S and age was performed for the long term survivors, and both early breast cancer (pre-treatment), and normal women control groups [see Fig. 7.5]. There was no statistically significant difference in the value of the slope between any of the groups. An analysis of variance for the difference in elevation of the slope demonstrated no significant difference between the control groups of normal women, and women with early breast cancer, but long term survivors had levels of serum DHEA-S significantly lower than either control group (long term survivors: breast cancer controls, $F = 12.4$, $p = 0.001$; long term survivors: normal women, $F = 5.8$, $p < 0.025$).

Serum DHEA-S of the poor prognosis survivors (at survival) was also significantly lower than normal postmenopausal women ($F = 9.14$, $p < 0.01$), but there was no significant difference in serum DHEA-S of the poor prognosis survivors (pretreatment) when compared to the remainder of the breast cancer control group [see Figs. 7.5 and 7.6].

Figure 7.5

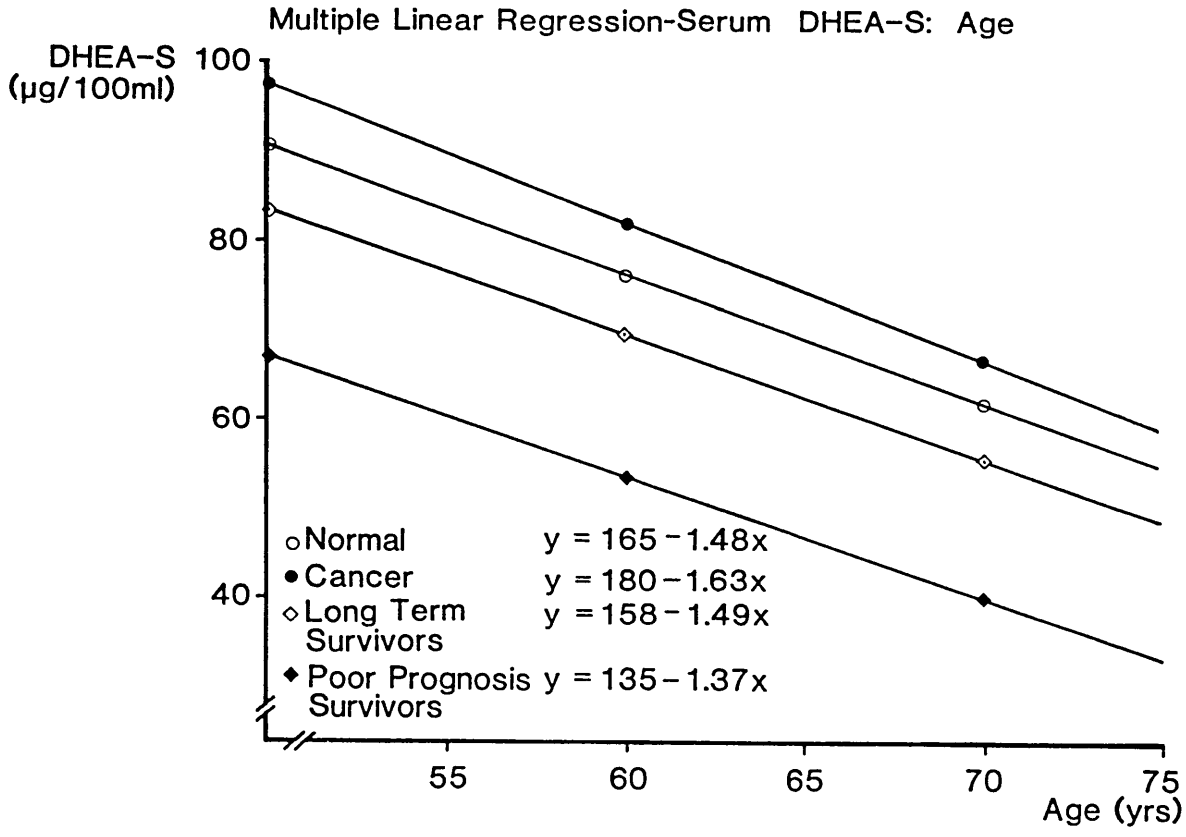
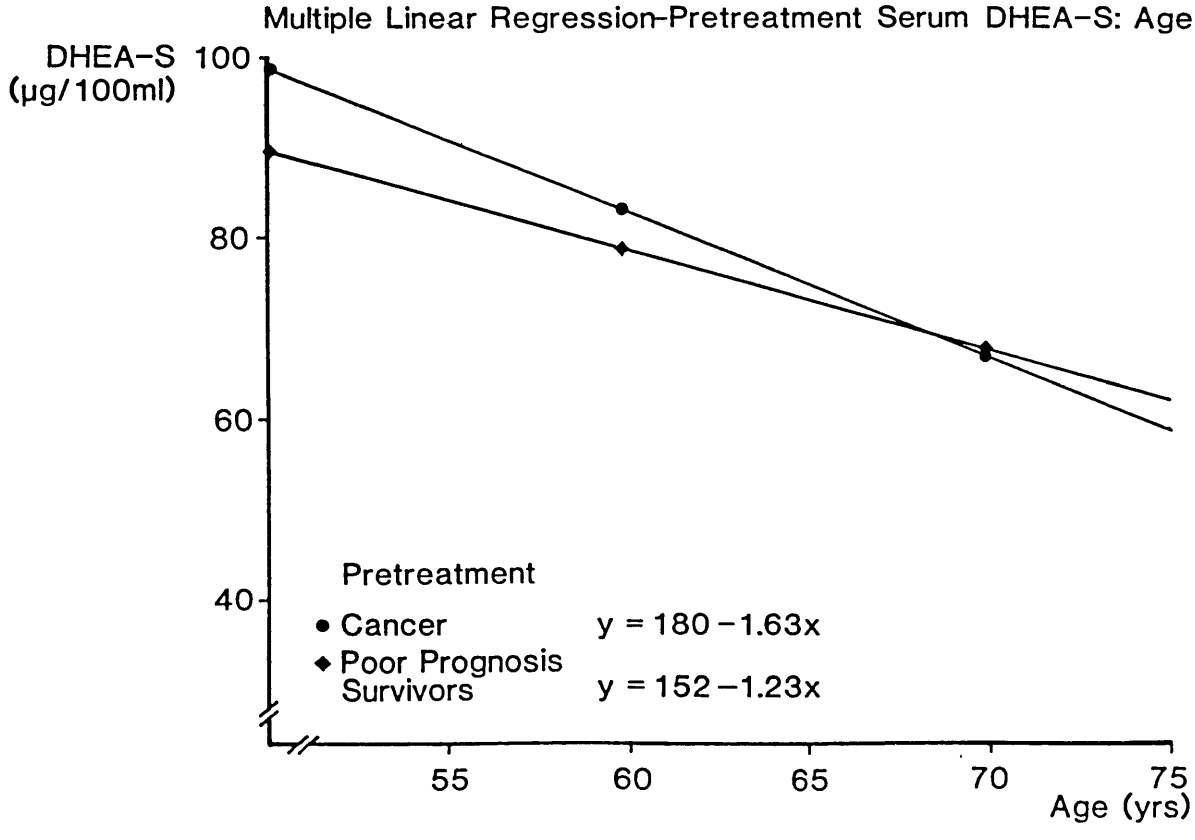


Figure 7.6



5. Serum DHEA-S: tumour stage

A linear regression analysis was performed on serum DHEA-S and tumour size, tumour TNM stage, histological grade, and fixation. There was no correlation between serum DHEA-S and these factors for any of the groups under study.

6. Correlation:- serum DHEA-S: other hormones assayed

Serum DHEA-S was significantly correlated to serum levels of the other androgens studied [see Table 7.5]. In the long term survivors, serum DHEA-S was also significantly correlated to serum free Thyroxine ($r = 0.256$, $p < 0.01$), but this correlation was not present in normal women, or the breast cancer controls.

There was no correlation, in any of the cohorts studied, between serum DHEA-S and oestrogens, SHBG binding capacity, or serum prolactin.

Cohort	serum DHEA r	serum DHEA p	serum Adiol. r	serum Adiol. p
Poor prognosis survivors (survival)	0.125	N.S.	0.332	N.S.
Breast cancer controls	0.661	0.001	0.662	0.001
Normal women controls	0.615	0.001	0.488	0.001

r = Pearson's correlation coefficient

Table 7.5 Correlation:- serum DHEA-S and other androgens

7. Life table analysis: serum DHEA-S in breast cancer controls

A life table analysis was performed for pretreatment DHEA-S in the breast cancer control group using clinical data supplied by the Imperial Cancer Research Fund and Guy's Hospital [see Fig. 7.7].

There was no correlation between pretreatment serum DHEA-S and clinical recurrence over the 100 months of follow up.

Figure 7.7

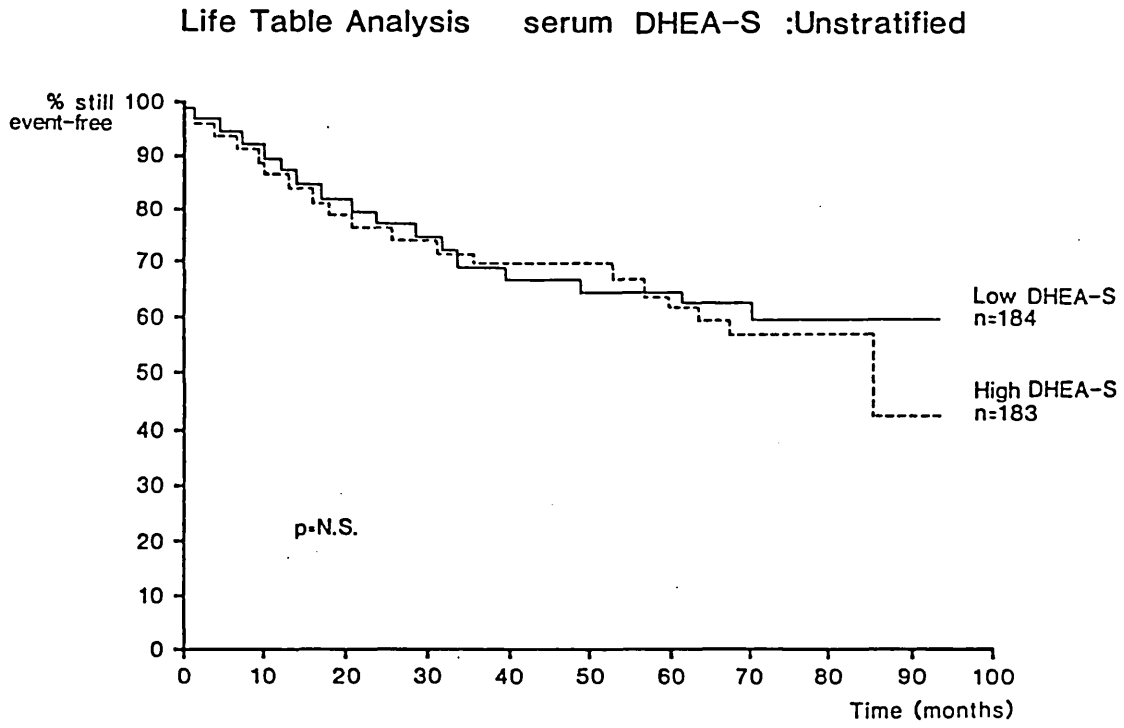
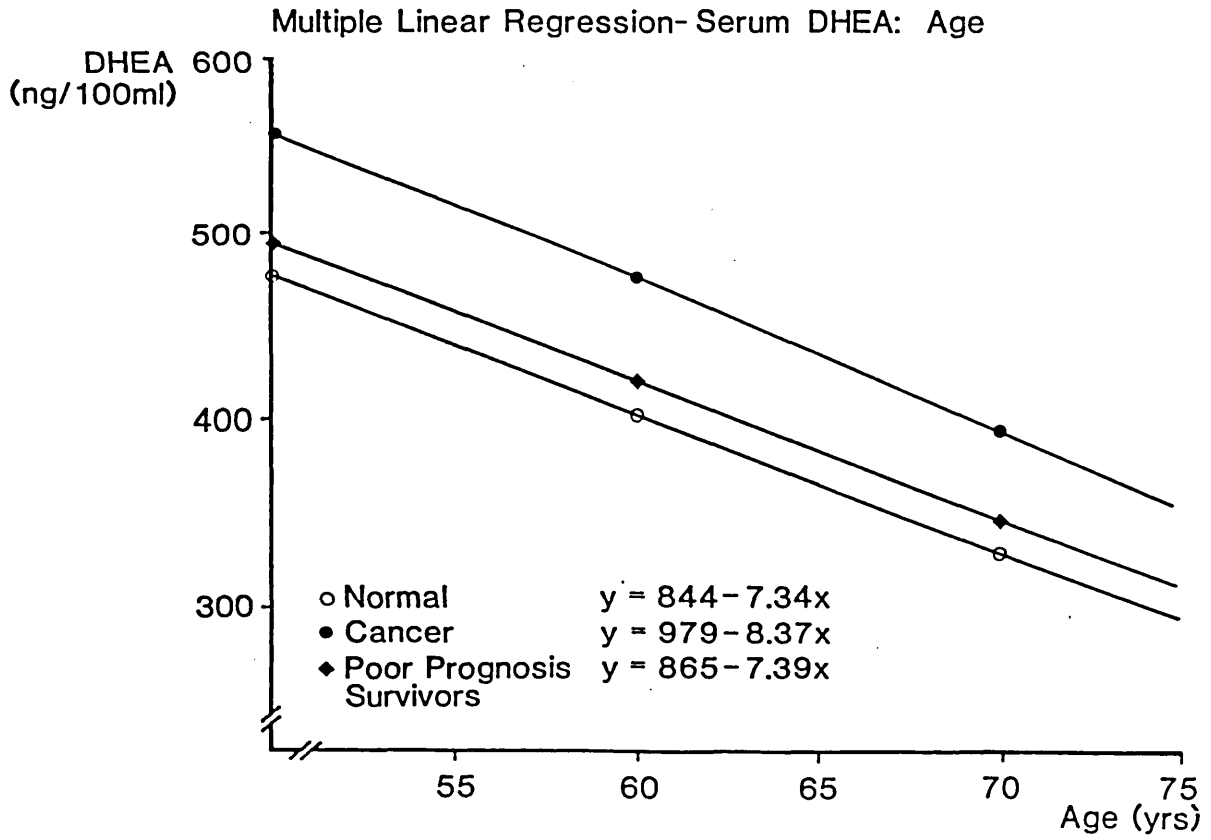


Figure 7.8



7.10.2 Serum Dehydroepiandrosterone (DHEA)

1. Quality Control

Duplicate samples and quality controls were included in each assay batch to ensure accuracy. The within-batch coefficient of variation was 8.5%, and the inter-batch coefficient of variation was 11.2%.

2. Normal values

Serum DHEA values in the normal women control group (mean ± S.D. = 3.9 ng/ml ± 2.5) were within the normal range for postmenopausal women quoted in other studies [see Table 7.6].

Author	serum DHEA mean ± S.D. (ng/ml)
Thomas et al (1976)	5.4 ± 1.6
Vermeulen (1976)	1.97 ± 0.43
Meldrum et al (1981)	2.24
Moore (1984)	4.01 ± 2.47

Table 7.6 Serum DHEA in normal postmenopausal women

3. Serum DHEA: age, height, weight

There was no correlation between serum DHEA and height, weight, ideal body weight, or Quetelet's Index, but serum DHEA is moderately correlated to age (poor prognosis survivors (at survival), $r = -0.151$, $p = N.S.$; poor prognosis survivors (pretreatment), $r = -0.015$, $p = N.S.$; breast cancer controls, $r = -0.294$, $p < 0.01$; normal postmenopausal women, $r = -0.217$, $p = 0.05$).

4. Serum DHEA: poor prognosis survivors: controls

Serum DHEA was analysed both by multiple linear regression on age, and by a standard non-parametric test (Mann Whitney).

A linear regression analysis of serum DHEA against age on the poor prognosis survivors (at survival) and both the breast cancer, and normal women control groups is shown in Figure 7.8. There was no statistically significant difference in the slopes between the three cohorts, nor between the elevation of the slopes, using an analysis of variance.

Comparison of the four cohorts by a non-parametric test also demonstrates no significant difference in mean serum DHEA (poor prognosis survivors (at survival), mean ± S.D. = 4.28 ng/ml ± 2.34; poor prognosis survivors (pretreatment),

mean ± S.D. = 4.73 ng/ml ± 3.23; breast cancer controls, mean ± S.D. = 5.33 ng/ml ± 3.4; normal women controls,mean ± S.D. = 3.94 ng/ml ± 2.56).

5. Serum DHEA: tumour stage

A linear regression analysis was performed on serum DHEA and tumour size, tumour TNM stage, histological grade, and fixation. There was no correlation between serum DHEA and these variables in either the poor prognosis survivors, or breast cancer control group.

6. Correlation: serum DHEA: other hormones assayed

Serum DHEA was significantly correlated to serum DHEA-S and serum Adiol. [see section 7.10.1, Table 7.7].

Cohort	Correlation r	p
Poor prognosis survivors (at survival)	0.468	$p < 0.05$
Breast cancer controls	0.769	$p < 0.001$
Normal women controls	0.398	$p < 0.001$

r = Pearson's correlation coefficient

Table 7.7 Correlation: Serum DHEA: Serum Adiol.

There was no correlation between serum DHEA and serum free thyroxine, oestrogens, SHBG binding capacity, or prolactin.

7. Life table analysis: serum DHEA in breast cancer controls

A life table analysis was performed for pretreatment serum DHEA and clinical recurrence, in the breast cancer control group, using clinical data made available by ICRF and Guy's Hospital. There was no correlation between pretreatment serum DHEA and clinical recurrence over the 100 month period of follow-up.

7.10.3 Serum Androstenediol (Adiol.)

1. Quality Control

Duplicate samples and quality controls were included in each assay batch to ensure accuracy. The within-batch coefficient of variation was 6.5%, and the between-batch coefficient of variation was 9.7%.

2. Normal values

Serum Adiol. values in the normal postmenopausal women in this study were within the range found in previous studies [see Table 7.8].

Author	serum Adiol. mean ± S.D. (ng/ml)
Moore (1979)	0.40 ± 0.24
Adams et al (1981)	0.33
Bird et al (1982)	0.94 ± 0.41

Table 7.8 Serum Adiol. in normal postmenopausal women

3. Serum Adiol.: age, height, weight

There was no correlation between serum Adiol. and height, weight, ideal body weight, or Quetelet's Index, but serum Adiol. was moderately correlated to age (poor prognosis survivors (at survival), $r = -0.079$, $p = N.S.$; poor prognosis survivors (pretreatment), $r = -0.216$, $p = N.S.$; breast cancer controls, $r = -0.262$, $p < 0.01$; normal postmenopausal women, $r = -0.197$, $p < 0.05$).

4. Serum Adiol.:- poor prognosis survivors: controls

As there was a moderate correlation between serum Adiol. and age in the normal women and breast cancer control groups, these were analysed by linear regression on age [see Fig. 7.9]. There was no significant difference in either the slopes or the difference in elevation of the slopes using an analysis of variance.

