UNIVERSITY OF SOUTHAMPTON.

FACULTY OF MEDICINE.

DEPARTMENT OF CELLULAR PATHOLOGY.

THE USE OF FINE NEEDLE ASPIRATION CYTOLOGY FOR THE IMMUNOLOGICAL DEMONSTRATION OF OESTROGEN RECEPTOR AND PROLIFERATING CELL ANTIGENS, AND THE CYTOLOGICAL GRADING OF BREAST CARCINOMA.

by Ann Cuthbert.

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ABSTRACT.

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THE USE OF FINE NEEDLE ASPIRATION CYTOLOGY FOR THE IMMUNOLOGICAL DEMONSTRATION OF OESTROGEN RECEPTOR AND PROLIFERATING CELL ANTIGENS, AND THE CYTOLOGICAL GRADING OF BREAST CARCINOMA.

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Fine needle aspiration cytology has enabled cellular material to be collected pre-operatively and in recurrent and inoperable breast carcinoma, for the immunological identification of oestrogen receptors and proliferating cell antigens.

Previously, oestrogen receptor analysis had only been available on resected tumour tissue, using a biochemical technique. Immunocytochemistry enabled the oestrogen receptor antigen to be identified in individual cells. Preserving their pathology, identifies the cells as benign or malignant and ensures only positive malignant cells are included in the final analysis. It is the aim to identify those tumours most likely to respond to the anti-oestrogen therapy Tamoxifen.

Assessment of other antigens concerned with the biology of breast cancer, led to the demonstration of the proliferating cell antigens Ki67 and EGFR. Ki67 enabled discrimination between slowly or rapidly dividing populations of cells, with the aim of identifying tumours with a poor or particularly good clinical prognosis.

In order to assess the consequences of cell proliferation it was necessary to devise a grading system tailored specifically to cytological specimens. The histological grading system was inadequate for the complete assessment cytological fine needle aspiration samples, as it requires the identification of architectural patterns, which do not pertain to discohesive cells.

All of these parameters described are an aid to the diagnosis and assessment of breast cancer. With the introduction of the breast screening programme for women aged fifty to sixty-five years in the United Kingdom, many new techniques are being employed to aid the pathologist in the identification of ever smaller breast tumours.

Fine needle aspiration cytology is the ideal technique for collecting sufficient cellular material for morphological diagnosis and biological assessment, in both symptomatic carcinomas and tumours from the breast screening patient.

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1.0 INTRODUCTION.

In the United Kingdom breast cancer is the commonest form of cancer among women. Every year there are approximately 24,000 new cases and 15,000 deaths from the disease. Some sight increases have been achieved in survival following treatment for breast carcinoma, using advances in surgery, radiotherapy, chemotherapy and hormone therapy, but early detection by breast screening has been shown to be the only effective method of reducing mortality. (Forrest 1986).

The clinical stage at which symptomatic breast carcinomas are detected is important for prognosis as is the histological grade, although the assessment of individual cases can be extremely subjective. Less is known about prognostic factors in screen detected carcinomas. A reliable method of demonstrating the rate in which breast tumours grow and proliferate would be helpful in deciding the prognosis of the disease and the extent of therapy required.

One of the most widely used markers of prognosis and response to treatment is oestrogen receptor (ER) status. The immunocytochemical demonstration of ER has provided an accurate method assessing this status. Fine needle aspiration cytology (FNAC) has provided a method of collecting unfixed cellular material in an atraumatic way, making the immunocytochemical assessment of ER applicable to many clinical situations, in screen detected lesions, preoperatively and in advanced or recurrent disease.

1.1 FINE NEEDLE ASPIRATION.

FNAC as the name suggests employs a fine gauge needle (18-23 gauge) to aspirate a sample of cells from a solid tumour. A 20 ml. syringe produces the negative pressure to allow the aspiration of cells from the lesion. FNAC was first employed in the United States in 1927, to obtain cells from a variety of anatomical sites, but despite this was not practiced widely for a number of years. (Martin and Ellis 1930, Stewart 1933). FNAC of the breast for the diagnosis of breast neoplasms, was pioneered in Sweden in the 1960's. (Frazen and Zajicek 1967).

FNAC has been used in Southampton for the pre-operative diagnosis of breast cancer since 1980. A 23 gauge needle is used with a 20 ml. syringe, placed in a commercially

available 'Cameco' syringe pistol. The needle is introduced into the lesion and the plunger drawn back with the aid of the pistol, creating negative pressure in the barrel. The needle is advanced and retracted repeatedly in a number of planes and the negative pressure released before the needle is withdrawn from the lesion. The needle is then removed and air drawn into the syringe in order to expel the material onto glass slides. At the Southampton symptomatic breast clinic, the cytological diagnosis is available when the women visits the clinic the following week, together with the clinical assessment and the mammography result. This allows the patient to discuss the diagnosis with the surgeon and the specialist breast care nurse. (Smallwood et al 1984). It is to the patient's benefit that all the treatment options should be considered and an informed choice be made wherever possible, as to the type of therapy to be carried out. (Scalon et al 1984).

The procedure is considered to be relatively painless, and is well tolerated by the vast majority of patients. No local anaesthetic is used (Zajdela et al 1974), as this might disrupt the cytological structure of the cells to be retrieved. Practice is required to be proficient in performing the FNAC technique and in preparing good cytological smears from the material. Correct technique is essential for the success of this sampling procedure (Frable 1983).

FNAC of a discrete breast lump may reveal fluid rather than the semi-solid material of a solid lesion. This denotes a cyst which is the commonest abnormality of the female breast. The cyst is aspirated until dry and the breast is examined immediately afterwards for any residual mass (Forrest et al 1975). If a residual mass is found a second aspirate is indicated. Routine cytological examination of cyst fluid has not proved valuable (Patey et al 1953). However Forrest et al reported 4 cases of breast cancer at the site of a cyst: all four cyst aspirates had contained gross amounts of blood. (Forrest et al 1975).

Where possible one FNA (Fine Needle Aspirate) sample is used to produce a number of preparations on microscope slides for different analytical techniques. An FNA from a suspected clinical carcinoma would be expected to yield five microscope slides of material for morphological diagnosis and another five slides for immunocytochemical techniques. This is not possible on every occasion and morphology takes precedence over special techniques if there is insufficient material for both. At least two slides are taken routinely into liquid nitrogen for oestrogen receptor assessment: they can be retrieved for conventional staining if necessary, in order to obtain a morphological diagnosis for the patient. It is, however impossible, to carry out oestrogen receptor

immunocytochemical analysis (ER-ICA) on slides which have been air dried or alcohol fixed rather than frozen in liquid nitrogen.

1.2 IMMUNOCYTOCHEMISTRY.

Immunocytochemistry originated in the 1940's and 1950's when Albert Coons and his colleges first labelled antibodies with fluorescent dye and used them to localize specific antigens in tissue. (Coons et al 1950).

Antibodies are produced by immunising an animal, with the specific antigen to be studied. Rabbits, rats, swine and mice have all been used for this purpose. After a period of time the donor animals serum will contain a mixture of antibodies. These oligo-clonal antibodies will react with different amino acid sequences in the hapten, the original peptide used for immunization. (Polak and van Noorden 1984).

The development of monoclonal antibodies enables more specific identification of antigenic molecules. The antibodies are raised by immunizing mice, after which lymphocytes from the spleen, the source of the antibodies, are fused with mouse myeloma cell lines in culture. (Gerdes et al 1983). This fusion allows the antibody containing cells to grow and divide in culture producing a greater quantity of the antibody. Antibodies to specific fragments of molecules or epitopes may be produced in this way and this method allows a continuous supply of standard antibodies. It may be necessary to employ a 'cocktail' of a number of different monoclonal antibodies, raised against different epitopes on the same antigenic molecule, to produce the best immunocytochemical stain. (Polak and van Noorden 1984).

The direct technique was replaced by an indirect immunofluorescence method. (Coons et al 1950, Coons et al 1955). Here the primary antibody is not conjugated but a second layer antibody is added, which is raised to the gamma-globulin of the species which donated the first antibody, the second layer being conjugated to the dye. Enzyme labelling of the secondary antibody was introduced using a horseradish peroxidase molecule. (Nakane and Pierce 1966). The peroxidase is visualised by a histochemical reaction using 3'3 diaminobenzidine tetrahydrochloride (DAB), producing a permanent brown end-product. (Graham et al 1966).

Further development of the indirect technique has produced a very sensitive double immunoglobulin bridge (Mason et al 1969) or peroxidase anti-peroxidase (PAP) method. (Hsu et al 1981). This involves a third layer antibody which is formed from two peroxidase molecules, forming a stable peroxidase anti-peroxidase complex. This leaves two unbound peroxidase molecules for demonstration by DAB. (Heyderman 1979). This method results in 100 to 1000 times higher sensitivity than the indirect technique. It allows a much higher dilution of the primary antibody, reducing non-specific staining. The tertiary antibody is immunologically bound together and not chemically conjugated, as in the previous methods, therefore it loses none of its enzymatic activity. (Polak and van Noorden 1984).

The Strepavidin Biotin Complex (ABC) is even more sensitive than the PAP technique. Avidin is a large glycoprotein found in egg white, which has a high affinity (four binding sites per molecule), for biotin. Biotin is a vitamin of low molecular weight found in egg yolk, which can be used to label antibodies in the same way as peroxidase but in higher molecular proportions. In this method the primary unconjugated antibody attracts the biotinylated secondary antibody. The third layer is made up from a complex of the avidin and biotinylated peroxidase which have been reacted together in such proportions so that some biotin binding sites on the avidin molecule remain free to react with the biotin labelled secondary antibody. The peroxidase is then developed by DAB as before. With this method a large amount of peroxidase is attached to each primary antigen site, increasing sensitivity. (Hsu et al 1981).

Immunocytochemistry is an important analysis procedure and may be used to great effect providing the limitations of the method are understood. Selecting the appropriate method and attention to protocol detail will produce accurate analysis, as will appropriate fixation of the specimen and the treatment of endogenous peroxidase, which may well be a normal occurrence in the tissue. Positive and negative controls should always be included.

1.3 FEMALE BREAST TISSUE.

Histologically the breast is a relatively simple organ with only two main epithelial cell types. However, the control of the tissue by hormonal influence renders the situation much more complex. The breast remains quiescent until puberty when under the

influence of steroid hormones, particularly oestrogens, the glandular epithelium proliferates. Both ductal and lobular structures can be distinguished, the lobular cells taking on a secretory appearance. Myoepithelial cells surrounding these structures are also increased in number under the hormonal stimulation. Opinions have differed in the past as to whether myoepithelial and epithelial cells have the same origin. Although some have postulated a smooth muscle origin in view of the contractile nature of the myoepithelial cell, the evidence points to a common stem cell for both cell types. (Vorherr 1974).

This dilemma may potentially be solved using tissue culture methods. However, cells from the breast epithelium have proved difficult to nurture in tissue culture, for a variety of reasons. The proliferative stages are under hormonal control and there is a need for other growth factors for in vitro growth systems. There is also difficulty in disaggregation of breast tissue, which consists of epithelial strands interspersed with stromal fibrous elements, these can prove difficult to separate in the laboratory. (Whitehead 1986). Similar problems are encountered in producing tissue culture specimens from breast tumours. Contamination with fibroblasts may be a problem in many breast cancer cell lines, raised from primary tumours. Therefore metastatic disease sites, have been used to harvest breast tumour cells, in particular from body effusions. Fibroblasts are a rare occurrence in effusions although reactive mesothelials may occasionally cause problems. However, most breast cancer cell lines have been developed from this type of metastatic breast tissue, including the cell line MCF7. (Soule et al. 1973). MCF7 cells are the type used for controlling the oestrogen receptor analysis using reagents from Abbott Laboratories. (See section 2.6.1.).

With the development of new breast cell lines which retain many more of the features of breast tumour tissue than anaplastic cells from metastatic deposits, there will be a better understanding of the nature of breast carcinoma. The interaction of breast tissue with hormones and other drug therapies used in the control of breast cancer, may also produce clinical benefit. (Whitehead 1986).

As information on breast tumour tissue increased there was a need for a system to characterize the nature of breast tumours. A system was developed over 30 years ago and has been updated by a number of subsequent committees from the Committee of International Union against Cancer (UICC) in Geneva. (Beahrs 1984). Since tumours may be discovered at different stages of the disease process a system is required to characterise the cancer and evaluate it. This system sets out the procedures for classifying the primary (T)umour, by the size of the diameter and by attachment to

skin or the chest wall; (N)odal involvement, their site and whether they are attached to one another and distant (M)etastases. TNM status may be used as a clinical diagnostic classification or as a post surgical pathological classification.

1.4 HISTOLOGICAL CLASSIFICATION.

Carcinoma of the breast may present in a wide variety of histological patterns. The morphological classification of invasive breast cancer has been used for a number of years and a variety of classification schemes have been employed.

The categories used in this study are those employed routinely in Southampton for the description of breast neoplasms. These have been identified as major histological types. (Sakamoto 1987).

Infiltrating ductal carcinoma, with no special features accounts for 70-80% of breast carcinomas. Mixed histological patterns may occur but if the tumour contains 50-90% of cells showing no special features, it should be considered as a ductal carcinoma, although the special patterns will be described. Infiltrating ductal carcinoma may be stellate, multinodular or circumscribed. There may be extreme variations in the pattern with large or small, or tightly cohesive or loosely aggregated bodies of cells. (See photograph A.) Tightly cohesive islands may have irregular shapes, occasionally with central lumina. Interlacing aggregates of frequently spindled cells may also be seen. It is these infiltrating ductal lesions which can be divided into three histological grades according to their histological pattern, nuclear pleomorphism and mitotic rate. (see section 1.7.). Intraduct carcinoma, has been identified separately from the infiltrating ductal carcinomas and can be distinguished histologically by the intact basement membranes surrounding the malignant epithelium.

The special types of breast carcinoma are not usually included in the grading system, but are described by their histological characteristics. It is important to identify them as this may affect the clinical treatment and prognosis.

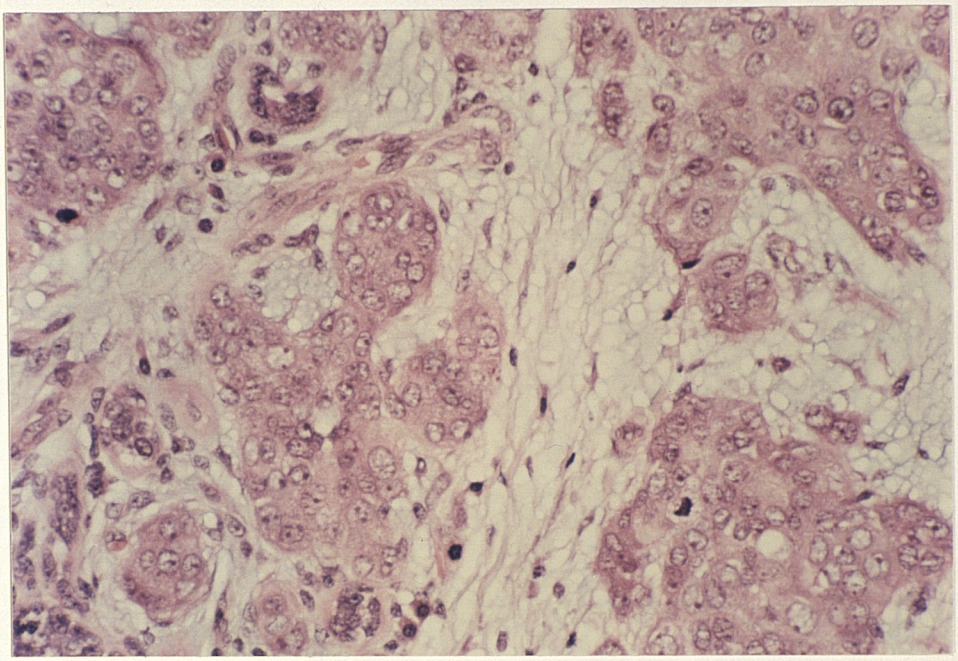
Tubular carcinoma is identified histologically by tubular structures which may be round or oval, characteristically distorted to form a tear-drop shape. The tubules are lined by a single layer of orderly epithelium. These tubules are distributed through a characteristic fibrous stroma. (See photograph B.)

Medullary carcinoma is characterised by syncitial islands of cells which crowd adjacent breast tissue without invading and with no apparent capsule. The cells have large nuclei and copious cytoplasm. Most typically there is a prominent lympho-plasmacytic infiltrate around and between the tumour islands. (See photograph C.)

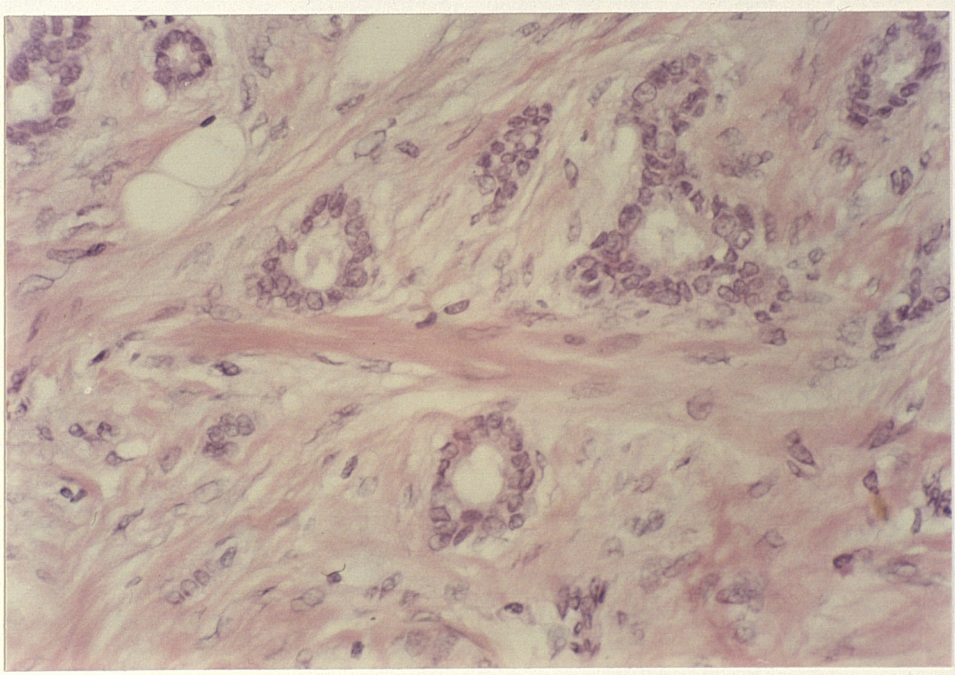
Lobular carcinoma is unique among breast tumours, in that it may be undetectable by observation or palpitation of the excised breast tissue. It may present as a well defined schirrhous mass or a poorly defined area of induration. Histologically it appears as a area of diffuse infiltrating small round cells, lying in lines, 'indian file', between collagen bundles. (See photograph D.)

Other types of breast carcinoma identified in this study are rare and include papillary carcinoma, which may be identified by the papillary structures seen in the low power photograph E. Mucinous (or colloid) carcinoma have also been included in this study: the vast majority of the tumour mass is composed of pools of mucinous material in which aggregates of tumour cells appear to be floating.

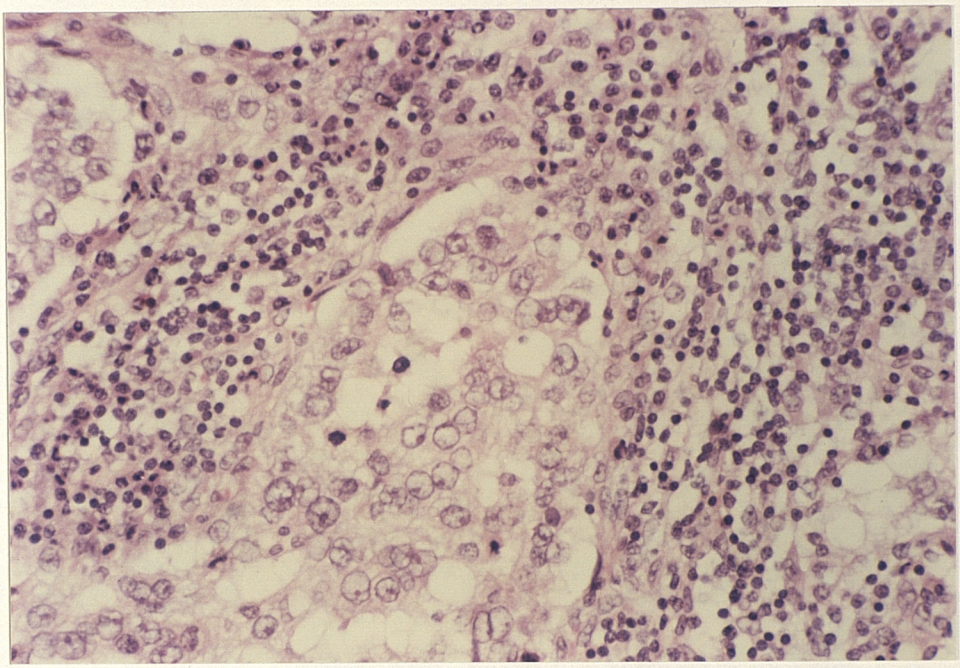
Breast carcinomas are classified according to the predominant cell pattern. However there are cases where two or more cell patterns may prevail in the one tumour. For the purposes of classification and grading it is necessary to concentrate on the prevalent cytological features. Although this system poses problems when considering the topic of heterogeneity. Heterogeneous expression of cellular antigens may well be demonstrated when cells from histologically and cytologically different tumour clones are found in the same specimen.



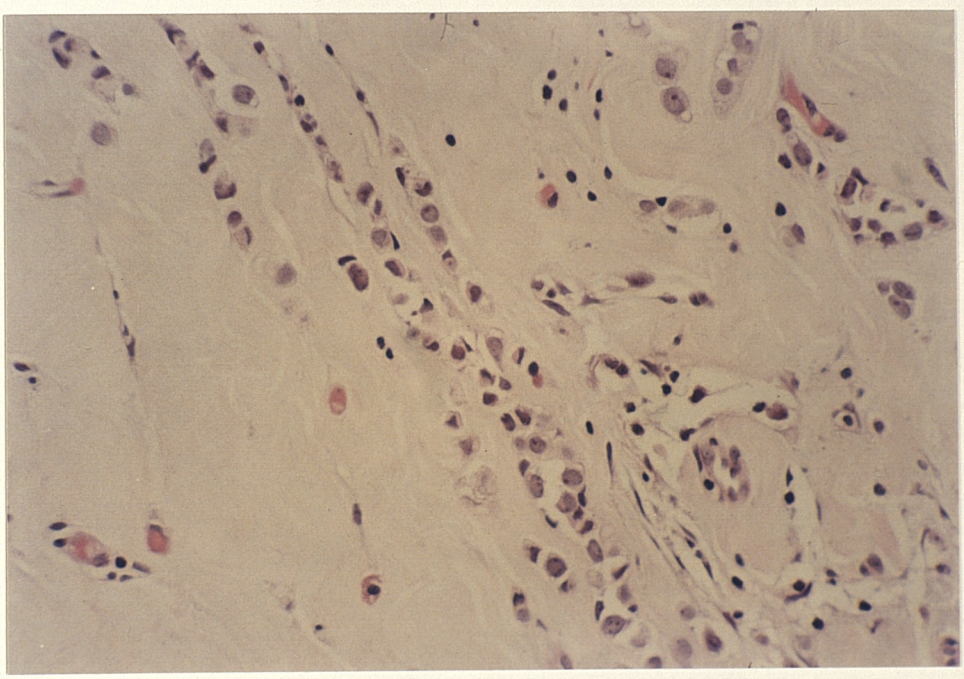
PHOTOGRAPH A : Infiltrating Ductal Carcinoma.x400.



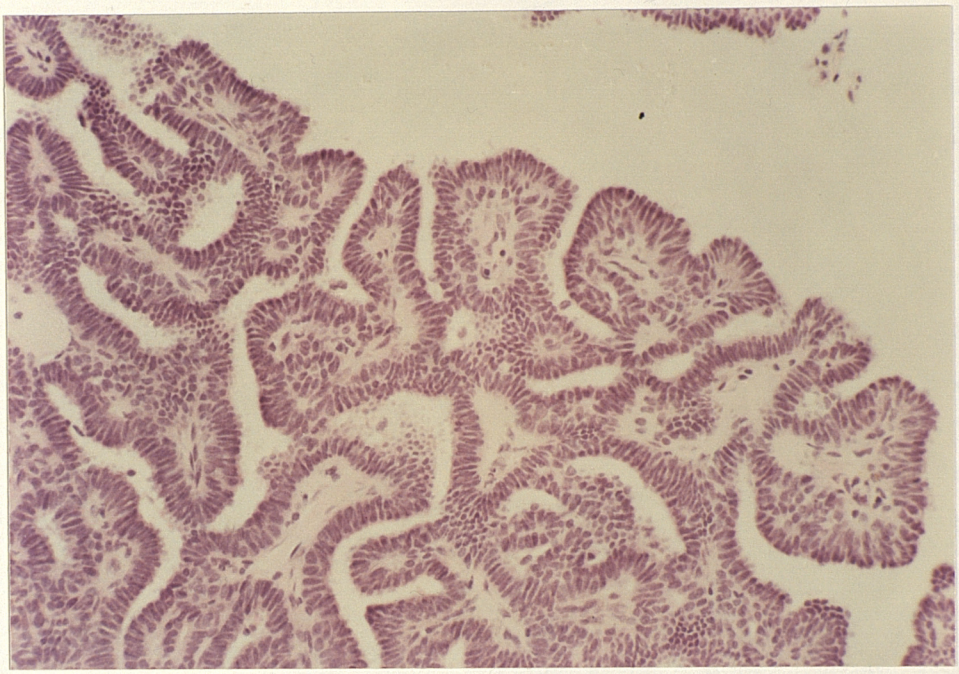
PHOTOGRAPH B : Tubular Carcinoma.x400.



PHOTOGRAPH C : Medullary Carcinoma.x400.



PHOTOGRAPH D : Lobular Carcinoma.x400.



PHOTOGRAPH E : Papillary Carcinoma.x250.

1.5 HISTOLOGICAL GRADING.

In breast cancer, tumour differentiation had not been examined closely until 1925 when Greenbrough used the first histological grading system. He divided breast tumours into three grades of malignancy by assessing eight histological factors. The number of cases in this study was small but it showed a correlation between histological degree of differentiation and what he described as a 'cure'. When Patey and Scaff analysed this data in 1928 they found that only three factors were of prognostic value in breast carcinoma; tubular formation, variation in size and shape of the nuclei and hyperchromatism linked with mitotic figures. It was not until the 1950's that Bloom and then Bloom and Richardson in 1957 reassessed the grading of breast tumours. They placed most importance on the three factors previously described by Patey and Scaff (1928). For each of these three factors a score of 1 to 3 was assigned according to the degree of severity, one point for mild aberration, two for moderate changes and three for severe abnormalities. This produced a total score of between 3 and 9 points; a score of 3, 4 and 5 points equalled grade I; 6 and 7 points, grade II; 8 and 9 points, grade III.