Figure 7.9

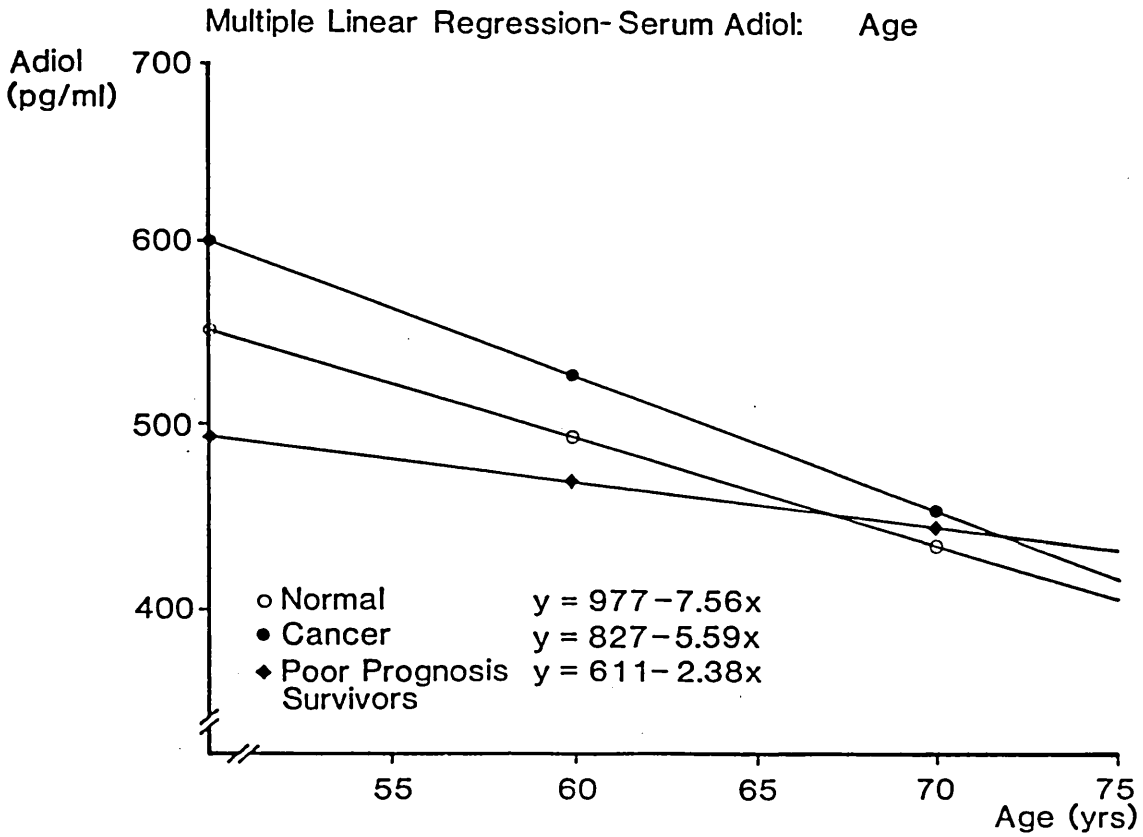
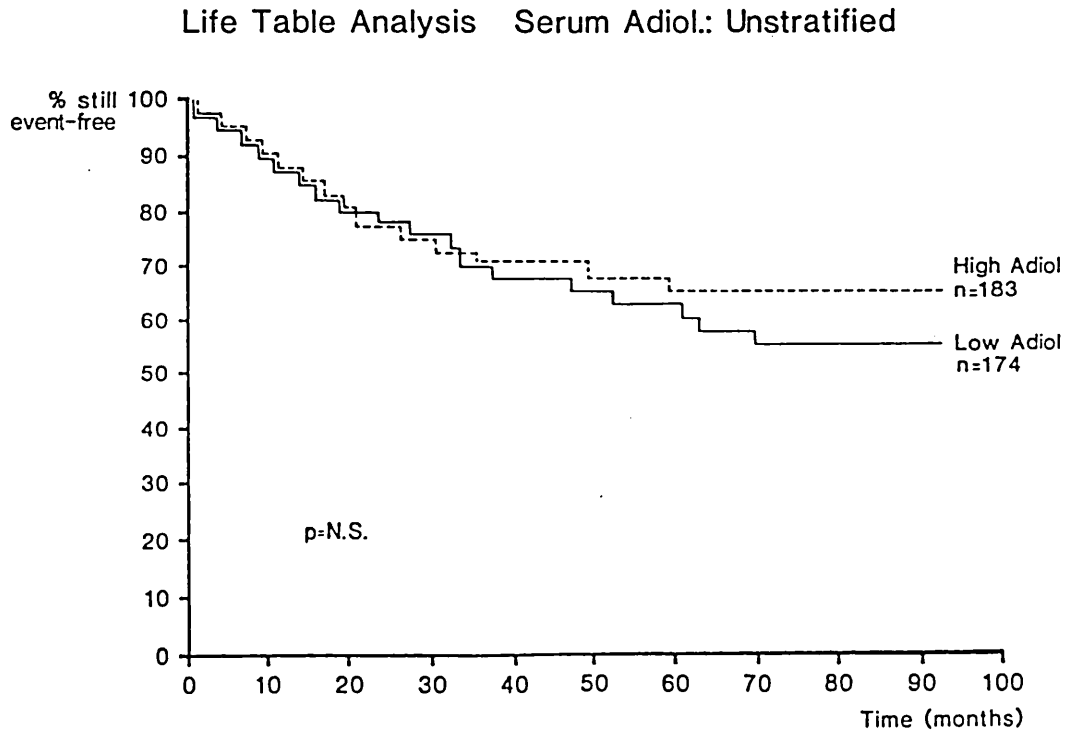


Figure 7.10



The cohorts were also analysed by a non-parametric test (Mann Whitney). There was no statistically significant difference between any of the four cohorts studied (poor prognosis survivors (at survival), mean ± S.D. = 0.46 ng/ml ± 0.25; poor prognosis survivors (pretreatment), mean ± S.D. = 0.51 ng/ml ± 0.32; breast cancer control group, mean ± S.D. = 0.575 ng/ml ± 0.34; normal women control group,mean ± S.D. = 0.48 ng/ml ± 0.27).

5. Serum Adiol.: tumour stage

A linear regression analysis of serum Adiol. in the poor prognosis survivors (at time of survival) and breast cancer control group, against tumour size, fixation, histological grade, and tumour TNM stage demonstrated no significant correlation.

6. Correlation:- serum Adiol.: other hormones assayed

Serum Adiol. was significantly correlated to both serum DHEA-S and serum DHEA [see sections 7.11.1 and 7.11.2].

There was no correlation between serum Adiol. and serum thyroxine, oestrogens, SHBG binding capacity, or prolactin.

7. Life table analysis:- serum Adiol. in breast cancer controls

A life table analysis was performed for pretreatment serum

Adiol. in the breast cancer control group [see Fig. 7.10]. Patients with elevated serum Adiol. had marginally improved disease free survival compared to patients with serum Adiol. less than the median serum hormone level, but this did not reach statistical significance.

7.11 Conclusions

7.11.1 Androgens and breast cancer

This study did not reveal any significant differences in serum androgens in postmenopausal patients with early breast cancer when compared to age matched normal women. There is disagreement between early studies, some of which reported decreased circulating androgens in women with breast cancer, and these discrepancies are most likely to have arisen from the wide range of analytical methods used. Despite this, there is however good comparability between the normal ranges for serum androgens assayed in this study, and those of previous authors. A further possible explanation therefore, is the recent observation that levels of serum DHEA may be up to 30% lower in the evening, than in the morning (Moore 1984). In the current study early breast cancer patients were bled in the morning whereas long term survivors and normal women were bled in the afternoon. The time of blood sampling is not quoted in previous studies and is unlikely to have been controlled. Thirdly, the levels of the serum androgens assayed in this study fell sharply with increasing age, and differences present in premenopausal women may be lessened, or absent, in postmenopausal women.

7.11.2 Serum DHEA-S and survival in breast cancer

The cohort of poor prognosis survivors were studied as they

represented patients in whom conventional clinical prognostic factors predicted a rapid recurrence of their disease. But the patients chosen for study within this group all had unexpectedly long survival, and it was postulated that this might be due to the action of a host related factor such as endocrine status. Previous studies on urinary androgens had demonstrated that patients with low androgen excretion were more likely to have early recurrence [see Section 7.5], and it was therefore anticipated that if androgenic status was an important factor in relation to recurrence, these patients with unexpectedly long survival might demonstrate normal, or even elevated levels of serum androgens. In fact this study showed poor prognosis survivors to have significantly reduced plasma DHEA-S (at the time of survival) compared to age matched normal women. There are several possible explanations for this finding.

Firstly, one cannot assume that low urinary androgen excretion is associated with low circulating androgens and vice-versa. Androgens are also excreted in the faeces, and increased faecal steriod excretion has been reported in women with breast cancer (Papatestas et al 1981). Secondly, serum androgens are reduced following surgery, but although this may persist for several months, it seems unlikely that this is the explanation in patients 2 to 6 years following surgery (Thijssen et al 1975, Wang et al. 1974). A further explanation for the low serum DHEA-S observed in the poor prognosis survivors is that this group, as well as representing a cohort of patients with

unexpectedly long survival, is nevertheless also a cohort of patients at very high risk of clinical recurrence. Low serum DHEA-S has been observed in several reports on women at high familial risk, as well as in women prior to the onset of clinical disease [see Section 7.3]. In this case, the observation supports the theory that low circulating androgens are associated with increased risk of recurrence, but does not support the initial hypothesis that the unexpectedly long survival in this group was related to a favourable androgenic status.

Significantly however, low serum DHEA-S was also observed in the long term survivors, when compared to either age matched patients with early breast cancer, or normal women. A similar argument can be followed to explain this unexpected finding. It seems unlikely from previous studies that a low circulating androgenic status is favourable to survival, but this cohort also is still at a very high risk of recurrence of breast cancer compared to a normal population. The low circulating androgens may therefore be more a reflection of high risk than the converse hypothesis, originally proposed, that long term survival is related to normal (i.e. favourable) serum levels of androgens.

If low serum DHEA-S is associated with high risk of recurrence, then a life table analysis of pretreatment serum DHEA-S in the breast cancer control group may have demonstrated a significant difference in mortality between

high and low serum hormone values. The study showed however, no survival advantage for patients with elevated pretreatment serum DHEA-S. Levels of serum androgens fall significantly after surgery, remaining depressed for an undefined period (Wang et al 1974). Pretreatment serum hormone levels may not therefore be an indication of serum levels six months or more following surgery. This is supported in this study by the finding that although the poor prognosis cohort had significantly depressed levels of serum DHEA-S at the time of survival, there was no significant difference between the same cohort's pretreatment serum hormone levels and those of the remainder of the early breast cancer controls.

7.11.3 Serum DHEA and Adiol. and survival in breast cancer

Although the study demonstrated a very considerable correlation between levels of circulating androgens, there was no evidence that either survival or recurrence was related to serum DHEA. In part this is probably explained by the fact that levels of DHEA in the serum are several hundred-fold lower than DHEA-S, the principal postmenopausal androgen, with the exception of testosterone. There was a small, albeit statistically insignificant, survival advantage in elevated pretreatment serum Adiol. seen in the life table analysis of the breast cancer control group. This is of interest, as it has been proposed that Adiol. binds to oestrogen receptors and can act as a weak oestrogen (van Doorn et al 1981).

7.11.4 The Moolgavkar model

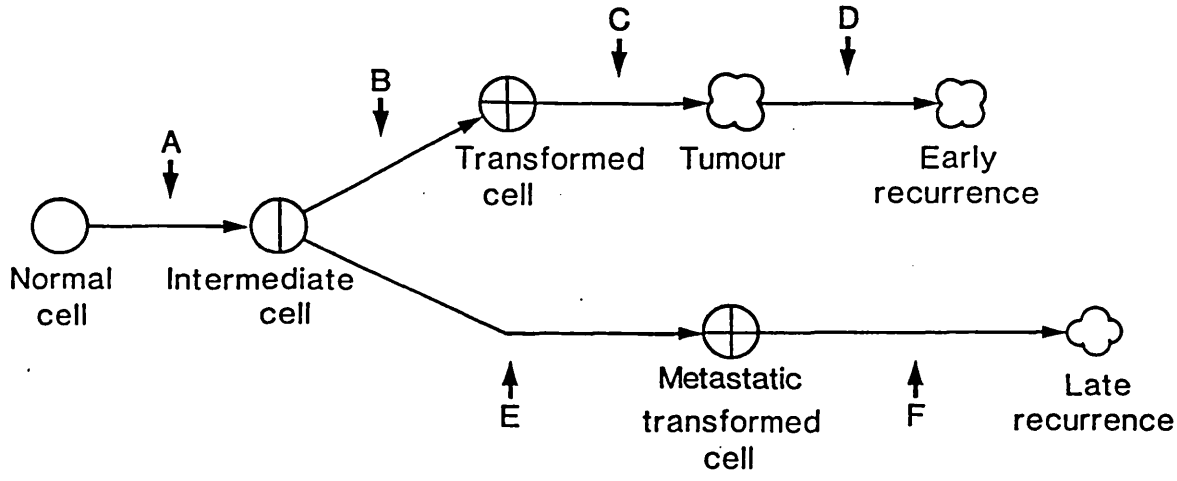
A mathematical model has been proposed, in order to facilitate an understanding of the role of hormones in the aetiology and clinical course of breast cancer [see Fig.7.11].

There is no direct evidence, either epidemiological or biochemical, to suggest that androgenic mechanisms act in the early development of breast cancer [see Fig.7.11(A)], although androgens may be capable of inducing breast growth and differentiation, as well as inhibiting it. The finding of low urinary androgen excretion in women at familial risk of breast cancer, and as long as nine years prior to the onset of clinical development of the disease, suggests that this may be related to increased transformation, perhaps via the action of androgens on 17β hydroxydehydrogenase [Fig.7.11(B) and (C)].

Low urinary excretion of androgens has been reported to be associated with increased risk of clinical recurrence and in the present study, if the poor prognosis survivors are interpreted as being a very high risk group, rather than a group with unexpectedly long survival, then there is further evidence that androgenic status may be related to recurrence although the mechanism remains uncertain [Fig.7.11(D)].

Figure 7.11

The Modified Moolgavkar Model



Low urinary excretion of androgens is associated with a poor response to endocrine therapy, but there is no experimental evidence linking urinary or serum androgens to tumour receptor status (Bulbrook et al 1960). It is more likely that the androgens are acting indirectly, either by competitive inhibition of steroid dehydrogenase increasing circulating oestradiol, or by androgens such as androstenediol binding with oestrogen receptors.

The Moolgavkar model was further modified in order to explain how the incidence of very late recurrence might be related to hormonal status. It was proposed that not only tumour cells metastasised to give rise to early recurrence, but perhaps intermediate cells could also metastasise, and continue to be acted upon by endocrine influences to give rise to late transformation and subsequent recurrent disease [Fig.7.11(E), (F)]. The finding of significantly low levels of serum DHEA-S in long term survivors suggests that an unfavourable androgenic environment continues to exist in these patients, in striking similarity to women at high familial risk and in women prior to the development of clinical disease.

This study of androgens in survivors of breast cancer does not support the concept that unexpectedly long survival is related to a favourable host androgenic status. It does however support the theory that continuing recurrent disease may be related to reduced levels of circulating androgens.

CHAPTER 8

PROLACTIN

Part I

Literature review

8.1 Introduction

8.1.1 Structure

With the similarity in structure between prolactin, human placental lactogen and growth hormone, the former proved difficult to measure until radio-immunoassays were available. All contain 190 amino acids with a molecular weight of around 22,000. The linear amino acid sequence of prolactin has been determined and about 20% of the amino acid sequence is the same as that of growth hormone.

8.1.2 Secretion and transport

Prolactin is secreted by acidophilic granules in the pituitary under the inhibitory control of the hypothalamus mediated by a prolactin inhibiting factor (PIF), and dopamine. A prolactin releasing factor has been postulated but thyrotrophin releasing hormone (TRH) also stimulates prolactin secretion. Oestradiol and thyroxine may exert a similar influence.

It seems probable that prolactin is transported unbound in the plasma as a free molecule with a relatively short half life of about 20 - 30 minutes.

8.1.3 Functions

Prolactin receptors have been reported in the breast, ovary,

testes, seminal vesicles, uterus, kidney, adrenals, liver and cerebral cortex, reflecting the very wide sphere of metabolic action. In the breast, prolactin is required for normal epithelial maturation, and also for the development of the breast during pregnancy. It also acts, with progesterone and placental lactogen, to control lactalbumin and lactose synthesis during lactation.

8.1.4 Aim of study

In the first part of this chapter the literature concerning prolactin and breast cancer is reviewed. In the second part, the results of a study on serum prolactin and survival in breast cancer are presented.

8.2 Prolactin and breast cancer

8.2.1 Animal models

The role of prolactin (or pituitary mammotrophin as it was then known) in experimental mammary carcinogenesis was first described by Furth in both mice and rats, although Lyons had previously noted that induction of oestrogen dependent tumours was prevented by hypophysectomy (Furth et al 1973, Lyons 1958).

Even in the rat model, in which much research has been performed, it is difficult to elucidate the precise role of prolactin. Prolactin is able to stimulate growth of new tumours and regrowth of tumours that have regressed following oestrogen deprivation by endocrine ablation, but conversely, high levels of oestrogen can cause tumour growth suppression. The inter-relationship of oestrogens and prolactin is complex. Prolactin is probably required in the initiation stage for the cells to be susceptible to oestrogens, but oestrogens correspondingly induce prolactin secretion also.

8.2.2 Human mammary cancer

The important, albeit illdefined, role of prolactin in animal models, notably the rat, stimulated research into its role in human breast cancer.

Specific receptor sites for prolactin have been discovered both in normal breast tissue and in breast tumours (Frantz et al 1974, Turkington et al 1973). Although about 20% of breast tumours may contain specific prolactin receptors the binding is low and its relevance to hormone responsiveness uncertain. Some tumours respond only to very high levels of circulating prolactin, and others only to low levels (Montague 1977). The relationship to oestrogen receptors (ER) might be considered of fundamental importance and though prolactin receptors have been observed to induce ER in the MCF 7 cell line, the precise relationship is not understood (Shafie and Brooks 1977).

8.3 Chronobiology of prolactin secretion

If the problems in understanding the role of prolactin in human breast cancer commenced with the unfortunate prior discovery of its potential importance in rodent mammary cancer, then they were compounded many times over by the more recent discoveries of its complex chronobiology.

8.3.1 Physiological factors

Human prolactin secretion follows a nycthemeral rhythm with peaks occurring at about 5.00 am and again, but lower, at about 6.00 pm. Approximately 50% of prolactin secretion occurs between midnight and 7.00 am. This rhythm appears to be governed by periods of rapid eye movement (REM) and non-REM sleep, with peak secretion occurring during the latter. As diet has been shown to modify REM sleep, one study compared prolactin secretion on a normal diet with that on a low carbohydrate-fat diet, and indeed mean 24 hr. prolactin was reduced, the effect occurring only in nocturnal secretion and not on the early evening peak (Hill and Wynder 1976). Prolactin secretion is increased with stress, including exercise, coitus and surgery (Stearns et al 1973, Rubens et al 1977).

Although the plasma half life of prolactin is very short, only 20 - 30 minutes, the half life in breast tissue is about 50 hours, and thus the effect of small physiological peaks in secretion may nevertheless be of importance.

In premenopausal women, prolactin secretion is also related to the menstrual cycle showing a slight increase in the luteal phase which may be related to the cyclical changes in oestradiol (McNeilly and Chard 1974, Franchimont et al 1976).

Plasma prolactin is also highly age dependent, rising to adult levels at puberty but falling gradually between ages 35 to about 65, before rising again in the eighth decade [see Fig. 8.1]. This relationship fits a cubic expression and shows a remarkable similarity both to oestradiol and androgen secretion, suggesting interdependence between these hormones.

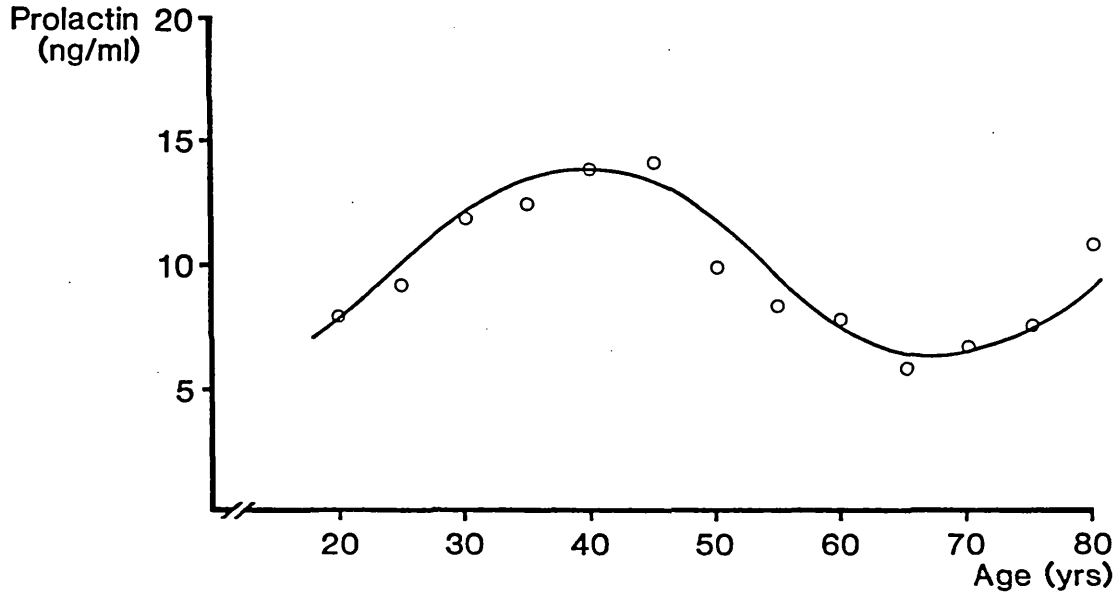
During pregnancy, levels of prolactin are significantly elevated, falling to approximately previous levels at about two weeks post-partum. Lactation however, produces a sharp rise varying from ten to one hundred fold.

In premenopausal women there is a significant decrease in plasma prolactin levels with increasing parity; this relationship being interdependent of age, and most marked within two years of birth of the last child. No relationship between prolactin and parity is seen in postmenopausal women (Kwa et al 1981).

There is no significant relationship between prolactin and height and weight.

Figure 8.1

Plasma Prolactin and Age (after Bulbrook et al.1981)



8.3.2 Pharmacological factors

A large number of non-physiological factors can also affect prolactin secretion, the most important of which are pharmacological.

Prolactin secretion is increased in women on the contraceptive pill, receiving oestrogen therapy, or who have an intra uterine device present (Robyn et al 1973, Fortuny et al 1973).

Hyperprolactinaemia is also associated with phenothiazine tranquilisers, amphetamines, anti-hypertensives such as reserpine and methyldopa, and tricyclic antidepressants. Despite a huge number of studies on breast cancer incidence in women receiving pharmacological agents associated with hyperprolactinaemia, there is no evidence to suggest an increased risk of breast cancer (for references see Bulbrook and Wang 1979).

Hypoprolactinaemia is associated with Bromocryptine treatment, a dopamine agonist, and often, but not invariably, with hypophysectomy. Stalk section has been shown to elevate rather than depress prolactin secretion (Turkington et al 1971).

8.4 Serum prolactin in women 'at risk'

Several studies have been performed with the object of correlating serum prolactin with known risk factors for breast cancer. These studies can be conveniently divided into those which have studied daughters of women with breast cancer, or in whom a very strong family history exists, and inter-population studies between geographical areas of high and low risk for breast cancer.

8.4.1 Familial risk

Henderson compared endocrine function in a group of adolescent girls whose mothers had breast cancer with girls having no family history. In the controls, prolactin levels were low, relative to oestradiol, in the follicular phase of the menstrual cycle, but the daughters of patients had a marginally significant elevation of oestradiol and prolactin (Henderson et al 1975) [see Table 8.1]. Unfortunately, when the study was repeated on the same cases, even the marginal significance was absent (Pike et al 1977).