Elston 1984 modified this technique by being more specific in the way in which each point was awarded:-

Tubular Formation: After scanning all parts of the tumour one point was awarded if the great majority was composed of recognisable tubular structures; a moderate amount of tubular formation scores two points; little or no tubular formation, cells growing in cords or sheets, three points.

Nuclear size and Pleomorphism: Assessment of variations in size and shape of nuclei. Nuclei that are small and dark staining with little increase in size score one point; cells that appear larger than normal, have vesicular nuclei with visible nucleoli, score two points; large pleomorphic vesicular nuclei with prominent nucleoli, three points.

Mitotic Rate: It is in this category that the method differs most from previous grading systems. Bloom and Richardson 1957 analysed both mitotic activity and hyperchromatic nuclei. However, hyperchromatic nuclei are extremely difficult to distinguish from cells which have undergone pyknotic degeneration. Therefore hyperchromatic nuclei are excluded and only mitotic figures were counted at a fixed magnification of 300 times. Less than one mitotic figure per field scores one point;

one to two per field, scores two points; three or more mitotic figures per field, scores three points. (Elston 1984).

In the same way as Bloom and Richardson, Elston adds the points together to achieve the final grade: 3,4 and 5 points, grade I; 6 and 7 points, grade II; 8 and 9 points, grade III. This system is not meant to assign mathematical accuracy but is meant to provide a framework to discipline observers into examining each category separately rather than giving an instinctive judgement. The method is still based on subjective interpretation therefore cross-checking between observers is preferable.

A grading system should also be applied to the FNAC tumour specimens, so they too may be used in the evaluation of other cytological techniques. However the histological grading system is not suitable for cytological specimens, since not all the features used to describe a histological sample are apparent in cytological specimens. Therefore Chapter 5, describes the current interest in producing a grading system suitable for cytology. It also develops ideas to improve the cytological grading systems recently described in the literature (Hunt et al 1990), evaluating the system for the assessment of FNAC specimens and correlates the data with the established histological grading system.

1.6 HETEROGENEITY.

It is known that histologically tumours may contain one or more distinct types of morphological structure. Egan (1982) described a series of 118 primary breast carcinomas, 25% of which had multiple histological types. It is known that these different histological entities may exhibit antigens to different degrees and therefore, in the case of the oestrogen receptor antigen, may well react differently in their response to manipulative hormonal therapy.

ER status also shows clonal heterogeneity, where one group or clone of cells may be receptor positive whilst another is negative. There are two schools of thought as to the way in which clonal heterogeneity develops within individual breast cancers. In the first theory, more than one cell may be affected by a carcinogen, viral or otherwise, or a random chemical event involved in carcinogenesis, resulting in a multifocal tumour which may exhibit different histological sub-types. Egan (1982), not only found a histological variance in 25% of his cases but the total number of cases with multifocal

disease, of the same histological type, in the same breast was 60% of the total number of women studied.

The second theory for clonal heterogeneity in individual breast cancers, suggests that the carcinoma develops monoclonally from a single cell transformed by a carcinogenic agent, but, as it continues to grow, individual karyotypes from the clone may become unstable, altering the phenotype of the cell. As these cells from the altered phenotypes continue to grow and to multiply new clonal sub-populations are established, changing the original homogeneous carcinoma to the heterogeneous state.

The implications of heterogeneity in therapy are wide reaching. Carcinomas may become resistant to therapy which was initially effective. This supports the theory that carcinomas continually change their phenotypic type, with the emergence of resistant clones. An established heterogeneous state may remain stable as long as the growth rates of the sensitive and resistant clones are similar. With therapy, however, the destruction of sensitive clones allows the more resistant ones to predominate. No further response will be achieved and although therapy was initially successful, disease progression will occur.

1.7 COMBINED CLINICAL, RADIOLOGICAL AND CYTOLOGICAL ASSESSMENT.

The combined triple assessment of the clinical opinion on palpating a breast lump, the radiological mammogram and FNA cytology, has proved extremely useful in producing an accurate diagnosis of cancer prior to surgical intervention. (Smallwood et al 1984).

With experience a firm diagnosis of malignancy can be made on cytological preparations providing an adequate sample has been obtained. It is necessary that there should be no false positives for the method to be reliable. In Southampton there have been no mastectomies or wide excisions performed for benign disease. (Smallwood et al 1984). Another group of workers have reported the same experience. (Gardecki et al 1980).

In 1981, shortly after the introduction of FNA in Southampton a new policy was adopted at the breast clinic incorporating a scoring system. (Smallwood et al 1984). At

the clinic the women were examined and assigned a clinical score. They underwent FNA of the breast lump on their first visit to the clinic and mammography was performed. Each of these assessments was assigned a score: -2 for a definitely benign tumour, -1 for a probably benign tumour, 0 for an equivocal or suspicious assessment, +1 for a probably malignant tumour and +2 for a definitely malignant tumour. Scores ranged from -6 to +6 and a score of +4 or more was required to proceed to mastectomy. In this particular study there were no false positives with the aspiration cytology and only two false negatives. (Smallwood et al. 1984). There may be problems with inadequate sampling where the preparations contain insufficient material for cytological diagnosis. In Smallwood's study 76 cases were inadequate, although cytology confirmed the diagnosis of malignancy in 76 of 99 cases (77%) of breast cancer. 70 of the 72 cases (97%) of benign lesions were diagnosed with adequate aspirates. FNA cytology gave an overall accuracy of 98.6% when the samples were adequate. In the same study mammography achieved an overall accuracy of 95.8% for diagnosing benign and malignant disease.

The combined scores for each patient for clinical examination, mammography and fine needle aspiration were divided into groups. All patients that scored between +6 and +4 were confirmed as having breast carcinoma on subsequent histology. 21 women were given scores between +1 and +3, of these 18 had a breast carcinoma and 3 had benign lesions. There were 91 women who scored between -3 and -6, 90 had benign disease and 1 was found to have a carcinoma. Lastly there were 40 women in the score group 0 to -2, 8 had breast carcinoma the remainder benign disease.

Before the study commenced patients were monitored for six months, 58 women underwent mastectomy and 45 of these required a preceding biopsy operation or a frozen section before definitive surgery. In the first six months of 1982 when the scoring system was implemented 63 mastectomies were performed, only 14 requiring the preceding open biopsy operation. Therefore there was a 74% reduction in the need for a two stage procedure in the surgical treatment of breast carcinoma. 72 of the 94 women patients benefited from the pre-operative counselling when diagnosis, proposed treatment and its consequences were explained in detail and an informed choice made by the patient.

Only those patients with scores of +1 and +2 cytologically have oestrogen receptor analysis by ER-ICA, with reported results. Those with 0 score cytologically wait to have the diagnosis confirmed by histology before a report is issued. This is a safe

guard procedure as both benign and malignant cells may immunostain as positive with ER-ICA.

1.8 BREAST CANCER SCREENING.

It has already been said that breast cancer is the commonest form of cancer in women in the United Kingdom. The mortality rate for United Kingdom is the highest in Western Europe and North America. Although breast cancer is rare before the age of 35, the incidence and mortality rate in the United Kingdom, between the age of 30 and 34, are 19.6 and 5.9 respectively per 100,000 women. By the ages of 50-54 these rates have risen to 145.9 and 73.7 per 100,000 women and continue to increase with age. For patients presenting with symptomatic breast cancer there has been some increase in survival following treatment due to advances in surgery, radiotherapy, chemotherapy and hormonal therapy. However the effectiveness of these treatments is dependant upon the stage of the tumour at the time of detection. Clinically the tumours may well be in the advanced stages of the disease at the time of presentation and detection, often already produced metastases in local lymph nodes or even distant parts of the body. Therefore, the only way to reduce the number of deaths from breast cancer, is to detect the tumours before the patient presents with clinical symptoms. In this way, smaller less well advanced tumours will be more susceptible to the range of treatments available, reducing the number of deaths. Breast cancer screening by mammography was thought to be the best way of achieving this aim. (Forrest 1986).

A number of studies in Europe and North America have addressed the sensitivity, specificity and acceptability of breast cancer screening. Many of these trials have shown the benefits of extending the life expectancy of the women involved but few have benefited from lengthy follow-up periods needed to examine the data on reduced mortality through the screening procedure. The Health Insurance Plan (HIP) New York, began in 1963 and ran for 23 years, used mammography and clinical examination and showed reduced mortality in women over 50 years, after just one year. (Strax 1984, Shapiro et al 1982). The screening programme included women from 40-64 years, but little benefit was observed for the younger women. In The Netherlands the first results of the Nijmegen Project (1975-1981), showed a reduced mortality of approximately 50%, in women of all ages over 35 years. This was achieved through a mass screening programme of the 30 000 eligible women in the town of Nijmegen. (Verbeek et al 1984). In 1977 the Ostergotland and Kopparberg

Counties, Sweden, used mammography alone and achieved reduced mortality after five years. (Tabar et al 1985).

Initial screening for breast cancer in the United Kingdom program, is carried out by viewing the breast tissue with a single mammogram. Women who are identified as having abnormal changes in the breast by this procedure go on to have further mammograms and aspiration cytology if appropriate prior to definitive treatment.

For any screening programme to be successful it should be concentrated on those members of the public who are most likely to benefit from the procedure, to achieve maximum efficiency. The most important risk factor associated with breast cancer is age. Studies so far have shown that the effect of breast screening on deaths from breast cancer have been of value for women 50 years and over. (Strax 1984, Shapiro et al 1982). For younger women its effectiveness is uncertain, largely because mammography is less likely to detect cancers in the dense shadowing of the pre-menopausal breast. The benefit of screening women over the age 65 is debatable for different reasons: firstly, there is a lower acceptance observed in the UK trial, secondly there is a increased risk of the women dying from other causes and finally breast cancer in the older age group tends to run a less aggressive course than when diagnosed in younger women. (Forrest 1986).

Other risk factors in breast cancer have been identified. Many of these are related to reproductive function and include early menarche, late menopause and late age of first full term pregnancy. Others include a family history of breast cancer, especially in a mother or sister, and a history of benign breast disease. The excess risk factor conferred by any one of these factors is less than two fold but a combination of two or more incurs a greater risk.

As use of risk factors other than age to identify women to be screened is not a practical proposition at the present time, the most effective use of screening resources is to offer an initial mammogram screen to all women between the ages of 50 and 64 years. However this does not exclude making screening available to older or younger women seen to be at specific risk from the disease.

1.9 HORMONE INTERACTION MECHANISMS AND CONTROL.

Oestrogen receptor status may be used as a predictor to estimate breast tumour dependency on the hormone oestrogen.

The way in which the uptake of oestrogen is facilitated by specific receptor sites has been a subject of controversy. Firstly, the definition of a specific 'receptor site', is characterised by an area within the cell or on the cell membrane, with a high affinity for a specific molecule. Oestrogen receptor sites have been demonstrated by immunocytochemistry in the nucleus of breast cells. The controversy concerns the way oestrogen travels through the cell and cellular membranes to the final nuclear receptor site. (Furnival 1986).

Greene et al (1984), described the direct transportation of oestradiol into target cell nuclei and suggested that any evidence for a specific cytoplasmic receptor was artificial and that, free receptor was released into the cytoplasm during the homogenization of the breast tissue. Earlier observations suggests that there were cytoplasmic oestrogen receptors, were based on the biochemical demonstration of oestrogen binding, which requires homogenization of the tissue for analysis.

However, in the same year as the observations of Greene et al (1984), Whitliff (1984), described oestrogen molecules bound first to a cytoplasmic receptor protein before undergoing changes enabling the combined molecule to migrate across the nuclear membrane. (Whitliff 1984). The effect of this was to induce gene transcription which had been shown by the production of mRNA within minutes of oestrogen uptake, followed by an increase in DNA and protein synthesis with mitosis in some cells. (Lippman et al 1976). This shows how oestrogen stimulates growth and division of breast cells containing ER sites. The hormone oestrogen does not act in isolation as progesterone is inextricably linked with the oestrogen cycle. (Horwitz et al 1978). MCF-7 cells derived from human breast cancer have been used to show the synthesis of progesterone receptor protein is controlled by oestrogens. (McGuire and Horwitz 1978). Cowan and Lippman (1982) observed that progesterone receptor is usually absent from human breast cancers which do not contain oestrogen receptor and suggests this modifying effect may occur in all breast epithelial cells. Yet, Saez et al (1981) examined the combined prognostic value of ER and PgR and found that in patients with resectable breast cancer, that the recurrence rates for cases with both receptors was significantly less than those who lacked both receptors.

Thus ER status can be used as a predictor to estimate tumour dependency on oestrogen and consequently the extent to which anti-oestrogen therapy would influence the prognosis of the breast carcinoma. In practice, in Southampton, it is the elderly women over 70 years of age who are most likely to benefit from ER status. It is these women who may be treated with an anti-oestrogen drug, as their primary therapy. Increasing age and frailty may preclude them from embarking other courses of treatment such as surgery and radiotherapy, especially when the anti-oestrogen therapy may adequately control their breast cancer for the remainder of their natural life. (Davies et al 1991).

Tamoxifen is an anti-oestrogen drug, a competitive inhibitor of oestrogen binding, which blocks binding of oestrogen at ER sites. This renders the tumour cells less sensitive to stimulation by circulating oestrogens. However, this may not be its only anti-tumour activity, as response to Tamoxifen therapy has been seen in some ER negative women. (Ingle 1984). Oestrogens are important in the female physiology as they maintain bone and prevent arteriosclerosis. The long term use of Tamoxifen has been difficult to assess and it is not certain whether Tamoxifen prevents or enhances osteoporosis and arteriosclerosis. This may be even more important when considering the outcome of clinical trials of Tamoxifen as a prevention rather than cure for breast cancer.

At the Royal Marsden Hospital, a blind randomised feasibility study was set up to assess, 20 mg. per day of Tamoxifen versus a placebo in 200 healthy women. (Powles et al 1989). In the pilot study a total of 435 women were accrued, over the age of forty with a family history of breast cancer. The trial was completed in 1988 with a high compliance rate and no unacceptable toxicity. There was a significant increase in hot flushes in the Tamoxifen treated group, (33% versus 17%). Bone mass and clotting factors were not affected. Lipid analysis, revealed in the Tamoxifen group, a significant decrease in serum cholesterol, low-density lipoprotein cholesterol (LDLC) and apolipoprotein B levels in the post-menopausal women. The pre-menopausal showed a smaller significant decrease, in the total serum cholesterol and LDLC only. (Powles et al 1990). It is elevated levels of total serum cholesterol and LDLC, which is associated with an increased risk for coronary heart disease. (Bush et al. 1988). This suggests that Tamoxifen may have a beneficial effect on lipoprotein and lipid profiles. It is the changes in the lipid and lipoprotein levels which suggest in this context Tamoxifen is having an oestrogenic effect on the protein synthesis in the liver, rather than an anti-oestrogenic effect. (Fex et al 1981). These oestrogenic effects in the liver raise the possibility of an increased risk of tumour at this site, or other target organs of

oestrogen ie. endometrium or even a colateral breast. One trial using 40 mg. of Tamoxifen per day over two years, twice the dosage of other adjuvant Tamoxifen trials, did show a significant increase in the number of endometrial carcinomas. (Fornander et al. 1989). This was not upheld whilst using 20 mg. Tamoxifen per day. (Stewart and Knight 1989). To date there is no evidence in adjuvant Tamoxifen trials of an increased risk of tumour at other sites, from those mentioned above. Furthermore, Tamoxifen induces synthesis of TGF-ß, which acts as an inhibitory growth factor in the breast. (Lippman et al 1986). For this reason women with breast cancer on Tamoxifen are less likely to experience a colateral tumour. (Cuzick and Baum 1985). To assess the implications of Tamoxifen fully, risk of cancer at other sites weighed against the potential benefits to lipid metabolism, and the implications in coronary heart disease, requires clinical trials on an international basis, running for at least ten years, to assess the incidence of breast cancer, much longer to fully understand the implications for mortality from the disease. (Fentiman 1990). With this aim, the Royal Marsden Hospital has won ethical approval to extend its pilot scheme, for the blind randomised Tamoxifen trial, recruiting upto 1000 women. (Powles et al. 1990).

Hawkins et al (1990) stressed ER analysis should be assessed before the onset of Tamoxifen therapy, as the drug might alter the subsequent demonstration of ER sites in the biochemical assay. However, demonstration of ER by immunocytochemistry avoids this problem. The antigen identifies an area on the ER site adjacent to the moiety concerned with the uptake of the oestrogen molecules or the anti-oestrogen drug.

Three distinct effects of Tamoxifen on cell cycle kinetics have been demonstrated in the breast cancer cell line MCF-7. Firstly, an irreversible accumulation of cells in the G0/G1 phase of the cell cycle, see section 1.10; secondly, a reversible accumulation in G0/G1. It was here that Sutherland et al (1983) demonstrated the effects of anti-oestrogens can be reversed by the addition of excess 17ß oestradiol. Thirdly, a cytotoxic effect only associated with the highest dosage of Tamoxifen resulting in rapid cell death. Hormonal agents such as Tamoxifen or a combination of hormonal agents may increase the response rate of breast tumours and even the duration of the response, but they appear to have no effect on the overall survival.

1.10 THE CELL CYCLE.

The cell cycle has been described as a series of phases. The first gap phase (G1) is a period of variable duration which separates the mitosis (M phase) from the previous cell cycle. The synthetic (S phase) is where the synthesis of DNA takes place. After the doubling of the genetic material the S phase is immediately proceeded by another period of inactivity the second gap phase (G2), followed by the next mitosis. A further phase in cell cycling is described as a resting phase (G0), which may be of variable length. Although not part of the true cell cycle, under suitable stimuli cells in G0 will re-enter the cycling process. (Hall and Levison 1990).

There are a number of ways in assessing cellular proliferation in the cell cycle, both simple cytological assessment with immunocytochemistry as well as with biochemical methods.

Mitoses can be identified with a simple histological or cytological staining method, but the mitotic phase is the only phase which can be established with standard morphological examination. Careful morphological examination is required in order to identify only typical mitoses and not those cells which just have pyknotic nuclei. It is preferable to state the number of mitoses per 1000 cells counted. If the result is to expressed as the number of mitoses in specified number of high power fields, it is important to ascertain the microscope is calibrated correctly and the exact size of each high power field is known. (Elston 1987).

DNA content is another way to approach the assessment of the rate of cell proliferation. The normal resting cell will carry the diploid geneome whereas during the S phase of the cycle the DNA content duplicates becoming transiently tetraploid prior to the next mitosis. This state can be taken into account and measured in a number of ways.

Thymidine labelling requires tritiated thymidine to be taken up by viable cells and to be incorporated into the DNA content during the S phase of the cell cycle. The in vivo administration of the radiolabelled thymidine is very rarely justified in clinical practice, however in vitro incubation of biopsy specimens with tritiated thymidine is possible prior to fixation. Studies on breast carcinoma show that thymidine labelling indices closely correlate with a number of pathological variables. (McGurrin et al 1987 and Kamel et al 1989).

DNA synthesis may also be assessed by incorporating a thymidine analogue such as bromodeoxyuridine. This is a molecule which can be identified by a specific antibody either in histological sections or by flow cytometry. The problems are similar to those encountered with the tritiated thymidine, with the need to administer the product in vivo or to incubate in vitro prior to fixation. (Hall and Levison 1990 and Hein van Dierendonck et al 1989).

The use of immunocytochemical methods to demonstrate proliferation goes beyond the demonstration of the uptake of bromodeoxyuridine. A particular advantage of immunocytochemical analysis is that the architecture of the specimen remains intact, allowing histological or cytological assessment of the sample. This is particularly important when the sample may have been contaminated with cells other than the tumour cell to be studied, or when there is a tumour cell population expressing degrees of heterogeneity within the sample. Ki67 is the antibody which is best known for the demonstration of proliferating cells. It marks cell nuclei in all cells in the cell proliferation cycle except those cells which are resting in G0. (Gerdes et al 1983).

Nuclear organiser regions is the name used to describe the loops of DNA which comprise the genes for ribosomal RNA together with their associated proteins. Although the function of these nucleic acid protein complexes is not fully understood, it is thought that they are possibly related to RNA polymerase I. Silver stained nucleolar regions (AgNORs) have been used to study a number of histological and cytological tissues, including breast tumours. The silver stained regions within the nucleus are associated with the acrocentric chromosomes and were at first thought to be associated with ploidy. In fact it has been proven that AgNORs correlate more closely with cell proliferation. (Dervan et al 1989 and Sivridis et al 1990).

Proliferating cell nuclear antigen (PCNA), is a nuclear protein associated with the cell cycle. A new monoclonal antibody PC10, has been developed to recognise a fixation and processing resistant epitope on the PCNA antigen. In non-Hodgkin's lymphomas a linear relationship was established between Ki67 and PCNA immunostaining. This suggests PCNA immunological demonstration may be used as an index of cell proliferation, as with the Ki67 antibody. (Hall et al 1990). In gastric carcinomas PC10 has been used as an independent prognostic indicator, with low grade PC10 tumours showing a better prognosis than high grade PC10 tumours. (Jain et al 1991). However, Hall et al. 1990, have reported a loss of relationship between PCNA expression and cell proliferation in some breast and gastric carcinomas. Further investigation is required to clarify this situation. The immunostaining described above has been carried

out on formalin fixed, paraffin wax processed material, but the PC10 antibody will also be appropriate for cytological or frozen histological material.

1.11 THE AIMS OF THE STUDY.

The aims of the study were to :-

I. To produce a rapid and reliable assessment of oestrogen receptor content in breast tumour cells, employing the techniques of fine needle aspiration and immunocytochemistry.

Thus oestrogen receptor analysis would no longer require a histological biopsy for assessment. Cytological and immunocytochemical analysis would provide a technique which could be used pre-operably or in recurrent or inoperable disease. This would bring assessment of oestrogen receptor status to a wider number of breast cancer victims, more importantly to elderly women, who are those most likely to benefit from this information.

II. To demonstrate rates of cell proliferation in breast tumours using immunocytochemistry.

The rate at which cells in tumours proliferate may well have a profound effect on tumour size, and be closely related to tumour grade. These factors may well influence the tumours potential to form local and distant metastases. Therefore cell proliferation rates may well effect the prognosis of the disease.

III. To assess oestrogen receptor and proliferating cell activity in symptomatic breast carcinomas and breast carcinomas detected in the breast screening programme.

The majority of the material available for assessing breast carcinomas has been from symptomatic patients with clinical symptoms of the disease. Information gained from these women is vital to understanding the nature of breast cancer. However, in the future in the United Kingdom and a number of other countries in the world, most breast carcinomas will be diagnosed via a breast screening programme. It will be important to use the variety of information gained to assess the nature of these early breast tumours identified in the breast screening programme.

2.0 INTRODUCTION TO OESTROGEN RECEPTOR ANALYSIS.

Hormones serve as biochemical regulators of tissue processes. Steroid hormones such as oestrogen follow a complex pathway to effect transformation of specific receptor proteins to a biochemically functional form, resulting in increased cell growth. The oestrogenic hormone enters the cell and binds to a specific cytoplasmic 'receptor protein', which is specific to the specific 'target' cell. This resulting steroid/protein complex migrates to the nucleus where it accelerates specific mRNA synthesis. In the case of the oestrogenic hormone it leads to cell and tissue growth. (Jensen and DeSombre 1973). The two step procedure can be explained by experiments in uterine cells, where the oestradiol-receptor complex found in the uterine nucleus, is derived by a temperature dependant process where association with the steroid hormone activates the cytosol receptor protein to migrate towards the nucleus. (Jensen and DeSombre 1973).

Immunocytochemical staining for oestrogen receptor shows nuclear localization of the receptor protein which tends to refute the hypothesis that suggests receptor proteins reside primarily in the cytoplasm in the absence of steroid hormone. (Jensen and DeSombre 1973 and Jensen 1984). The immunocytochemical results of King and Greene 1984, indicate that oestrogen receptor may reside primarily in the nuclei of the target cells. In biochemical analysis of oestrogen receptor any oestrogen receptor found in the cytosol fraction of a homogenate must represent oestrogen only loosely associated with the nucleus. Any oestrogen binding to its receptor leads to a tighter bond and the retention of the oestrogen in the nuclear portion of the of the cytosol. (King and Greene 1984).

2.1 PRODUCTION OF MONOCLONAL ANTIBODIES TO OESTROGEN RECEPTORS.

Greene 1980 produced specific oestrogen receptor monoclonal antibodies using affinity purified oestrogen receptor from MCF-7 human breast cancer cells. This was used to immunise Lewis rats, their spleenic lymphocytes were fused with mouse myeloma lines. King and Greene 1984 produced 13 monoclonal hybridomas lines in this way, using two different mouse myeloma lines. These 13 cell lines secreting antiestrophilin antibodies recognised different antigenic determinants on the human receptor molecule. (Greene et al 1984). Five monoclonal rat antibodies D547Spy, D58P3y, D75P3y, H222Spy and H226Spy have proven specific in their localization of

oestrophilin in the indirect peroxidase technique in human breast tissue and a variety of mammalian reproductive tissues, as well as cultured MCF-7 cells.

2.2 IMMUNOCYTOCHEMISTRY VERSES BIOCHEMICAL RADIOLIGAND BINDING DEXTRAN COATED CHARCOAL ASSAY, (DCC).

Before monoclonal antibodies to oestrogen receptor (ER) were developed, the adopted method of demonstration of oestrogen receptors was by biochemical assay. There were a number of methodologies for quantitative oestrogen receptor analysis including the sucrose density gradient, (King et al 1985) the dextran coated charcoal method, gel electrophoresis, hydroxylapatite and gel filtration.

The radioligand binding dextran coated charcoal (DCC) was the most widely used method of assessment of ER and has been used to compare the value of the immunocytochemical demonstration of oestrogen receptors. More recently the oestrogen receptor antibody has been incorporated into a biochemical enzyme immuno-assay (ER-EIA) which has superseded other biochemical assays. The dextran coated charcoal radioligand binding method employs a tritiated oestradiol molecules which become incorporated into the oestrogen receptor molecule. Tritiated oestradiol bound to the oestrogen receptor site is quantitated by eluting the radio-labelled ligand using a dextran coated charcoal. The quantity of oestrogen receptor is expressed in fentomols. per milligram of the total amount of cytosolic protein in the sample. (See section 2.6, Methods and Materials).

The method requires approximately 0.25 grams of tumour tissue for the DCC assay. (EORTC 1980). The sample should be taken as soon as possible after surgery and immediately frozen in liquid nitrogen to preserve the oestrogen receptor antigen. The oestrogen receptor enzyme immuno-assay ER-EIA, Abbott Laboratories, requires a similar amount of tissue collected in the same way. (See section 2.6, Methods and Materials).