A series of important studies has been carried out by Kwa, and colleagues at the Imperial Cancer Research Fund. In the first study, prolactin was assayed in 64 members of 9 families with a strong history of breast cancer. Values for the 64 kindred were higher than in the controls, or in women with breast cancer (Kwa et al 1974).

Author	Plasma prolactin
Henderson et al (1975)	increased in daughters of patients (accompanying increase in follicular oestradiol).
Pike et al (1977)	no significant difference (on same patients as above study).
Kwa et al (1974)	significantly elevated
Kwa et al (1976)	abnormal evening peak in luteal phase.
Kwa and Wang (1977)	above observation confirmed
Kwa et al (1978)	abnormal evening peak also associated with nulliparity and postmenopausal obesity.
Kwa et al (1981)	elevated in postmenopausal precancers.
Fishman et al (1978)	no significant difference
Boffard et al (1981)	no significant difference

Table 8.1 Plasma prolactin in women at familial risk of breast cancer

In the second study plasma prolactin was measured in 369 women who had participated in a screening project on the island of Guernsey. On this occasion no significant differences in mean plasma levels were observed in women at varying degrees of familial risk, but it was noted that in both controls and women 'at risk' there was an early evening peak in prolactin secretion. In the daughters of patients a more marked evening peak was observed in the luteal phase of the menstrual cycle (Kwa et al 1976).

A subsequent, and larger study, confirmed this finding, but a later investigation found that this abnormality was also associated with nulliparity, and postmenopausal obesity (Kwa and Wang 1977, Kwa et al 1978).

A further, and very large, prospective study was then conducted using 5100 women in the Guernsey screening programme (Kwa et al 1981). Breast cancer was subsequently diagnosed in 47 of these women at a median time of 5 years following blood collection. No significant difference in mean plasma prolactin was found between premenopausal 'pre-cancer' patients and controls, but the 'pre-cancer' cases exhibited a significant positive correlation to age and it was suggested that this might represent a marker for late menopause, a known risk factor. The postmenopausal 'pre-cancers' however, had significantly elevated plasma prolactin compared to controls.

Fishman studied hormone profiles in 30 young women with strong family histories of breast cancer and well matched controls (Fishman et al 1978). No statistically significant difference was found in plasma prolactin despite very careful analysis of sampling during the menstrual cycle. Blood samples were collected in the morning though, not the evening.

Boffard, similarly found no difference in plasma prolactin in a study of 52 adolescent girls with a family history and normal controls. In this study, however, it proved to be much more difficult to specify the phase of the menstrual cycle, although blood sampling was performed in the early evening (Boffard et al 1981).

8.4.2 Geographical studies

The incidence of breast cancer in British women is approximately eight times greater than that in Japanese women and epidemiological inter-population studies have frequently contrasted Japanese and Caucasian women. Kumaoka analysed plasma prolactin levels in adolescents, premenopausal, perimenopausal and postmenopausal Japanese and English women and could find no significant differences (Kumaoka et al 1976). Hill claimed lower prolactin levels in both Japanese and Bantu women compared with Caucasians but the significance was not impressive (Hill et al 1976). Lastly a pilot study comparing Japanese

and English women with an intermediate group, Hawaiian Japanese, also found no statistically significant differences (Hayward et al 1978).

8.5 Plasma prolactin in women with breast cancer

There are very large numbers of studies comparing plasma levels of prolactin in women with breast cancer and normal, healthy, controls; and there are very few conclusions to be drawn from them [See Table 8.2].

It is apparent from the preceding sections that normal prolactin secretion follows a complex series of rhythms that are themselves modified by physiological and pharmacological factors. Surgery is itself a potent cause of stress and elevated levels of prolactin are found up to 10 days or more post operatively (Rubens et al 1977). Any study designed to analyse subtle changes in prolactin secretion without accounting for these variables is likely to reach erroneous conclusions. In particular, studies in which blood sampling was performed on the day of operation and then compared to a healthy group of control cases should be censored.

There remain, fortunately, several studies in which care was taken in case selection and sampling time. Cole compared prolactin secretion in 11 premenopausal patients following mastectomy, in serial samples throughout the menstrual cycle, with normal controls and patients with breast cancer (Cole et al 1977). In all groups, luteal phase prolactin was higher than follicular phase, but the breast cancer patients exhibited a mid-cycle ovulatory peak, and the decline in secretion during the follicular phase was less

Author	Plasma prolactin
Dicky and Minton (1972)	no significant difference
Murray et al (1972)	elevated in advanced cancer
Boyns et al (1973)	no significant difference
Franks et al (1974)	no significant difference
Rolandi et al (1974)	elevated in advanced cancer
Kwa et al (1974)	no significant difference
Wilson et al (1974)	no significant difference
Sheth et al (1975)	decreased in early cancer
Safarty et al (1976)	elevated in early cancer
McFadyen (1976)	no significant difference. (matched for age, menopausal status and parity).
Cole et al (1977)	elevated in mid-cycle (see text)
Malarkey et al (1977)	decreased in postmenopausal (prior to surgery) elevated in premenopausal (prior to surgery)
Willis et al (1977)	elevated (in 44%) in recurrent disease
Tarquini et al (1978)	elevated in premenopausal early cancer

Table 8.2 Plasma prolactin in women with breast cancer

marked. These rather subtle changes did reach statistical significance.

Malarkey determined hourly plasma prolactin in pre- and postmenopausal patients prior to surgery. In 5 of the premenopausal patients the nocturnal levels of prolactin were elevated, a finding similar to that of Tarquini (Malarkey et al 1977, Tarquini et al 1978). Corresponding nocturnal levels of prolactin in postmenopausal patients were however decreased. If stress were the primary cause of these findings then it might be assumed that both pre- and post-menopausal subjects would react similarly.

8.6 The hypothalamic - pituitary - thyroid axis

Thyrotrophin releasing hormone (TRH) stimulates prolactin secretion and thus hyperprolactinaemia may accompany hypothyroidism. Following the many studies claiming to show an association between hypothyroidism and breast cancer risk [see Chapter 9], it was appropriate that studies should be designed to investigate any abnormality in prolactin secretion which might be mediated through the hypothalamic - pituitary - thyroid axis.

Mittra carried out TRH stimulation tests on three groups of 50 women each, with early breast cancer, advanced breast cancer, and normal controls, measuring basal levels of plasma prolactin and at intervals of 20 and 60 minutes following stimulation. Mean basal levels of prolactin and the response to stimulation was similar in all three groups, although patients with advanced cancer had marginally more elevated levels at 20 minutes following stimulation ($p = 0.05$) (Mittra and Hayward 1974).

Aldinger performed a similar study on 148 patients including both early and metastatic disease, and 45 control subjects. The conclusions were similar to the above study, although on this occasion the early breast cancers had a marginally more significant elevation ($p = 0.01$) (Aldinger et al 1978).

Both groups also assayed TSH, following TRH stimulation. Their findings were similar, namely that patients with

breast cancer had significantly elevated basal levels, and a significantly greater proportion of breast cancer patients exhibited an exaggerated response to TRH stimulation. (40% and 36% respectively). Ohgo, in a smaller study, observed parallel findings (Ohgo et al 1976).

Aldinger could find no correlation however between serum TSH and prolactin, and it was therefore concluded that the thyroid dysfunction was not mediated via any abnormality in prolactin secretion.

Mittra proposed that the subclinical state of hypothyroidism could alter the sensitivity of mammary epithelium to prolactin which might then act as a tumour promoter. In support of this theory is an observation from an earlier study that mammary growth induced by prolactin, in an animal model in which the pituitary was transplanted subcutaneously in the mammary gland, was suppressed by thyroxine (Meites and Kragt 1964).

The possible role of hypothyroidism in breast cancer is discussed, at length, in Chapter 9, but the studies performed to date on the hypothalamic - pituitary - thyroid axis give no indication that a disorder of prolactin secretion is primarily responsible for hypothyroidism observed in breast cancer patients.

8.7 Prolactin and breast cancer recurrence

Hypophysectomy results in regression of most hormone dependent breast cancers, or approximately 30% of unselected cases. It is probable that this response to endocrine ablation is due to reduction in circulating androgens and oestrogens, rather than any alteration in prolactin secretion. Firstly, response has not been observed to correlate with plasma prolactin levels, and secondly, stalk section increases plasma prolactin rather than depressing it, and is still associated with clinical remission (Bates et al 1976, Turkington et al 1971).

Further, albeit indirect, evidence comes from studies using drugs known to effect prolactin secretion. Bromocryptine is a dopamine agonist, and effectively depresses plasma prolactin, but has not been associated with breast cancer regression (Heuson et al 1972). In contrast, Stilboestrol in common with other oestrogens, stimulates prolactin secretion, but is associated with remission in breast cancer (Wilson et al 1974).

Plasma prolactin following TRH stimulation has been assayed in two small studies on patients with advanced cancer treated by Nolvadex. In the first study, responders showed a reduction, on treatment, in mean basal prolactin values as well as a reduction to TRH stimulation. The non-responders had only a reduction in TRH stimulated prolactin (Willis 1977).

Other studies have found no difference in either basal or stimulated prolactin between responders and non-responders either before or during treatment (McFayden et al 1979, Pannuti et al 1981).

8.8 Conclusion

The complex nycthemeral, monthly and age dependent cycles of prolactin secretion, and the pronounced effect of parity in premenopausal women might be expected to obscure all but gross deviations in plasma prolactin values that could be attributed to breast cancer. Nevertheless, it is apparent from the studies that have controlled the physiological variables, that subtle changes may be attributed to breast cancer.

It seems probable that these changes are not primary defects in pituitary function but a reflection of other alterations in the hormonal environment, possibly due to oestrogens. Against this proposition however it should be stated that although normal age dependent prolactin secretion follows very closely the pattern of oestradiol secretion, and oestrogens are known to stimulate prolactin secretion, no correlation has been observed between plasma oestradiol and plasma prolactin.

Part II

Endocrine study of long term survival

8.9 Introduction

The second part of this chapter presents the results for serum prolactin in the endocrine study of long term survival.

8.9.1 Patients

As in the previous chapter, four groups of patients were studied:

1. Long term survivors
2. Women with early breast cancer
3. Normal women
4. Poor prognosis survivors

The poor prognosis survivors, described more fully in the last chapter, were chosen from the larger cohort of women with early breast cancer. Either pre-treatment serum, or pre-treatment plasma prolactin assays were available for study on this group of patients who were then re-bled at the time of survival for comparison of hormone profiles.

8.9.2 Method

The method used for prolactin assay is the homologous double-antibody radioimmunoassay described by Kwa (Kwa et al 1973). Using a rat anti-human antibody calibrated

against an MRC reference preparation (71/222) a standard curve was produced. Plasma samples were assayed undiluted, and at four serial dilutions, using an automated serial dilutor, so that the points fell on the straight part of the 'S' shaped inhibition curve. The mean and standard deviation were calculated for each sample at each dilution, yielding as many estimates for the sample as there were points on the straight part of the inhibition curve. Full details of the plasma prolactin assay are given in Appendix 8.

Plasma prolactin assays for the normal women and a proportion of the breast cancer control group were performed by the Department of Clinical Endocrinology, Imperial Cancer Research Fund, using an identical method. Quality control samples and duplicates of controls were used to ensure accuracy of plasma prolactin assay in the patients, and remaining controls.

8.9.3 Results

The results of the plasma prolactin assays performed on the above patients are analysed as follows:

1. Quality control.

2. Comparison of normal values from other studies.

3. A linear regression on height, weight, Quetelet's Index, and parity.

4.(i) Comparison of hormone profile of long term survivors with age matched breast cancer and normal women control groups (Student's t test).

(ii) Comparison of pre-treatment plasma prolactin of poor prognosis survivors and breast cancer controls (Student's t test).

(iii) Comparison of plasma prolactin of poor prognosis survivors (at point of survival) with normal controls and long term survivors (Student's t test).

(iv) Comparison of poor prognosis survivors (histologically grade III), and poor prognosis survivors (pathological nodes $\geq$ 4).

5. Linear regression of plasma prolactin and pathological TNM stage, fixation, histological grade, and tumour size.

6. Correlation of plasma prolactin and other hormones assayed, in long term survivors, and breast cancer and normal women controls (Pearman's correlation coefficient).

7. Life table analysis on short term survival (ten years) of breast cancer control group using clinical data from the Breast Unit, Guy's Hospital (Log rank test).

8.10 Patients

Four groups of patients were studied:

8.10.1 Long term survivors

n = 144

mean age ± S.D. 65.4 years ± 9.0

mean survival ± S.D. 16.3 years ± 10.5 (range 10-3 years)

From a total cohort of 160, plasma prolactin was assayed in 144 postmenopausal long term survivors who had been disease-free for a period of ten years or more, and were clinically free of breast cancer at time of entry into study. No patients had received hormone therapy. Full details of these patients are given in Chapter 6.11, and Appendix 6.

8.10.2 Breast cancer control group

n = 346

mean age = 63.5 years ± 4.7

From a total cohort of 346 patients with early breast cancer, age matched controls were chosen in whom either pre-treatment plasma prolactin had been recently assayed or pre-treatment plasma was available for assay. Full details of clinical and pathological data on the entire cohort are given in Chapter 6.11 and in Appendix 6.

8.10.3 Normal women control group

n = 523

mean age ± S.D. = 63.6 years ± 5.75

From a large cohort of women screened in the Guernsey III study, age matched postmenopausal women were chosen as normal controls [See Chapter 6.11]. Plasma prolactin assay had been recently performed on these women. All were clinically and mammographically free of disease at time of entry into the study, and none had received recent hormone therapy.

8.10.4 Poor prognosis survivors

n = 31

mean age ± S.D. = 62.4 years ± 8.3

mean survival ± S.D. = 73.5 months ± 10.0

(range 49 - 94 months)

From the total cohort of postmenopausal women with early breast cancer a smaller cohort was chosen in whom, because of their poor prognosis due to histological grade or pathological lymph node status, the cumulative proportion surviving was equal to that of the long term survival group. Pre-treatment serum was available on this group of patients, all of whom were clinically free of breast cancer at time of entry into the study. A complete description of clinical and pathological data on this group is in Appendix 7.

8.11 Results

8.11.1 Quality control

Plasma prolactin was assayed on the long term survivors, poor prognosis survivors, and a proportion of the breast cancer and normal women control groups in a single assay batch. Duplicates and quality controls were included. The within-assay coefficient of variation was 12.5%.

8.11.2 Normal values

Plasma prolactin values obtained for the normal women (mean ± S.D., 9.5 ng/ml ± 5.0) in this study were similar to those of a large group of normal women studied by Bulbrook (Bulbrook et al 1981) [See Table 8.3].

Age (Years)	Mean plasma prolactin (ng/ml)
55 - 60	8.0
61 - 65	7.8
66 - 70	9.0
70 - 75	9.5
75 - 80	10.2

Table 8.3 Plasma prolactin in normal postmenopausal women

8.11.3 Plasma prolactin: age, height, weight

Plasma prolactin was analysed by 5 year age cohorts in the normal postmenopausal women. Hormone values were higher in the first and third decades following the menopause than in the second decade, a pattern also seen in the women with early breast cancer and in the long term survivors. There was no statistically significant correlation with age however [see Fig. 8.2].

There was no correlation between plasma prolactin and parity, height, weight, or Quetelet's Index in the patients or the controls.

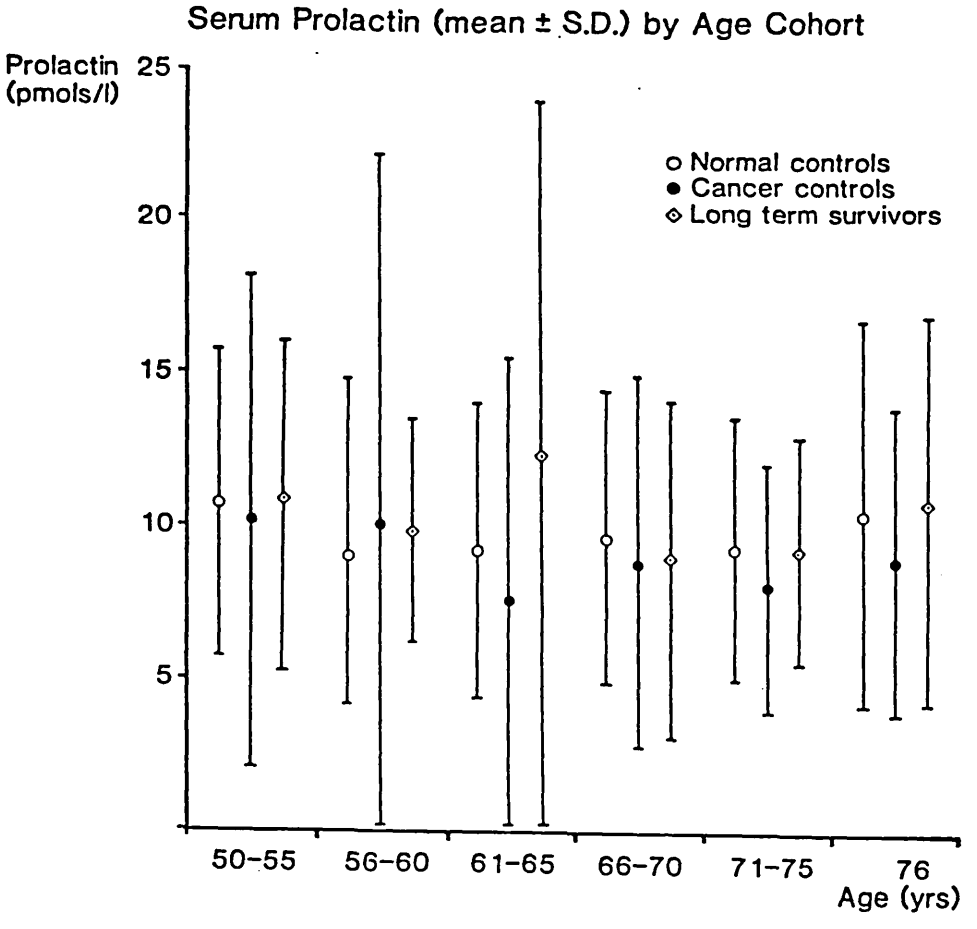
8.11.4 Plasma prolactin:- long term survivors: controls

Plasma prolactin in 141 long term survivors (mean ± S.D., 10.3 ng/ml ± 7.0) was compared to age matched controls of women with early breast cancer (mean ± S.D., 9.52 ng/ml ± 8.8) and normal women (mean ± S.D., 9.5 ng/ml ± 5.0). [See Fig. 8.2]. There was no statistically significant difference between survivors and controls, or between breast cancer and normal control groups (Student's t test).

Poor prognosis survivors: controls

The plasma prolactin at time of survival for 31 poor prognosis survivors (median 10.5 ng/ml) was compared to that of normal women (median 8.5 ng/ml) each survivor being

Figure 8.2


Serum Prolactin (mean ± S.D.) by Age Cohort
Prolactin (pmols/l)
25
20
15
10
5
o Normal controls
• Cancer controls
◇ Long term survivors
50-55
56-60
61-65
66-70
71-75
76
Age (yrs)

matched to ten controls (matched for age at time of survival). The survivors had elevated plasma prolactin compared to normal postmenopausal women ($p = 0.06$, Mann Whitney test). The pre-treatment plasma prolactin of 31 poor prognosis survivors (median 7.5 ng/ml) was compared to pre-treatment plasma prolactin of women with early breast cancer (median 7.2 ng/ml), each survivor being matched to ten controls (matched for age at time of mastectomy). The pre-treatment plasma prolactin of the poor prognosis survivors was not significantly different from that of age matched early breast cancer controls.

8.11.5 Plasma prolactin: pathological stage

A linear regression was performed on plasma prolactin and tumour and nodal TNM stage, histological grade, fixation and tumour size, for both long term survivors and the breast cancer control group. There was no association between plasma prolactin and pathological stage.

8.11.6 Plasma prolactin: other hormone assays

There was no statistically significant correlation between plasma prolactin and other hormones assayed (Free E_2, Total E_2, SHBG binding capacity, DHEA-S, Adiol, DHEA, or free T_4) in any of the cohorts studied.

8.11.7 Life table analysis: breast cancer control group

A life table analysis was performed on pre-treatment plasma prolactin in the breast cancer control group using clinical data and hormone assays from the Imperial Cancer Research Fund. The probability, p, was estimated from a two tailed test of probability for trend from the log rank test. For all patients (n=571) unstratified, there was no significant difference in disease-free survival between patients with plasma prolactin greater than the median, and those below [See Fig. 8.3]. When patients were stratified for parity, multiparous patients (n=419) showed a significant trend ($p = 0.03$) for prolonged disease-free survival associated with low plasma prolactin [See Fig. 8.4]. This was not seen in nulliparous patients.

When patients were stratified according to menopausal status, there was a significant increase in disease-free survival ($p = 0.02$) seen only in premenopausal patients (n=174) with low pre-treatment plasma prolactin [See Figs. 8.5 and 8.6].

The patients were then stratified both by parity and menopausal status. Premenopausal multiparous patients (n=174) demonstrated a significant inverse trend ($p = 0.006$) between disease-free survival and pre-treatment plasma prolactin.