The immunocytochemical demonstration of oestrogen receptor on cytological specimens is achieved with the oestrogen receptor immunocytochemical assay ER-ICA, Abbott Laboratories (see section 2.6, Methods and Materials). Fine needle aspiration is used to collect the cytological specimens although ER-ICA may also be used to demonstrate oestrogen receptor on frozen and paraffin sections prepared from

freshly fixed histological specimens. In this study the DCC assay has been used to compare the viability of the new ER-ICA method.

There are a number of points to be considered when comparing DCC and ER-ICA. In the DCC assay the quantity of oestrogen receptor is expressed as a percentage of the total cytosolic protein present, therefore the sample to be analysed should contain tumour tissue only and not the surrounding connective tissue and fat, which would alter the ratio of total protein to oestrogen receptor. (Poulson 1981). In practice as much excess fat as possible is trimmed macroscopically from the specimen, but stromal elements within the tumour paranchyma cannot be removed in this way. Also, benign areas in the specimen will contribute to the total protein measurement and may or may not bind oestradiol, thus altering the final oestrogen receptor value. (Hawkins et al 1977). FNA allows the microscopical examination of individual cells to confirm their malignant characteristics as well as their oestrogen receptor positivity.

A further disadvantage of the DCC assay is that only one small area of the tumour is sampled. (Scheres et al 1988, Steele et al 1987). Breast carcinoma cells show heterogeneous characteristics in their expression of oestrogen receptor antigens. Poulsen (1981) described histopathologically similar portions of the same tumour expressing oestrogen receptor values varying from 0 to 300 fmol./mg. protein. FNA is a technique in which material from many areas of the tumour may be sampled. A stabbing action in many different planes is used to sample as widely as possible. Of course FNA may also contain non-malignant cells but as Weintraub et al (1987), suggests, these may be readily identified in the cytological preparations and disregarded. Both benign and malignant breast cells may immunocytochemically stain positive for oestrogen receptor so it is equally important to identify these cells and not include them in the final analysis of the sample. (Giani et al 1986, Weintrab et al 1987).

Cellularity may also be a problem, especially when the fraction of neoplastic cells is estimated as a semi-quantitative value when calculating the final fentomols of ER per milligram of protein. (Helle et al 1988). Different histological types of tumour have a greater or lesser density of cells within the tumour paranchyma. Berger et al 1987 found a significant relationship between tubular formation and oestrogen receptor positivity, $p=0.003$, however Parham et al 1989, found no significant correlation.

Hawkins et al 1977 shows that the DCC assay can only demonstrate ER sites which are free from the oestrogen molecule. Any ER sites which have taken up endogenous

oestrogen circulating in the body are inaccessible to the assay. In the post-menopausal women this does not pose a problem but in pre-menopausal or peri-menopausal women with circulating oestrogens this could produce inaccurate ER results. (Saki et al 1976 and Parl et al 1988). The ER-ICA assay however demonstrates ER sites by using the ER antibody which demonstrates an epitope on the oestrogen receptor site which is adjacent to, but not the actual site of oestrogen uptake. (Scheres et al 1988). Thus ER-ICA can demonstrate the ER site regardless of whether it has bound molecules of oestrogen.

Many groups of workers have set out to make direct comparisons between the DCC assay and ER-ICA. (Reiner et al 1986, Flowers et al 1986). Reiner et al (1986) showed concordant results in 80% of cases. Only 2 cases were positive with DCC but negative with immunocytochemistry, but both these cases fell below 20 fmol./mg. protein. Underwood et al (1983) however showed discrepancies in correlation between the DCC and the ER-ICA on frozen sections in the 15-80 fmol./mg. protein range.

Research has progressed from comparing DCC with frozen section ER-ICA samples, (Reiner et al 1986, McCarty et al 1985, Cudahy et al 1988, Azavedo et al 1986 and King et al 1985), to incorporate many different types of histological and cytological specimens. 'Tru-cut' needle biopsy specimens taken from the tumour produce a core biopsy which is frozen for ER analysis leaving the remainder of the specimen for conventional histological confirmation of the cancer. (Pietribasi et al 1986). Cytological 'touch' imprint specimens from the cut surface of tumour have also been used for ER analysis. (Masood et al 1987 and Marchetti et al 1987). Importantly, FNAC is now being used for ER-ICA analysis. (Lozowski et al 1986, Develeeschower et al 1986 and Masood et al 1989).

Progress has also been made in the biochemical analysis of ER sites. Where laboratories were using DCC for their biochemical assay many have converted to the Abbott Laboratories enzyme-immuno oestrogen receptor assay (ER-EIA). The two methods have correlated very well in their estimations of oestrogen receptor. (Riccoban et al 1988, Hanna et al 1989 and Thorp 1987). Anderson et al (1988), however felt ER-EIA slightly over estimated the ER values.

2.3 CORRELATION OF ER WITH PROGESTERONE RECEPTOR STATUS.

Oestrogen and progesterone steroid hormone pathways are inextricably linked, progesterone synthesis is the end product of oestrogen activity. (Hawkins et al 1978). Bonneterre et al (1986) also found a correlation between prolactin receptors PRLR and ER, and with PRLR and progesterone receptors PgR. PgR like ER are almost exclusively located in the nucleus of the cell and show the same heterogeneous characteristics as with ER. (Helin et al 1988).

PgR have in the past been elucidated by the DCC method of analysis. (Gelbfish et al 1987, Ruder et al 1989, Helal et al 1988, Lacombe et al 1989). However this suffers from the same intrinsic problems as the ER-DCC assay. It is unable to detect bound receptor sites in patients with endogenous progesterones; there is no confirmation viable tumour is present; dilution with stromal and benign elements produces false low values and there is no information about tumour heterogeneity. (Giri et al 1988).

Immunocytochemistry allows PgR to be demonstrated in a similar way to ER-ICA with a progesterone antibody supplied by Abbott Laboratories. (Skoog et al 1989). PgR like the ER antigen may be measured using a biochemical DCC assay. Demonstration of PgR by immunocytochemistry alleviates the problems encountered through using tumour tissue, in the same way as described for the ER analysis. (See section 2.2) Although earlier studies have shown that PgR may well be a better predictor of endocrine response than ER. (Sutton et al 1987, Raemakers et al 1987). Hawkins et al 1987 found no additional benefit in measuring PgR activity, however the median follow-up for this study was only five years. Prognostic significance may be established over a longer time interval.

2.4 ER AND PgR DEMONSTRATION IN PARAFFIN WAX.

Demonstration of both the ER and PgR antibodies have been achieved in conventional formalin fixed paraffin embedded breast carcinoma tissue. This has been compared to staining in frozen sections and correlated with the biochemical DCC assay and ER-EIA. (Anderson et al 1986). Fixation is an important prerequisite to good ER staining in paraffin sections. Coagulating fixatives ie. Bouin's and Carnoy's preclude ER staining, where as cross-linking fixatives such as formaldehyde and gluteraldehyde preserve antigenicity, although reduced. (Anderson et al 1988). Enzyme pretreatment enhanced the reduced activity. (Cheng et al 1988). This DNA method exposed nuclear

antigenic sites to immunostaining. (Shintak and Said 1986, Shousha et al 1989). Raymond and Leong (1989) suggest that the time spent in fixative is critical to the outcome of the ER staining, their method requires 1.5 hours in buffered formalin for the best results. In most cases this rules out the use of archival material where the fixation time cannot be guarantied. Anderson et al (1990) suggest that, immunostaining archival material for oestrogen receptor antigens, is a viable proposition. Although their results show that ER-DCC and ER-ICA are equivalent prognosticators but that ER demonstrated in the paraffin sections is somewhat less sensitive.

2.5 PRE-OPERATIVE ASSESSMENT OF ER, RECURRENCE AND SURVIVAL.

The most important advantage of FNAC in ER-ICA analysis is that it renders the procedure a pre-operative technique. (McClelland et al 1987). Because of this the method may be used to in determining treatment regimes prior to surgical intervention.

Women beyond 70 years, some of whom may be unsuitable for surgery due to other medical conditions, may benefit from measurement of ER status for hormonal therapy. (Davies et al 1990). In recurrent disease, FNA is an easily repeatable technique which may be used to monitor hormonal therapy. However, Hawkins et al (1990) concluded that there was no relationship between response to systemic therapy and a change in ER concentration. The anti-oestrogen drug therapy Tamoxifen may cause a problem when measuring ER status with the biochemical assay. The drug is used to block the oestrogen receptor sites, this action thus precludes these ER sites from being counted in the assay. However, since the ER-ICA immunocytochemical assay demonstrates an epitope on the oestrogen receptor away from the site of oestrogen up-take, ER are being measured regardless of whether they have been occupied by oestrogen or not.

Andry et al (1989) suggested ER positive patients fared better after recurrent disease, this was interpreted as a consequence of their responsiveness to hormonal therapy. Prolonged recurrence free survival for at least five years after diagnosis was associated with higher ER levels. (Shek et al 1989). The trend persisted after adjustment for other prognostic factors; node status; TNM stage; menopausal status and type of systemic therapy. ER concentration, nodal status and tumour necrosis was found to be the best prediction of survival by Shek and Godolphin (1988).

The ER status and lymph node status of the patient are well recognised as predictors of prognosis, but tumour proliferative rate and tumour ploidy must also be significant. The percentage of cells in the S-phase of the cell cycle may be related to ploidy. McGuire (1987), found that diploid tumours had significantly less cells in the S-phase than in an aneuploid population. Amplification of other oncogenes in the erb-B family ie. Cerb-B 2 and HER-2/neu have been implicated in tumours with poor survival. (Berg et al 1990, McGuire 1987).

2.6 MATERIAL AND METHODS

2.6.1 Fine Needle Aspiration Cytology technique.

The FNAC was obtained with a 22 gauge needle attached to a 20 ml. hypodermic syringe, positioned in a 'Cameco' syringe holder. Material collected from this procedure was expelled onto clean glass slides and spread using a 50 mm. coverslip. The unfixed slides were placed immediately into a slide box, before allowing them to dry, and then into a container of crushed solid CO_2 at -68°C or liquid nitrogen at -196°C, for transportation to the laboratory, where the slides were stored at -80°C prior to immunostaining. Preliminary trials had shown that it was essential to freeze the slides before they dried.

2.6.2 Breast FNAC from symptomatic patients.

Breast FNA from 337 patients with breast carcinoma, who had presented to the symptomatic breast clinic of the Royal South Hants. Hospital, Southampton, were stained with ER-ICA, obtained from Abbott Laboratories, Diagnostic Division, Moorbridge Road, Berkshire. The FNAC was either obtained during surgery or preoperatively at the clinic. In 105 cases tissue samples obtained at surgery, were immediately frozen and stored in liquid nitrogen, ready for transportation to the Tenovus Institute, Heath Park, Cardiff. CF4 4XX., for the radioligand binding assay.

2.6.3 Breast FNAC from the Breast Screening patients.

The Breast Screening Programme in Southampton and Salisbury Districts has been running since September 1988. In the first 20 months of the programme 145 cancers were detected from women between 50 and 64 years of age. 77 of these cases provided sufficient material for ER-ICA analysis. Because of the small nature of the screen detected lesions, the method of collection of cytological material had to be modified in a number of cases. Where there were solid palpable lesions FNA cytology could either be performed at the assessment clinic or from the operative specimen. Other lesions could prove more difficult, consisting of only a mammographic abnormality with microcalcification. When this was the case the operative specimens were sliced fresh, in the operating theatre, into 5 mm. slices, using a specially designed perspex

instrument. The slices were X-rayed, identifying the specific areas of microcalcification within the specimen. Poly-L-lysine coated microscope slides were moistened with TRIS buffered saline and pressed to the cut surface of the specimen, in order to facilitate the cells from the surface of the specimen adhering to the glass slide. Although we have never to our knowledge encountered difficulties with FNAC specimens staying on the microscope slides, there have been problems with the type of 'touch' preparations described above. It seems that the act of spreading the material on the glass slide provides better bonding than just touching the cells onto the glass microscope slide. Having perfected the method of collection of different types of cytological specimen in this group of breast screened women, we were able to go ahead and access the information as it accumulated.

2.6.4 Oestrogen receptor immunocytochemical assay. (ER-ICA).

In preparation for ER-ICA staining, the slides were taken from the -80°C freezer and placed immediately, without allowing them to dry, into the fixative solution, 3.7% formaldehyde in TRIS buffered saline (TBS), for 15 minutes. Slides were then washed in TBS for 5 minutes followed by enhancement of the antigen by placing the slides in methanol at between -10°C and -25°C for 3 to 5 minutes and then into acetone at between -10°C and -25°C for 1 to 3 minutes. After a rinse in TBS at room temperature for 5 minutes, the slides were ready to continue with the immunostaining procedure as described in the manual provided with the ER-ICA kit. The material to control the ER-ICA assay, provided by Abbott Diagnostics, consists of a microscope slide containing two areas of cells; one for each of the primary ER antibody and one for the control antibody. These cells were derived from the MCF-7 tumour cell line and will immunostain strongly for oestrogen receptor.

Negative controls for each patient test were achieved by incubating a duplicate FNA slide with a control rat antibody. All test and control slides were treated with normal goat serum as a blocking agent to prevent nonspecific binding of successive reagents. Test slides were incubated with the rat monoclonal antibody against oestrogen receptor (ER) and the control slides with the normal rat antibody. Goat anti-rat Ig forms the bridging antibody between the primary ER antibody and the horseradish peroxidase anti-peroxidase (PAP) complex. The free peroxidase portion of this molecule reacts with the chromogen substrate 3'3 tetrahydrochloride diaminobenzidine (DAB) to form the brown coloured end product. All slides were counterstained with 1% Harris's

haematoxylin to delineate the negative nuclei without masking the positive end product.

2.6.5 Biochemical radioligand binding, dextran coated charcoal assay.

Tissue for this assay was prepared by removing excess fat and blood from the specimen. It was frozen in liquid nitrogen and pulverized into a powder. Homogenisation in buffer produces the cytosol for the biochemical reaction. A solution of tritiated oestradiol was added in equal quantities and incubated for 16 hours at $4^{o}C$. Oestradiol bound to the ER sites in the tissue cytosol was separated from the free steroid by the addition of a dextran coated charcoal solution. After centrifugation the supernatant is decanted and a scintillating mixture added to count the ER bound radioactive oestradiol molecules. Scatchard analysis was used to determine ER in fentomols., expressed as a percentage of the total cytosolic protein content. (Personal communication, B.Francis, Tenouvus Institute, Cardiff; Cooke et al 1979). Tumours were considered oestrogen receptor positive if they contained more than 10 fentomols. of specific oestradiol binding per milligram cytosol protein.

2.6.6 ER analysis by enzyme immuno-assay. (ER-EIA).

Abbott Laboratories also produce an ER-EIA system for the demonstration of ER. ER-EIA replaced the biochemical DCC assay, for the tissue analysis of ER at the Tenovus Institute, Cardiff, using monoclonal antibodies in a solid phase enzyme immunoassay based on the 'sandwich' principle. (Miles et al 1968). In this method inert beads are coated with anti-ER rat monoclonal antibody and are incubated with homogenised tissue cytosols, prepared in the same way as for the DCC assay. Controls and standards are included where appropriate. ER present in these cytosols is bound to the anti-ER on the beads, known as the 'solid phase'. Unbound material is removed by washing and the beads are incubated with anti-ER conjugated with horseradish peroxidase: washing removes the excess. An enzyme substrate solution hydrogen peroxidase and ortho-Phenylenediamine. 2HCl is used to develop a colour and measure the amount of conjugated ER. 1N sulphuric acid is used to stop the enzyme reaction. The colour is read on a spectrometer at 492 nm. The intensity of colour is proportional to the concentration of ER within the standard constructed for each assay. Comparison with the radioligand binding, DCC assay has shown very good correlation between the two methods. (Nicholson et al 1986).

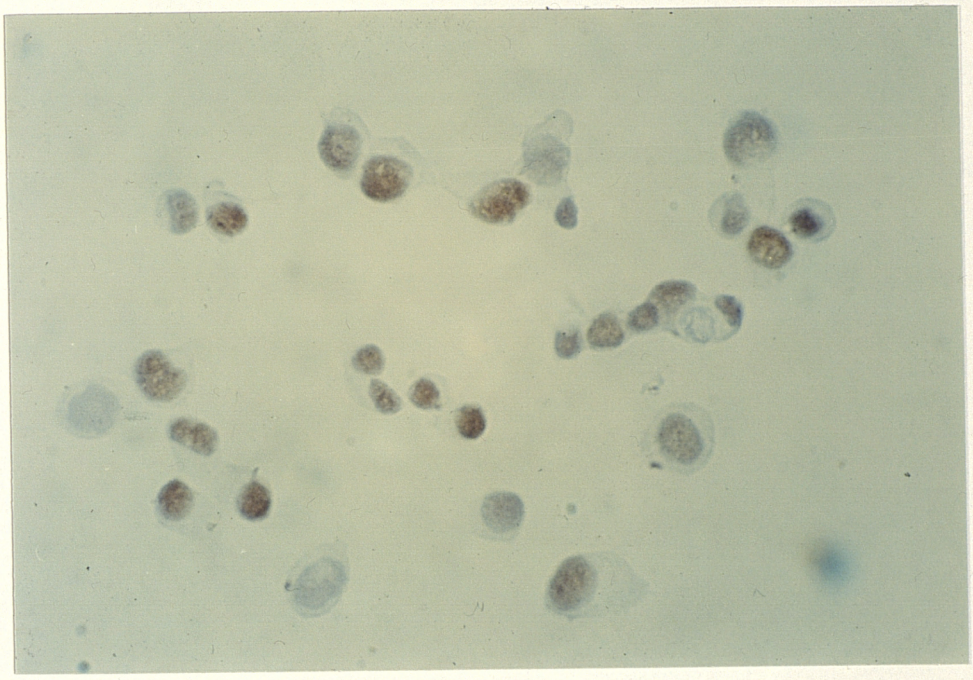
2.6.7 Assessment of ER-ICA slides.

Cells were designated ER-ICA positive if the brown reaction product of DAB was observed in the nucleus, (see photograph F), and negative if the nuclei showed haematoxylin staining only, (see photograph G). Any benign cells in the preparations were noted and their positive or negative staining reaction recorded, (see photograph H). Breast carcinomas may exhibit a combination of ER-ICA positive and ER-ICA negative staining cells, which may be distributed throughout the preparation, (see photograph I). However in some cases clonal heterogeneity was observed (see photograph J).

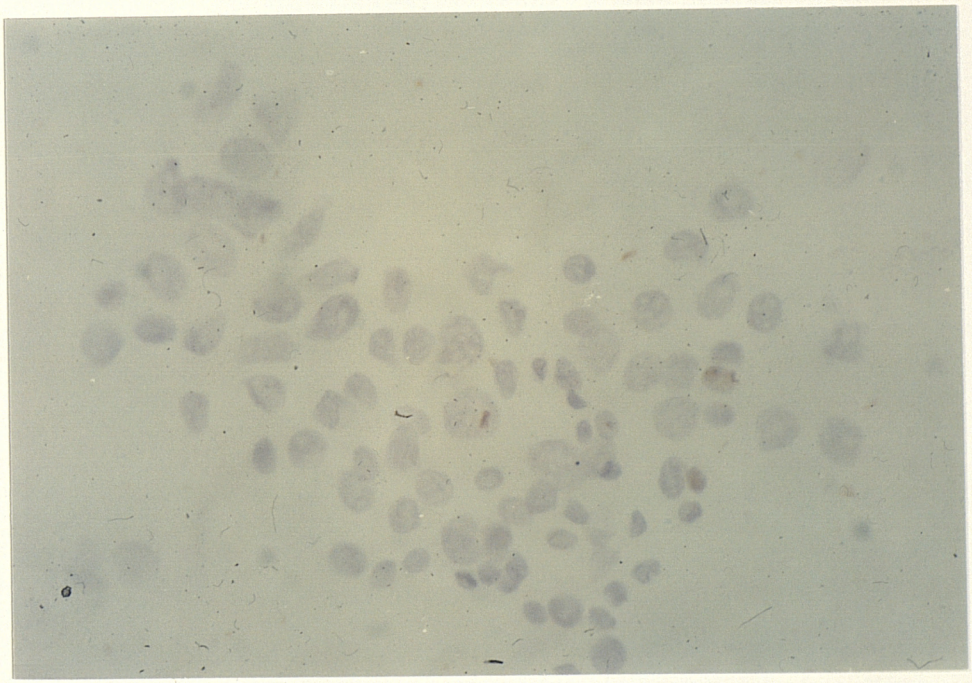
Each test slide was compared with its control slide and any artificial or cytoplasmic staining noted. The heterogeneity of the staining pattern was also assessed in the first 136 cases examined: +/- indicating <25%, + 25-75%, ++ >75% and +++ 100% of the tumour cells in the preparation showing positive staining in their nuclei. In the overall assessment, +/- (<25%) was taken as a negative result. The proportions of cells were assessed approximately but not counted.

2.6.8 Histological tumour grade.

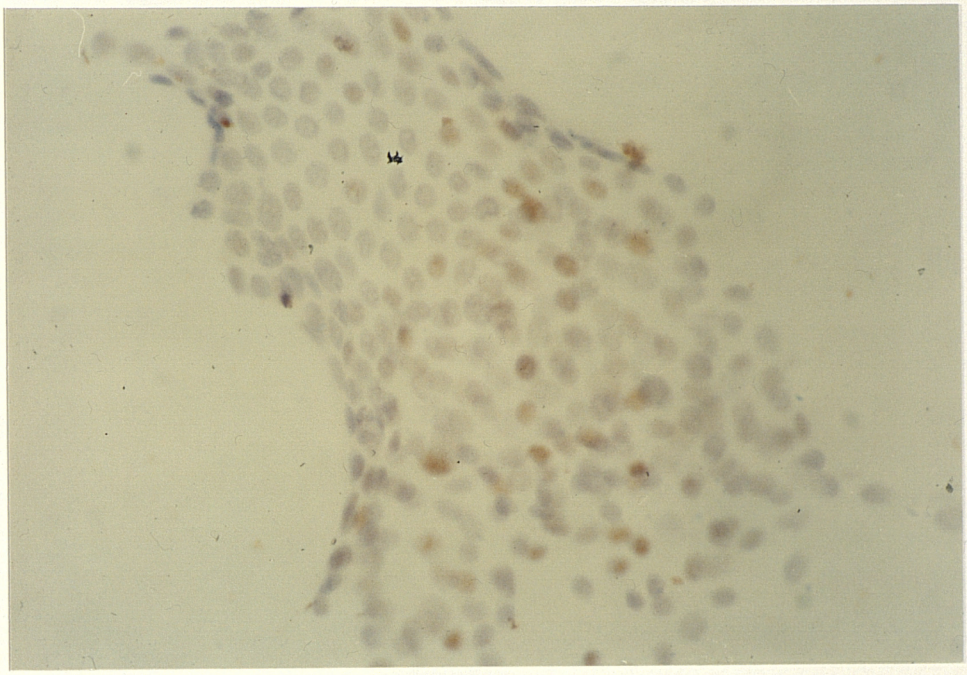
Tumours were graded by the method recommended by Elston et al (1984).



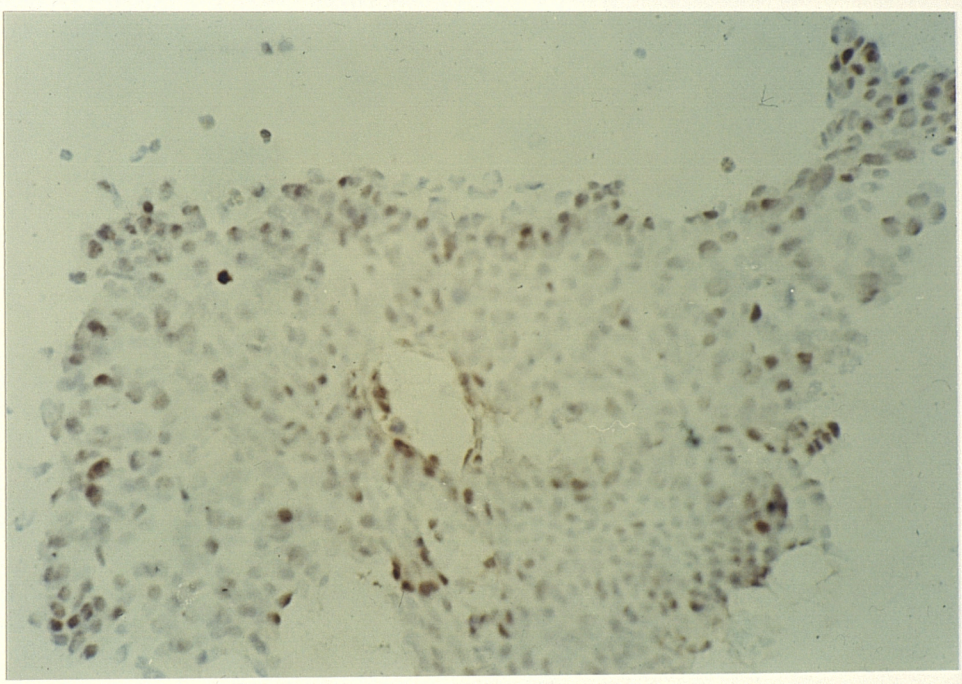
PHOTOGRAPH F : ER-ICA Positive.x400.



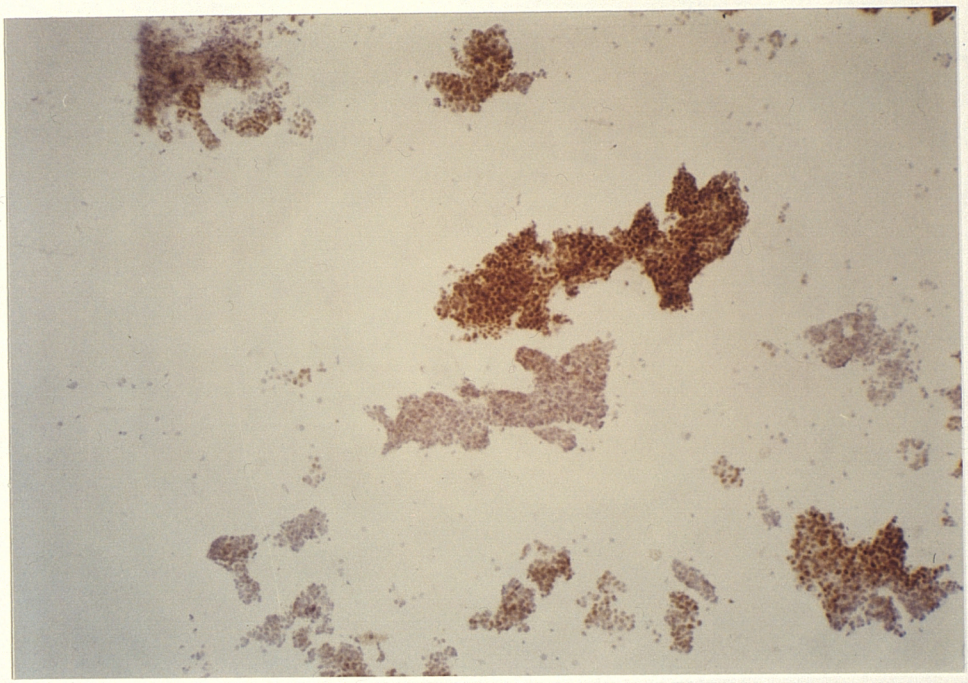
PHOTOGRAPH G : ER-ICA Negative.x630.