Figure 8.3

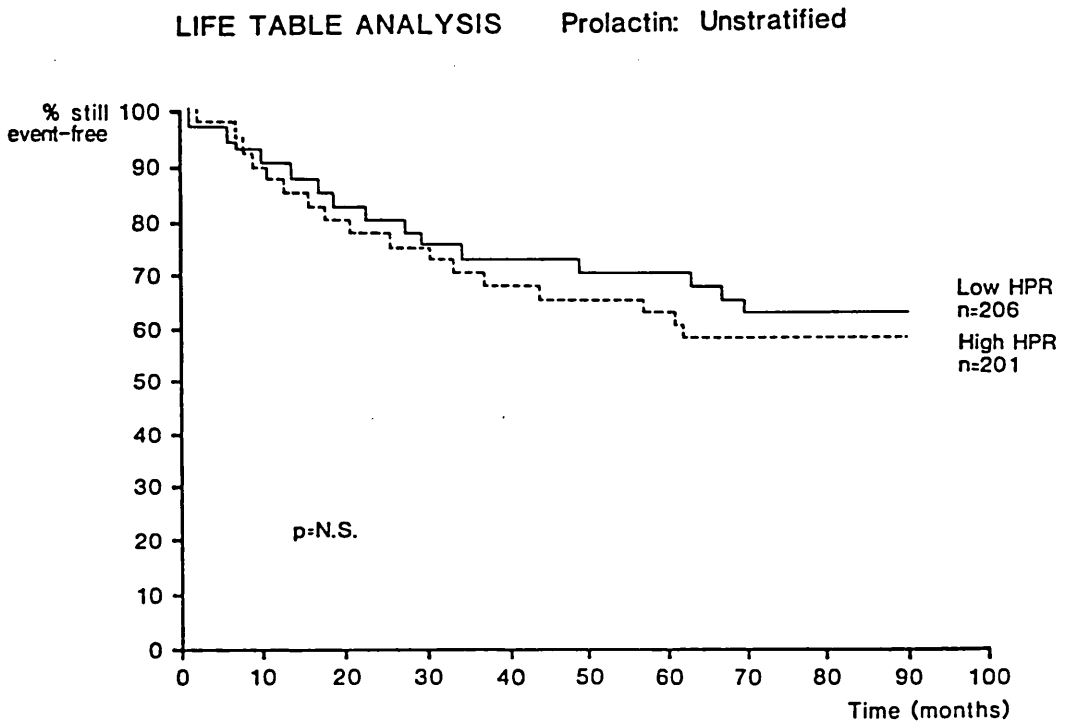


Figure 8.4

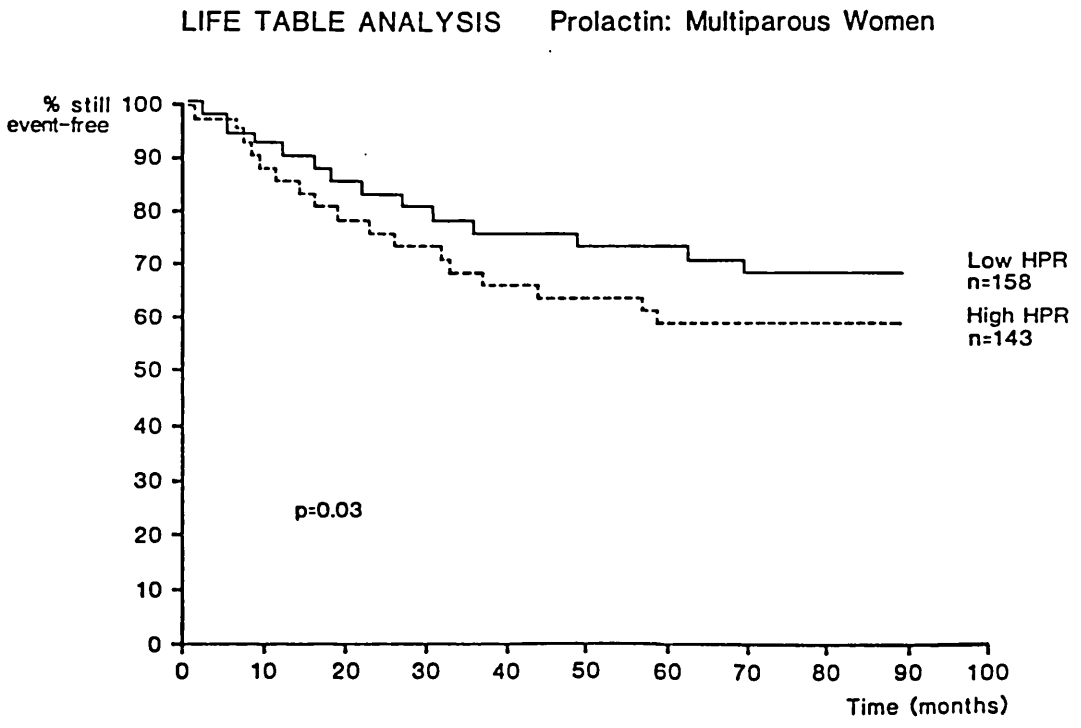


Figure 8.5

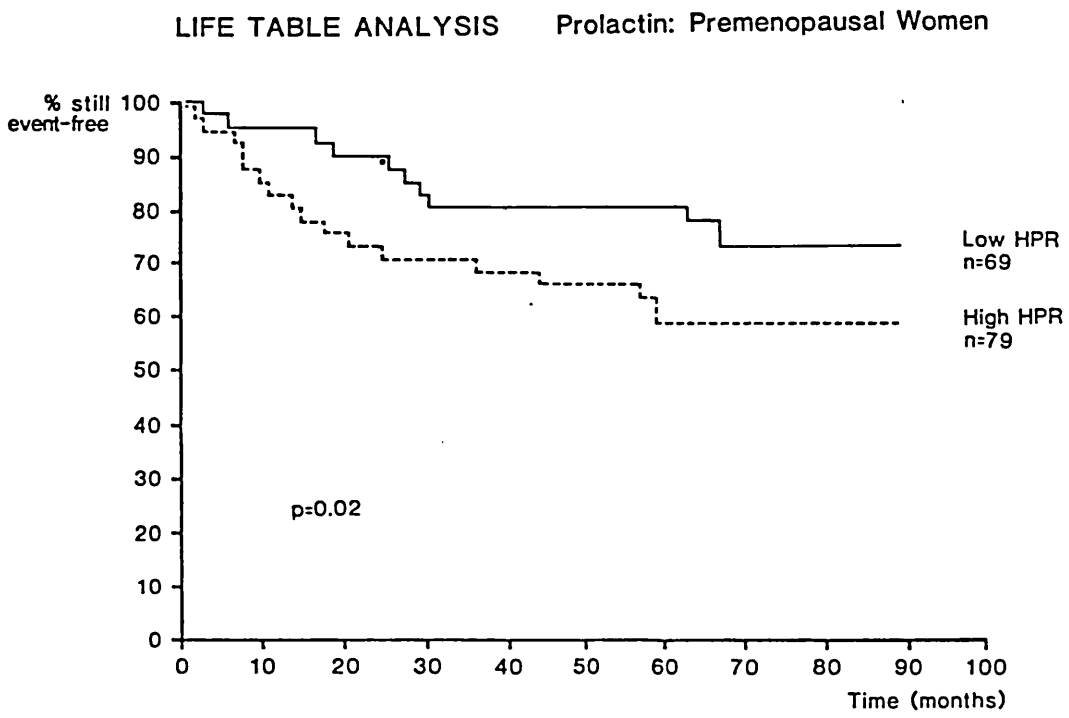
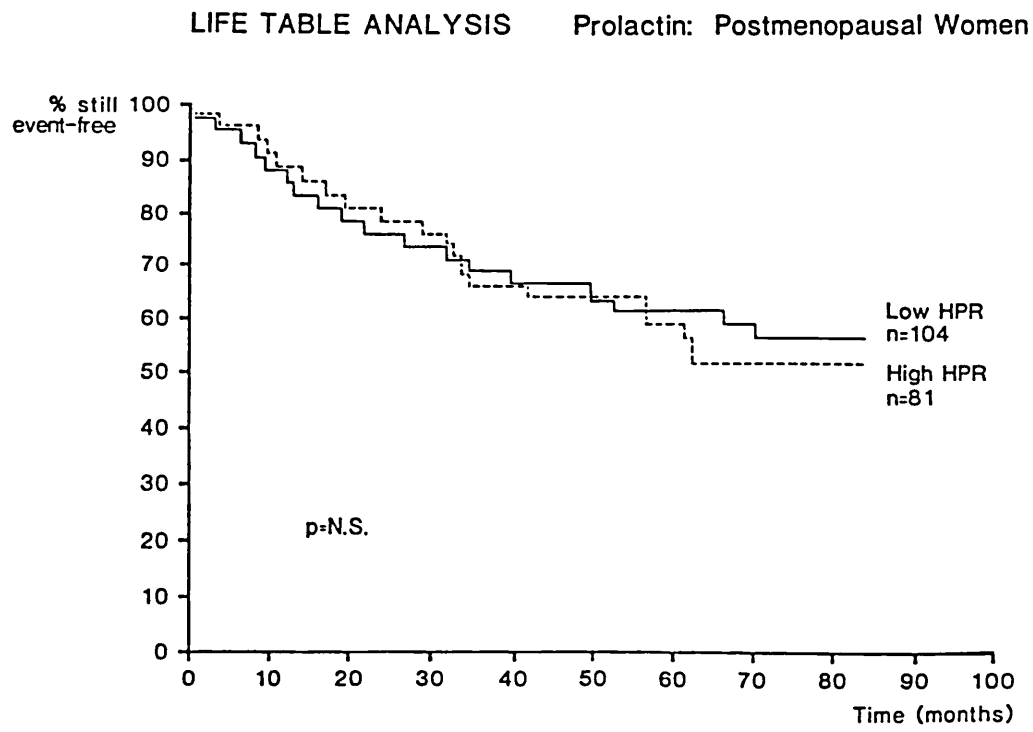


Figure 8.6



8.12 Conclusions

8.12.1 Sampling

The literature review highlighted the importance of timing of sampling due to the complex chronobiology of prolactin secretion. Due to the very large numbers of patients in this study from diverse sources it was impossible to fix a precise time for sampling. However, the evening peak at between 6.00 - 8.00 p.m. observed by Kwa in women at risk, nulliparae, and obese women is more pronounced in premenopausal than postmenopausal women (Kwa et al 1977). Prolactin secretion is otherwise stable between 9.00 a.m. - 6.00 p.m. during which time all blood sampling in this study was performed, and it is therefore unlikely that the results of this study are prejudiced by fluctuations in nycthemeral rhythms.

8.12.2 Physiological variables

Plasma prolactin is age dependent, and although the variation seen in normal women in this study is small, the results are analysed by age cohorts to minimise this effect. It has been demonstrated that although substantial atrophy of breast epithelium occurs at the menopause breast lobules may reappear in the eighth and ninth decades (Jensen 1981). Whether the increase in serum oestradiol and serum prolactin observed in women in this study during these

decades is responsible for this late development is not known, although it seems plausible. Similarly, it was difficult to detect any effect on late breast cancer recurrence due to this, when an analysis of late recurrence by age was performed on the women studied in Chapter 3, although such an analysis is complicated by the alteration in the incidence curve at the time of the menopause [see Chapter 3].

There was no correlation, in this study, between plasma prolactin and parity, height or weight, although they have been found to be correlated in previous studies in premenopausal women [see Section 8.5]. A more important physiological variable, though difficult to quantitate, is stress. Plasma prolactin rises following surgery due to the metabolic response to trauma. It might be anticipated that the breast cancer control group were under greater stress at the time of blood sampling than either the normal women attending a screening clinic, or survivors attending a follow-up clinic. However, the concordance in plasma prolactin between normal women, women with breast cancer, and long term survivors would seem to indicate that stress has not influenced the result.

8.12.3 Plasma prolactin and survival

There is no evidence from this study that plasma prolactin exerts an important influence on survival. The long term survivors had no significant difference in plasma prolactin

to the age matched normal women, although a possible short-term survival advantage of low pretreatment plasma prolactin is borne out by the life table analysis of the women with breast cancer. The influence is more marked in multiparous than nulliparous women, and in premenopausal rather than postmenopausal women. Care must be taken however, in the interpretation of probability tests on trends in relatively small subgroups of patients (Cuzick, pers. comm.). If prolactin does exert an inverse effect on survival, then the study suggests that this is independent of pathological stage. However, analysis of the small cohort of poor prognosis survivors, both at time of mastectomy, and subsequently at time of survival, does not support such an influence. Indeed, at the time of survival, the poor prognosis cohort have marginally elevated plasma prolactin suggesting that they may be compatible more with a high risk cohort than an exceptional survival cohort [see also Chapter 7].

8.12.4 Prolactin and breast cancer

There is no clear indication as to how prolactin might influence breast tumours, though their possession of specific receptors suggest that such mechanisms exist even if clinically unimportant.

In a mouse mammary cancer model Röpcke has demonstrated how synergism between high physiological levels of prolactin and normal, or low physiological levels of oestrone, can

nevertheless lead to an increased incidence of mammary carcinomas (Röpcke 1973). Although this is a good example of synergism occurring, the dangers of extrapolating events from the mouse model have already been discussed.

There was no correlation in this study between prolactin and serum oestrogens, androgens or thyroid function, suggesting no obvious role of prolactin acting via a simple synergistic pathway.

Prolactin augments oestrogen receptor production in the human MCF 7 cell line which could represent another mechanism of action [see Section 8.2]. It might then be anticipated however, that elevated plasma prolactin would correlate with a survival advantage, which cannot be the overall conclusion from this study.

8.12.5 The modified Moolgavkar model

The modified Moolgavkar model was proposed earlier in this thesis as a basis on which to explain the mechanism of action of hormones on breast cancer development, and its subsequent clinical course.

Unfortunately, the paucity of reliable data on prolactin virtually precludes any conclusions being drawn, let alone their application to a mathematical model. If prolactin exerts an important influence on either breast cancer development or its clinical course then it still remains to be proven.

CHAPTER 9

THYROID FUNCTION

Part I

Literature review

9.1 Introduction

9.1.1 Thyroid hormone and the normal breast

Although breast maturation is predominantly stimulated by oestrogens and progestins, thyroxine together with cortisol, insulin and prolactin are required for normal development.

The mammary gland is also one of the organs, apart from the thyroid gland, that is able to concentrate inorganic iodine. The primary function of this is in lactation, where iodine is actively secreted into breast milk resulting in levels as much as twenty times those of normal blood. But there is no experimental evidence to show that the normal breast is able to synthesise thyroid hormones, although it has been demonstrated that thyroxine may be formed by iodination of tyrosine from a variety of proteins by non-specific peroxidases, including lacto-peroxidase, and there is experimental evidence for extrathyroidal thyroxine formation (Taurog and Gamble 1966, Taurog and Evans 1967).

9.1.2 Thyroid hormone and breast cancer

There is similarly no direct evidence to support thyroxine formation in breast cancer, although rat mammary cancers possess peroxidase, and are able to concentrate iodine to a much greater degree than normal mammary tissue; this latter ability correlates with oestrogen receptor concentration (Anderson et al 1975, Thorpe 1976).

Breast cancer cells from the human MCF7 cell line have also been found to possess nuclear T_3 receptors, in addition to receptors for oestrogen, progesterone, testerone, cortisol and prolactin (Burke & McGuire 1978, Burke et al 1977). The precise function of T_3 receptors is unknown, although they are likely to be concerned with cell differentiation.

There is therefore, an undoubted link between thyroid hormone and normal breast development and possibly also with the breast cancer cell. The very large literature on thyroid disease and thyroid function in breast cancer development and pathogenesis is reviewed in Part I of this chapter, and the endocrine study of long term survival is presented in the second part.

9.2 Thyroid disease in the aetiology of breast cancer

The relationship between thyroid function and breast cancer has been in dispute since Beatson first used thyroid extract as adjuvant therapy in 1896.

The evidence to link them comes primarily from three sources:- demographic, epidemiological, and from thyroid function tests in patients with breast cancer.

9.2.1 Demography

Stocks was probably the first to demonstrate a conclusive relationship between breast cancer mortality and endemic goitre due to iodine deficiency (Stocks 1924). Western Europe, certain areas of the United States of America, and South West Asia all had a high incidence of both goitre and breast cancer, whilst Belgium, Japan, Iceland and Chile have a correspondingly lower incidence of both.

A subsequent study, analysing data from four continents confirmed Stocks' initial observations (Spencer 1954), Bogardus & Finley 1961). The explanation is however less clear.

Eskin demonstrated that, when iodine uptake in rat mammary tissue is blocked by perchlorate, histological changes of atrophy and focal hyperplasia occurred, despite thyroxine administration. He concluded that iodine deficiency may be

more relevant than hypothyroidism per se (Eskin 1967, 1970, 1975).

In a study of 1000 autopsy cases, 25% of which had had breast cancer, and the remainder, non-cancer controls, a significant proportion of the breast cancer cases had thyroid atrophy with anterior pituitary, ovarians, and endometrial hyperplasia, compared to the control cases (Sommers 1955). It was therefore proposed that initial thyroid disease stimulated pituitary gonadotrophin release with resultant ovarian and endometrial hyperplasia. The excess oestrogenic stimulation could also then be responsible for the incidence of breast cancer.

9.2.2 Epidemiology

The epidemiological evidence linking thyroid disease and breast cancer is confusing, and fraught with problems, the greatest of which is sampling.

Of the reviews that support a relationship between breast cancer and thyroid dysfunction, some are based on breast cancer discharge data from general, or cancer hospitals, or thyroid dysfunction admission or discharge date. Others are based on Cancer Registries, or life insurance data [see Table 9.1]. Only one study was prospective (Levy and Levy 1951).

The main conclusion of these reports is that hypothyroidism is associated with an increased risk of up to ten-fold for breast cancer. Only one report (Wanebo et al 1966) finds an increased risk with hyperthyroidism.

Author	Conclusion
Levy & Levy (1951)	Increased risk with hypothyroidism
Repert (1952)	" " " "
Dessaive (1956)	" " " "
Chalstrey & Benjamin (1966)	" " " "
Wanebo et al (1966)	Increased risk with hypothyroidism
Schottenfield (1968)	No risk
Moossa et al (1973)	"
MacFarlane et al (1980)	"
Hedley et al (1981)	"
Kalache et al (1983)	"

Table 9.1 Epidemiological studies on thyroid disease and breast cancer risk

Unfortunately, there are almost as many reports that refute such a link. It is notable however, that amongst the latter reports, they tend to be more recent, to include prospective studies, and superior handling of statistical data that mostly uses life table analysis. Even this though, does not prevent confusion.

Hedley examined prospectively, the incidence of breast cancer in a study of 2523 patients registered in a thyroid follow-up system. Forty two cases were suspected of developing breast cancer, of which 4 patients were rejected

because of wrong diagnosis, 7, because of diagnosis prior to hospital attendance for thyroid dysfunction, and a further 9 patients were rejected because breast cancer was diagnosed simultaneously with thyroid disease.

Of the remaining twenty-two patients the observed incidence of breast cancer was 1.54% (± 0.42 S.E.) compared to an expected incidence of 1.5%, i.e. there was no indication of increased risk (Hedley et al 1981).

Cuzick subsequently reanalysed the data arguing that a proportion of the patients excluded from statistical analysis because of simultaneous diagnosis, should not have been, because the diagnosis would have been made shortly afterwards regardless of the hospital admission. Recalculating relative risk with conservative estimates then puts the observed incidence at 1.7, and the opposite conclusion is drawn to that of the original authors, with a significance level of $p < 0.01$ (Cuzick 1981).

In the only study examining thyroid function in women at high risk of breast cancer, Bulbrook could find no variation in plasma levels of T_3 and T_4 between controls and patients at risk, or who subsequently developed breast cancer (Bulbrook et al 1981).

However, they also measured the urinary androsterone and etiocholandrone ratio (5x/5β ratio) which is a sensitive index of thyroid function (Hellman et al 1959). This was

significantly different from controls in one subset of the population studied. These were peri- and post-menopausal women with a family history of breast cancer who subsequently developed breast cancer themselves. In this group the 5x/5β ratio was significantly lower, indicating a degree of hypothyroidism.

9.3 Thyroid disease and the clinical course of breast cancer

Although the role of thyroid disease in the aetiology of breast cancer is confusing, there is some evidence to suggest that it may adversely affect the subsequent clinical course of the disease (Backwinkel & Jackson 1964, Liechty et al 1963, Moossa et al 1973).

The classical study is that of Moossa, of 71 breast cancer patients with thyroid dysfunction. Of these, 51 patients were followed up for a minimum of 10 years. Twenty-one patients had pre-existing hyperthyroidism treated by either surgery or radioactive iodine. Their 10 year survival rate was significantly ($p < 0.01$) worse than a comparable, but larger, control group. Twenty-eight patients had pre-existing non-toxic goitres, of whom 13 had had a partial thyroidectomy. Their 10 year survival was similarly, significantly reduced ($p < 0.01$).

The patients were biochemically euthyroid at the time of primary treatment of their breast cancer, though it might be assumed that a proportion of the hyperthyroid patients may have become hypothyroid over the subsequent 10 years.

In the other studies of the impact of thyroid dysfunction on the subsequent clinical course of breast cancer, only hypothyroidism was associated with decreased survival (Backwinkel and Jackson, Liechty et al 1963).

9.4 Thyroid therapy and breast cancer

A study on the prevalence of breast cancer in women on thyroid therapy received wide publicity in the United States (Kapdi and Wolfe 1976). The study, on patients undergoing mammography for screening, noted that the prevalence of breast cancer appeared to increase with increasing duration of hormone therapy. The paper lacked vital data on the nature of the thyroid disease being treated, the biochemical thyroid status and much else. However, it caused such widespread alarm, that the American Thyroid Association had to make an unprecedented statement in all leading medical journals urging caution. A subsequent case control study by Shapiro on 659 patients with breast cancer compared with 1719 controls could find no evidence to support the suggestion that thyroid supplementation increases the risk of breast cancer (Shapiro et al 1980).