PHOTOGRAPH H : ER-ICA Positive Benign Breast Cells.x400.



PHOTOGRAPH I : Heterogeneity.x250.



Photograph J: Clonal Heterogeneity.x100.

2.7 RESULTS

The cytological examination of the May-Grunwald Giemsa, (See Appendix A.) and Papanicoloau (See Appendix B.) stained material on each case, confirmed malignancy. The morphology of the cells was also preserved in the ER-ICA stained material therefore it was possible to recognise both benign and malignant cells and make a visual assessment of the heterogeneity expressed. The staining pattern was assessed in the first 136 cases stained for ER-ICA. Most of the slides fell well within the broad groups described above, relatively few being on the borderlines. It was rare to find all the cells staining, so the group 100%, +++ was amalgamated with those assessed as ++ for most of the purposes in the study. Within observer variation was 85%. On a third reading, disagreement was resolved by the decision to disregard negative staining confined to the edge of the preparation, sometimes dried before fixation.

2.7.1 ER-ICA in symptomatic and breast screening patients, correlation with the radioligand binding assay.

The radio ligand binding assay was available for comparison in 105 cases, (Figure 1, Table 1). Five cases from the 105 were patients from the breast screening programme: all of these five cases were ER-ICA positive and all had radio ligand binding assay results of more than 60 fento.moles/mg. protein. (166, 173, 244, 249, 490 fento.moles/mg. protein of oestrogen receptor binding sites, respectively). The predictive value for a positive result was 94% but sensitivity only 69%, (see table 1).

$$\text{Positive Predictive Value} = \frac{\text{Number correctly identified as positive}}{\text{Total number of cases said to positive}} \times 100$$

$$\text{Sensitivity} = \frac{\text{Number of cases correctly identified as positive}}{\text{Number of positive cases}} \times 100$$

As can be seen from Figure 2, 66% of FNAC negative with ER-ICA fell within the lower levels of the radioligand binding, being lower than 60 fmol./mg. protein. There were insufficient corroborative results from the breast screening women to produce an independent correlation of ER-ICA with the radio ligand binding assay in both symptomatic and breast screening women.

There were six cases negative for ER-ICA but with values for the DCC/ER-EIA assays greater than 60 fentomol/mg. protein. Four of these six cases actually contained some positive staining, but less than 25% of the total number of nuclei in the sample, and three of these four also contained positive staining in benign cells in the aspirate analysed. The other two cases with values of 228 and 336 fentomol/mg. of cytosolic protein, would be expected to immunostain positive with the ER-ICA assay. However, the only explanation that can be offered for this discrepancy, assuming the biochemical values were correct, was a technical error in the analysis of the cytological specimen. A delay in the collection or fixation of the specimen was the most likely explanation for this problem, particularly since one of the two specimens contained brown cytoplasmic staining. Cytoplasmic staining has not been considered as a positive result in this study. It is important the slides for ER analysis should be cooled to below -80°C as rapidly as possible, any delay results in loss of antigenicity.

TABLE 1. COMPARISON OF ER-ICA WITH THE RADIOLIGAND BINDING ASSAY, IN SYMPTOMATIC AND BREAST SCREENED PATIENTS.

	RADIOLIGAND BINDING ASSAY		TOTAL
	<10 fmol./mg. protein.	>10 fmol./mg protein.	
ER-ICA POSITIVE	3	47	50
ER-ICA NEGATIVE	24	21	45
TOTAL	27	68	105

X^2 with correction for continuity = 29.3 $p < 0.001$

2.7.2 ER-ICA correlation with the age of the patient, in symptomatic women.

327 breast FNAC were stained for ER-ICA analysis, 271 produced satisfactory staining results, 56 cases had insufficient cells for assessment of the ER-ICA staining. The correlation of ER-ICA staining with patients age is shown on Figure 2, Table 2. The age of 50 years has been taken as an indication of menopause, 50 to 64 years indicates the group of women who would have been eligible for breast screening, however, they presented with a symptomatic breast lump before the breast screening programme was set up in the Southampton area prior to September 1988; or after September 1988, prior to an invitation to attend for a screening mammogram. 73% of women under the age of 50 had ER-ICA negative tumours and 59% of strongly positive tumours were in women over 50 years of age. When the women over 65 years of age were considered separately 67% were ER positive. The overall correlation between women over and under 50, with ER-ICA status was statistically significant.

TABLE 2. CORRELATION OF ER-ICA WITH THE AGE OF THE PATIENT, SYMPTOMATIC WOMEN.

AGE	ER-ICA POSITIVE	ER-ICA NEGATIVE	TOTAL
<50 years	12	33	45
50 to 64 years	35	45	80
>=65 years	98	48	146
TOTAL	145	126	271

$X^2 = 26.9$ with two degrees of freedom $p < 0.001$

2.7.3 ER-ICA correlation with women from the breast screening programme.

In the first 22 months of the breast screening programme, 77 cases had satisfactory ER-ICA staining. 19 of these cases were from pre-operative FNAC specimens, 44 from immediate FNAC of the operative specimen and 15 were the 'touch' imprint specimens. From these 77 cases, 37 (48%) were ER positive and 40 (52%) were ER negative.

FNA specimens were collected at the initial assessment clinic or from the tumour specimen immediately after surgery. However, it was important to analyses the way in which the breast screened specimens were collected, as this will have been achieved in a variety of different ways as described above. Of the pre-operative breast screened FNAC 57% were ER positive; 16 cases (67%), where the FNAC was performed on the operative specimen were ER positive; but when the aspiration of the specimen was delayed for upto 30 minutes only 39% gave ER-ICA positive results. This type of delay can be experienced when the tissue is sent immediately for X-ray to ensure the surgery is complete. In the case of the 'touch' imprints, where only 22% of cases were ER positive; the specimen will experience a delay of at least 45 minutes whilst the tissue specimen was X-rayed intact to assess the extent of microcalcification and then the tray of slices of breast tissue were sent for X-ray, to identify those slices containing the areas of microcalcification, those areas most likely to contain the tumour cells. Examining the figures for the assessment of the different types of collection FNAC specimens, it would seem there was a distinct difference between the pre-operative FNAC and production of 'touch' imprint specimens, however there were insufficient numbers in each of the groups for statistical analysis of the data to produce any significant result.

Figure 3 and Table 3, shows the comparison of ER-ICA status between symptomatic women aged 50-64 and the breast screened women. 37 breast screened women (48%) were ER-ICA positive, a group of 80 age matched women with symptomatic breast carcinomas, showed a similar ER positive rate, (44%). As might be expected there was no statistical significance between the numbers of women ER positive and negative.

TABLE 3. CORRELATION OF ER-ICA WITH BREAST SCREENED WOMEN, AGED 50 TO 64 YEARS.

AGE	ER-ICA POSITIVE	ER-ICA NEGATIVE	TOTAL
Breast Screened 50 to 64 years	37	40	77
Symptomatic 50 to 64 years	35	45	80
Total	72	85	157

$X^2 = 0.32$ with one degree of freedom $p < 0.5$

2.7.4 ER-ICA correlation with TNM stage and lymph node status, in symptomatic women.

There was no statistical correlation between ER-ICA and TNM stage (Figure 4), or with tumour size alone (Figure 5, Table 4.) or with lymph node status alone (Figure 6, Table 5)., on 150 patients who have had surgery. There was no evidence to suggest there was any correlation in tumour size or lymph node status the in breast screening population.

TABLE 4. CORRELATION OF ER-ICA WITH TUMOUR SIZE ALONE, IN SYMPTOMATIC WOMEN.

TUMOUR SIZE	ER-ICA POSITIVE	ER-ICA NEGATIVE	TOTAL
T1	18	18	36
T2	50	46	96
T3	6	12	18
TOTAL	74	76	150

$X^2 = 2.14$ $P < 0.3$

TABLE 5. CORRELATION OF ER-ICA WITH LYMPH NODE STATUS ALONE, IN SYMPTOMATIC WOMEN.

LYMPH NODE	ER-ICA POSITIVE	ER-ICA NEGATIVE	TOTAL
POSITIVE	36	36	72
NEGATIVE	38	40	78
TOTAL	74	76	150

$X^2 = 0.051 \quad p < 0.3$

2.7.5 ER-ICA correlation with histological grade of tumour, in symptomatic women.

The correlation of ER-ICA staining with the histological tumour grade is shown in Figure 7 and Table 6. It demonstrates 56% of the high grade ductal carcinomas were ER-ICA negative and 62% of the low-moderate grade tumours were ER-ICA positive, which showed a weak statistical significance. Non-invasive and tumours of special type from the symptomatic group are shown in Table 7.

TABLE 6. CORRELATION OF ER-ICA WITH THE HISTOLOGICAL GRADE, IN 138 DUCTAL CARCINOMAS, FROM SYMPTOMATIC BREAST PATIENTS.

HISTOLOGICAL GRADE	ER-ICA POSITIVE	ER-ICA NEGATIVE	TOTAL
Grade I	7	2	9
Grade II	35	24	59
Grade HI	31	39	70
TOTAL	73	65	138

$X^2 = 5.19, \quad p < 0.1$

TABLE 7. NON-INVASIVE AND SPECIAL TYPES OF BREAST TUMOURS, FROM THE SYMPTOMATIC GROUP. (not included in Table 6).

BREAST TUMOUR	ER-ICA POSITIVE	ER-ICA NEGATIVE	TOTAL
INTRADUCT	0	3	3
LOBULAR	2	2	4
TUBULAR	0	0	0
PAPILLARY	1	0	1
MEDULLARY	0	2	2
MUCINOUS	1	1	2
CARCINOID	0	1	1

NB. The mucinous group in Table 7 above indicates one mucoid carcinoma, ER-ICA positive and one colloid carcinoma, ER-ICA negative.

2.7.6 ER-ICA correlation of histological grade of tumour, in the breast screening population.

There were 77 breast screening tumours with ER-ICA analysis in the first 22 months of the screening programme, having omitted the 'touch' imprint results from the analysis, 68 were histologically graded, 13 of these were breast tumours of special type 54 were ductal carcinomas. There was a significant difference in the ER positivity of the Grade I, II and the Grade III tumours, (See Figure 8 and Table 8). Non-invasive tumours and those of special type, from the breast screening group are shown in Table 9.

TABLE 8. CORRELATION OF ER-ICA WITH THE HISTOLOGICAL GRADE, IN 54 DUCTAL CARCINOMAS FROM BREAST SCREENING WOMEN.

HISTOLOGICAL GRADE	ER-ICA POSITIVE	ER-ICA NEGATIVE	TOTAL
Grade I	8	7	15
Grade II	11	9	20
Grade III	5	14	19
TOTAL	24	30	54

$X^2 = 3.92$ with two degrees of freedom $p < 0.1$

TABLE 9. NON-INVASIVE AND SPECIAL TYPES OF BREAST TUMOURS, FROM BREAST SCREENING WOMEN. (not included in Table 8).

BREAST TUMOUR	ER-ICA POSITIVE	ER-ICA NEGATIVE	TOTAL
INTRADUCT	2	4	6
LOBULAR	1	1	2
TUBULAR	2	3	5
PAPILLARY	0	0	0
MEDULLARY	0	0	0
MUCINOUS	0	0	0

2.7.7 ER-ICA heterogeneity correlated with age and histological grade, in symptomatic patients.

The extent of the heterogeneous nature of breast tumours from symptomatic patients is demonstrated in Figure 9. Heterogeneity from the symptomatic patients has been compared with the two parameters proven to be statistically significant, previously in this chapter, namely age of the patient and histological grade of the tumour.

Figure 10, demonstrates a moderately strong correlation with the age of the patients. The women over 65 years of age accounted for 82% of all those with more than 75% of their tumour staining positive with ER-ICA.

TABLE 10. HETEROGENEITY IN SYMPTOMATIC PATIENTS COMPARED WITH AGE.

ER-ICA	-	+/-	+	++	+++	TOTAL
< 50 YRS	14	0	0	1	0	15
50-64 YRS	17	2	5	5	2	31
> 64 YRS	15	1	9	23	1	49
TOTAL	46	3	14	29	3	95

KEY:- - ER-ICA Negative
+/- < 25% ER-ICA Positive
+ 25-75% ER-ICA Positive
++ > 75% ER-ICA Positive
+++ 100% ER-ICA Positive

$X^2 = 25.36$ with eight degrees of freedom $p < 0.01$

Figure 11., shows a strong statistical correlation with histological grade of the tumour.

TABLE 11. HETEROGENEITY IN SYMPTOMATIC PATIENTS COMPARED WITH HISTOLOGICAL GRADE.

ER-ICA	-	+/-	+	++	+++	TOTAL
GRADE I	1	2	0	1	0	4
GRADE II	10	1	4	13	0	28
GRADE III	22	1	3	9	2	37
TOTAL	33	4	7	23	2	69

KEY:- - ER-ICA Negative
+/- < 25% ER-ICA Positive
+ 25-75% ER-ICA Positive
++ > 75% ER-ICA Positive
+++ 100% ER-ICA Positive

$X^2 = 26.2$ with eight degrees of freedom $p < 0.001$

2.7.8 Analysis of ER-ICA with the patient survival data.

Data on the recurrence dates and survival figures were collected for comparison with the oestrogen receptor status.
380 patients were recruited into the scheme, from the symptomatic and breast screened women who had received ER-ICA analysis, since February 1987, the last follow-up date being July 1991. Of these 380 women, 59 have died upto July 1991, 49 from breast carcinoma, 2 from other causes and 4 where cause of death was not stated in the medical notes.

Statistical assessment was divided into four groups. Firstly, all the patients were considered as a whole and divided into those women fifty years of age and under;

those women over fifty years of age; and finally the women over the age of seventy years, who were given Tamoxifen as their primary course of treatment. Each of these groups were considered in comparison with their ER-ICA status and the survival data. However there were insufficient events, patient deaths, for any of the groups to produce any statistically significant figures. Therefore the statistical assessment progressed to consider the groups in comparison with the recurrence data, or the date of first recurrence of their primary breast disease.

2.7.9 Analysis of ER-ICA with patient recurrence data, in all age groups.

The whole group of 380 women considered against their recurrence data, showed a positive statistical significance, $X^2 = 5.615$, $p < 0.01$ see Figure 12.
Appendix J. provides the full numerical information used to produce the recurrence analysis graph in Figure 12.

2.7.10 Analysis of ER-ICA with patient recurrence data, in selected age groups.

The recurrence data was also examined with a number of selected age groups to assess the significance, of firstly, pre- and post-menopausal women, above and below fifty years of age; and secondly, women over seventy years of age, whose primary course of treatment was the drug Tamoxifen. There was no statistical significance identified in any of the age groups, when they were compared with their survival or recurrence data, however it is possible to observe a trend. The positive trend towards a significant result indicates, that it might be possible to observe a significant occurrence in the future. This will depend upon accurate follow-up data, collected over a greater length of time, a full ten years of data would be the ideal.

2.7.11 Analysis of ER-ICA status compared with the response to Tamoxifen.

In Southampton, the department of Cytology and the University Surgical Unit combined their information to assess ER status and patient response to the drug Tamoxifen. It was a prospective study of 56 patients, over 70 years of age, all of whom had clinically symptomatic breast cancer.

The age of the patients ranged from 70-94 years with a median of 79 years. 35/56 (65%) were ER-ICA positive, 14/56 (25%) were ER-ICA negative and 7/56 (12%) had unsatisfactory ER-ICA results. The patients with satisfactory ER-ICA staining were followed in the symptomatic breast clinic for a median of 24 months, ranging from 9-23 months. The response to the Tamoxifen therapy shows a strong statistical correlation within the ER-ICA positive and negative groups, shown below in Table 12 below and Figure 13.

TABLE 12. ER-ICA STATUS COMPARED WITH THE RESPONSE TO TAMOXIFEN.

RESPONSE	ER-ICA POSITIVE	ER-ICA NEGATIVE	TOTAL
COMPLETE	12	0	12
PARTIAL	15	1	16
STATIC	5	0	5
PROGRESSIVE	3	13	16
TOTAL	35	14	49

$X2 = 32.48$ with two degrees of freedom $p < 0.001$

Of the ER-ICA positive patients 91% responded to Tamoxifen or they had static disease, whilst in the ER-ICA negative only 7% of patients responded or had static disease. There were three patients in the ER-ICA positive group whose disease progressed, one died 1 month after starting Tamoxifen treatment; this patient had an advanced disease at diagnosis (T4 N2). The two other women responded initially to the Tamoxifen therapy, but relapsed whilst on treatment at 12 and 18 months and were treated by mastectomy. In the ER-ICA negative cohort, there were 13 women with disease progression; three died, seven received mastectomies, three had radiotherapy and two were treated with chemotherapy. The one patient who responded in the ER-ICA negative group, relapsed at 20 months and went on to receive a mastectomy for

her advancing disease. The median time to progression was 4 months in the ER-ICA negative group. (Davies et al 1990).

2.8 DISCUSSION

The results of this study have shown that fine needle aspiration may be used to demonstrate ER in breast cancer nuclei, using the ER-ICA monoclonal antibody technique. Comparison with biochemical radioligand binding, DCC assay or ER-EIA has shown a high specificity for a positive result. This was undoubtedly because the cut-off point for a ER-ICA positive result was set at more than 25% of the nuclei showing positive staining. However there was a lower sensitivity, suggesting that less reliance should be placed on a negative result. This is consistent with previous studies.(Hawkins et al 1988). However, biochemical assay, using the radioligand binding DCC assay or the ER-EIA technique, cannot necessarily be used as the "gold standard" for ER status, since it has several features inherent to the method which may make it less accurate than the direct visualisation of nuclei reacting with a monoclonal antibody.

Benign cells may express ER, an unknown quantity with biochemical assay, but are excluded from the final analysis when identified by ER-ICA staining; similarly, necrotic material and stroma may cause significant contamination of material for biochemical assays, but when aspirated in cytological preparations is easily identified. However, our study has shown that neither of these can fully explain the main difference between the two techniques, lower sensitivity of the ER-ICA staining. Stromal contamination may have reduced the numerical value for radioligand binding assay, and may occasionally have been responsible for a negative result, three cases were negative by radioligand binding but positive by ER-ICA staining. Benign cells expressing ER and ER in less than 25% of tumour cells have elevated the numerical value for radioligand binding assay, in a number of cases, but appear not to have affected the close correlation between high radioligand binding levels and ER-ICA positivity in tumour cells. The main area of disagreement was negative staining in twelve tumours with low levels of radioligand binding, between 10 and 60 fentomols./mg. protein. It was difficult to know whether this was due to the presence of benign cells in the material for the DCC/ER-EIA, lower sensitivity of the ER-ICA technique or failure to collect samples quickly, preserving the ER antigen for ER-ICA analysis. It is important to note, that the perceived greater sensitivity of the biochemical analysis, may in practice be of little advantage to the patient, who only requires a clinical response to the disease. Quality of life cannot be measured in fentomols.

Experience with the technique suggested that the lower sensitivity may be related to difficulties with antigen preservation. The ER antigen appears to be extremely fragile and is partially or totally destroyed if allowed to dry at any time. The drying artefact often leads to negative ER-ICA staining at the periphery of the smears, which was not interpreted as true negativity. Extreme care in the preparation and the fixation of the slides is emphasised and must not be underestimated.

Although ER-ICA staining does not provide a numerical value for ER status, without time consuming quantification of results, (Flowers et al 1986), it has been possible to ascribe a semi-quantitative value for tumour heterogeneity. Tumour heterogeneity is clearly important, and radioligand binding assays have been shown to vary with tissue taken from different parts of the same tumour.(Devleeschouwer et al 1988). Many of the studies with immunocytochemistry have used visual methods of assessing heterogeneity and intensity of staining, (Flowers et al 1986, King et al 1985 and Reiner et al 1987), correlating the indices with radioligand binding. The current study has identified a relatively small number of cases with a well defined population of ER-ICA negative cells among the positive tumour cells; long term follow-up is needed to tell whether or not this group of tumours has any clinical significance. It may well be that tumour heterogeneity changes during the development of the tumour, where previously ER positive clones of tumour cells are gradually depleted by the anti-oestrogen therapy, leaving only the ER negative tumour cells to proliferate. The presence of significant numbers of ER negative cells within a previously ER positive tumour may well affect the response of the tumour to hormonal manipulation.

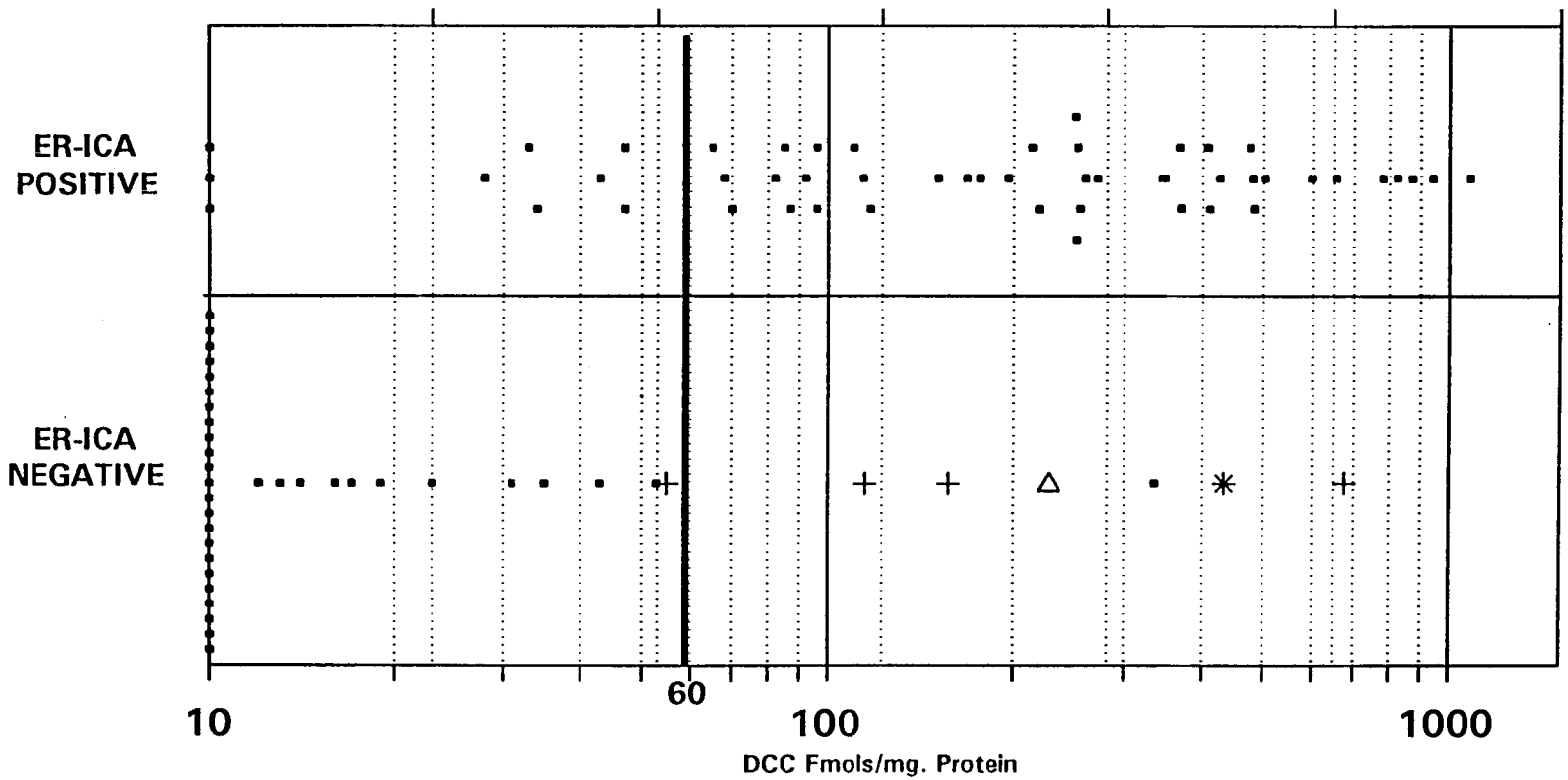
There was a striking correlation between ER-ICA positivity and patient's age above 65 years. It was previously considered that radioligand binding assay would underestimate ER levels in the pre-menopausal women because binding of tritiated oestradiol to receptor in tissue homogenates may be blocked by endogenous oestrogen. (Petterson et al 1985). This study has shown negative ER-ICA staining in all but twelve of the tumours from pre-menopausal women, even though the monoclonal antibody is known to react with a site remote from the site of hormone binding.

When referring to the statistical information from the survival analysis, the hazard calculation shows, pre-menopausal women under the age of fifty years are twice as likely to suffer a recurrence as the post-menopausal women at and over fifty years of age. The same hazard analysis for women who are ER-ICA negative as compared to those who are ER-ICA positive, shows ER-ICA negative women are one and a half times more likely to suffer a recurrence than those who are positive.

Although no correlation was found in this study between clinical stage or lymph node status, supporting previous studies with radioligand binding, (Shek and Godolphin 1989), there was a correlation between ER-ICA staining and tumour grade, less strong than with age, but statistically significant. Elston's grading scheme was shown to have a good correlation between combined Grade I and II ductal carcinomas and ER-ICA positivity, and between Grade III tumours and ER-ICA negativity. A previous study from Southampton showed a large proportion of high as opposed to low grade tumours in women under the age of 35, (Ashley et al 1989) using a slightly different modification of the Bloom and Richardson's grade, supporting the present study in which there were more ER-ICA negative tumours in pre-menopausal women, correlating with higher tumour grade.

This study has shown that there are undoubted technical problems in applying the ER-ICA technique to fine needle aspirates, possibly resulting in a lower sensitivity than biochemical assays. It is possible that adaptation of the technique for permanent paraffin sections could overcome this problem in the long term, Anderson et al 1988, providing a reliable assessment of ER status, but it is not known whether similar problems might be encountered if initial fixation had been delayed. Fine needle aspiration is likely to continue to have an important role in the pre-operative assessment of ER status, in inoperable cases, such as elderly patients treated with Tamoxifen alone (Davies et al. 1990) and in recurrent disease.