Conversely, Emery and Trotter treated patients with breast cancer with Thyroxine in addition to conventional therapy, but found no difference in survival (Emery and Trotter 1976). Similar studies have reached the same conclusion (Stoll 1965, O'Bryan et al 1974).

9.5 Thyroid function in breast cancer patients, and women at risk

There are many reports over the last 30 years of the results of Thyroid Function Tests in women with breast cancer. As methods have become more sophisticated, understanding has improved, regrettably however, not in proportion to the biochemical advances made.

9.5.1 ^{131}I Uptake

The earliest studies utilised ^{131}Iodine uptake as an index of thyroid function. Stoll studied ^{131}Iodine uptake in 183 patients, divided into those with clinical disease, minimal clinical disease, and disease-free for 5 years. He found no significant difference in ^{131}I uptake between these groups, but did note that uptake was generally lower in those with more advanced disease. Other studies have however reached variable conclusions [see Table 9.2].

9.5.2 Protein Bound Iodine

During the 1960's six reports were published of Protein Bound Iodine in patients with breast cancer [see Table 9.3].

Author	^{131}Iodine uptake
Edelstyn et al (1958)	significantly reduced
Reeve et al (1961)	no change
Lencioni et al (1962)	reduced (not significant)
Stoll (1965)	reduced in advanced disease

Table 9.2 ^{131}Iodine uptake in women with breast cancer

Author	P.B.I.
Carter (1960)	Significantly raised
Dargent et al (1962)	Significantly raised
Lencioni (1962)	No significant difference
Myhill (1966)	Significantly raised (Early cancer) Normal (Advanced cancer)
Schottenfield (1968)	Normal
Sicher & Waterhouse (1967)	Normal

Table 9.3 Protein Bound Iodine in patients with breast cancer

Of the six studies, four reported raised levels of PBI in women with breast cancer, although in one study this was only found to be significantly different from normal controls for those women with advanced disease. In the other two studies, no difference was noted between cancers and controls. In retrospect, these findings are not surprising, as PBI is an insensitive discriminator of thyroid function.

9.5.3 Thyroid Stimulating Hormone

With the advent of a radioimmunoassay for thyroid stimulating hormone (TSH) and the thyrotrophin-releasing hormone (TRH) stimulation test, a more sensitive method for assessing thyroid function became available and a clearer trend emerges [see Table 9.4].

Mittra and Haywood studied three groups of 50 patients each, with early breast cancer, with advanced disease, and normal controls. Although a majority of the subjects had TSH levels below the sensitivity of the assay prior to TRH stimulation, there was a significantly raised TSH in both early and advanced disease ($p < 0.02$ and $p < 0.01$ respectively). Following TRH stimulation both groups of cancer patients had significantly elevated TSH when compared to normal controls (Mittra and Hayward 1974).

This study was repeated with a similar finding of elevated TSH levels in women with breast cancer, though this only reached significance in women with advanced disease (Rose and Davis 1979). In a further control group with colonic carcinoma, no significant difference in TSH levels was found. This interesting observation will be discussed later.

Author	TSH
Mittra & Hayward (1974)	significantly elevated
Perry et al (1978)	significantly elevated
Adami (1978)	8% significantly elevated
Aldinger et al (1978)	36% significantly elevated
Rose & Davis (1979)	significantly elevated (advanced cancer)
MacFarlane et al (1980)	no difference
Saito et al (1977)	no difference (in Japanese women)

Table 9.4 Thyroid stimulating hormone (TSH) in women

Similar studies also demonstrated raised TSH levels in women with breast cancer, though Macfarlane found no difference in thyroid function between cases and controls (Penny et al 1978, Adami 1978, Aldinger et al 1978, Macfarlane et al 1980).

The general finding of elevated levels of TSH suggests relatively low levels of circulating thyroid hormones are present in breast cancer, and the pattern of TRH stimulation further suggests that this is not due to a primary pituitary or hypothalmic disorder.

9.5.4 T_3, T_4, Free T_4

Following upon the previous studies, the majority of the authors subsequently measured total T_3 and T_4, the circulating thyroid hormones [see Table 9.5].

Author	T_3	T_4	TFI
Perry et al (1978)	-	-	reduced
Rose & Davis (1979)	reduced	normal	-
Adami et al (1979)	normal	normal	-
Zumoff (1981)	elevated	elevated	-
Thomas et al (1983)	-	-	reduced

Table 9.5 Serum T_3, T_4 and Free T_4 in women with breast cancer

Rose and Davis found a negative correlation between TSH and T_3 in patients with early cancer, with reduced levels of T_3 in both early and advanced disease. T_4, however, was normal (Rose and Davis 1979). In support of this study, two other authors report lower free T_4 indices in breast cancer patients (Perry et al 1978, Thomas et al 1983).

Adami found both T_3 and T_4 to be normal, but Zumoff even found T_4 to be raised in breast cancer patients (Adami 1979, Zumoff 1981).

To date, only two studies have reported measurement of free T_4, both finding significantly lower values in patients with breast cancer (Perry et al 1978, Thomas et al 1983).

The present evidence thus strongly suggests that women with breast cancer are hypothyroid with respect to normal controls. However, the depression of thyroid status is relative, the majority of women being clinically euthyroid.

Although it is known that chronic disease and disseminated malignancy may be associated with reduced levels of plasma T_3 and T_4 it is unlikely that this is the sole explanation (Bermudez et al 1975 and others). Firstly, Rose and Davis could find no similar alteration in thyroid function in patients with non-disseminated colonic carcinoma, and

secondly there was no significant difference in T_3 depression between patients with early cancer, compared to those with advanced disease.

9.5.5 Thyroid function and women at risk

Hellman first demonstrated that in hypothyroid patients excrete less androsterone in the urine, relative to aetiocholanolone. The ratio of androgen excretion 5 α : 5 β is now recognised as a sensitive index of thyroid function (Hellman et al 1959).

In a prospective study of women at risk of breast cancer because of familial history, Bulbrook found no significant abnormality in thyroid function in the majority of women using the androgen excretion ratio, and measurement of plasma T_3 and T_4. However for women aged 46-50 years with a family history of breast cancer the 5 x : 5 β ratios were lower than in normal controls. Measurement of total T_3 and T_4 showed no differences between the groups. The results of this study were thus not wholly consistent, although measurement of free T_4 might have provided a more sensitive index of subtle alterations in thyroid function (Bulbrook et al 1981).

9.5.6 Thyroid function and surgery

In order to assess the validity of studies of thyroid function and breast cancer recurrence it is necessary to

examine the effect of surgery on thyroid function. From the many relevant studies, the following concensus is reached.

Total T_4 rises during surgery reaching a peak approximately 48 hours later, and returning to normal or slightly above normal values approximately one week later. In contrast total T_3 falls markedly during surgery and then returns much more slowly to normal values (Kirby et al 1973, Adami et al 1978, Chan et al 1978). Free T_3 follows an inverse pattern to total T_3 but returns to normal within one week of surgery.

The free thyroxine fraction (FT_4) is thought to be the most important determinant of thyroid status. Thyroxine exists in equilibrium with plasma proteins, about 15% being bound to thyroid binding pre-albumin (TBPA) and most of the remainder to thyroid binding globulin (TBG) and albumin. Although TBG is quantitatively more important it remains unchanged following surgery, whereas TBPA is markedly depressed (Surks et al 1964, Kirby et al 1973). It is therefore likely that altered binding capacity to TBPA is an important cause of the altered thyroxine equilibrium. Elevated free T_3 and free T_4 in turn exert a negative feedback on the hypothalamus inhibiting TRH and TSH release. Several authors confirm that neither TRH nor TSH alter following surgery (Chan et al 1978, Kirby et al 1973).

The part played by stress and adrenal cortical hormones is unclear. One might anticipate a rise in trophic hormones if this were the primary event, and yet clearly this does not occur. Also it has been noted that 80 i.u. ACTH given to healthy volunteers for 2 days causes no alteration in free T_4, nor TBPA binding capacity (Surks et al 1964).

9.5.7 Thyroid function and recurrence

Following the early clinical reports that hypothyroidism was associated with decreased survival following mastectomy, several prospective studies have been published in which thyroid function has been assessed biochemically, before the commencement of treatment, and at intervals thereafter.

In the first of such studies, serum TSH and free T_4 were measured in 40 women with breast cancer, and 40 normal women (Perry et al 1978). Prior to surgery TSH was significantly elevated and free T_4 reduced, when compared to normal controls. In the 29 remaining patients available for follow-up at six months (the loss mainly being due to recurrent disease) the TSH was elevated further, though not significantly, and the free T_4 was significantly depressed, when compared both to controls, and to pre-treatment. In a study with such small numbers of patients it is regrettable that 4 of the cancer patients were clincally hypothyroid at the time of surgery, and that 25% of the cases should not be available to follow-up.

The second study suffers from similar drawbacks. Adami measured a wide spectrum of biochemical function including serum TSH, T_3, reverse T_3, T_4, T_3 resin uptake, and free T_4 index (FTI). The FTI is calculated from the product of T_4 and T_3 resin uptake expressed as a percentage (Adami et al 1979). The first serum sample was taken pre-treatment, and the second at a variable time of follow-up between 7 and 27 months, twelve patients being followed up for a maximum of 27 months. No significant differences were found in any of the parameters of thyroid function measured.

In the third, and best designed, of the studies, Thomas analysed the urinary 5α : 5β androgen ratio in 269 patients followed up for periods of up to 85 months (Thomas et al 1983). A significantly higher rate of recurrence was found in those patients with a low urinary 5α : 5β ratio (indicating hypothyroidism) with a similar trend being observed for serum TFI, though this only reached statistical significance in postmenopausal patients. The study was however weighted numerically in favour of patients with recurrence.

In a separate study the same authors note that there is a marked fall in the FTI following mastectomy, which at one year still does not approximate to pre-treatment levels. Although this is in contrast to the previous study by Perry, there was no correlation between recurrence and the TFI over a period of one year (Thomas, personal comm.)

The overall conclusion from these studies is therefore that hypothyroidism may be associated with earlier recurrence following treatment.

9.6 Thyroid function and hormone metabolism

If thyroid dysfunction is able to influence either the aetiology, or the clinical course of breast cancer, then it is likely that it does so through its complex effects on the metabolism of other hormones. Interest has therefore centered on it actions on androgens, oestrogens and prolactin.

9.6.1 Androgens

The principal metabolic effect of thyroid hormones on androgen metabolism is the control of the testosterone clearance rate. In hypothyroidism the metabolic clearance of testosterone is increased, as its conversion to androstenedione. It is likely that this is as a consequence of its action on hepatic 5 α reductase (Gordon et al 1969). The result is reduced urinary excretion of androsterone relative to aetiocholanolone, a sensitive indicator, of thyroid dysfunction [see section 9.5]. Thus hypothyroidism, by increasing testosterone conversion to androstenedione may indirectly increase oestrogen production from this hormone also.

9.6.2 Oestrogens

Hypothyroidism influences the metabolism of oestrogens principally by augmenting 16 α hydroxylation, thus increasing oestriol production (Fishman et al 1965). The

same authors have also demonstrated an identical augmentation of 16 hydroxylation in patients with breast cancer [see Section 6.4].

9.6.3 Sex Hormone Binding Globulin

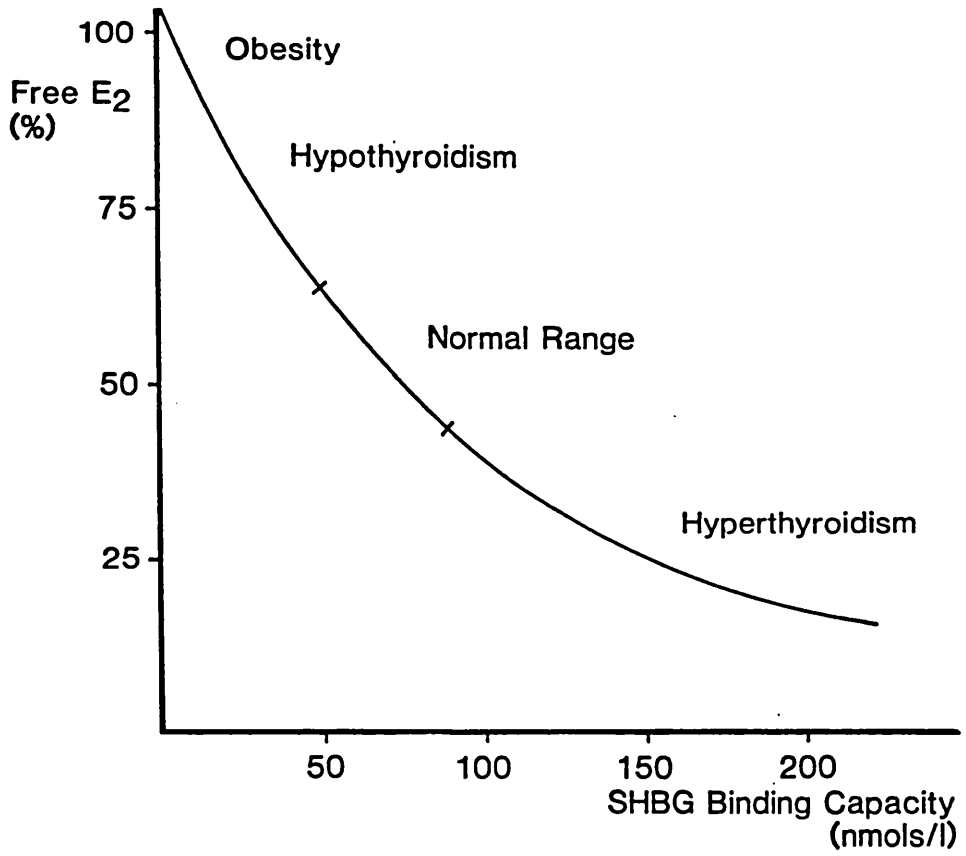
Serum SHBG levels are markedly influenced by thyroid hormones, being significantly elevated in hyperthyroidism and depressed in hypothyroidism [see Fig.9.1]. Hypothyroidism may exert an important oestrogenic effect by means of this mechanism.

9.6.4 Prolactin

The relationship between the thyroid and the pituitary is complex, but elevated levels of TRH often associated with hypothyroidism have been shown to increase prolactin secretion, as well as TSH (Noel et al 1972). The theory that thyroxine may inhibit the effects of prolactin on mammary epithelium has been dealt with more fully in Chapter 8.

Figure 9.1

Thyroid dysfunction and SHBG Binding Capacity



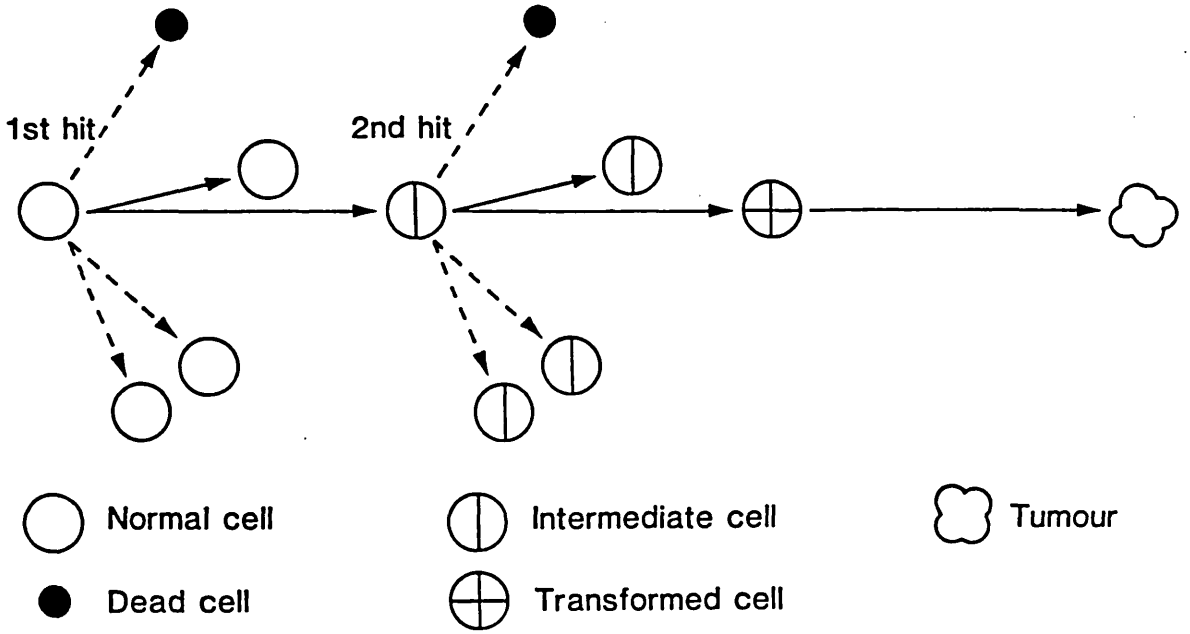
9.7 Conclusions

There is strong demographic evidence that hypothyroidism at an early age is associated with an increased risk of subsequent breast cancer. Although the hypotheses of Eskin and of Sommers may partly explain this association an alternative hypothesis can be presented on the basis of the Moolgavkar model [see Fig.9.2].

Thyroxine is a prerequisite for normal differentiation and maturation of breast tissue in late childhood and adolesence, and thyroid deficiency occuring at puberty will thus delay differentiation, increasing the probability of intermediate cell production. This may be influenced further by the irregular menses, and possible excess oestrogenic action associated with them, found in hypothyroidism. Subsequent replacement therapy may not complete the maturation process and although there is no direct experimental evidence for this, it has been shown that the thyroid gland does not revert to histological normality following iodine replacement therapy for childhood goitres (Clements 1960). This latter finding has been used to support Sommers' hypothesis, but the incidence of endemic goitre has declined very substantially over the past thirty years, though the incidence of breast cancer in the same regions remains stable. This fact may be accounted for in the model because the carcinogenic effect of hypothyroidism would act predominantly at puberty, and a decrease in breast cancer mortality would not be anticipated for at least 20 years.

Figure 9.2

A two stage model for breast carcinogenesis (Moolgavkar, 1980)



There is epidemiological evidence to support the theory that hypothyroidism post-puberty may still exert an influence on subsequent breast cancer risk, but this evidence is by no means conclusive. It is interesting that no studies have taken the duration of thyroid dysfunction as an important parameter. If the explanation of the action of thyroid hormones on breast epithelial cells according to the Moolgavkar model is correct, then the earlier the onset of thyroid dysfunction the more likely it is to exert an influence on subsequent breast cancer risk. As most of the studies presented were retrospective one may only presume that the cases analysed probably had thyroid dysfunction occurring later than the third decade.

The Moolgavkar model is further explored in Part II.

Part II

Endocrine study of long term survival

9.8 Introduction

The second part of this chapter presents the results for thyroid function in the endocrine study of long term survival.

9.8.1 Patients

Three groups of patients are studied. The long term survivors are compared to two age-matched control groups, patients with breast cancer and normal women.

9.8.2 Method

Serum free T_4 was measured by radioimmunoassay using materials available from Amersham UK. This method utilises a radioactive tracer ^{125}I thyroxine of high specificity that does not bind to the serum T_4 binding proteins but does do so to antibodies to T_4. T_4 is in equilibrium in the serum with the binding proteins thyroxine binding globulin, thyroxine binding prealbumin, and albumin. When the tracer and antibody are mixed with serum, free T_4 competes with the tracer to bind with the antibody. Separation of the antibody fraction is then by means of centrifugation as they are bound to polymer particles. The proportion of tracer bound to antibody is then inversely proportional to serum free T_4 and can be estimated using a standard curve calculated from the reference standards. The assay is described in full in Appendix 9.

9.8.3 Results

The results of serum free T_4 are analysed as follows:

1. Quality control

2. Linear regression on age, height, weight, ideal body weight and Quetelet's Index

3. Comparison of long term survivors and control groups by age cohorts (Students t test).

4. Linear regression analysis of serum free T_4 on pathological nodal status, tumour TNM stage, tumour histological grade, and tumour size.

5. Correlation of serum free T_4 and other hormone assays in this study (Pearson's correlation coefficient).

6. Life table analysis on short term survival (ten years) of breast cancer control group (Log rank test).

9.9 Patients

Three groups of patients were studied:

9.9.1 Long term survivors

n = 146

mean age ± S.D. = 62.0 yrs ± 11.9

From the total cohort of 160 patients, serum free T_4 was assayed in 146 long term survivors who had been disease-free for a period of ten years or more, and were clinically free of breast cancer at time of entry into study. No patients had received hormone therapy. Full details of these patients are given in Chapter 6.11, and Appendix 6.

9.9.2 Breast cancer control group

n = 71

mean age ± S.D. = 65.0 yrs ± 14.0

From the total cohort of patients with breast cancer, serum free T_4 was assayed in 71 patients. The full clinical and pathological details of this cohort are described in Chapter 6.11, and Appendix 6.

9.9.3 Normal women control group

n = 91

mean age ± S.D. = 63.0 yrs ± 6.2

From a large cohort of normal women screened for breast cancer in the Guernsey III study, age-matched post-menopausal women were chosen as controls. All women were clinically, and mammographically free of disease at time of entry into study, and none had received recent hormone therapy.

9.10 Results

9.10.1 Quality control

The Amerlex Free T_4 assay gave extremely reproducible results. Duplicates and controls were assayed in each batch to give a within assay coefficient of variation of 2.5%, and a between assay coefficient of variation of 7.2%. Duplicates differing by more than 2 x S.D. from the mean, and samples values beyond the stated normal range (8-23 pmols/l) were repeated.

9.10.2 Free T_4: age, height, weight

Analysis of 91 normal postmenopausal controls (mean age 63 years ± 6.2, range 55-85 years) demonstrated a significant correlation with age ($r = 0.445$, $p < 0.001$), free T4 rising with increasing age [See Table 9.6]. This is in contrast to a further group of 60 premonopausal normal women (median 53 years, range 35-55 years) in whom free T_4 was shown to decrease with advancing age ($r=0.290$, $p=0.01$).