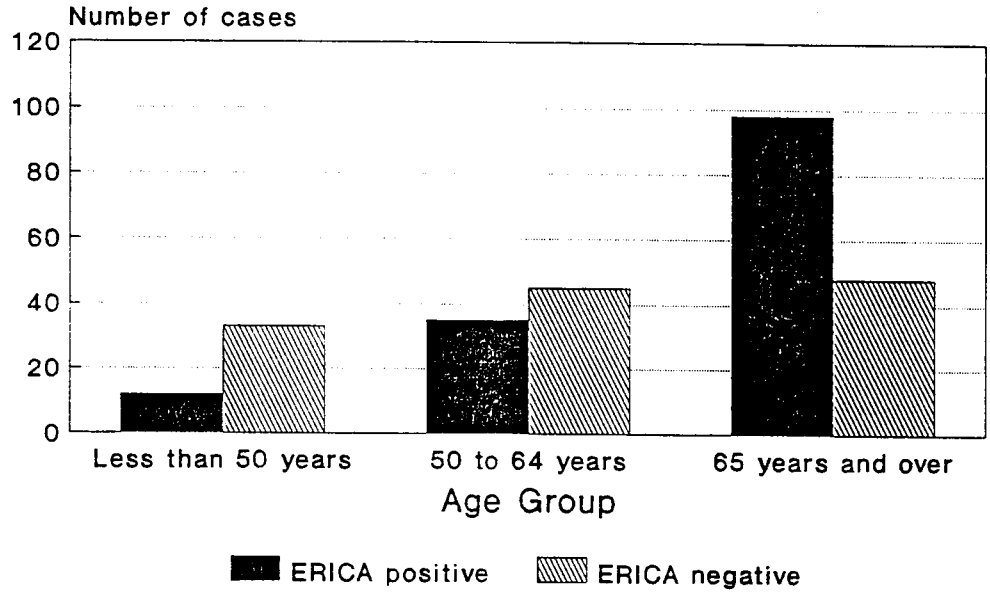
Figure 1. ER-ICA Compared with the Biochemical Radioligand Assay



Chi-Squared = 29.3, p < 0.001

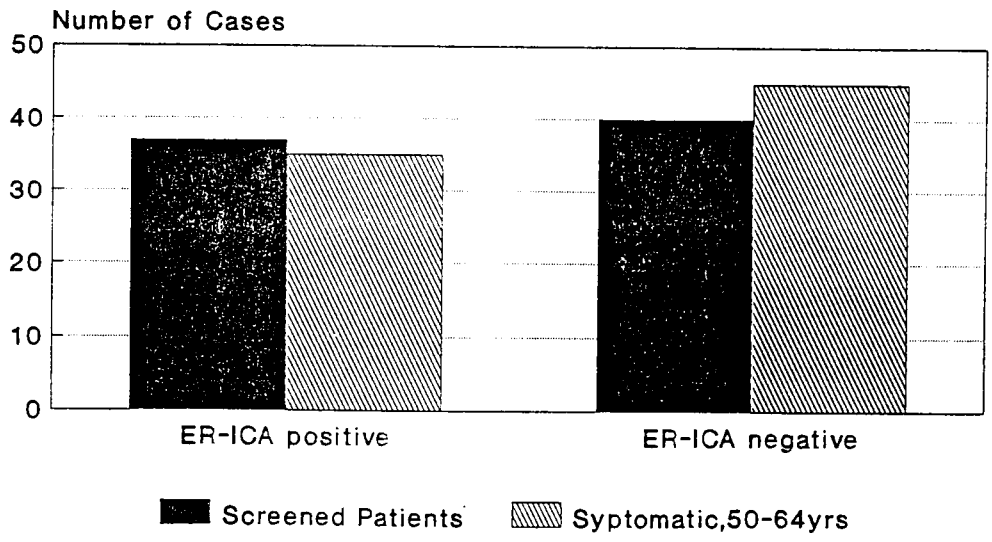
N.B. values < 10 fmols, Plotted at 10.

Figure 2
Age compared with ER-ICA



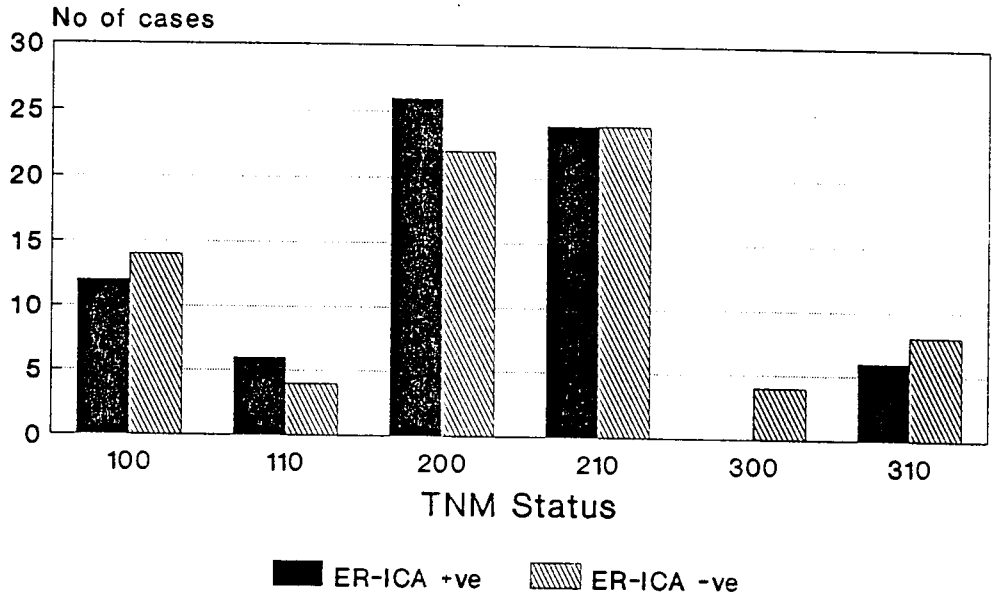
Chi-Squared = 26.9; $p < 0.001$

Figure 3
Breast Screened Patients compared with symptomatic patients aged 50 to 64 years



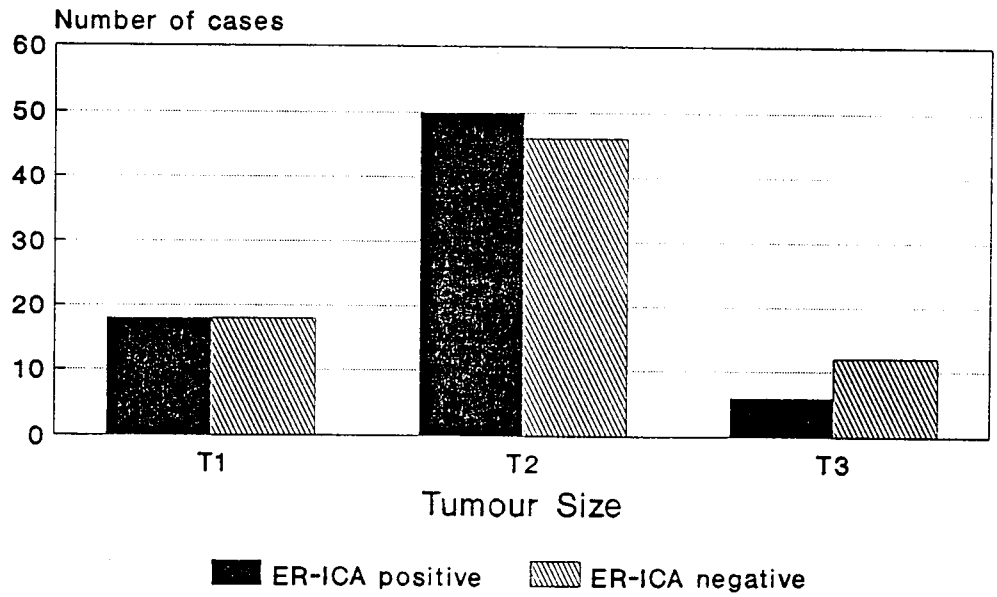
Chi squared = 0.32, $p < 0.5$

Figure 4
Tumour size,lymph node status vs. ER-ICA



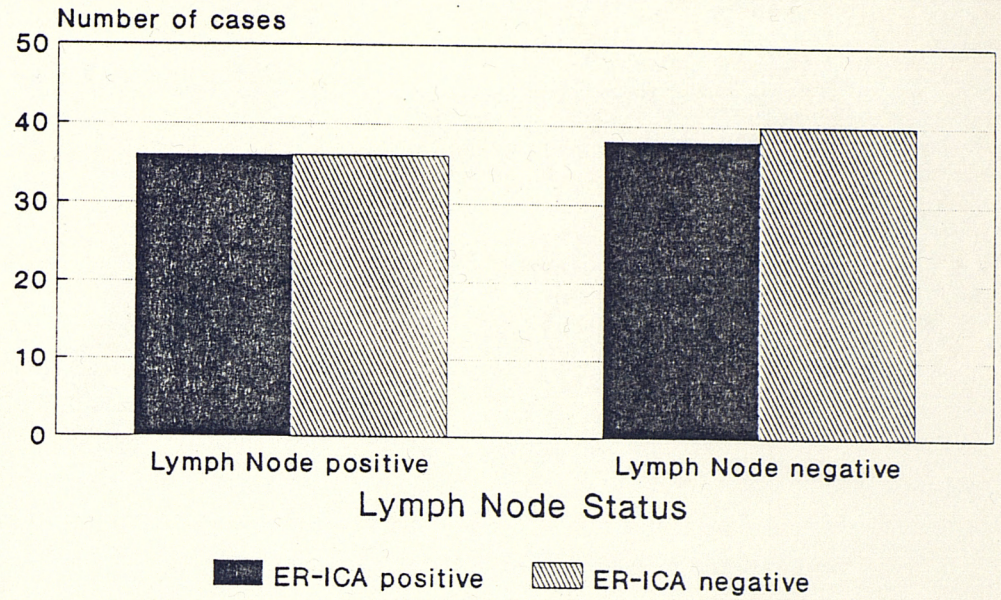
Chi-squared = 5.28, $p < 0.5$

Figure 5
Tumour size compared with ER-ICA



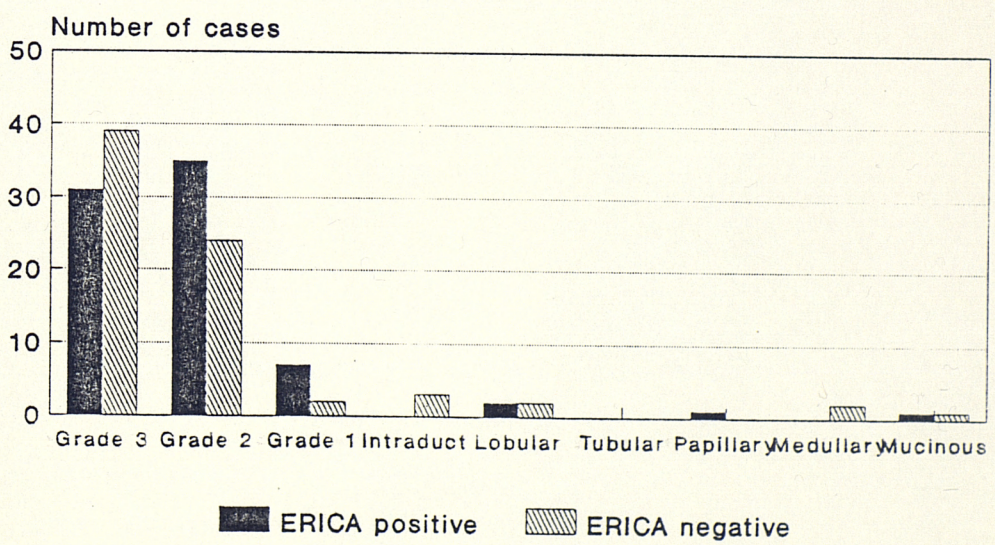
Chi-Squared = 2.14, $p < 0.3$

Figure 6
Lymph Node Status compared with ER-ICA



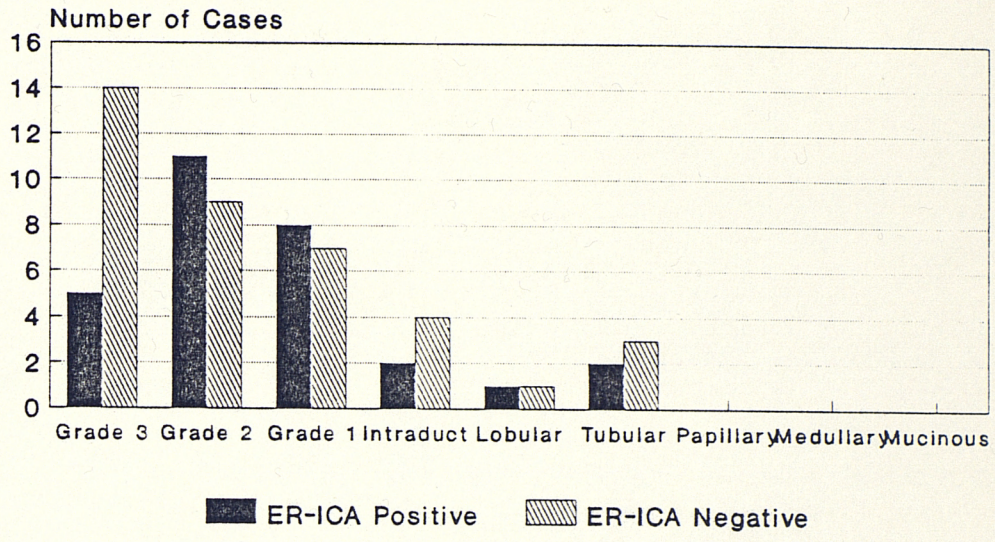
Chi-Squared = 0.05, $p < 0.3$

Figure 7
Histological Grade of symptomatic patients compared with ER-ICA



Chi-Squared = 5.19, $p < 0.1$

Figure 8
Breast screened patients, histological grade compared with ER-ICA



Chi-squared = 3.92, $p < 0.1$

Figure 9
Demonstration of Heterogeneity

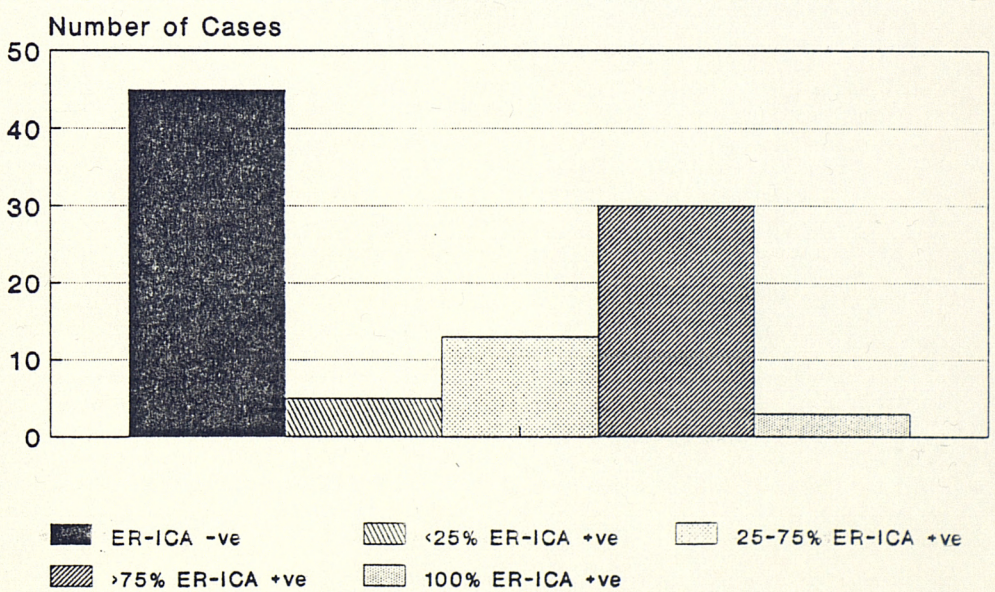
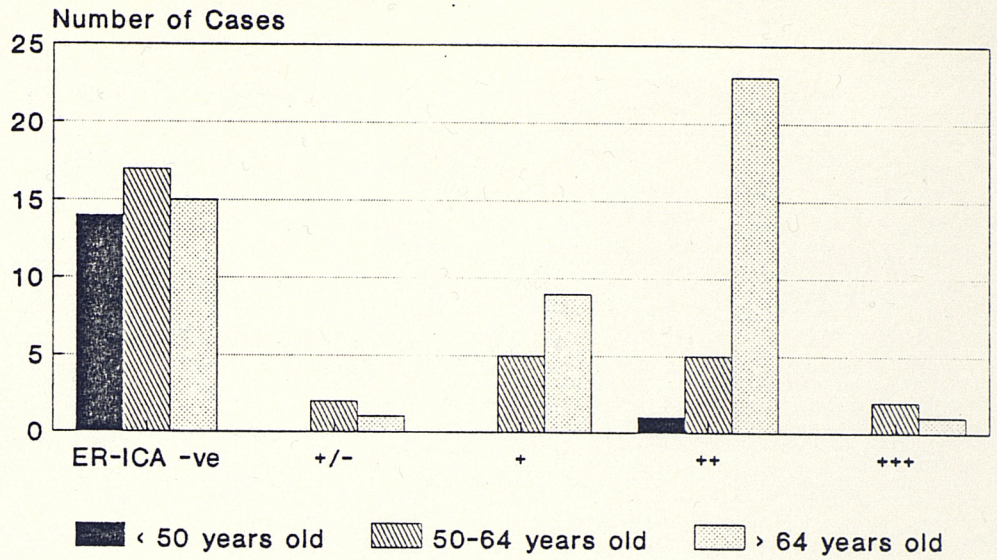


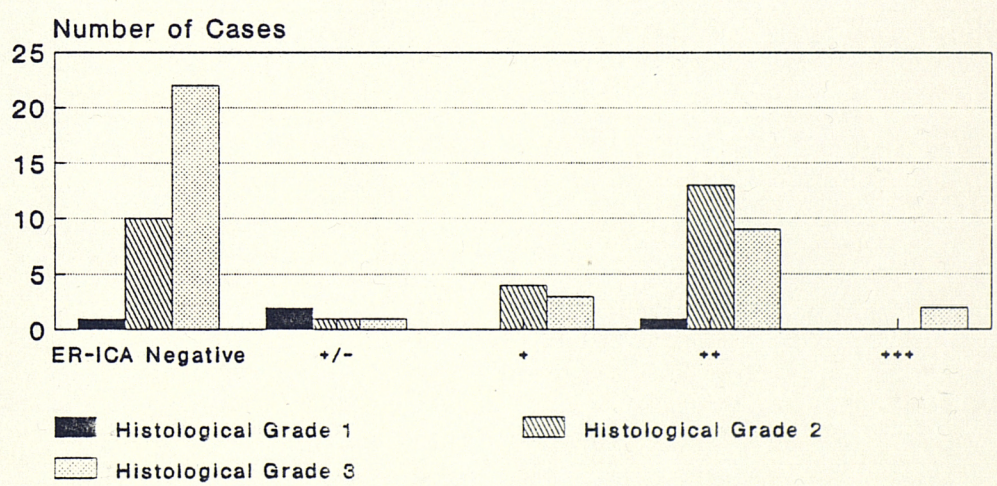
Figure 10
Heterogeneity compared with age.



Chi-squared = 25.36; p < 0.01

KEY : +/- = < 25% ER-ICA +ve; + = 25-75% ER-ICA +ve; ++ = >75% ER-ICA; +++ = 100% ER-ICA +ve

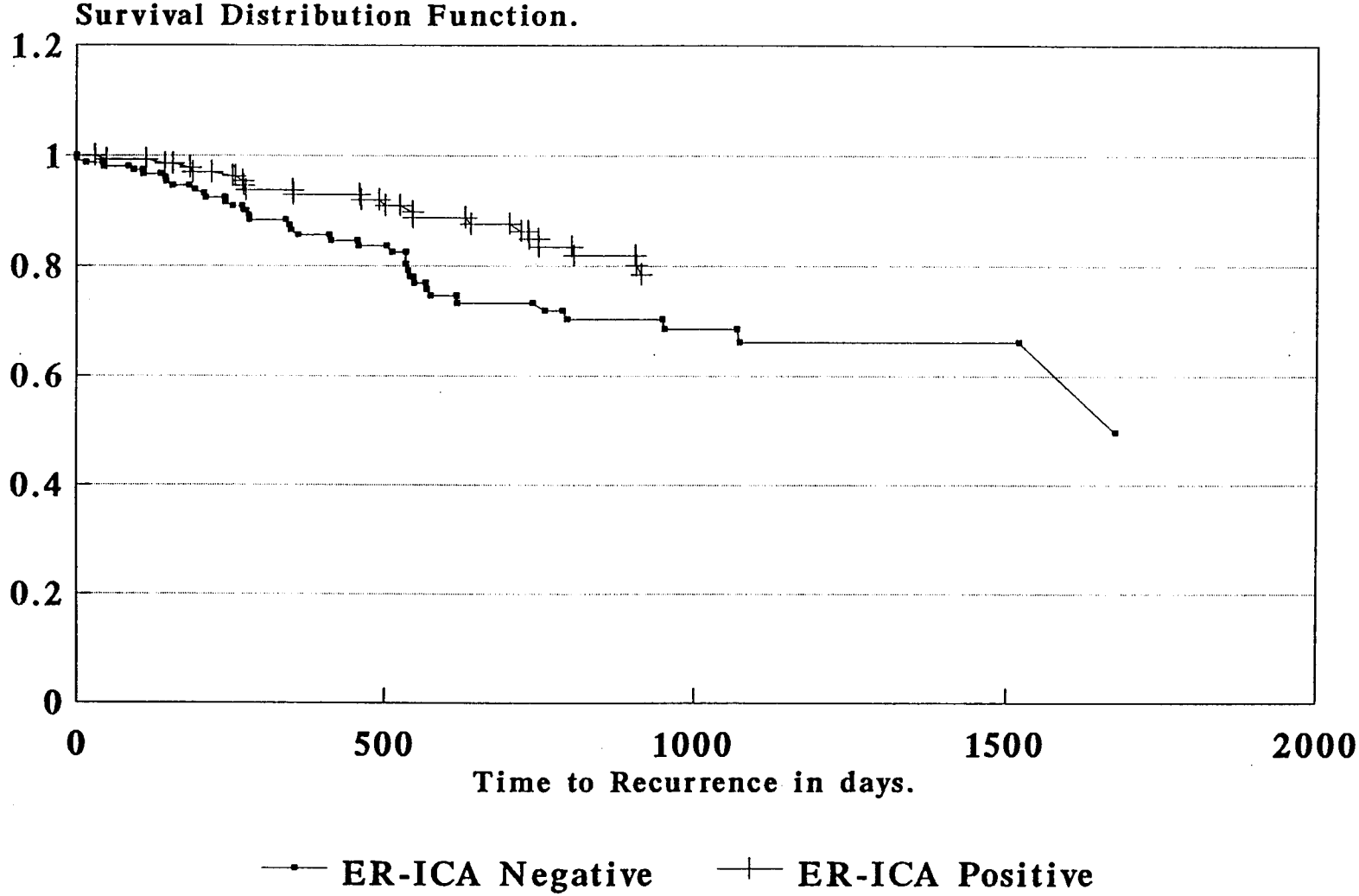
Figure 11
Histological grade compared with heterogeneity



Chi-squared = 26.2; p < 0.001

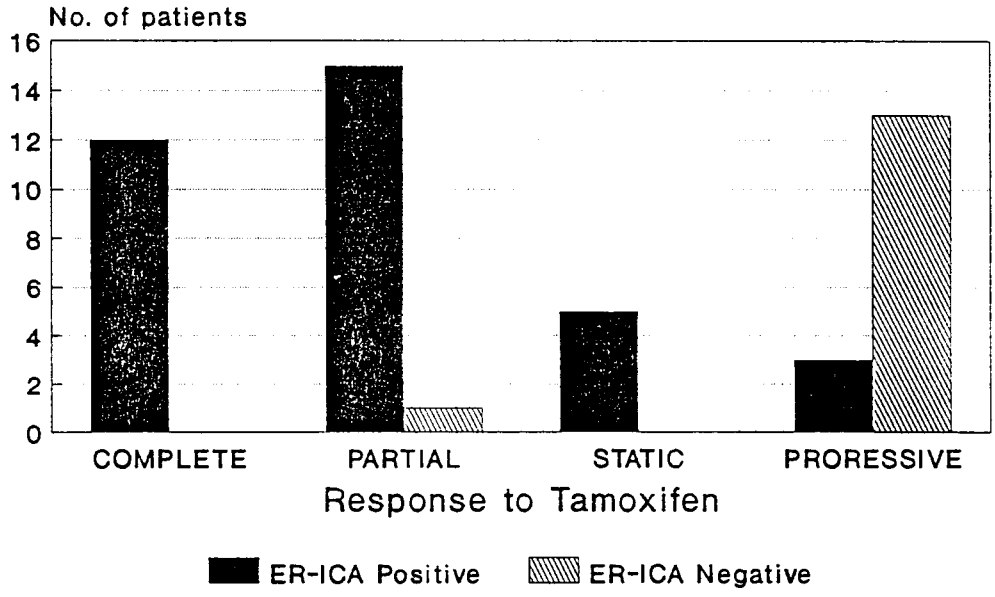
KEY : +/- = <25% ER-ICA positive; + = 25-75% ER-ICA +ve; ++ = >75% ER-ICA +ve; +++ = 100% ER-ICA +ve

Figure 12. Breast Cancer Recurrence Analysis



Chi-squared = 5.516; $p < 0.01$

Figure 13. ER-ICA Compared with Tamoxifen Response.



Chi-squared = 32.48; $p < 0.001$

3.0 INTRODUCTION TO THE DEMONSTRATION OF PROLIFERATING CELLS.

Cell proliferation is a fundamental biological process, so that understanding proliferation should give insight into the cellular cytological process. (Hall et al 1990). Conventional assessment involves the histological grading (Elston 1984) of tissue sections, or cytological grading of cells. (Hunt et al. 1990). The demonstration of proliferating cells by immunocytochemistry using the monoclonal antibody Ki67 would enhance the understanding of cell cycling systems.

The monoclonal antibody Ki67 demonstrates a nuclear antigen only present in proliferating cells, in G1, S, G2 and M phases. It does not immunostain cells in the resting phase G0. (Gerdes et al. 1983 and 1984). Hein van Dierendonck et al 1989 has described the exact nuclear distribution of Ki67 in human breast cancer cells, MFC-7. Their results have shown that the antigen demonstrated by Ki67 belongs to a group of antigens associated with the organization of chromosomes in metaphase and anaphase and with proteins located near to the cortical regions of prenucleolar bodies and nucleoli.

3.1 Ki67 LABELLING AN INDEX TO BREAST CANCER.

Ki67 immunostaining has been correlated with a variety of other parameters assessed in breast cancer, to determine its importance as an indicator of disease extent and prognosis.

Mitotic activity has in the past been the conventional way of assessing proliferative activity. (van der Linden et al 1987). There has been shown to be a strong correlation between Ki67 immunostaining and mitotic activity. (Marchetti et al 1990). However only a minor fraction of proliferating cells demonstrated in this way by Ki67, will be in mitosis. (Kuenen-Boumeester et al 1988).

A number of groups of workers have compared histological grade with Ki67 staining. There work has shown a considerable scatter, for the values of Ki67, so that the percentage values of Ki67 overlapped between the histological grades I, II and III. However the differences between the groups in each grade were statistically significant. (Gerdes et al 1986, Betta et al 1989, McGurrin et al 1987 and Lelle et al

1987). Others have postulated that grading breast cancers by a nuclear cytological grading system might provide a more accurate correlation, (Lash et al 1986, Bauer et al 1986, Bacus et al 1989)., than using the histological system which combines the assessment of the nucleus with observations on the maintenance of architectural patters within the tumour.

Lymph node status is the other major prognostic indicator in the assessment of breast carcinoma, but comparison of lymph node staging with Ki67 binding revealed no association in the majority of cases. (Bouzubar et al 1989, Barnard et al. 1987). However (Lelle et al. 1987) did suggest the average number of Ki67 positive staining cells was higher in cases with lymph node metastases.

Other parameters which have been assessed include, the age of the patient. A weak negative correlation was reported by Barnard et al (1987), which may reflect the state of the disease in younger women. Tumour size was not related to Ki67 activity, whereas tumour necrosis found most frequently in rapidly proliferating tumours was significantly associated with tumour grade. (Barnard et al. 1987). However, Bouzubar et al (1989) found that the large tumours did tend to have a disproportionately high number of cases with greater than 20% of the tumour cells staining positive with Ki67. Ki67 did not correlate with age at mastectomy, menopausal status or tumour size. (Bouzubar et al 1989).

Comparison with oestrogen receptor status has produced a variety of results. No correlation was found by McGurrin et al (1987), although eight of nine patients with Ki67 labelling over 30% were ER negative, no significant correlation was found by Barnard et al (1987) or Bouzubar et al (1989). However another author has found a significant very close inverse correlation, yet in this study there were some cases with both a high number of percentage positive staining in the Ki67 as well as high ER expression in most or all of the tumour cells. (Gerdes et al. 1987). Charpin et al (1988) show that Ki67 values are elevated when ER and PgR are absent.

Tumours with statistical higher values for Ki67, had a high mitotic rate, high nuclear and histological grade and were ER negative. (Crispino et al 1989). There was no statistical difference between Ki67 labelling and tumour size, lymph node invasion, age at diagnosis or menopausal status. (Crispino et al 1989). However Barnard et al (1987) did suggest a weak negative correlation between Ki67 and age of the patient.

3.2 ALTERNATIVE METHODS FOR DEMONSTRATION OF Ki67 VALUES.

Charpin et al 1989 used an image analysis system to analyse Ki67 and make comparisons between this and ER and PgR receptors. Most significantly they found no significant difference in the type of specimens used to demonstrate the Ki67 values, whether imprints, frozen sections or FNA specimens. They concluded that FNA constitutes a convenient method of cell sampling.

Ki67 labelling also compares favourably with tumour ploidy. The median Ki67 score was significantly higher in DNA aneuploid tumours than in diploid tumours (Isola et al 1989), and the thymidine labelling index, when tritium labelled thymidine estimates the number of tumour cells in the S phase of DNA synthesis, shows similar strong correlation. (Kamel et al 1989).

3.2.1 Alternative methods for the demonstration of the proliferating cell antigen.

More recently a new monoclonal antibody has been developed, named PC10, to demonstrate the proliferating nuclear cell antigen (PCNA). This research was carried out in non-Hodgkin's lymphomas, and shows a strong relationship between Ki67 and PC10. (Hall et al 1990). However in the same paper a reference is made to some unpublished data, by Barnes et al, expressing some difficulties with the correlation between cells in the S-phase and PCNA immunoreactivity. In this situation there appear to be more PCNA reactive cells than would be expected. One other observation made by Hall et al (1990), was seen in breast lobules adjacent to breast tumour. It involved histopathological normal tissue adjacent to the tumour cells, in some but not all of the cases, immunologically detectable PCNA was markedly increased. This observation may be due autocrine or paracrine growth factor influence on the PCNA gene expression, by increasing the PCNA mRNA stability and consequently PCNA expression. Growth factors secreted by the tumour may actively recruit surrounding benign cells into the accumulation of PCNA protein, without necessarily inducing DNA synthesis in the host cell. (Williams et al 1989).

3.3 METHODS AND MATERIALS.

Specimens for the Ki67 analysis were collected from the symptomatic breast clinic at the Royal South Hants. Hospital at the same time as those collected for ER-ICA analysis, and stored in the same way prior to immunostaining. (See section 2.6).

3.3.1 Collection of specimens from the breast screened patients.

Specimens from the breast screened patients were collected from the surgical specimen in the theatre as soon after surgery as possible. Slides were collected for ER-ICA and Ki67 into liquid nitrogen as for the symptomatic patients. One air dried slide was collected to be stained May-Grunwald Geimsa, to assess the presence of tumour cells prior to immunostaining. The preparations were either FNA specimens or touch imprints of the cut surface of the surgical specimen.

Poly-L-lysine coated microscope slides have helped to retain material for immunostaining. Cells from FNAC of tumour specimens do not usually detach themselves from the microscope slides, however, if the tumour is small it is possible to aspirate from the lump whilst at the same time aspirating quantities of fat and benign cells from the surrounding area. It is the fat cells which may preclude the rest of the aspirated cells from remaining on the microscope slide. Poly-L-lysine can produce a more tenacious surface for the cells to attach to. Imprint preparations of the cut surface of the visually detectable tumours, proved helpful in increasing the yield of cells. When micro-calcification was present in the surgical specimen, the specimen was sliced at 5 mm. intervals using a special knife holder. The slices were X-rayed and the imprint preparations taken from the areas of calcifications. Immunostaining of FNA and imprint slides requires substantial quantities of reagent, approximately 150-300 μl. Slides with a fatty component repel the aqueous reagents, so it is feasible some cells amongst the fatty component will be inadequately incubated with one of the reagents producing negative results.

3.3.2 Immunocytochemistry for the demonstration of proliferating cells.

In preparation for demonstrating proliferating cells by the monoclonal antibody Ki67 (Dakopatts Ltd.), the slides were taken from the -70°C freezer and placed into dry acetone for 20 minutes fixation. The slides were then rinsed in TRIS buffered saline

(TBS) for 5 minutes prior to immunostaining by the Strep ABComplex method. (See Appendix E).

Blocking, with methanolic hydrogen peroxide, (see Appendix F), to prevent non-specific binding of successive reagents was omitted from this procedure as it interfered with the immunostaining of the Ki67 antibody. The alternative procedure using sodium azaide, (see Appendix G), was discarded for the same reason.

A frozen section of tonsil tissue was used as a positive control. Ki67 demonstrates the nuclei in the proliferating follicles of the tonsil.

Test slides were incubated with the mouse monoclonal primary antibody for 30 minutes at room temperature, diluted 1:50 with TBS. Then washed in TBS three times for three minutes. The secondary stage, biotinylated sheep anti-mouse antibody (Dakopatts Ltd.) was incubated for 30 minutes at room temperature, washed three times for three minutes in TBS. Finally, the tertiary antibody Strep ABComplex HRP, (Dakopatts Ltd.), labelled with horseradish peroxidase. The horseradish peroxidase molecules were demonstrated by the cromogen substrate solution 3'3 diaminobenezidine tetrahydrochloride (DAB), forming the brown coloured end product. All slides were counterstained with a weak solution of Harris' haematoxylin, approximately 1% for 10 minutes. (See Appendix E).

3.3.3 Assessment of Ki67 slides.

All slides were examined on an Olympus microscope using the times 40 objective. The control slide for each case was examined for endogenous peroxidase staining and compared with the equivalent test slide. The control slides, for individual patient test, were achieved by omitting the primary antibody in the Strep ABComplex immunostaining procedure.

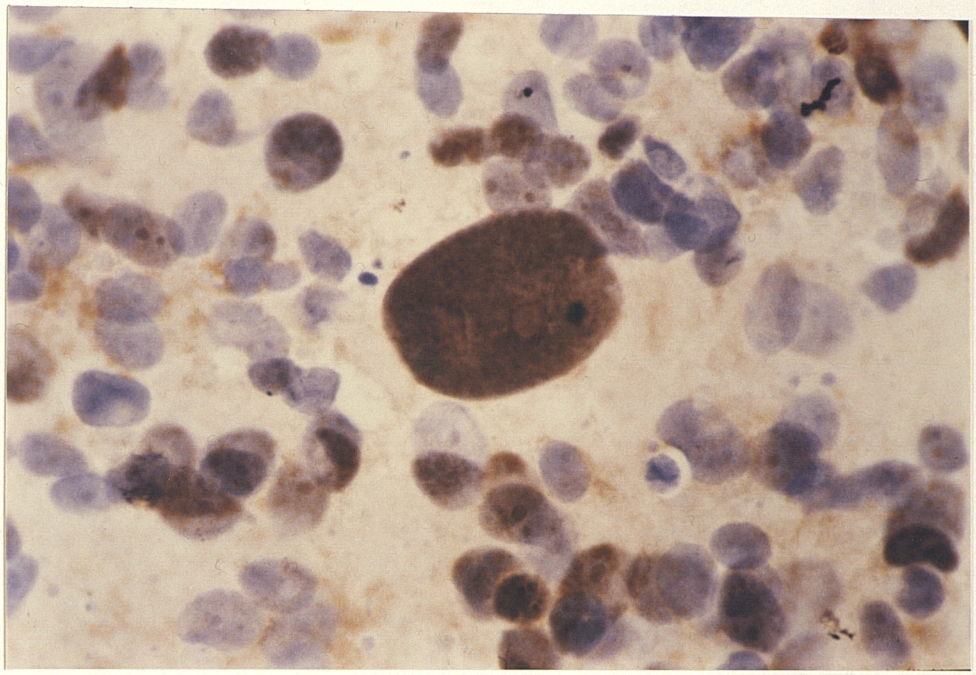
A minimum of 100 cells were counted for every case. Positive cells were identified as described by Hein van Dierendonck et al (1989). This may be shown by positive staining in the mitotic figures, granular staining aborizing throughout the nuclei, (see Photograph K), or more strongly over the nucleoli, (see Photograph L). At the beginning of the study a microscope with an extension tube was used to ensure the accuracy of the counting system. Each individual Ki67 positive and negative cell nuclei was recorded by tracing their image onto graph paper, with experience this

procedure was nolonger necessary. Cell nuclei were counted visually using a cell counter to record the positive and negative numbers.

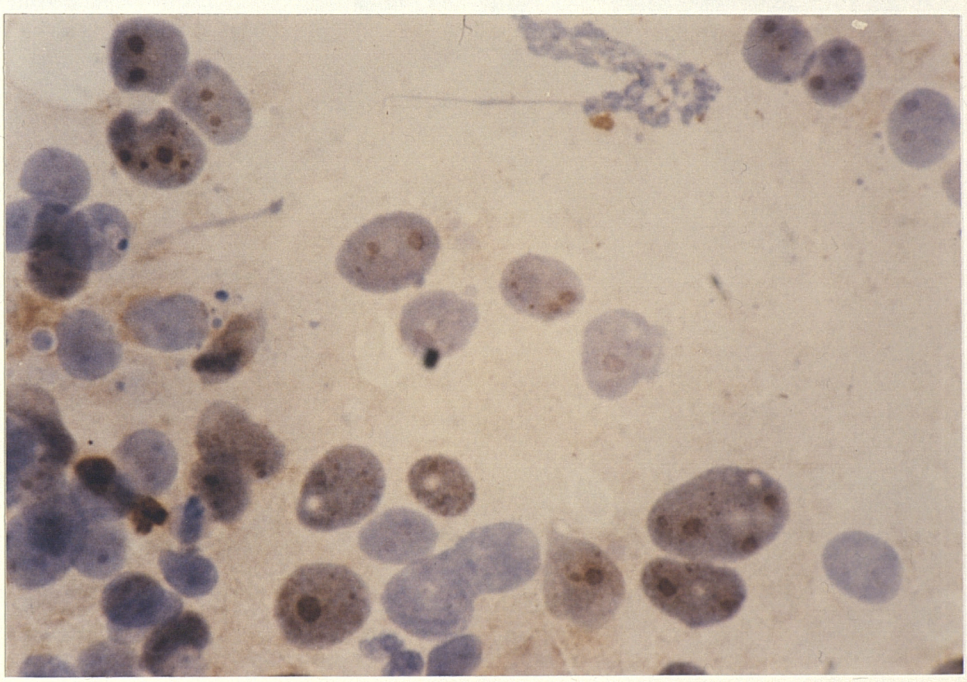
The Ki67 value is expressed as the percentage of positive nuclei to the total number of positive and negative nuclei counted.

$$\frac{\text{n - positive nuclei}}{\text{n - positive nuclei + n - negative nuclei}} = \text{\% Ki67 value}$$

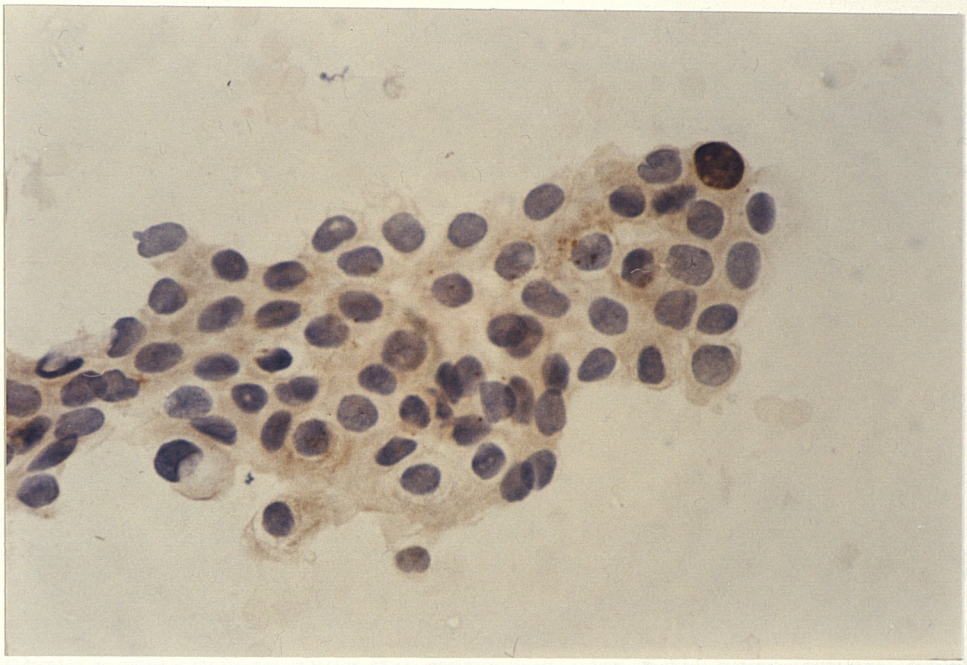
Observer error was assessed by comparing the Ki67 values counted by two independent microscopists, examining the same specimen preparation. For statistical purposes the Ki67 values were grouped into three bands, >15% Ki67, 15-30% Ki67 and >30% Ki67, representing the low, moderate and high Ki67 grades. Photographs M and N show the range of Ki67 immunostaining observed, less than 15% and greater than 30% Ki67 staining, respectively. Full agreement was obtained when assessing the results between observers in these bands.



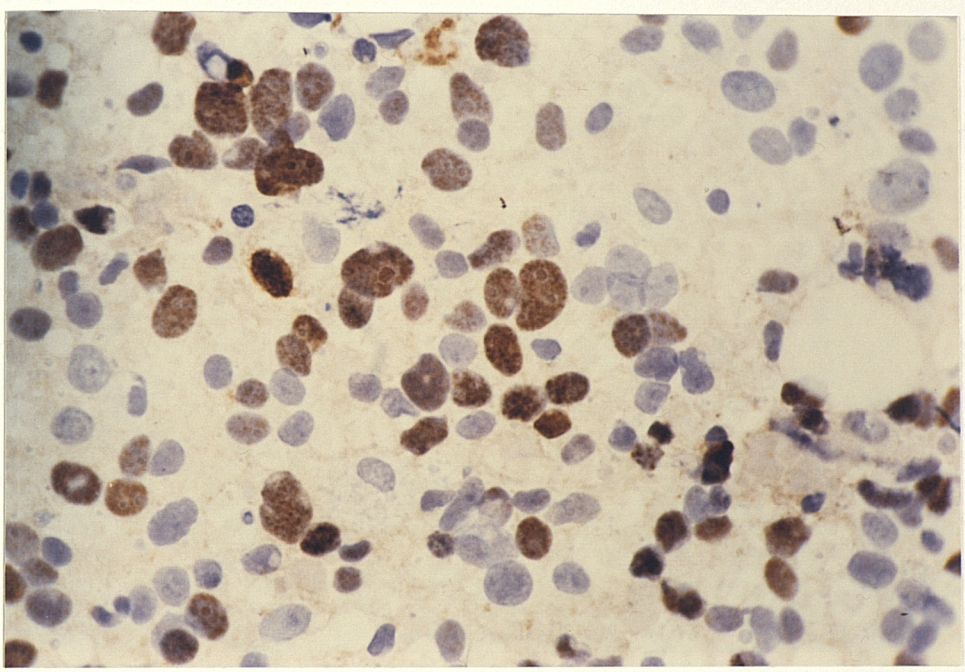
PHOTOGRAPH K : Positive KI67 Staining Arborizing Throughout The Nuclei.x630.



PHOTOGRAPH L : Positive KI67 Staining More Strongly In The Cell Nucleoli.x630.



PHOTOGRAPH M : Positive Ki67 Staining, <15% Ki67.x630.



PHOTOGRAPH N : Positive KI67 Staining, >30% Ki67.x400.

3.4 RESULTS.

A total of 128 symptomatic breast patients had satisfactory Ki67 immunostaining, 114 of these cases had satisfactory ER-ICA staining for comparison and 68 cases had a histological grade. There were insufficient numbers of breast cancers of special type to provide a statisical analysis, therefore they have not been demonstrated in this chapter. In the breast screening group 31 cases of the breast screened patients had satisfactory ER-ICA analysis for comparison and 28 cases had histological grades, the 3 other cases were breast tumours of special type.

3.4.1 Correlation of Ki67 with ER-ICA staining, in symptomatic patients.

The Ki67 values ranged from 1% to 57%, with a mean value of 8% Ki67 for ER-ICA positive tumours and 18% Ki67 for ER-ICA negative tumours.
56% of ER-ICA negative tumours fell above the 15% Ki67 level, whilst, 83% of ER-ICA positive tumours were on or below the 15% Ki67 value, see Figure 14 and Table 13. These results showed a strong positive correlation between ER-ICA positivity or negativity and the percentage of Ki67 staining, below 15%, between 15 and 30% and above 30%.

TABLE 13. COMPARISON OF Ki67 WITH ER-ICA, IN SYMPTOMATIC WOMEN.

% Ki67	ER-ICA POSITIVE	ER-ICA NEGATIVE	TOTAL
< 15%	53	22	75
15 -30%	8	18	26
> 30%	3	10	13
TOTAL	64	50	114

X^2 with 2 degrees of freedom = 19.2 $p < 0.001$

3.4.2 Correlation of Ki67 with histological grade in symptomatic patients.

The comparison of Ki67 and histological grade showed a weak positive statistical correlation, see Figure 15, Table 14. Those cases below 15% Ki67 were distributed throughout the range of histological grades, however 94% of Ki67 values between 15-30% and all of the Ki67 values above 30% were found to be histological grade II or III.

TABLE 14. COMPARISON OF Ki67 WITH HISTOLOGICAL GRADE.

%Ki67	HISTOLOGICAL GRADE			TOTAL
	I	II	III	
< 15%	5	24	13	42
15-30%	1	4	11	16
> 30%	0	2	8	10
TOTAL	6	30	32	68

X^2 with 4 degrees of freedom = 11.04 $p < 0.05$

3.4.3 Correlation of Ki67 and age of the patient.

The comparison of Ki67 with the age of the patient is shown in Figure 16, Table 15. There was a good statistical correlation between the two, 77% of women over 64 years of age have less than or equal to 15% Ki67 values, whilst, 59% of young women under 50 years of age have Ki67 values greater than 15% Ki67.

TABLE 15. COMPARISON OF Ki67 WITH THE AGE OF THE SYMPTOMATIC PATIENTS.

%Ki67	PATIENT AGE IN YEARS			TOTAL
	< 50	50-64	> 64	
< 15%	9	21	51	81
15-30%	7	13	12	32
> 30%	3	7	5	15
TOTAL	19	41	68	128

X^2 with 4 degrees of freedom = 8.85 $p < 0.05$

3.4.4 Correlation of Ki67 with TNM stage, and tumour size and lymph node status alone in symptomatic patients.

There was no statistical correlation between Ki67 and TNM stage (see Figure 17). Correlation of Ki67 and tumour size alone, Table 16, Figure 18 and lymph node status alone, Table 17, Figure 19 showed similar results, with no statistical significant correlation.

TABLE 16. COMPARISON OF Ki67 AND TUMOUR SIZE.

%Ki67	SIZE OF THE TUMOUR			TOTAL
	T 1	T 2	T 3	
< 15%	4	30	5	39
15-30%	5	10	4	19
> 30%	1	8	0	9
TOTAL	10	48	9	67

X^2 with 4 degrees of freedom = 5.82 $p < 0.3$

TABLE 17. COMPARISON OF Ki67 WITH LYMPH NODE (LN) STATUS.

% Ki67	LN LN POSITIVE	NEGATIVE	TOTAL
< 15%	22	17	39
15 -30%	10	9	19
> 30%	5	4	9
TOTAL	37	30	67

X^2 with 3 degrees of freedom = 0.07 $p < 0.3$

3.4.5 Comparison of Ki67 in breast screened patients with ER-ICA and histological grade.

There were 31 patients with breast carcinoma from the breast screening unit, who had satisfactory results for the ER-ICA analysis. This showed a weak statistical correlation $p < 0.05$, not as great as that shown in the symptomatic group of patients, see section 3.4.1. There was no significant correlation between Ki67 and histological grade, in the breast screening group see Figure 20, Table 18 and Figure 21, Table 19, respectively.

TABLE 18. Ki67 COMPARED WITH ER-ICA IN BREAST SCREENED PATIENTS.

% Ki67	ER-ICA POSITIVE	ER-ICA NEGATIVE	TOTAL
<=15%	14	13	27
> 15%	0	4	4
TOTAL	14	17	31

X^2 with correction for continuity = 4.21 $p < 0.05$

TABLE 19. Ki67 COMPARED WITH HISTOLOGICAL GRADE IN BREAST SCREENED PATIENTS.

%Ki67	HISTOLOGICAL GRADE			TOTAL
	I	II	III	
<= 15%	10	8	7	25
> 15%	0	1	2	3
TOTAL	9	8	8	28

X^2 with 2 degrees of freedom = 2.5 $p < 0.5$

3.4.6 Correlation of %Ki67 values in breast screening unit (BSU) patients and symptomatic breast patients aged 50-64 years.

There were insufficient patients from the breast screening unit, in the over 30% Ki67 group to include the group in the statistical analysis, shown above in figures 19 and 20. The one BSU case identified in the over 30% Ki67 group, was histological grade III ductal carcinoma, ER negative and 37% Ki67 positive.

Since there was only one BSU patient who recorded a Ki67 value of over 30%, it was appropriate to compare the Ki67 results from the breast screened women with an age matched group from the symptomatic breast carcinomas. (see Table 20 and Figure 22).

TABLE 20. PERCENTAGE Ki67 COMPARED IN BREAST SCREENING UNIT (BSU) PATIENTS AND SYMPTOMATIC BREAST PATIENTS AGED 50-64 YEARS.

% Ki67	SYMPTOMATIC PATIENTS	SCREENED PATIENTS	TOTAL
< 15%	21	26	47
15 -30%	13	3	16
> 30%	7	2	9
TOTAL	41	31	72

X^2 with 2 degrees of freedom = 8.29 $p < 0.025$

Table 20 and Figure 22 shows a good statistical correlation between the aged matched groups, this must be because the number of cases in the <15% Ki67 were very similar and the number of cases in the 15-30% Ki67 and >30% Ki67 values were small and had an insignificant effect on the final correlation.

3.5 DISCUSSION.

The results of this section of the study has shown FNA specimens may be used successfully to demonstrate other monoclonal antibodies, than those against ER. In this case the monoclonal antibody Ki67 has identified proliferating cells to great effect.

The collection of FNA material for this study followed the same procedure as for the material collected for the demonstration of ER, (see Section 2.6). Both sets of slides were collected in the same way for ease and convenience in the busy clinic environment. However there is no evidence to suggest that the Ki67 is susceptible to destruction in the same way as the ER antigen. In fact this is quite to the contrary, the demonstration of the Dakopatts Ki67 antigen has been adapted for use in paraffin sections, where the specimen requires no special treatment prior to fixation.

The collection of material for Ki67 staining was not a problem although the immunostaining procedure proved to be more difficult. The Strep ABC method was used because of its reputation for high sensitivity. A normal procedure when using this technique would be to block for endogenous peroxidase, to prevent it being demonstrated in the specimen, methanolic hydrogen peroxidase was used, (see appendix F). However this method interfered with the antigen and inhibited all positive staining. An alternative procedure using sodium azide was implemented, (see appendix G), this produced the same negative results. Finally the immunostaining was carried out without any blocking system employed for endogenous peroxidase. This was proved satisfactory, with strong positive staining in the nuclei of the cells, in the range of 1-57% Ki67 from every cellular specimen. The immunostaining of endogenous peroxidase did not prove to be a problem, with only in a minority of cases could some brown background immunostaining be observed. Breast tumour FNAC are usually cellular but free from inflammatory cells such as polymorphs and histiocytes and without proteineous background material. There may be some red blood cells as some tumours may be vascular in nature, but this fails to cause a problem with inappropriate staining of endogenous peroxidase.

Assessment of the Ki67 slides caused some problems, in that however carefully FNA material is spread onto the glass microscope slides, the nature of the specimen determines the way in which the cells are spread, this does not always result in the perfect monolayer of cells. Whilst this does not prove to be a problem in the gross assessment of the less than or greater than 25% positive staining in the ER-ICA assay, counting individual cell nuclei can be more difficult.