Neither the breast cancer control group (mean age 65 years ± 14) nor the long term survivors (mean age 62 years ± 11.9) showed any correlation between free T_4 and age.

There was no correlation between serum free T_4 and height, weight, ideal body weight, or Quetelet's Index, for any of the groups under study.

Age (yrs)	Normal Women mean ± S.D	Breast Cancer Controls mean ± S.D	Long Term Survivors mean ± S.D	Survivors: Normal P
55 - 60	11.8 ± 1.6	13.1 ± 2.0	14.4 ± 4.1	0.006
61 - 65	11.9 ± 1.9	13.6 ± 4.0	14.3 ± 4.2	0.01
66 - 70	12.7 ± 2.5	13.9 ± 2.5	14.1 ± 3.8	0.15
71 - 75	14.6 ± 3.3	13.5 ± 5.2	14.5 ± 2.6	N.S.
76	18.3 ± 5*	11.4 ± 4*	13.9 ± 3.5	N.S.
Number	91	71	146	
Mean Age ± S.D.	63 yrs ± 6.2	65 yrs ± 14	62 yrs ± 11.9	

* less than 10 observations
p from two tailed t test

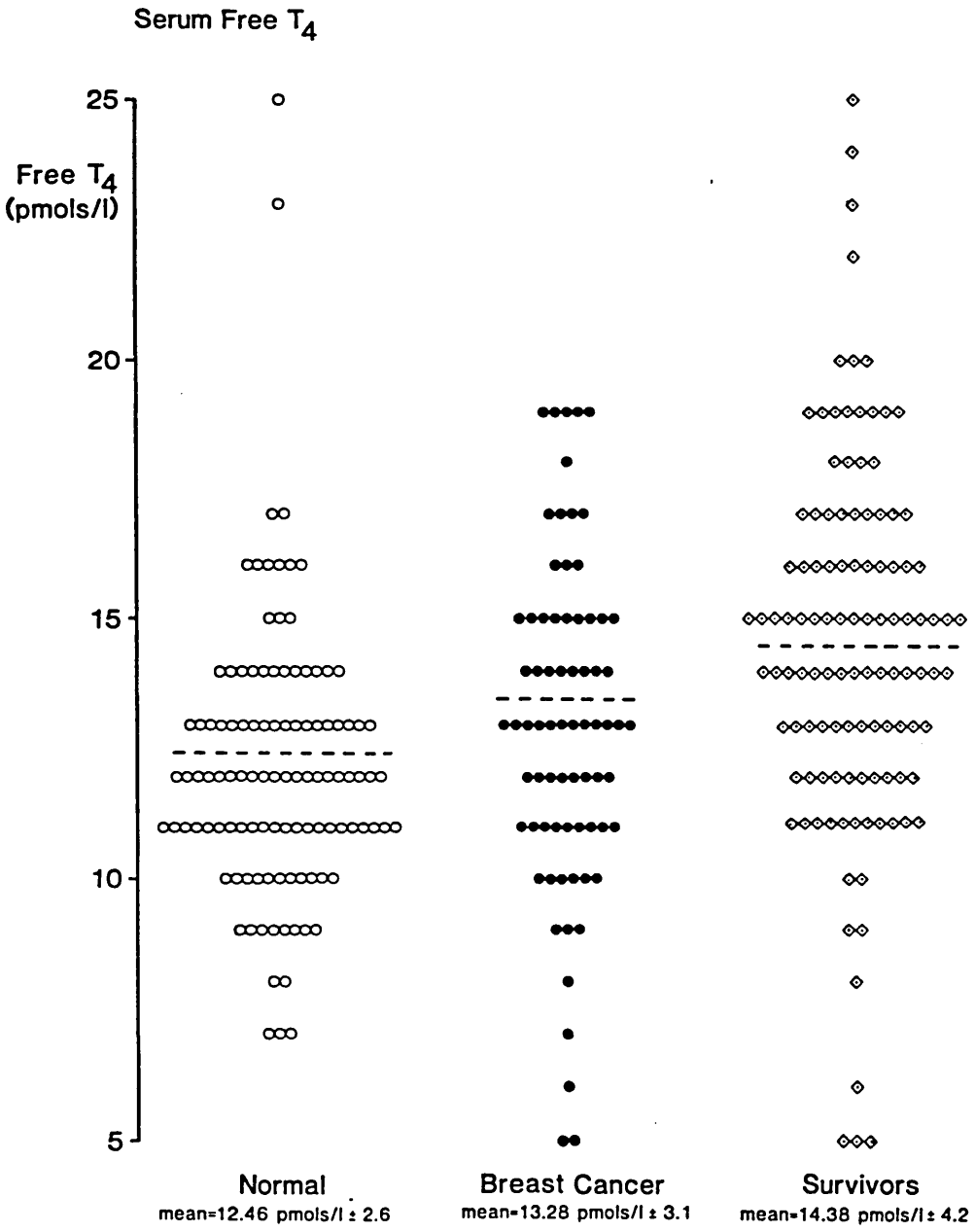
Table 9.6 Free T_4 (pmols/l) in survivors and controls by age cohort

9.10.3 Free T_4: long term survivors: controls

The mean serum free T_4 in 146 long term survivors (mean ± S.D. = 14.4 pmols/l ± 4.2) was significantly higher than in 91 normal women (mean ± S.D. = 12.5 pmols ± 2.6) of equivalent age ($t = 3.88$, $p < 0.001$) [see Fig.9.3].

There was no significant difference in serum free T_4 between normal women and breast cancer patients, nor between the long term survivors and women with breast cancer (mean ± S.D.= 13.3 pmols/l ± 3.7).

Figure 9.3


Serum Free T_4
25
Free T_4
(pmols/l)
20
15
10
5
Normal
mean=12.46 pmols/l ± 2.6
Breast Cancer
mean=13.28 pmols/l ± 3.1
Survivors
mean=14.38 pmols/l ± 4.2

In view of the significant correlation between serum free T_4 and age in normal women, the analysis was repeated by age cohort [see Table 9.6]. Long term survivors aged between 55 and 65 years have significantly elevated free T_4 compared to the control groups, but this is not present in survivors aged 66 years and over [see Fig. 9.4].

9.10.4 Free T_4: pathological stage

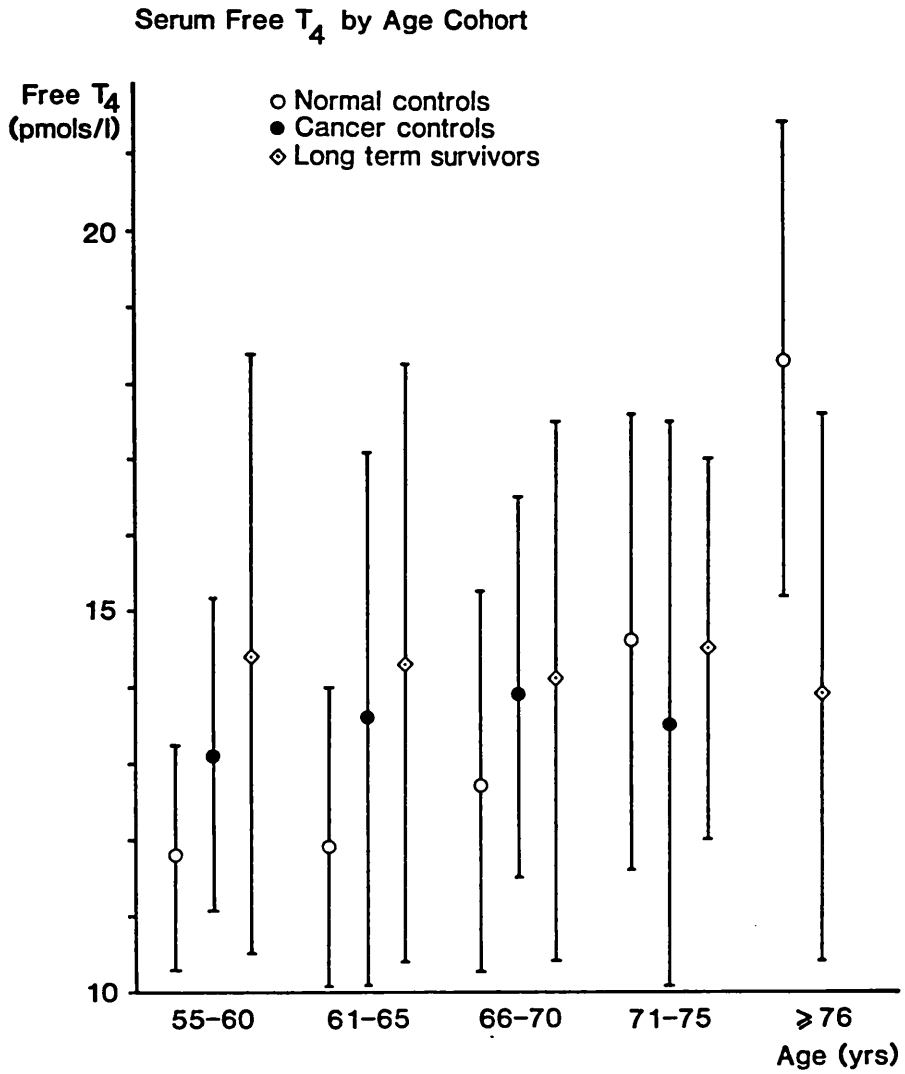
A linear regression analysis was performed on serum free T_4 and nodal involvement, with the breast cancer control group graded as no pathological nodal involvement, one to three nodes involved, and four or more nodes involved. There was no significant trend. Linear regressions on tumour histological grade, tumour TNM stage, and tumour size also showed no significant correlation with Free T_4.

9.10.5 Free T_4: other hormones

Serum free T_4 was significantly correlated with serum DHEA-S in the long term survivors ($r = 0.256$, $p = 0.01$), but this correlation was not present in the control groups.

There was no significant correlation however, with either oestrogens, SHBG binding capacity, or prolactin.

Figure 9.4

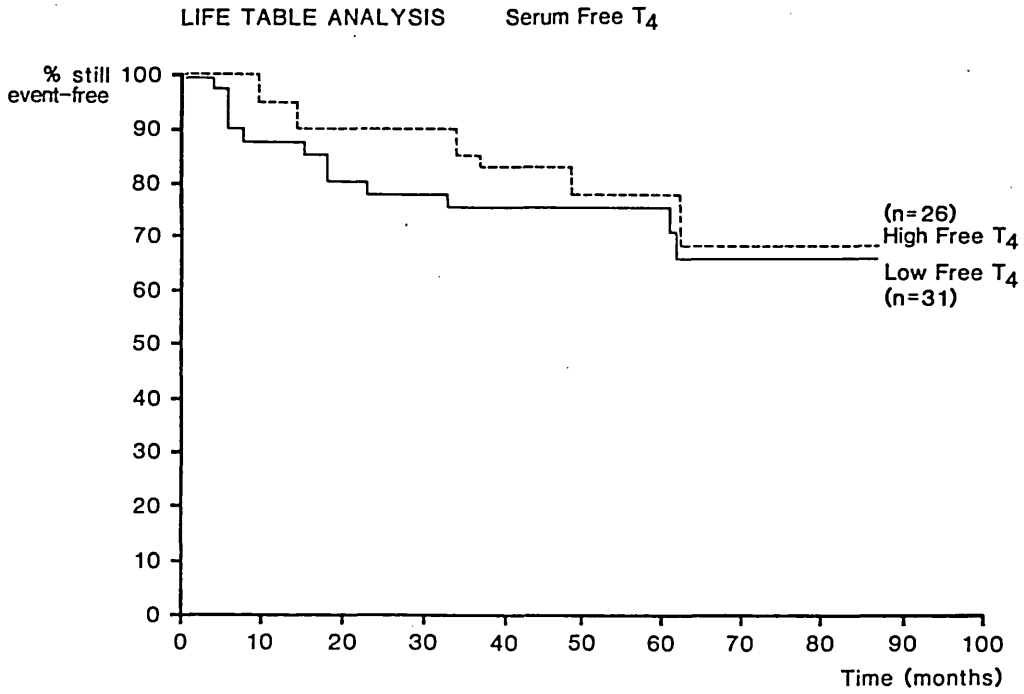

Serum Free T_4 by Age Cohort
Free T_4 (pmols/l)
○ Normal controls
● Cancer controls
◇ Long term survivors
20
15
10
55–60
61–65
66–70
71–75
⩾76
Age (yrs)

9.10.6 Free T_4 life table analysis

A life table analysis was performed on the 71 breast cancer controls using clinical recurrence data from the I.C.R.F. The patients were stratified by those above and below median free T_4 (13.5 pmols/l) [See Fig.9.5].

There was no statistically significant survival advantage between the two groups.

Figure 9.5


LIFE TABLE ANALYSIS
Serum Free T_4
% still event-free
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
90
100
Time (months)
(n=26)
High Free T_4
Low Free T_4
(n=31)

9.11 Conclusions

9.11.1 Thyroid function and women with breast cancer

Several previous studies have demonstrated that women with breast cancer have a depressed level of thyroid function as compared to normal women [Tables 9.2, 9.3, 9.4]. However, the depression, although statistically significant, remains within the normal range of thyroid function, whether assessed as T_3, T_4, free T_4 or as a thyroid function index, and thus the changes detected are subtle rather than gross.

Where the mean age of the subjects has been quoted in these reports, the age range has been significantly lower than in the current study. It has been shown that whereas free T_4 decreases slightly with age until the menopause, it then assumes a significant upward trend. The failure to detect any significant difference in free T_4 in the older breast cancer control group in this study may therefore be due, at least in part, to the fact that a subtle cancer correlated trend in premenopausal women might be lost within the age correlated trend of postmenopausal women.

There is no accepted explanation as to why a primary depression of thyroid function should be observed in women with breast cancer, although low levels of serum T_3 have been reported in a variety of systemic illnesses where the generalised catabolic state may affect thyroid binding

pre-albumin (Oppenheimer et al 1963, Bellabarba et al 1968, Lutz et al 1972, Portnay et al 1974). In these circumstances, TSH levels are not elevated, even after TRH stimulation (Maturlo et al 1980). When comparing patients with advanced breast cancer with those with disseminated colonic cancer, Rose and Davis found significantly elevated plasma TSH in 15% of the breast cancer patients, but only 1% of the colonic cancers (Rose and Davis 1978).

If the depression in thyroid function in breast cancer observed in some studies is primarily due to systemic illness, then one would predict an association with pathological stage. In neither this study, nor the majority of previous ones, was any correlation seen between thyroid function and pathological stage, although Thomas finds a significant trend with histological grade (Thomas et al 1983).

9.11.2 Thyroid function and recurrence

Studies, notably those of Moossa and Thomas, have demonstrated an unequivocal correlation between depressed thyroid function and early recurrence [sections 9.3, 9.4]. It is therefore of interest that the long term survivors in this study have significantly elevated levels of serum free T_4 when compared to age matched controls, although it should be noted once again, that this difference represents only subtle changes in thyroid function.

The biological explanation of these findings may lie either indirectly, with the action of thyroid hormones on other hormones, or on the breast cancer cells directly. The influence of thyroid hormones on androgens, oestrogens, oestrogen binding and prolactin has been described previously [Section 9.6]. Although no correlation has been observed, in this study, between free T4 and SHBG binding capacity, this lack of correlation has been previously noted (Thomas, pers. comm.). SHBG binding capacity does however correlate strongly with serum T_3 and T_4, and thereby indirectly with free oestradiol (Rose 1979). The correlation between free T_4 and serum androgens noted in this study is interesting as previous authors have equated it solely with urinary androsterone and aetiocholanolone. The finding confirms the strong influence of thyroid hormones on androgen metabolism.

At a cellular level, the ability of human breast cancers to concentrate iodine has been discussed earlier as has the possession in some tumours of T_3 receptors [section 9.1]. Iodine concentrating ability is associated with higher levels of oestrogen receptor, and so an indirect link can be proposed between the possibility of thyroxine production by a breast tumour possessing T_3 receptors, and the survival advantage known to be conferred by the posession of oestrogen receptors.

9.11.3 The modified Moolgavkar model

In conclusion, the actions of thyroid hormones on the genesis and subsequent clinical course of breast tumours can be explained on the basis of the proposed mathematical model [See Fig 9.6].

The influence of hypothyroidism is strongest around puberty and adolesence (A), where the lack of thyroid hormone impedes breast development and maturation leading to an increased incidence of intermediate cells, perhaps augmented by an excessive oestrogenic influence from anovulatory, or short menstrual cycles.

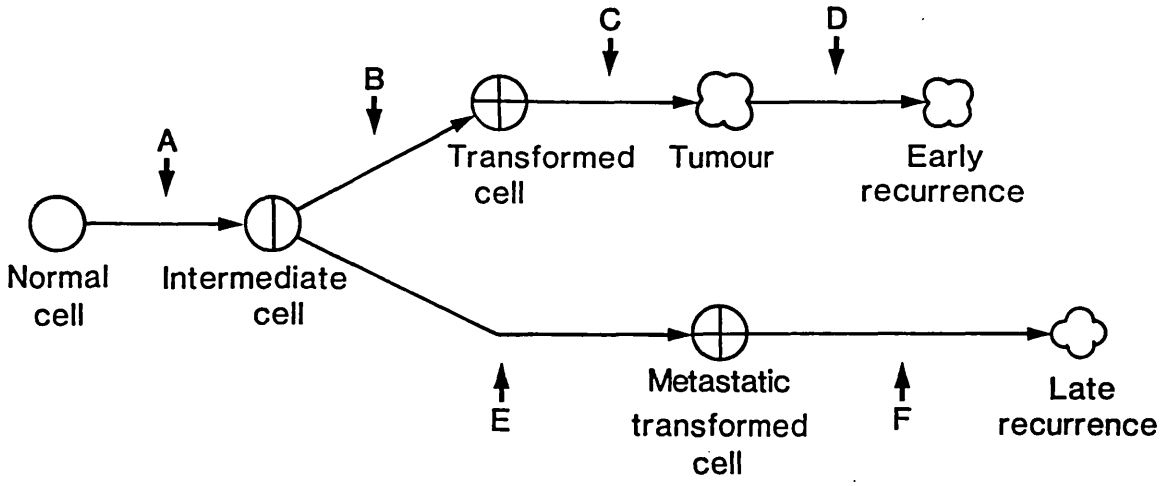
The influence of hypothyroidism on breast cancer risk over the subsequent decade or two (B) might be anticipated to decline, particularly after pregnancy and the associated boost to epithelial cell maturation.

After the establishment of transformed cells, and during pre-clinical cancer development (C) it is possible that subtle changes in thyroid metabolism may affect the rate of tumour growth, but this is not proven.

Following clinical tumour appearance and possible treatment, thyroid hormones almost certainly influence early recurrence (D) perhaps by acting via other hormones modifying

Figure 9.6

The Modified Moolgavkar Model



metastatic tumour cell metabolism, or by a direct action on such cells, some of which may possess thyroid receptors.

Life table analysis of thyroid function and clinical recurrence in the breast cancer control group did not reveal any influence of thyroid function on short term recurrence in this study. The number of clinical recurrences was however small, and it is anticipated that a very much larger cohort might be required to show what is probably a subtle effect.

Lastly, evidence from this study showing some long term survivors to still have a significant elevation in free T_4 compared to normal women, suggests that even late recurrence (F) might be linked either directly, or indirectly, with thyroid metabolism.

CHAPTER 10

CONCLUSION

10.1 Introduction

It has been estimated that 60% of the women who survive ten years after initial therapy for breast cancer, will nevertheless die of recurrent disease (Mueller & Jeffries 1975). The aim of this thesis was to investigate tumour, and host related factors that might influence long term survival in order to identify patients still at risk, and assess whether a biological basis exists for further therapy.

In brief, the conclusion of the thesis must be that prediction of late recurrence (whether local or distant) is not possible with any degree of accuracy, and secondly, that long term survival does not principally depend upon the hormonal status of the host.

10.2 Prognostic indices

Blamey has suggested that the most effective method of predicting individual patient survival is by means of a prognostic index (Blamey et al 1979). The results of this study demonstrate that the tumour associated prognostic variables used in the prediction of 5 year survival have progressively less impact, until at 10 years, only tumour fixation, and pathological lymph node status have any measurable effect. As the influence of both these factors continues to wane, it is not possible to construct a prognostic index for long term survival.

10.3 Endocrine status

This study failed to demonstrate any major influence of endocrine status, as assessed by serum hormones, on long term survival, although several of the findings may have clinical relevance.

10.3.1 Oestrogens

It was noted that long term survivors had significantly elevated SHBG binding capacity compared to normal women, and this has been associated with the possession of tumour oestrogen receptors (Murayama et al 1979). As it is known that a long disease-free interval is similarly associated with endocrine dependence, there is an 'a priori' reason for anticipating benefit from the use of an oestrogen receptor blocking agent in long term survivors.

There are now many trials in progress assessing the role of adjuvant endocrine therapy in early breast cancer. Early results with Tamoxifen suggest that it can delay evidence of recurrence, notably in postmenopausal women, in patients with oestrogen receptor positive tumours, and in those with four or more involved axillary nodes (Fisher et al 1981, Ribiero and Palmer 1983, Baum 1982). These trials will not of course demonstrate any benefit to long term survival unless follow-up is continued for a further decade or more.