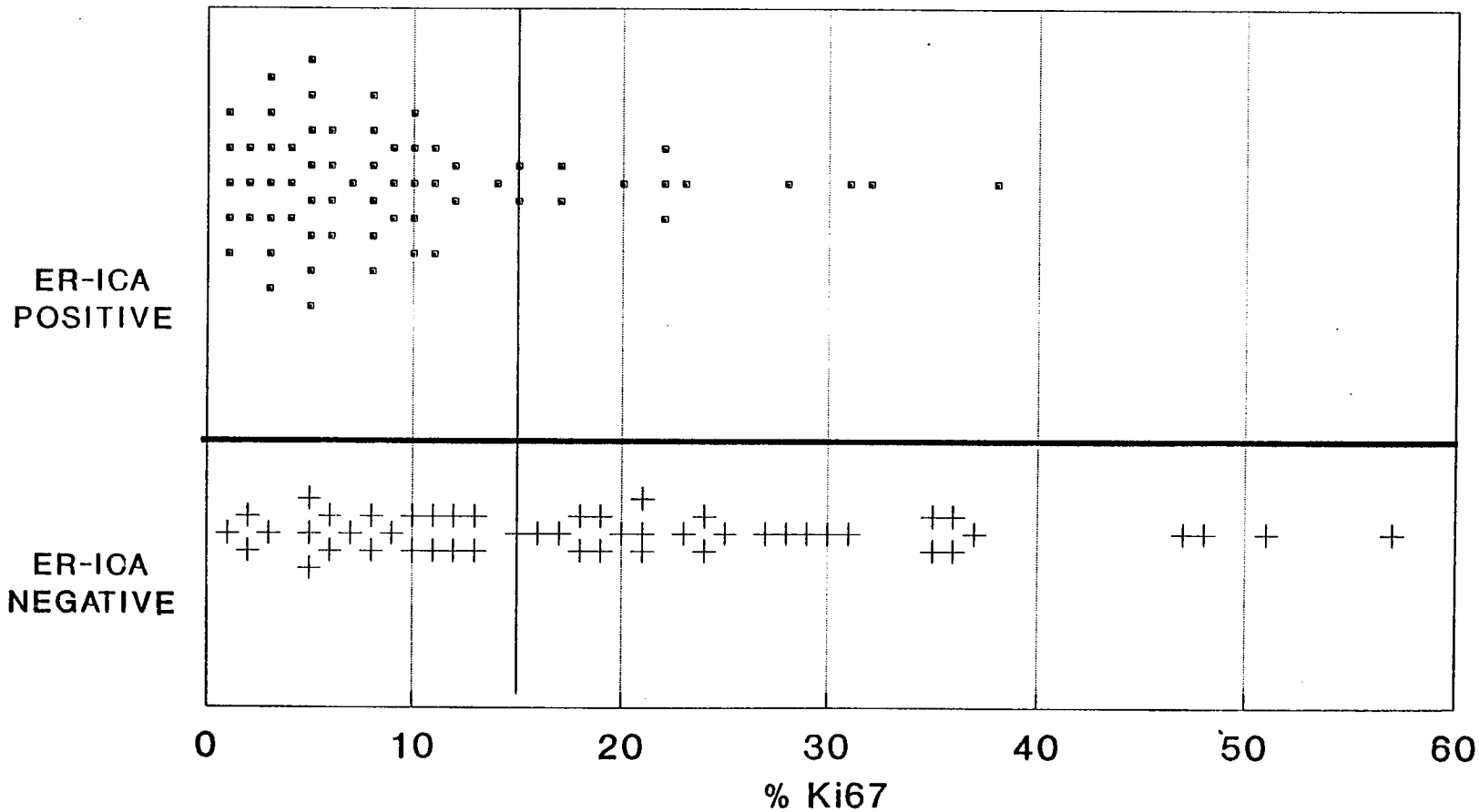
The statistical correlation between ER-ICA positivity and negativity and Ki67 values above and below 15% was very strong indeed, $p < 0.001$. Comparison of Ki67 with the age of the patient showed a strong correlation $p < 0.01$, similar to that seen with the ER-ICA assay. There was no correlation found between Ki67 and TNM stage or between lymph node status alone and tumour size alone.

When considering the patients from the breast screening unit, there was no significant correlation between Ki67 and ER-ICA or histological grade of the excised tumour. This may have been because of the small size of the population examined, only 26 patients had material adequate for both ER and Ki67 status, because of the small size of many of the tumours. The size of tumours identified was extremely variable, from tissue adjacent to micro-calcifications to tumours greater than 5 cm. in dimension. In the first round of the screening programme, as well as picking up very small impalpable lesions, there will also be a number of larger palpable tumours which would have been classed as symptomatic had the patient detected the tumour herself. It is said that the practice of accurate breast self examination is not widespread amongst women of this age group. (Forrest 1986).

In the same way as preparations from symptomatic patients, FNA and imprint specimens from breast screening patients are prone to air-drying at the periphery of the preparation prior to freezing or fixation. Although this is not as pronounced with the fatty specimens. All these phenomenon may contribute to more unsatisfactory preparations and reduced quality of ER-ICA and Ki67 results in some cases from breast screened patients.

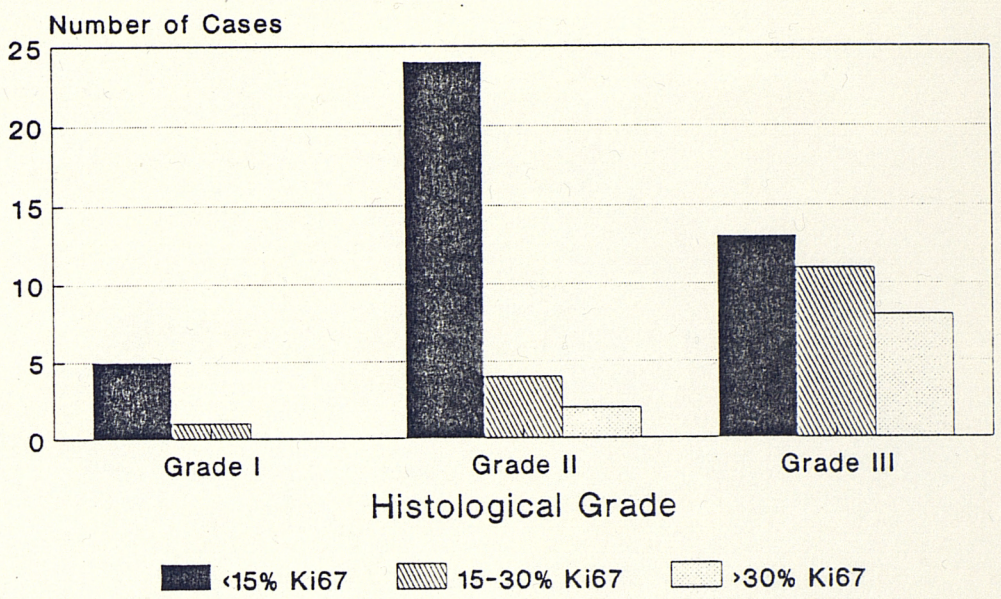
Other antibodies have been suggested for the demonstration of proliferating cell antigen. The monoclonal antibody PC10 demonstrates the proliferating cell antigen (PCNA). PC10 has been proven to have a linear relationship with Ki67 antibody in non-Hodgkin's lymphoma. (Hall et al 1990). However, the same correlation was not observed in breast tumours. (Barnes et al, unpublished data, quoted by Hall et al 1990). In some breast and pancreatic tumours there is apparent deregulation of PCNA with increased expression in the tissues adjacent to the tumours.

Figure 14. ER-ICA Compared with Ki67 in Symptomatic Patients



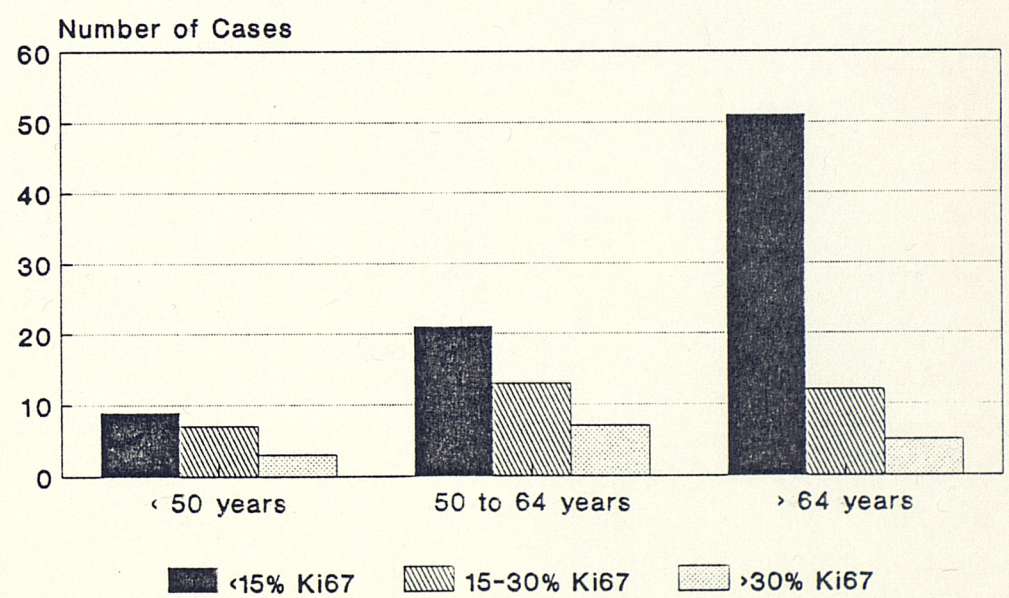
Chi-Squared = 19.2, $p < 0.001$

Figure 15. Ki67 and Histological Grade in Symptomatic Patients



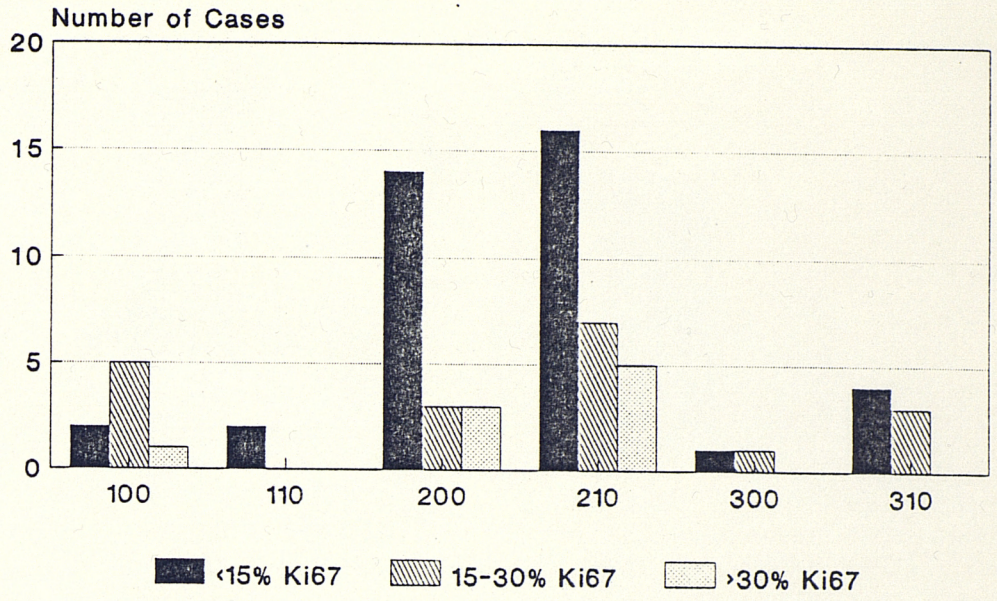
Chi-Squared = 11.04 $p < 0.05$

Figure 16. Ki67 Compared with Age in Symptomatic Patients



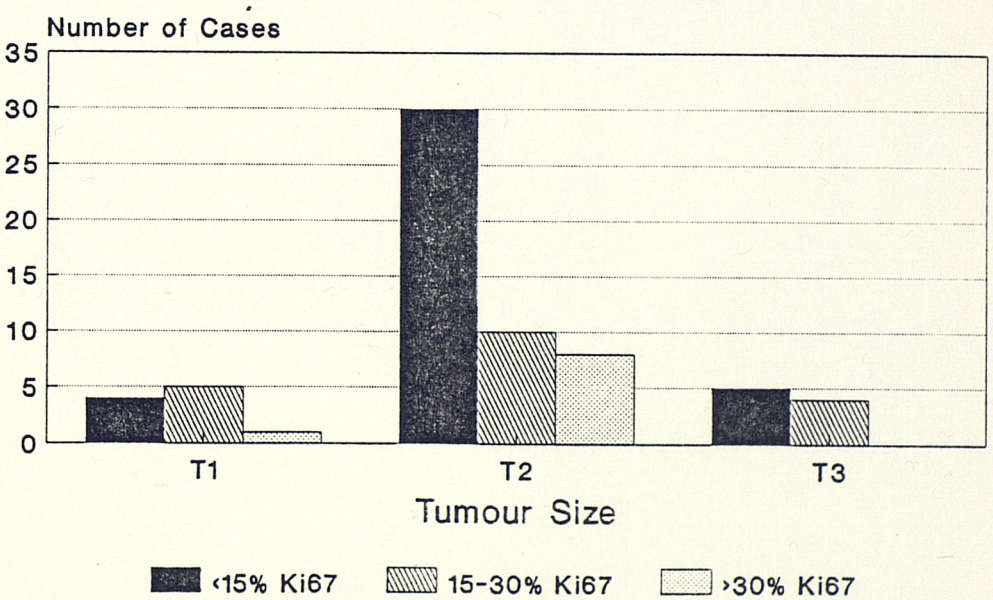
Chi-squared = 8.85, $p < 0.05$

Figure 17. Ki67 Compared with TMN Status in Symptomatic Patients



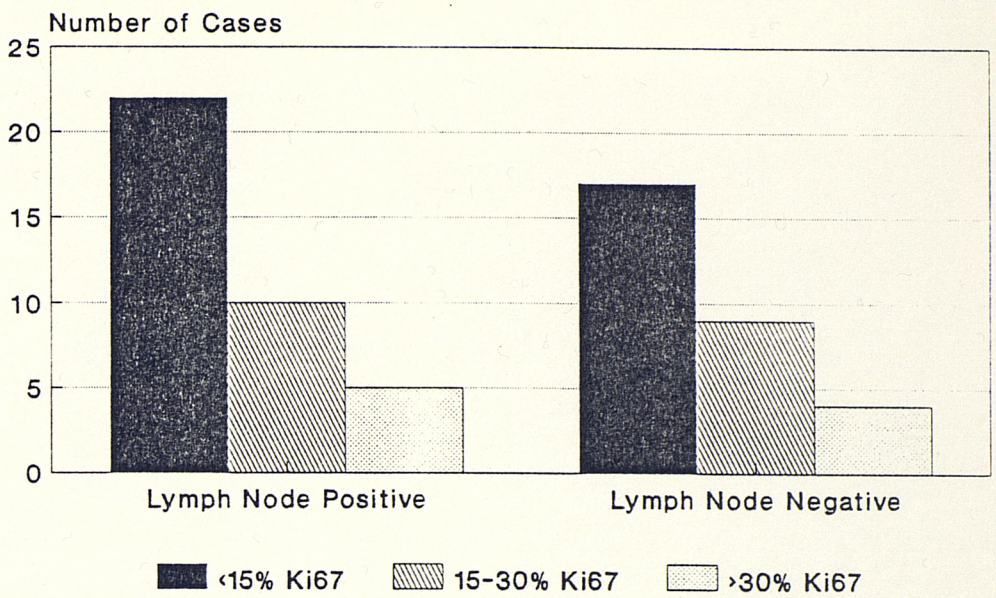
Chi-Squared = 10.73 $p < 0.3$

Figure 18. Ki67 Compared with Tumour Size in Symptomatic Patients



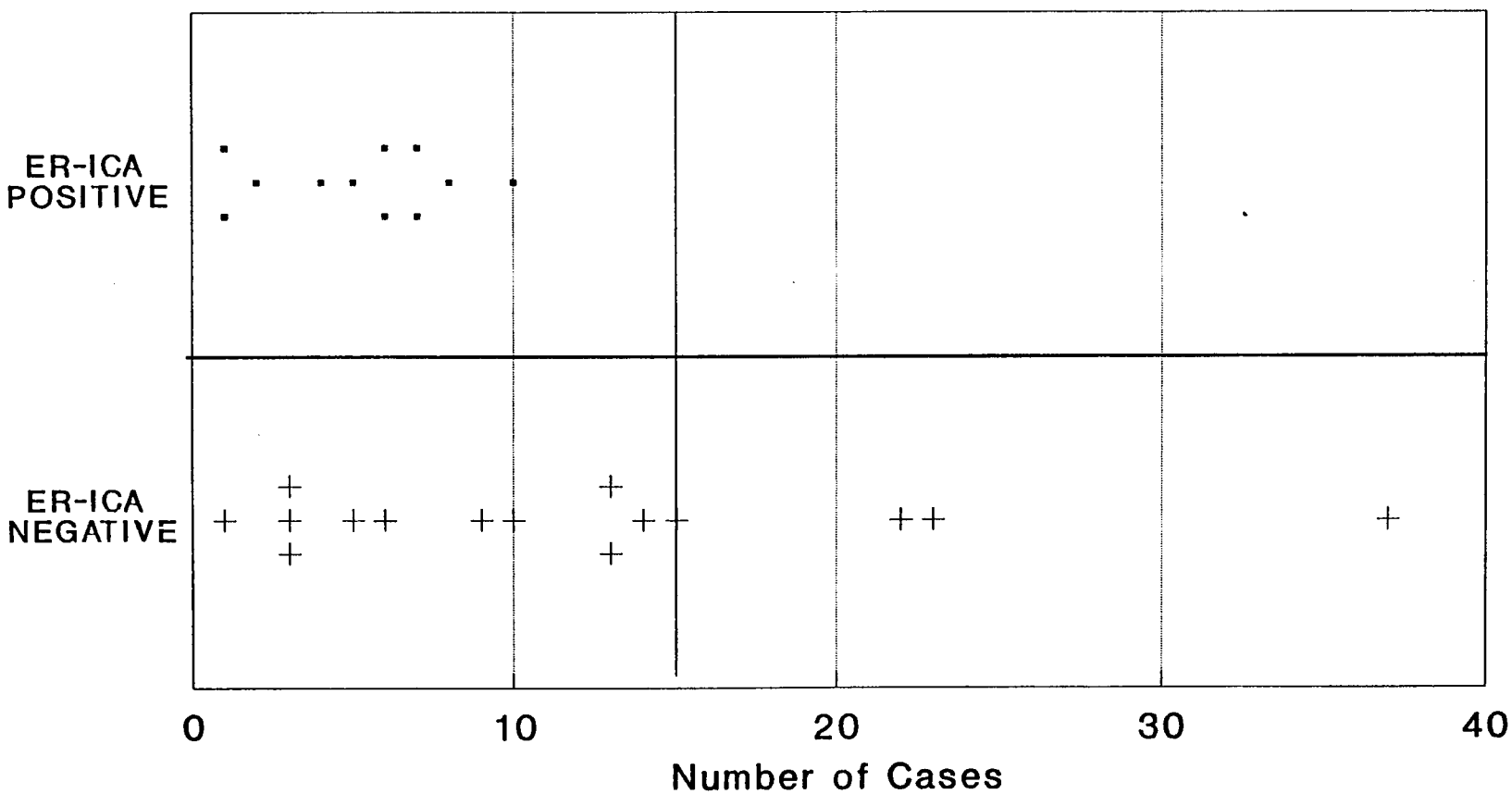
Chi-Squared = 5.82 $p < 0.3$

Figure 19. Ki67 Compared with Lymph Node Status in Symptomatic Patients



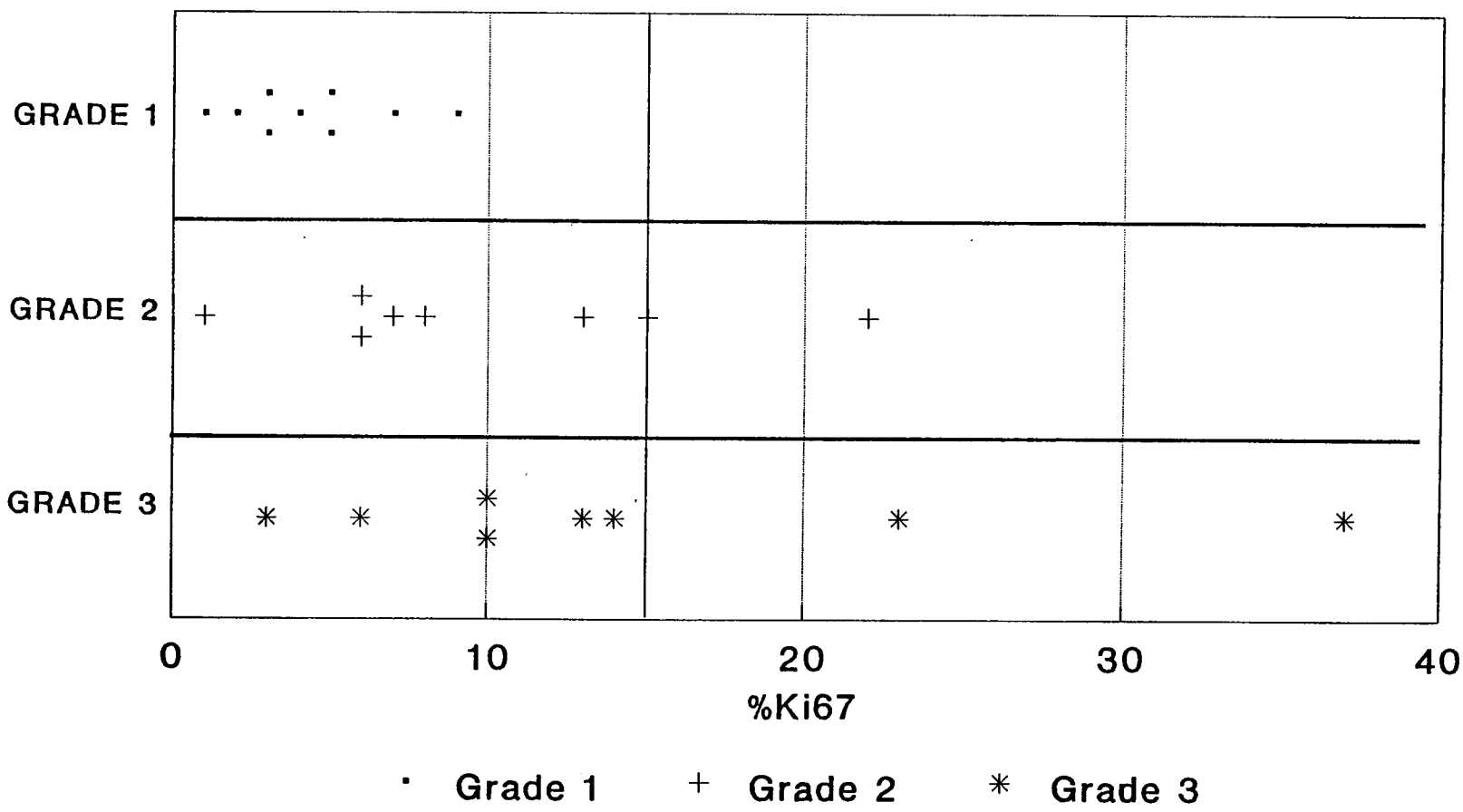
Chi-Squared = 0.07, $p < 0.3$

Figure 20. Ki67 Compared with ER-ICA in Breast Screened Patients



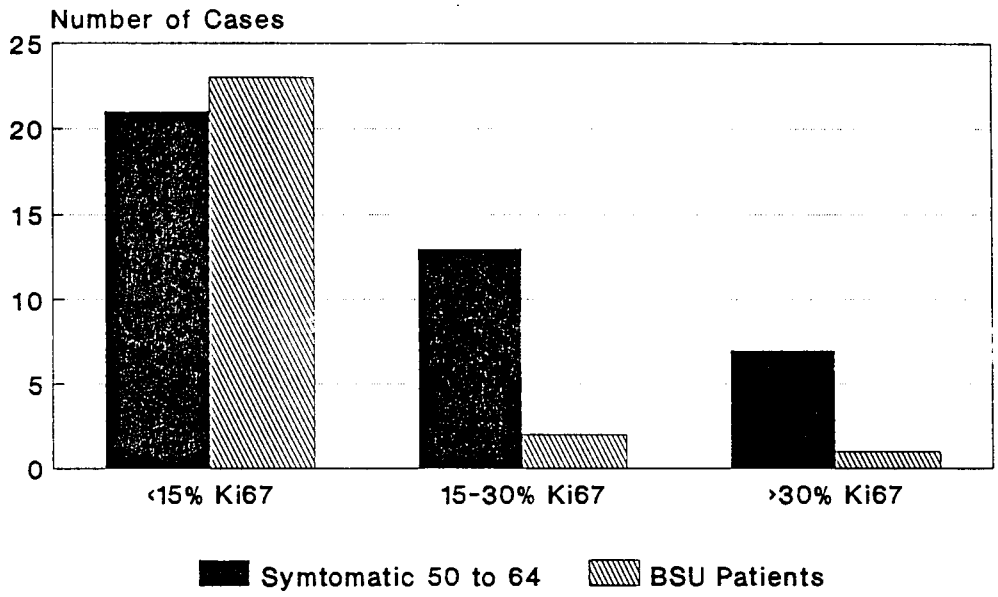
Chi-squared = 1.72, p < 0.2

Figure 21. Ki67 Compared with Histological Grade in Breast Screened Patients



Chi-squared = 3.53, $p < 0.2$

Figure 22. Ki67 Compared with BSU and Symptomatic Patients 50-64 Years



Chi-Squared = 9.68 $p < 0.01$

4.0 INTRODUCTION TO EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR).

Epidermal growth factor (EGF) is a low molecular weight polypeptide (6000 daltons), composed of 53 amino acid residues. (Cohen 1983). EGF is known to accelerate cell proliferation through binding with its specific receptor, epidermal growth factor receptor (EGFR). (Carpenter and Cohen 1979). EGF also has a significant homology with transforming growth factor alpha (TGF-alpha). Oestrogens may stimulate the release of growth factors such as TGF-alpha from hormone sensitive breast cancer lines and therefore may mediate the growth response. The EGRF has an external domain which binds EGF and TGF-alpha, a transmembrane section and a cytoplasmic section which contains tyrosine-specific protein kinase. (Coussens 1985). However polypeptide growth factors and their receptors, including EGFR are not only involved in the control of normal growth patterns but are implicated in pathogenesis of breast cancer. (Moller et al 1989, Tsutsumi et al 1990).

EGFR is also structurally related to the neu oncogene, named HER-2 by Coussens et al (1985), v-erb-B by Downward et al (1984) or c-erb-B2 by Yamamoto et al (1986). C-erb-B2 is a normal cellular gene present on chromosome 17, which has a close sequence homology with the epidermal growth factor receptor. (Coussens et al 1985). This gene was originally identified as an activated oncogene in rat neuroblastoma cell lines and was called neu. (Padhy et al 1982). It is the amplication of the gene on chromosome 17, by C-erb B-2, and the over expression of the protein formed, which has been postulated in the oncogenesis of breast carcinoma. (Borresen et al 1990, Downward et al 1984).

4.1 EGFR CORRELATED WITH ER AND OTHER CLINICAL PARAMETERS.

There is a strong inverse relationship between EGFR and ER expression in breast carcinoma. The co-expression of both receptors is rare and most tumours are positive for one or other of the receptors but not both. (Cappelletti et al 1988, Sainsbury et al 1985, Delarue et al 1988, Toi et al 1989 a and b, Nicholson et al 1989 Wrba et al 1988 and Battaglia et al 1988). Others have also observed the same negative correlation with the progesterone receptor (PgR). (Battaglia et al 1988).