10.3.2. Androgens

There is currently at least one study investigating aminogluthimide as adjunct therapy in early breast cancer, although there are no published results to date (Powles 1982). Aminoglutethimide, with concomitant hydrocortisone administration, is associated with significant depression of urinary and serum oestrogens, but maintenance of serum androgens, with the exception of serum DHEA-S. Surgical adrenalectomy suppresses both serum oestrogens and androgens, and as there is no significant difference in clinical response rates, the therapeutic value of preserving androgen secretion is uncertain (Santen 1981).

This study demonstrated both poor prognosis patients with unexpectedly long survival, and long term survivors, to have significantly depressed serum androgens. There is some indication therefore that aminoglutethimide might be of benefit in adjuvant therapy for long term survivors.

10.3.3 Prolactin

Although bromoergocryptine effectively reduces plasma prolactin, there is no clinical evidence that it is of therapeutic use in advanced breast cancer. Neither is there evidence from this, or previous studies, that clinical recurrence is related to plasma prolactin. It would therefore seem inappropriate at the present time to consider manipulation of prolactin metabolism as an effective means

of adjuvant endocrine therapy.

10.3.4 Thyroid function

Clinical trials in the use of L-thyroxine as adjuvant therapy in early breast cancer have shown no benefit. Although this study lends some credence to the longstanding hypothesis that hyperthyroidism is associated with delayed recurrence, there is, as yet, insufficient evidence on which to base a further clinical trial of adjuvant thyroxine therapy.

10.4 Future prospects

This thesis, in a study of tumour associated variables, and host endocrine status, has not produced conclusive evidence concerning the biology of long term survival. There are areas of further research that may still be profitable. When a satisfactory method for the demonstration of tumour endocrine receptors in formalin fixed material is available, a retrospective study could assess whether receptor status has a prognostic significance extending beyond ten years. Secondly it is probable that immune competence is of importance in long term survival in breast cancer, but to date, there is neither the ability to detect immune incompetence in such a population, nor to treat it effectively.

Within the present framework of our knowledge, the only means currently available to reduce the continuing late mortality of breast cancer is to attempt a second line of adjuvant endocrine, or low toxicity chemo-therapy, perhaps as patients enter their second decade of survival. There would appear to be justification in establishing a multi-centre clinical trial for this purpose, utilising tamoxifen and aminogluthimide in a multi-factorial design, with consideration to be given also to a short course of cyclophosphamide, as in a current CRC adjuvant trial for early breast cancer.

APPENDIX 3

DATA MANAGEMENT

Introduction

The production of this thesis required storage and manipulation of very large amounts of clinical, pathological and hormonal data on several thousand patients. Some of this data was available from computer storage, albeit in several different locations, and it was therefore decided, at an early point in preparation, that all data for this thesis would be computerised using the facilities of the department of computing, Imperial Cancer Research Fund (ICRF)'. Clinical and pathological data on the patients in this study were transferred from computers at the ICRF Breast Unit, Guy's Hospital, the Department of Medical Computing, Charing Cross Hospital, and the South West Thames Regional Cancer Registry, to the Digital 1020 deck at ICRF.

Data-base management

The 1022 data-base management system is a sophisticated method for fast storage, sorting and retrieval of very large amounts of data.

A data-base is composed of a large number of records, each record containing the values of the items of information required, termed attributes. The collection of records, each having the same attributes, is called a data set. A group of data-sets, possibly each composed of different attributes, makes a data-base.

This data base system was used for the management of all data in this thesis, differing data sets being constructed for each cohort of patients under study.

Statistics

After initial sorting within the 1022 data base management system, the data was manipulated in a basic statistics system, Minitab, written by the Department of Statistics, University of Pennsylvania. This programme was used for simple parametric or non-parametric tests, correlation, and linear regression. Where hormonal data was not normally distributed a log transformation was carried out which resulted in a Gaussian distribution being obtained. A parametric test (Student's t test) was then applied. The exact tests used are specified within each results section. More comprehensive statistical analysis was performed using the BMDP system of the Department of Biomathematics, University of California, Los Angeles. Life table analyses were performed on this programme for survival distribution observed over varying time periods, plotting survival, hazard, and related functions. Life table analysis was also performed on the 'Surv-c' program from the Department of Cancer Epidemiology, ICRF, Oxford.

Where hormonal data was correlated with age, analysis was by regression, with comparison of slopes and intercepts of regression lines by covariance analysis (Snedecor 1956). Graphics were plotted both on the above statistical

programmes, and on 'MLAB', a programme designed by the Department of Mathematics, for advanced mathematical modelling.

APPENDIX 6

OESTROGENS

Patients

The clinical and pathological data on the long term survivors, and early breast cancer controls studied in this and subsequent chapters, are outlined in Figs. 6A to 6D. The figures under the histograms refer to the number of patients in each subgroup.

Serum oestrogen and SHBG binding capacity assays

Materials

(1) Total oestradiol

1. Diethyl ether - anaesthetic grade

2. Phosphate buffered saline pH 7.2 (Dulbecco's method)

NaCl	10g
KCl	0.25g
Na_2HPO_4	1.44g
KH_2PO_4	0.25g

made up to 1.0 litre with distilled water.

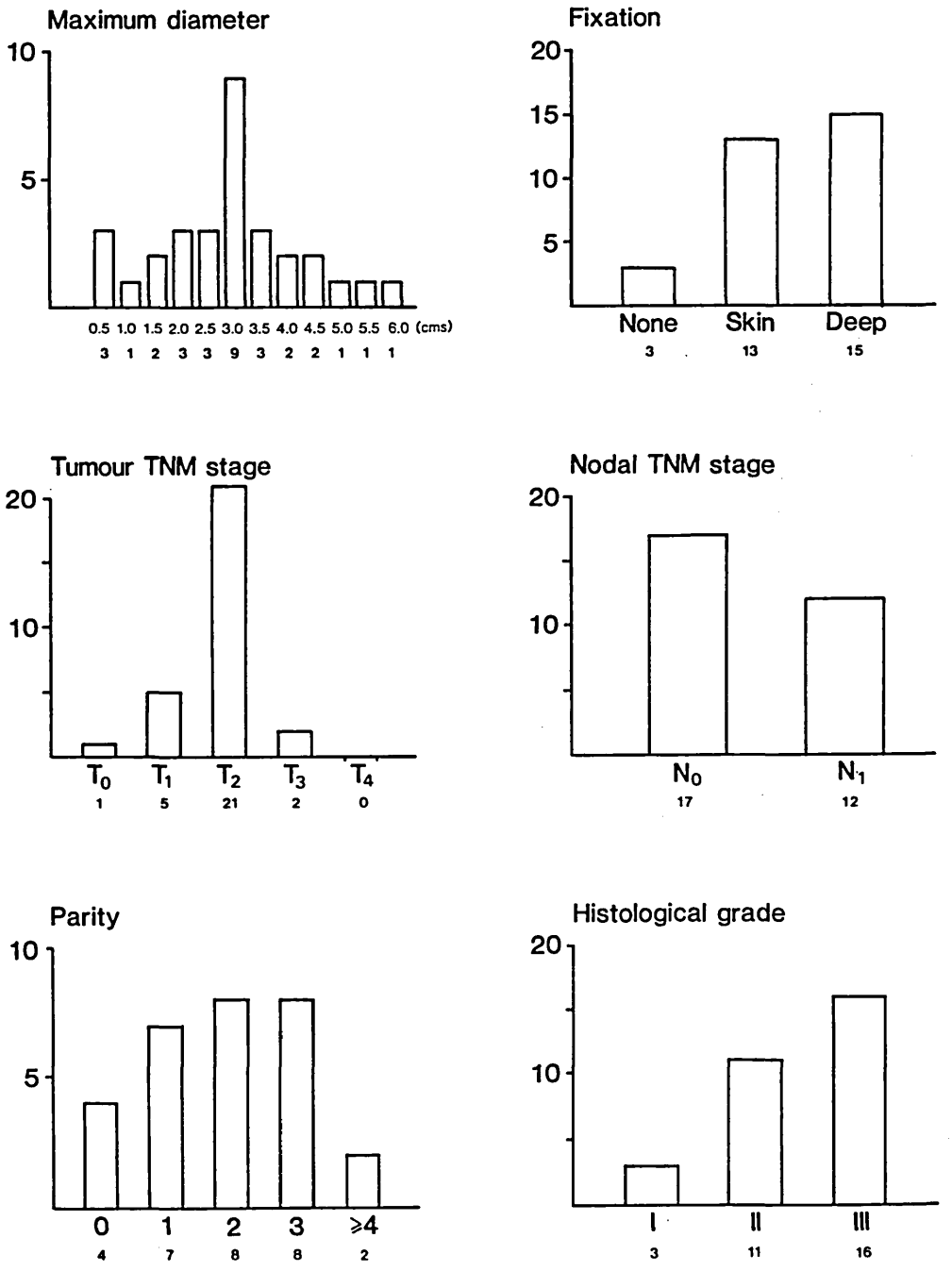
3. Dextran-coated charcoal suspension in buffer.

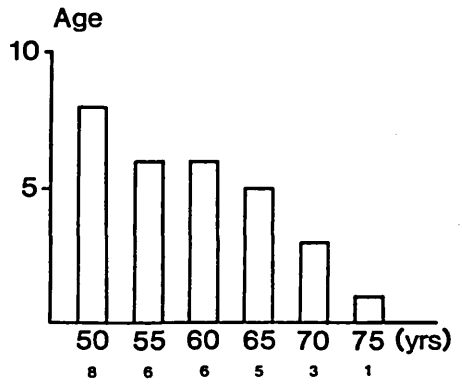
0.5% Norit charcoal

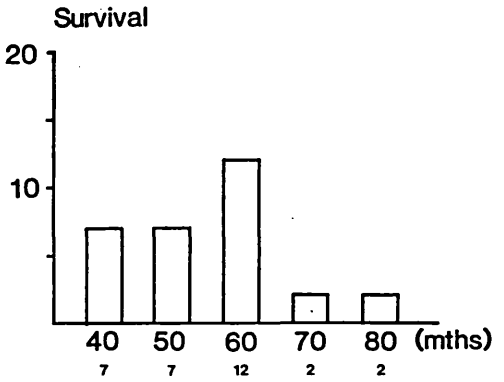
0.05% Dextran T-70

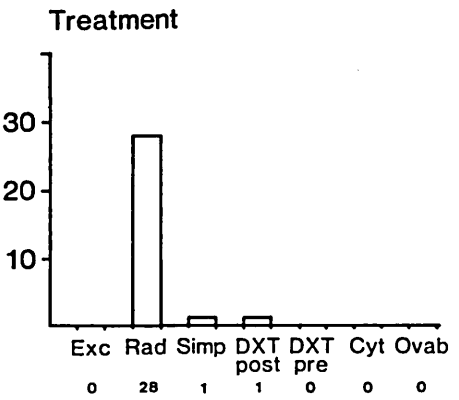
Figure 7 A

Clinical and Pathological Data: Poor Prognosis Survivors









Abbreviations

Exc	Excision or quadrantectomy
Rad	Radical mastectomy
Simp	Simple mastectomy
DXT (post)	DXT (post operative)
DXT (pre)	DXT (pre operative)
Cyt	Cytotoxic chemotherapy
Ovab	Ovarian ablation

Clinical and Pathological Data: Breast Cancer Controls

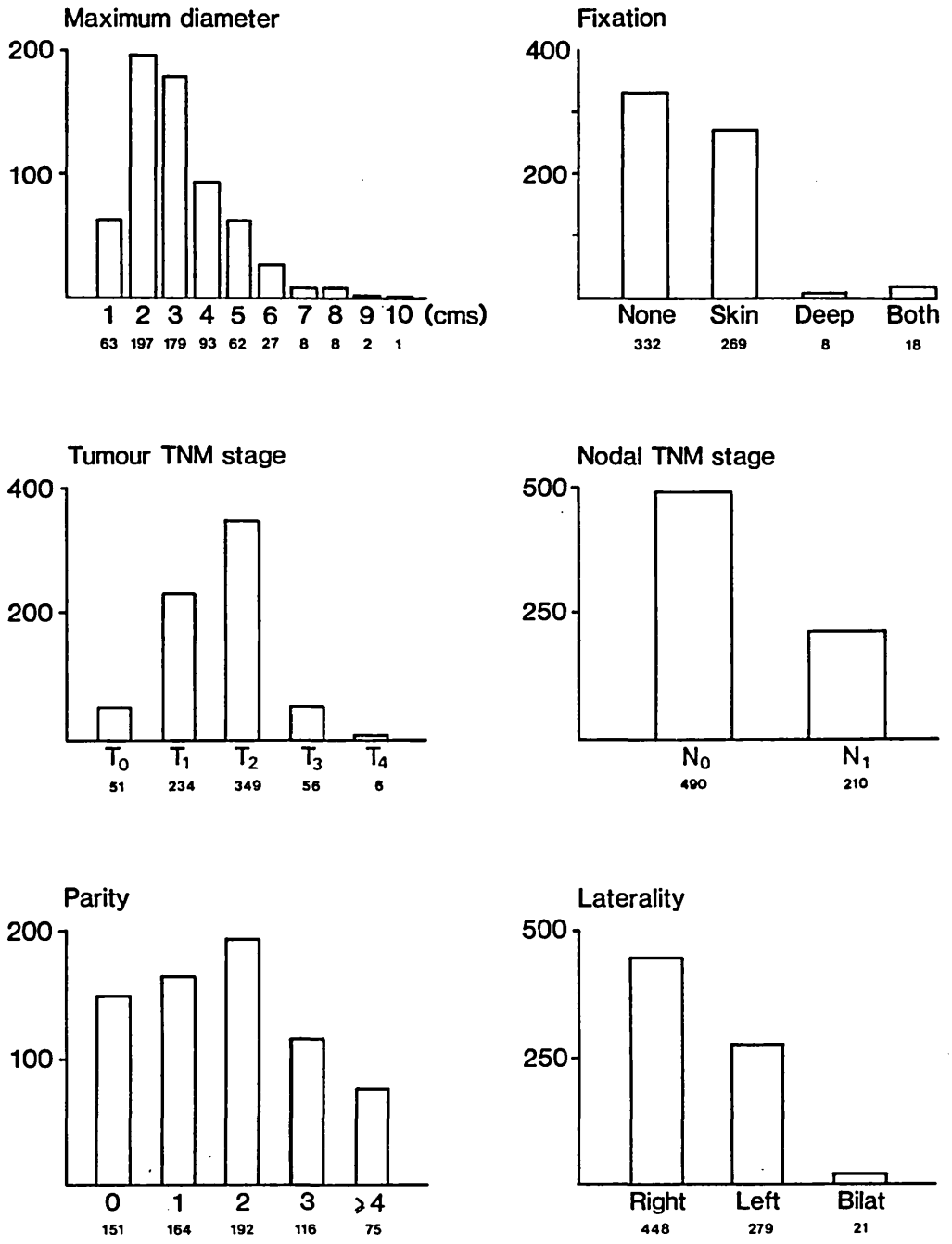
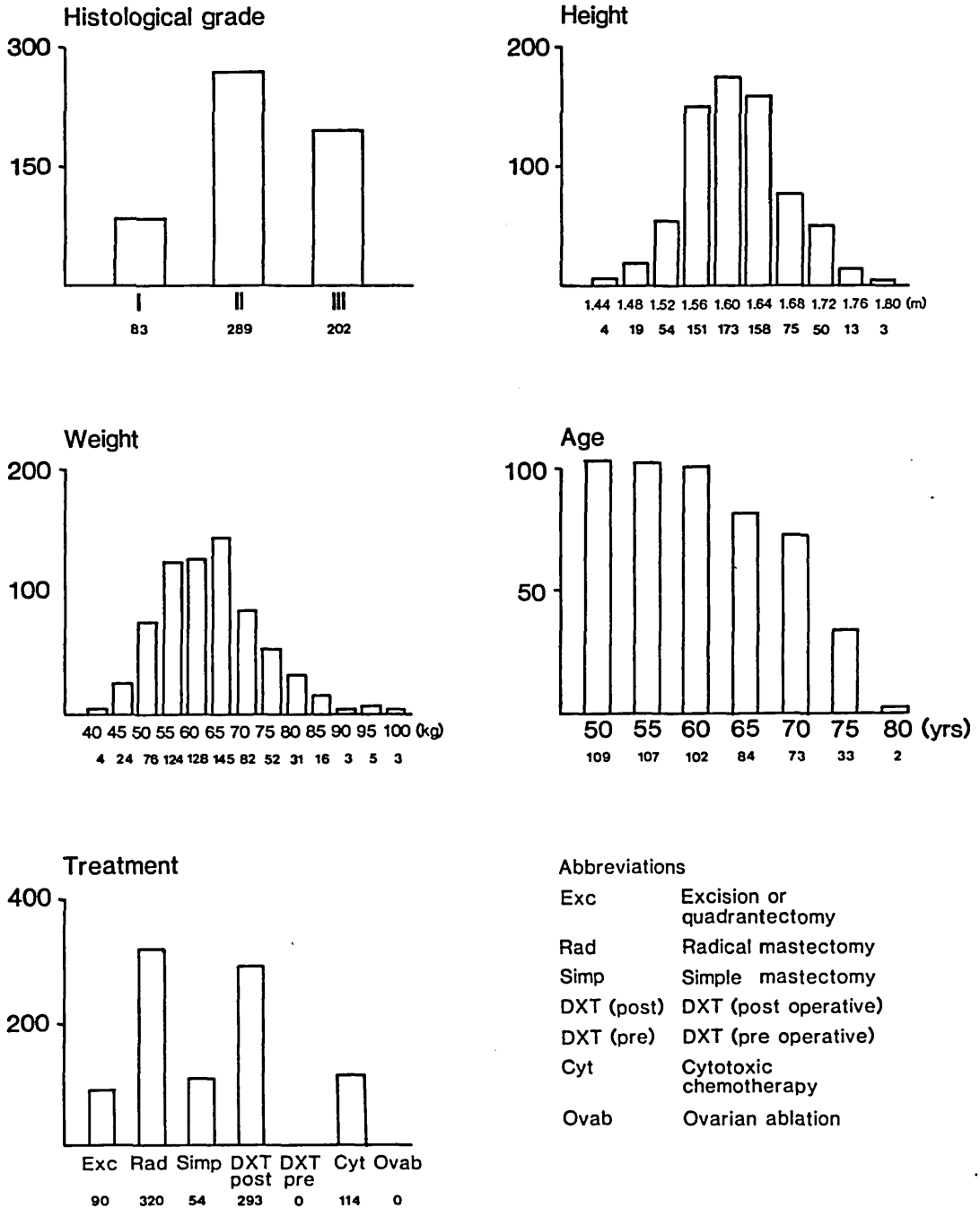


Figure 6 D



Abbreviations

Exc	Excision or quadrantectomy
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Simp	Simple mastectomy
DXT (post)	DXT (post operative)
DXT (pre)	DXT (pre operative)
Cyt	Cytotoxic chemotherapy
Ovab	Ovarian ablation

4. 2,4,6,7 (H^3) Oestradiol (Amersham)

   Purified using Lipidex column chromatography

5. Cold oestradiol

6. Antibody-label solution.

   2 parts antibody (at dilution required to give 50% - 70% displacement) with 1 part label solution (6-7000 cpm/50µl). The oestradiol antiserum was prepared at ICRF by immunisation of New Zealand white rabbits with a 6-oxo-carboxymethyloxine derivative of E_2 conjugated to bovine serum albumin by the mixed anhydride reaction. The cross reactivity of this antiserum is low, and shown in Table 6.A.

7. Scintillation fluid - Aquasol.

8. Various stock standard solutions

   a) 20 mg/100 mls ethanol
   b) 500 ng/ml ethanol
   c) 1000 pg/ml buffer

9. Working standards.

   Solution (c) was diluted in buffer to give a range of standards from 2.5 to 120 pg/200 µl buffer.

Hormone	% cross reactivity
Oestradiol 17β	100.0
Oestrone	0.9
Oestriol	2.2
Androstenedione	0.1
Testosterone	0.1
17 α hydroxyprogesterone	0.1
Progesterone	0.1
Cortisol	0.1
Cholesterol	0.01

Table 6.A Cross Reactivity of ICRF E_2 antiserum

Methods

Blood samples were taken at the time of annual clinical follow-up for the long term survivors. Normal controls had blood taken at the time of screening, and breast cancer controls on admission to hospital and prior to any treatment being undertaken.

Blood samples were centrifuged within 4 h of venesection and sera stored at -20°C until use.

The following assays were performed:

(1) Serum total oestradiol assay (Bulbrook et al 1978)

200 µl buffer containing 1300 cpm tritiated steroid was added to extraction tubes and a similar aliquot to counting tubes for a total recovery count. 3 ml serum was added to the extraction tubes and allowed to equilibrate for 30 min. 7.5 ml diethyl ether was added and extracted by vigorous shaking for 3 min, followed by freezing in solid CO_2 and acetone. The ether was decanted into conical centrifuge tubes and refrozen to ensure that no water was present. The ether was then evaporated under nitrogen and the extract taken up in 500 µl solvent mixture and applied carefully to a previously prepared Lipidex 5000 chromatography column. The columns were washed with:

1 x 500 µl solvent
1 x 1000 µl solvent
3 x 1000 µl solvent discarding eluent

The next 10 ml was collected and evaporated under nitrogen. 600 µl buffer was added to each tube and vortexed and then 200 µl was pipetted into duplicate assay tubes, and 150 µl to counting vials for estimate of recovery.

Standard curve:- standards ranging from 0-200 pg/200 ml of buffer were prepared and a standard curve constructed.
Total count and non-specific binding:- duplicate tubes were set up containing 600 and 300 µl respectively of buffer and 50 µl (7000 cpm) of label solution.

The contents of all tubes were mixed, incubated at 37°C for one hour, and left overnight at 4°C. All samples were placed in an ice bath for 10 min.
300 µl of ice-cold water containing well stirred dextran-coated charcoal suspension was added to all except 'Total count' tubes [see Table 6.B].
These were then Centrifuged at 2000g for 15 min.
500 µl of supernatant was removed to the mini-counting vials and 4.5 ml of scintillation fluid added, before mixing and placing in scintillation spectrophotometer and counting to 1% efficiency or 10,000 cpm. The results of unknown samples were calculated by interpolation of the values into the the standard curve.

Tube	Buffer	Std/Sample	Label	Antibody	DCC	Total Vol.
Total	600	-	50	-	-	650
Non-specific binding	300	-	50	-	300	650
Zero	200	-	50	100	300	650
Std/Sample	-	200	50	100	300	650

Table 6.B Total E_2 method

(2) Free oestradiol

Dialysing membranes are required in addition to the stock solutions and reagents described for total oestradiol. The membranes were manufactured from Visking tubing following boiling in 95% ethanol, 3% sodium bicarbonate plus EDTA, and finally distilled water.

Ultrafiltration vials were made comprising an internal glass tube over which the membrane was stretched, being held in place by latex tubing. Glass scintillation counting vials were used as the external tube, each containing three filter paper discs at its base [see Fig. 6.E]. The [^{3}H] labelled steroid is purified on a Lipidex (Packard) column.

(3) Sex hormone binding globulin

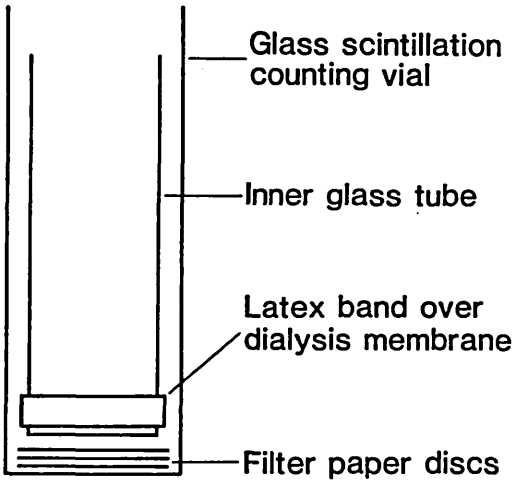
1. 5 α [1,2,4,5,67-3H] Dihydrotestosterone purified on Lipidex chromatography column using hexane: chloroform (50:50) mixture as eluting solvent. Solution should contain 80,000 cpm (Amersham).

2. Non-labelled 5 α DHT recrystallised from methanol and prepared as 680 nmol/l solution in acetone.

3. Cibachrome Blue F3G-A (Serva Biochemicals).
   Sepharose 6B (Pharmacia Fine Chemicals).
   Sephadex LH-20 suspended in Tris buffer.

Figure 6 E

Ultra Filtration Vial



4. Tris buffer (pH 7.4)

Hydroxymethyl-aminomethane	30.285 g
Calcium chloride	5.55 g
Distilled water	5.0 l

(2) Serum free oestradiol assay (Hammond et al 1980)

Triated steroid (80,000 cpm) was added to test tubes in a chloroform: hexane solution (50:50) and the solvent evaporated in a heating block under nitrogen. 7000 cpm of ^{14} glucose in distilled water was added, followed by 450 µl of serum. The test tubes were mixed in a vortex mixer and incubated at 37°C for 1 h and then mixed again and left for a further 1 h at room temperature.

During this time the ultrafiltration vials were thoroughly cleaned and dried and three filter paper discs placed at the base of each vial. 200 µl of incubate was added, in duplicate, to the inner vial, which was capped and centrifuged at 3000 g for 1 h at 37°C.

After centrifugation the inner vial was carefully removed and 30 µl transferred to a scintillation vial also containing three filter paper circles inside the vial. 350 µl of water were added to both sets of scintillation vials, which were then mixed vigorously. Scintillation fluid was added and the [^{3}H:^{14}C] ratio for 'inside' and 'outside' vials counted. [^{14}C] spill was checked by counting vials containing [^{14}C] and water only.

Calculation

After correcting for background counts and [^{14}C] crossover to [^{3}H] the calculation was as follows:

$$\text{non-protein bound steroid} = \frac{[^{3}\text{H}]\ \text{steroid (cpm)}_{\text{OUTSIDE}}}{[^{14}\text{C}]\ \text{glucose (cpm)}} \div \frac{[^{3}\text{H}]\ \text{steroid (cpm)}_{\text{INSIDE}}}{[^{14}\text{C}]\ \text{glucose (cpm)}} \times 8$$

(3) Sex hormone binding globulin (SHBG) assay (Iqbal and Johnson 1977)

The two tier column was prepared by soaking Sephadex LH-20 in Tris buffer overnight prior to application of the gel to the column. Following further rinsing with Tris buffer, Cibachrome Blue coupled to Sepharose 6B was added to the column. Uncoupled dye was removed by washing the column with Tris buffer.

100 µl of serum was diluted with 400 µl of buffer and a 400 µl aliquot transferred to another tube containing labelled 5 x dihydrotestosterone (DHT) (80,000 cpm) and 0.068 nmol of unlabelled DHT. The contents were mixed and incubated for 1 h at 4°C. A 5% albumin solution in Tris buffer was used to monitor non-specific binding.

An aliquot (100 µl) of each incubate was transferred to a scintillation vial together with 2.7 ml Tris buffer and set aside for the 'Total' count. Two aliquots (100 µl) were pipetted on to the surface of the two tier column and washed sequentially with 100 µl, 200 µl, 500 µl and 2 ml of Tris buffer.

All eluates were collected in scintillation vials and radioactivity counted for 'Totals', 'Blanks', and samples.

Calculation

SHBG binding capacity was calculated from the known specific activity of the added label as follows:

$$\text{SHBG binding capacity (nmol/l.)} = \frac{\text{specifically bound (cpm)} - \text{non-specifically bound (cpm)} \times 860}{\text{Total (cpm)}}$$

Coding

Serum samples for analysis of both cases and controls were coded and randomly distributed between assay batches. All assays were performed in duplicate, and samples from quality control pools were included in each batch.

APPENDIX 7

ANDROGENS

Patients

The clinical and pathological data on the poor prognosis survivors studied in Chapter 7 are described in Figs. 7A and 7B. The figures under the histograms refer to the number of patients in each subgroup.

Serum Androgen Assays

Materials

1. [^{3}H] DHEA-S, [^{3}H] Adiol., and [^{3}H] DHEA were obtained from Amersham International plc.
   Non-radioactive DHEA-S, Adiol., and DHEA were obtained from Steraloids Ltd. and recrystallised from methanol prior to use.

2. Autisera were produced by the ICRF by immunisation of New Zealand white rabbits with 7-oxo-carboxymethyloxime derivatives of DHEA and Adiol. The cross-reactivity of these sera are shown in Tables 7.A and 7.B and are seen to be extremely low.

3. Dextran-coated charcoal suspension

Norit A charcoal	0.5%
Dextran T-70	0.05%

4. Phosphate buffered saline (0.16M)

Clinical and Pathological Data: Poor Prognosis Survivors

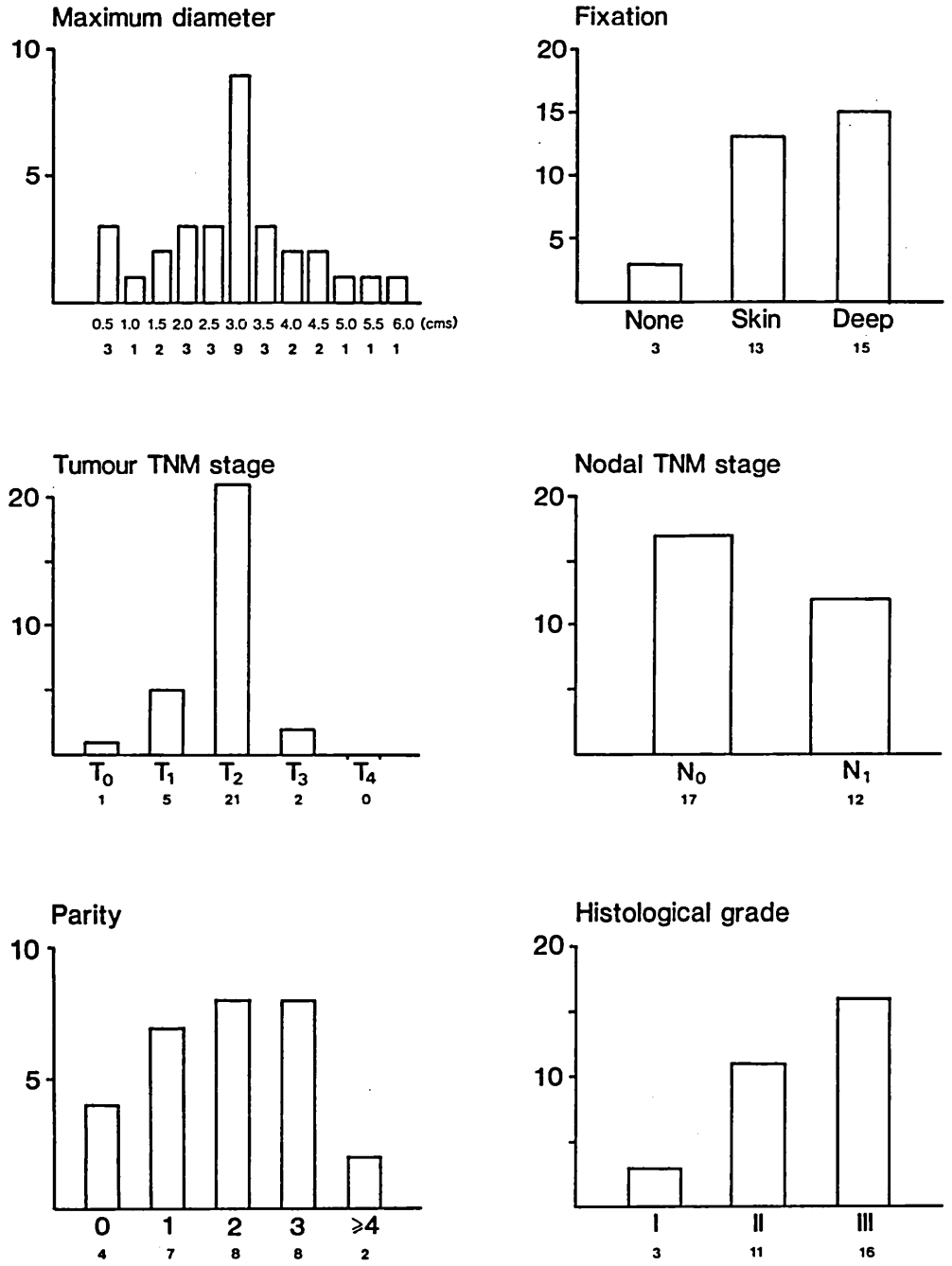
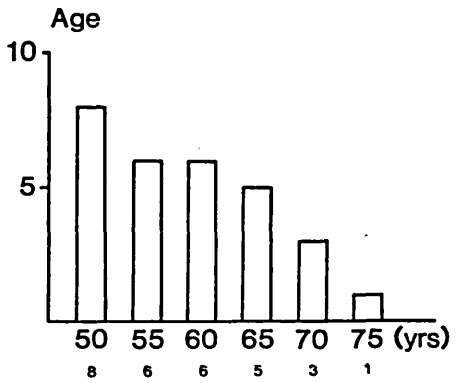
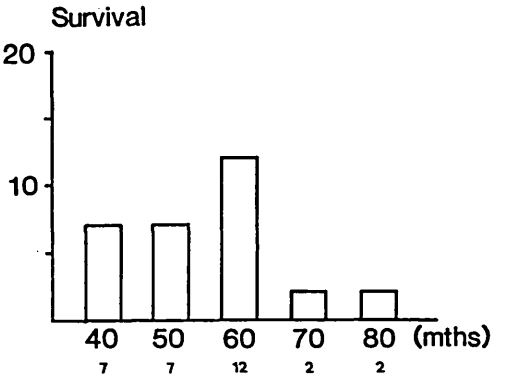
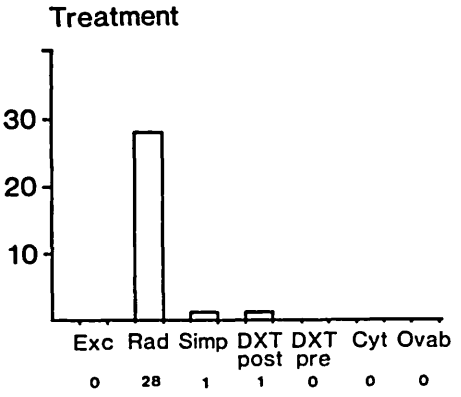


Figure 7 B







Abbreviations

Exc	Excision or quadrantectomy
Rad	Radical mastectomy
Simp	Simple mastectomy
DXT (post)	DXT (post operative)
DXT (pre)	DXT (pre operative)
Cyt	Cytotoxic chemotherapy
Ovab	Ovarian ablation

Steroid	Cross reaction %
5-Androstene-3β,17β-diol (androstenediol)	100.000
5-Androstene-3β-ol-17-one (DHEA)	0.025
5-Androstene-3β-ol-17-one (DHEA sulphate)	0.006
5-Androstene-3β-16a-diol-17-one (16a-hydroxy DHEA)	0.05
4-Androstene-3β,17β-diol	1.11
4-Androstene-17β-ol-3-one (testosterone)	0.05
5a-Androstane-17β-ol-3-one (dihydrotestosterone)	0.166
5a-Androstane-3a,17β-diol	0.005
5β-Androstane-3a,17β-diol	0.005
5a-Androstane-3β,17β-diol	6.05
4-Pregnene-11β,17β,21-triol-3,20-dione (cortisol)	0.0007
4-Pregnene-3,20-dione	0.001
5-Prenene-3β-ol-20-one (pregnenolone)	0.010
5-Cholestene-3β-ol (cholesterol)	0.003
1,3,5(10)-estratrien-3,17β-diol (oestradiol)	0.08

Percent cross-reaction is expressed as the ratio of A/B x 100, where A = the amount of androstenediol and B = the amount of competitor required to reduce binding of [3H] androstenediol by 50%. Where, due to limited solubility of a steroid, this inhibition of binding was not possible, the ratio at the maximum inhibition of binding was used.

Table 7.A
Specificity of Antiserum to Androstenediol-7-CMO-BSA

Steroid	Cross reaction %
5-Androstene-3β-ol-17-one (DHEA)	100.000
5-Androstene-3β-ol-17-one KS04 (DHEA sulphate)	6.325
4-Androstene-3, 17-dione (androstenedione)	1.900
5-Androstene-3β,17β-diol (Adiol)	2.200
17β-hydroxy-4-Androstene-3-one (Testosterone)	0.060
5a-Androstane-17β-ol-3-one (DHT)	0.100
5a-Androstane-3β,17β-diol	0.200
5a-Androstane-3β,17β-diol	0.010
5a-Androstane-3a-ol,17-one (androsterone)	0.114
5a-Androstane-3a-ol,17-one KS04 (Androsterone sulphate)	0.024
5β-Androstane-3β-ol-17-one (aetiocholanolone)	0.026
5-Pregnene-3β-ol-20-one (pregnenolone)	0.008
4-Pregnene-11β,17a,21-triol-3,20dione (cortisol)	0.001
4-Pregnene-11β,17a,21-triol-3,20-dione (progesterone)	0.036
5-Cholesterol-3β-ol (Cholesterol)	0.006
1,3,5(10)Estratrien-3-ol-17-one (Oestrone)	0.330
1,3,5(10)Estratrien-3,17β-diol (E2)	0.020

Percent cross-reaction defined as in Table 7.A

Table 7.B Specificity of antiserum to Dehydroepiandrosterone-7-CMO-BSA

5. Assay buffer

1g gelatin

1g sodium azide

1l phosphate buffered saline

6. Various stock standard solutions (BDH chemicals)

7. Scintillation fluid - Aquasol.

Methods

(1) Serum DHEA-S Assay

25µl plasma samples were added to acetate buffer containing 8000 cpm of tritiated DHEA-S. This mixture was extracted with ethyl acetate to remove non-conjugated steroids and lipids, and the aqueous phase was then heated at 120°C for 1½ h to hydrolyse DHEA-S. After cooking, the hydrosylate was extracted with isopentane which was then evaporated at 45°C.

The residue was dissolved in 1 ml of RIA buffer and left for a minimum of 1 h, or overnight. Aliquots of 0.5 ml were taken for estimation of recovery, and triplicates of 50µl for assay. 50µl aliquots of seven standards were taken in serial dilution from 4ng/ml to 63pg/ml.

Extracts and standards were pipetted into assay tubes into which were added 50µl of tritiated DHEA-S (12,000 cpm), 50µl

of antibody, and 350µl of buffer. To assess non-specific binding 350µl of buffer was added to 50µl of labelled DHEA-S.

All assay tubes were left to incubate at 37°C for one hour and then at 4°C for a further hour. 300µl of ice cold dextran-coated charcoal was added to all assay tubes (except 'total') and following vortexing, they were left at 4°C for a further 30 min. The tubes were centrifuged at 2000g for 15 min and then 500µl of supernatant was transferred to a counting vial and 4.5ml of scintillation fluid added. Tritium was counted to 1% accuracy and calculation of serum samples estimated from the standard curve using a computer programme.

(2) Serum DHEA and Androstenediol assay

DHEA and Androstenediol were extracted on Lipidex 5000 chromatography columns. The gel was poured into 12cm glass columns to a height of 6 cm with a filter paper disc at the base of the column to prevent leakage. Hexane: chloroform (50:50) mixture was used to wash the column prior to use.

Serum samples of 200µl were pipetted into extraction tubes and 6,7-^{3}H Adiol (10,000 cpm) or 6,7-^{3}H DHEA (10,000 cpm) added. The extraction tubes were incubated at room temperature for 30 min following which 10 ml ethyl ether was added and the tubes vortexed. The aqueous layer was frozen

in a solid CO_2 and acetone bath and the ether was then decanted into a conical centrifuge tube and evaporated to dryness. The extract was dissolved in buffer and applied to the chromatography column for separation using a hexane: chloroform (93:7) eluting mixture. A 200µl aliquot was taken for recovery estimation and duplicate 200µl aliquots for sample estimation.

The radioimmunoassay was performed as for DHEA-S using specific antisera for DHEA or Adiol.

APPENDIX 8

PROLACTIN

Plasma Prolactin Assay

Materials

1. Purified human prolactin (MRC reference preparation, MRC 71/222) containing the equivalent of 0.3 µg highly purified prolactin.

2. Anti-human prolactin (hPrl) sera derived from immunisation of a highly inbred strain of Long-Evans derived rats.

3. ^{125}I labelled hPrl was prepared using a Chloramino T method on 10µg amounts of freeze dried lyophilised hPrl, resulting in an iodination efficiency of 60-90%. This was applied to a Sephadex G 75 chromatography column to obtain the monomeric form of labelled hPrl.

Sampling

Blood sampling was performed on long term survivors and normal women between 2.00 - 5.00 p.m., and on women with breast cancer between 8.30 - 10.30 a.m. The poor prognosis survivors were re-bled, at the time of survival, between 2.00 - 5.00 p.m. The blood was placed in non-heparinised containers and centrifuged within 2 hours. Plasma was stored at -20°C until hormone assay was carried out.

Method (Kwa et al 1973)

A homologous double-antibody radioimmunoassay method was used. All plasma samples were assayed undiluted and at four serial dilutions. The results were checked for parallelism with the reference preparation. The assay was carried out so as to have the greatest discriminatory power in the region between 12.5 and 25.0 ng/ml; i.e. the dilution of the specific antiserum and the amount of label were so chosen as to result in an inhibition curve of plasma samples of the indicated range of prolactin levels in undiluted plasma in which at least four points would fall on the straight part of the S- curve. (Maximal binding by antiserum excess ranges from 80 to 92% of total radioactivity in the bound fraction; for the inhibition curves antiserum dilution resulting in 60% of this maximum binding is employed; at minimal inhibition 48-55% of total radioactivity is precipitated, at maximal inhibition 4-8% remains in the 'bound' form. The straight part of the S-curve ran from 10-14% to 42-46% in the four serial dilutions).

The mean prolactin level and the standard deviation expressed as a percentage of the mean were calculated for each individual plasma sample at dilutions 2°, 2^{-1}, 2^{-2}, 2^{-3} and 2^{-4}, by extrapolation of each point considered to fall on the straight part of the plot nearest to the two points on the reference curve. This yielded as many estimates of each sample as there were points on the straight part of the

plot. An automated serial dilutor was used which enabled all plasma samples to be processed in a single batch, together with the internal laboratory reference preparations (in nine serial dilutions).

APPENDIX 9

THYROID FUNCTION

Serum Free Thyroxine Assay

Serum samples were taken as previously described [see Appendix 6].

Materials

1. ^{121}I thyroxine derivative solution.
2. T_4 antibody (Amerlex).
3. Reference standards of serum free T_4.

Method

100µl aliquots of both serum samples and reference standards were pipetted into tubes. 500µl of the ^{125}I thyroxine was then pipetted into these tubes, plus two additional ones in order to obtain the 'Total' count. 500µl of T_4 antibody was added to each tube and then all tubes were vortexed and left standing at 37°C for 1 h. All tubes were centrifuged at 1500 g for 15 min, the supernatant was decanted and the tubes were then counted in a gamma counter for the time required to accumulate at least 20,000 counts in the lowest reference standards.

Calculation

After subtraction for background counts a standard curve was produced by a log-log plot and the best straight line drawn through the results obtained for the reference standards. The unknown samples were calculated from the plot.

Duplicates of samples, and known controls were included in each batch. All reference standards were also calculated in duplicate.

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