It has also been postulated that the presence of EGFR indicates a poor prognosis and survival in primary breast cancers. (Sainsbury et al 1987). EGFR does not correlate with the number of lymph nodes with metastases (Grimaux et al 1989), but correlates well with the degree of lymphatic invasion. (Toi et al 1989, Battaglia et al 1988). Ki67 positive stained nuclei, which reveals the proportion of cycling cells, was significantly higher in EGFR positive tumours than in EGFR negative patients. (Toi et al 1989).

Like ER, EGFR is also present in benign breast tissue. (Barker et al 1989). EGFR is expressed in variable patterns in lobular, ductal and myoepithelial cells, stromal fibroblasts. (Moller et al 1989). All of these previous studies on EGFR have either used frozen section material for immunocytochemistry, or others have used biochemical methods for the demonstration of EGFR. Immunocytochemistry on FNA specimens would only help to advance the assessment of breast carcinoma prior to surgery and in recurrent disease.

4.2 PRODUCTION OF AN ANTIBODY TO EGFR.

Waterfield et al (1982) isolated a monoclonal antibody of the IgG class using an epidermoid carcinoma cell line A431, known as EGFR1. The antigen recognised by EGFR1 is a cell surface molecule of MW.175 000, which can be specifically linked to EGF. EGFR1 is directed against an antigenic site to the human EGF receptor and EGFR1 does not inhibit EGF binding under a variety of assay conditions. (Waterfield et al 1982).

4.3 MATERIALS AND METHODS.

4.3.1 Demonstration of EGFR on FNAC material.

For the demonstration of EGFR on FNA specimens, extra slides were taken from suitable cases with plenty of cellular material, collected in the same way as described for ER and Ki67 staining on FNA of breast carcinomas. (See section 2.6).

4.3.2 Demonstration of EGFR on frozen sections from breast tumour tissue.

EGFR was also demonstrated on frozen section material from breast tumour. Small pieces of breast tumour tissue retrieved during surgery and snap frozen in liquid nitrogen and stored in liquid nitrogen prior to cutting frozen sections. Frozen sections were collected onto poly-L-lysine coated microscope slides, in order to help retain the sections during immunostaining. FNA and frozen section specimens were fixed in dry acetone for 20 minutes before staining with the Strep ABComplex method, (see Appendix E).

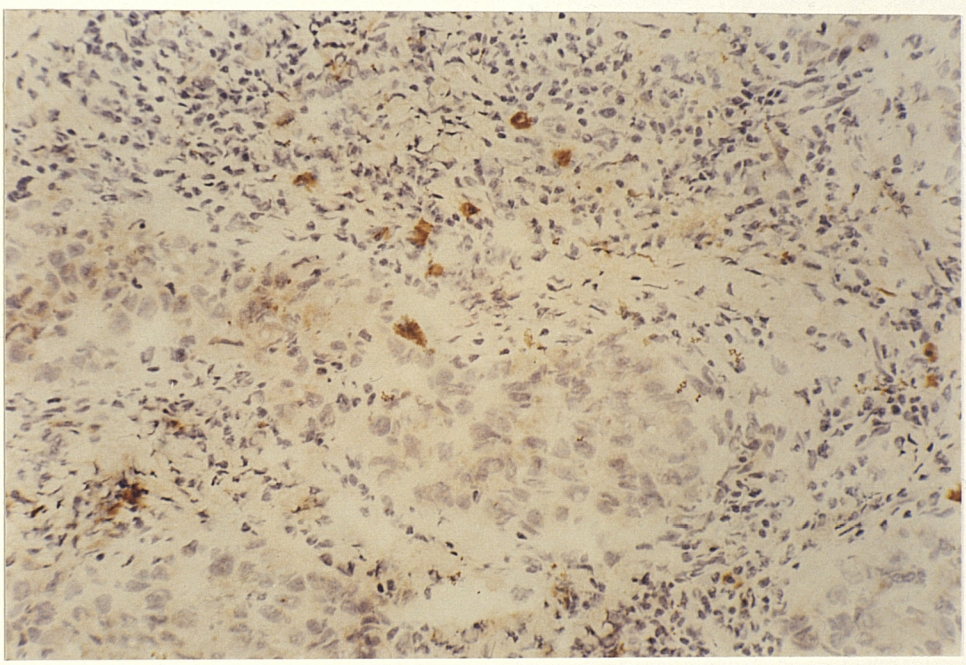
4.3.3 Controls for EGFR immunostaining.

Frozen sections of normal tonsil tissue were used for positive control material.

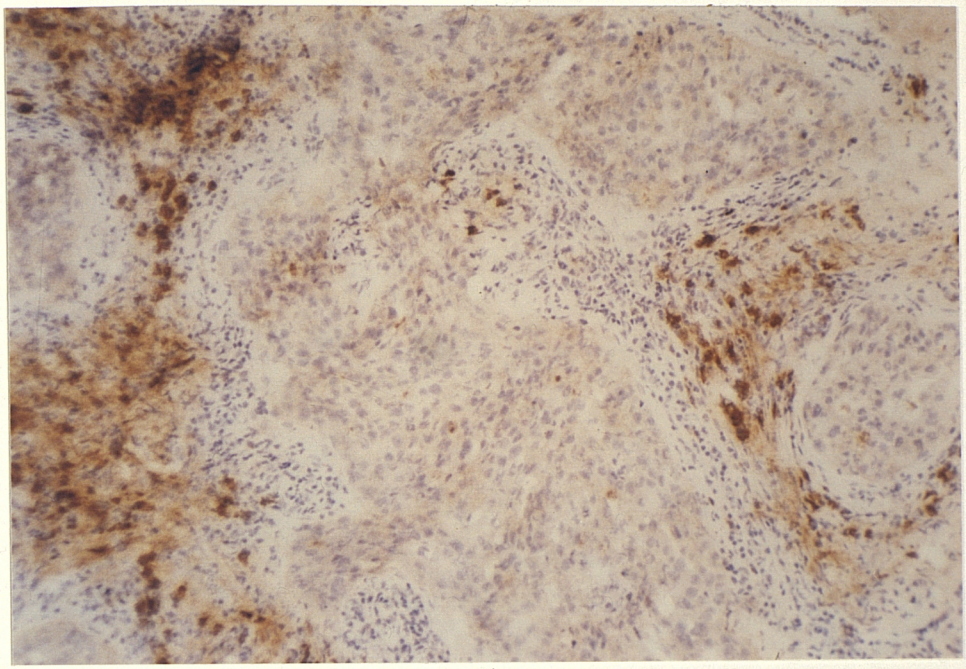
4.4 RESULTS.

Demonstration of EGFR can be seen in the photograph O of a frozen section of breast carcinoma. The membrane antigen is shown staining a number of cells in the stromal element of the tissue, these may be either stromal fibroblasts or myoepithelial cells. None of the nests of tumours cells, seen in the photograph P, exhibited the same strong positive staining.

In this preliminary study of frozen section and FNA specimens, four tumours have been immunostained for EGFR. The photograph of the specimen described above represents the strong immunostaining in the stromal element of the section and not in the epithelial tumour cells. FNAC samples, corresponding to the same patients demonstrated in the frozen section material, were immunostained for EGFR, all of the four cases showed negative immunostaining for EGFR. The reason for the negativity in the FNAC samples is most likely due to the epithelial cells being selectively aspirated by the FNAC technique.



PHOTOGRAPH O : Positive EGFR Staining In Stromal Breast Cells.x400.



PHOTOGRAPH P: Breast Tumour Cells Staining Negative With EGFR.x400.

4.5 DISCUSSION.

The immunostaining of EGFR by the Strep ABComplex was a disappointing experience with the small number of frozen section and FNA specimens tried with this method. The positivity of stromal fibroblasts or myoepithelial shows that the immunocytochemical method is working well on the frozen section material, although the epithelial cells consistently did not stain. The stromal elements, demonstrated in the frozen section material, are rarely aspirated in FNA specimens. The act of aspirating cells from the solid tumour through a fine gauge needle selectively aspirates clusters and groups of epithelial cells as well as numerous single cells. The percentage of clusters and single cells will depend on the type of tumour aspirated. Stromal fragments, myoepithelial cells and fibroblastic nuclei are aspirated in benign lesions but less often in malignant tumours.

It might be expected that membrane antigens would be more difficult to stain in FNA specimens because of the architectural loss described above, than the strong nuclear antigens of ER and Ki67. However in the routine laboratory practice the membrane antigen human milk fat globule (HMFG2) has been used successfully on cytological material, to identify single and groups of malignant cells amongst the normal cellular content of body fluids.

None of the four samples chosen for EGFR staining showed positive staining for EGFR in the malignant tumour cells identified on the frozen sections. However two of these cases did demonstrate positive staining in the stromal element of the breast tissue. The FNA specimens from these four cases all showed negative staining for EGFR.

Further work is in progress to assess EGFR in breast FNA specimens. Our results are inconsistent with previous workers who demonstrated EGFR in epithelial as well as stromal cells, however all of the studies have been performed on histological sections or tissue specimens. None of the studies have evaluated the use of FNAC for this technique. In this study EGFR negativity in the FNA specimens is consistent with the lack of staining of epithelial cells in frozen sections, which has yet to be explained.

EGF is known to accelerate cell proliferation by binding to its specific receptor site, EGFR, (Carpenter and Cohen 1979). Therefore it would be a valuable addition to a panel of antibodies for the assessment of breast cancer. However, since FNAC is the method of choice for the primary diagnosis of breast tumours, its potential for the

demonstration of EGFR is restricted until it may be used reliably on FNAC specimens as well as frozen and paraffin wax sections.

5.0 CYTOLOGICAL PRE-OPERATIVE GRADING AND ASSESSMENT OF BREAST CARCINOMA.

Fine needle aspiration (FNA) of breast tumours provides material for the pre-operative assessment of carcinoma. Conventional morphological diagnosis might be complimented by a system of cytological grading of FNAC material, particularly if it could be shown to relate to parameters such as ER and Ki67 status. Such a grading system could be useful for pre-operative assessment and in inoperable and recurrent disease.

5.1 HISTOLOGICAL GRADING OF BREAST TUMOURS.

Histological grading of post-operative surgical specimens is used to provide information as to the tumour grade. This relies upon a description of the patterns of tumour growth to identify the special types of breast cancer, ie. tubular, lobular, papillary, mucinous and medullary carcinoma. This is followed by a numerical grading process on the majority of breast carcinomas, ductal carcinomas, of no special type. Patterns of tumour growth, glandular differentiation and nuclear characteristics are taken into account. (Sakamoto 1987). It is usual, but not essential, to exclude tumours of special type from the histological grading system. In this study histological grading was only applied to infiltrating ductal carcinomas. Histological grading has been shown to correlate well with survival, ER and Ki67 status. (Hawkins et al 1987, Crispino et al 1989).

5.2 CYTOLOGICAL GRADING OF BREAST TUMOURS.

A similar technique might be applied to cytological FNA specimens from breast tumours. Experienced cytologists may be able to identify features suggesting several of the special types of breast cancer on the cytological specimen alone, including papillary, tubular medullary and mucinous carcinoma. Lobular carcinoma is virtually impossible to assess because it is composed of small uniform round cells with intracytoplasmic mucin vacuoles, not dissimilar those which might be found in invasive ductal carcinoma. However, it is not possible to diagnose the special types with certainty by FNAC because the final histological diagnosis depends on the purity of the special type features within the tumour. Therefore a cytological grading system

must encompass all types of carcinoma, without excluding or identifying special types of carcinoma.

Black et al (1957) studied the nuclear structure of cells in a variety of different tumours including breast cancer in order to observe nuclear variability amongst different tumours as well as the relationship between nuclear structure and survival. Since this time with advent of fine needle aspiration many more people have become interested in observing nuclear morphometry in cytological aspirates. Boon et al (1982) were interested in any advantage over conventional cytodiagnosis in cytologically doubtful cases. Others have been more interested in producing diagnostic scoring systems to relate to prognosis. Kuenen-Boumeester et al (1984), Zajdela et al (1979), Mouriquand et al (1986) and Wallgren et al (1976) have concentrated on comparing the findings from the cellular grade with the grade obtained from histological sections.

In the past some workers have been most concerned with describing the size of the nuclei in the tumour cells. Zajdela et al (1979), described two types of breast carcinoma "small nuclear type" where only 7% of the cell nuclei measured more than 12 μm, and the "large nuclear type" where only 7% of the cells had nuclei of a diameter less than 12 μm. The nature of the small cell nuclei in low grade carcinomas proved very difficult to distinguish from benign breast lesions. (Zajdela et al 1979, Thomas et al 1989). Distinction between benign breast cells and those from low grade or well differentiated carcinomas also proved difficult for those using computer assisted morphometric analysis. (Boon et al 1982). Therefore it has been shown to be necessary to include a wide variety of parameters in the grading system in order to improve the reproducibility of the technique. However the more parameters that are introduced the more complicated the procedure is to apply to routine cytological diagnosis of breast cancer. Wallgren et al 1976 opted for three discrete bands of nuclear size, <12 μm, 12-19 μm and >19 μm. Four bands for the expression of the nucleoli, just discernible, clearly discernible, enlarged and prominent as well as observing necrotic matter, mitotic frequency, size of cells clusters, mucoid matter and lymphocytes. In this work all of the predictors apart from the presence of mucus and of lymphocytes, were shown to be significant indicators when compared with data for the five and ten year survival of the patients with breast carcinoma.

Kuenen-Boumeester et al (1984) wanted to develop their theories further by combining the morphometric studies with the use of lymph node status. However they found that they are both independently related to survival and in fact the grading system they devised was more closely related to prognosis than when lymph node status was

assessed alone. More recently, Hunt et al (1990) showed cytological grading of breast carcinoma to be a feasible proposition using parameters such as nuclear diameter, nuclear pleomorphism, the presence of multiple easily visible nucleoli, the degree of cell clustering and cellular necrosis; for the assessment of cytological grade. Thus restricting the number of parameters to be assessed. It is the method of this group of workers on which we have based the morphometric studies of our own.

5.3 METHODS AND MATERIALS.

5.3.1 Evaluation of specimens suitable for cytological grading of breast tumours.

A collection of 129 breast carcinomas, from screen detected and symptomatic women, with ER-ICA and or Ki67 values were used for the cytological grading project. 11 cases were rejected with insufficient material or poorly preserved and stained cells inadequate for the assessment of nuclear characteristics. All evaluation was undertaken on the May Grunwald-Geimsa stained material, because this is the main type of preparation made for FNAC in Cytology at Southampton General Hospital. Adequate alcohol fixed Papanicoloau stained material was not available in all of the cases. Smears were assessed by two observers, [A.C.] and [L.C.], using Leitz microscopes, the x40 objective and x10 eyepieces. Both microscopists agreed prior to examination only to observe well fixed, spread and stained areas of the preparations. Clusters of cells may be difficult to evaluate. The periphery of the smear, where the material dried most quickly, was the easiest area in which to assess the groups of cells.

5.3.2. Microscopy techniques for cytological grading of breast tumours.

The first readings were taken using four of the characteristics described by Hunt et al. (1990). Firstly, cellular pleomorphism graded as: (1) mild, (2) moderate and (3) severe; secondly, the appearance of the nucleoli graded as: (1) inconspicuous, (2) multiple conspicuous nucleoli and (3) multiple large angulated nucleoli; thirdly, the degree of cell cohesion graded as: (1) very cohesive groups, (2) loosely cohesive groups and (3) a free cell pattern; fourthly, nuclear size following Hunt's recommendations, comparing nuclear size with adjacent red blood cells graded as: (1) largest nuclear diameter, <2 red blood cells, (2) largest nuclear diameter, 2-4 red blood cells and (3) largest nuclear diameter, >4 red blood cells. The latter method for assessing nuclear size was replaced in the final analysis of the data in this study by direct numerical measurement of the nuclear diameter, (see section 5.3.4). The number of points assigned to each case were combined and divided into three cytological grades. Grade I = 4,5 and 6 points, grade II = 7,8 and 9 points and grade III = 10, 11 and 12 points.

5.3.3. Microscopy techniques for cytological grading of breast tumours, omitting architectural detail.

We also looked at these values omitting the parameter assessing the degree of cellular clustering or cellular cohesion. Hunt et al (1990) found this parameter very difficult to assess. "Most clusters accumulated at the edges of the smears , with few clusters in the centres. It would appear clustering depends on some degree to the method of spreading of the smear". This was less of a problem in this series because of the technique used to prepare the slides. The blood film technique of preparing a smear, is not entirely suitable for FNA cytology work, and may result in all the clusters of cells at the tail-end of the smear. To ensure an even distribution of cells throughout the smear, a small drop of the material from the FNA should be placed onto a glass microscope slide, a coverslip is placed flat on the material and drawn down, distributing the cells as evenly as possible over the preparation. The coverslip is employed to reduce spreading artefact, caused through too much pressure as the slide is prepared; a coverslip will break before too much pressure is applied. However, since the cell clustering was largely related to histological architecture and may be altered by artefact during FNAC, it was decided to concentrate on cytological grading of nuclear morphometry.

Omitting a parameter in the grading system required a redistribution of the points for the final grading and were allotted as follows: grade I = 3,4 and 5 points, grade II = 6 and 7 points, grade III = 8 and 9 points.

5.3.4. Microscopy method for assessing the accurate size of tumour cell nuclei using an eye-piece graticule.

Although comparison of nuclear diameter to red blood cell size was a comparatively easy technique to perform, it was found that greater accuracy could be obtained by counting the nuclei with an eye-piece graticule. It was decided to assess twenty nuclei from each of the 118 cases of breast carcinoma with adequate cellular preparation, using the Leitz microscope and an eye-piece graticule. A better comparison could be made in the case of pleomorphic nuclei in the breast tumours involved. Cells for assessment were chosen from rapidly air-dried, well fixed areas of the smear, often found at the periphery of the specimen. Here it was possible to avoid over-lapping or cells clustered into three dimensional balls of cells, a monolayer being preferred for the counting procedure. Intact cytoplasm was used as a guide to the viability of the

cells to be counted, avoiding anisokaryosis. Points were awarded for nuclear size as follows:- for example,

Nuclei counted, μm = 8,6,5,7,5,5,7,7,5,5 = 60 mean = 5.8

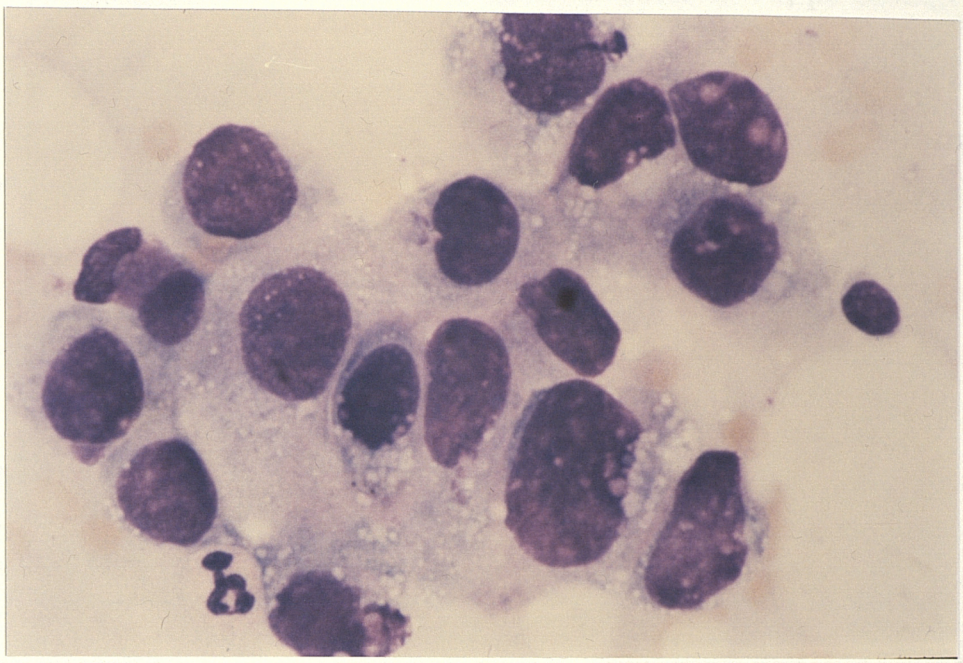
6,5,5,6,7,5,6,8,5,4 = 57 mean = 5.5

Cell size was graded as follows: (1) = an average of <5 μm, (2) = an average of between 5 and 7 μm and (3) an average of >7 μm.

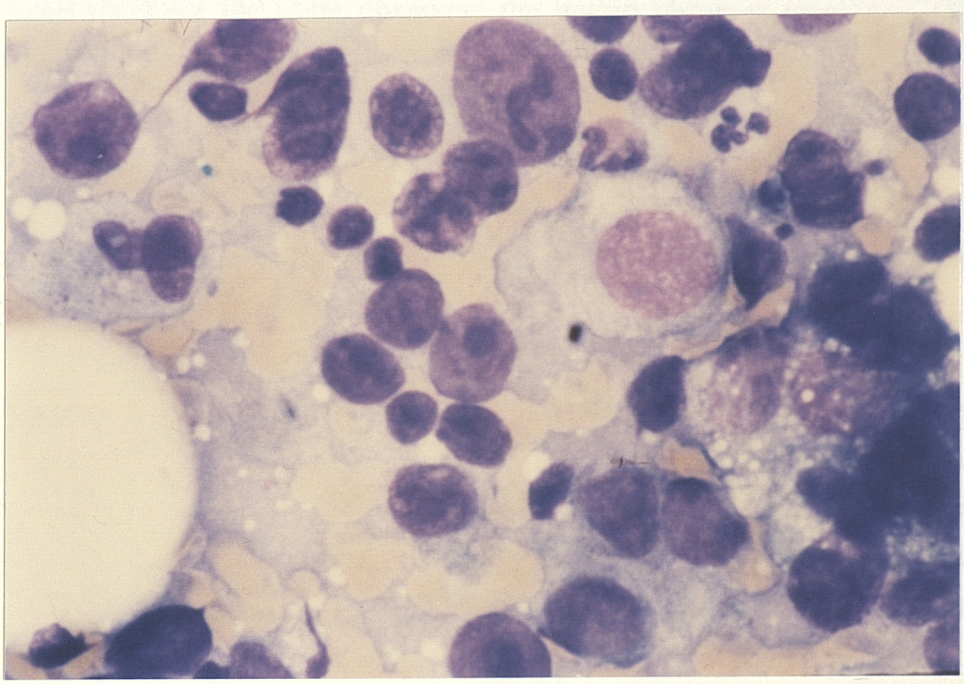
Having awarded the points for nuclear size, pleomorphism and nucleoli, the points were graded as Grade I, a total of 3 and 4 points, Grade II, 5,6 and 7 points and Grade III, 8 and 9 points. It is this method for the assessment of nuclear size which was used exclusively for the evaluation of the results for cytological grade, (see section, 5.4.).

5.3.5 The assessment of nuclear pleomorphism.

Nuclear pleomorphism is a difficult parameter to evaluate consistently. It is possible to begin the assessment using the individual figures obtained for the evaluation of nuclear size. The range of numbers will identify the difference in nuclear size, however the parameter of pleomorphism should also take into account nuclear shape and irregularity. The assessment of shape and irregularity was assessed by eye, with the most uniform and smooth nuclei assessed at the bottom end of the scale and nuclei with indented irregular nuclear boarders at the top end of the scale. Irregularity and range of nuclear size were identified as mild, moderate and severe. Photographs Q and R, show severe nuclear pleomorphism, (three points) and multiple, prominent irregular cell nucleoli, (three points) respectively.



PHOTOGRAPH Q : Breast Carcinoma Cells Showing Severe Cellular Pleomorphism. MGG.x630.



PHOTOGRAPH R : Breast Carcinoma Cells Showing Prominent Nucleoli. MGG.x630.

5.4 RESULTS.

5.4.1. Calculation of between and within observer observation, percentage error.

Before correlating the cytological grading system with other parameters such as oestrogen receptor status and proliferative cell activity it was necessary to assess the accuracy of the microscopy performed. The diameters of the breast cell nuclei were compared with the following formulae:-

$$\% \text{ ERROR} = \frac{\text{ERROR}}{\text{AVERAGE VALUE}} = \frac{2(M1 - M2)}{(M1 + M2)}$$

The mean value for the twenty nuclei counted in each case were evaluated. These mean values were correlated in each of twenty paired cases were assessed firstly between [A.C.] and [L.C.]. The percentage error between observers, was 12%, and the range of error in the twenty cases ran from 0% to 30%. Ten similarly paired cases were counted twice by the same observer: the within observer error was also 12%, ranging from 3 to 21%.

5.4.2. Assessment of cytological grading for breast tumours with and without the architectural considerations of cellular cohesion.

All the available samples for the cytological grading system were assessed using the methods described in section 5.3.2 and 5.3.3. It can be seen from Table 21, there was no alteration in the Grade III tumours with or without cell cohesion included in the cytological grading system and only two cases moved between the Grade I and Grade II groups. Section 5.4.3 shows the statistical analysis of both of these parameters.

TABLE 21. COMPARISON OF CYTOLOGICAL GRADE OMMITTING AND INCLUDING CELL COHESION.

CYTOLOGICAL GRADE	WITH CELL COHESION	WITHOUT COHESION
GRADE I	22	24
GRADE II	70	68
GRADE III	26	26
TOTAL	118	118

5.4.3 Correlation of cytological grading with and without cell cohesion included, with Ki67 analysis in symptomatic patients.

Correlation with the proliferating cell antigen Ki67 were similar, with or without cell cohesion included. There was little difference to the value obtained for X^2, and no difference to the value for p. (See tables 22 and 23).

TABLE 22. COMPARISON OF CYTOLOGICAL GRADE, INCLUDING CELL COHESION WITH %Ki67 STAINING.

%Ki67	CYTOLOGICAL GRADE			TOTAL
	I	II	III	
< 15%	19	47	11	77
15-30%	3	15	9	27
> 30%	0	8	6	14
TOTAL	22	70	26	118

%Ki67 correlated with cytological grade including cell cohesion.

X^2 with four degrees of freedom = 11.37 $p < 0.025$

TABLE 23. COMPARISON OF CYTOLOGICAL GRADE, OMITTING CELL COHESION, WITH %Ki67 STAINING.

%Ki67	CYTOLOGICAL GRADE			TOTAL
	I	II	III	
< 15%	21	45	11	77
15-30%	3	15	9	27
> 30%	0	8	6	14
TOTAL	24	68	26	118

%Ki67 correlated with cytological grade omitting cell cohesion. (See Figure 23).

X^2 four degrees of freedom = 12.82 $p < 0.025$,

Since there was no statistical difference between including or excluding cell cohesion in the analysis, all further correlations were based on the assessment of cytological grading with the exclusion of the parameter for cell cohesion.

The most striking observation between Ki67 staining and cytological grade was the decreasing percentage of low proliferation rate tumours, (< 15% Ki67), with increasing cytological grade: Grade I = 87%, Grade II = 66% and Grade III = 42%. (See Figure 24).

5.4.4 Correlation of the cytological grading of the breast tumours with the histological grade in symptomatic patients.

There was no correlation between cytological grade and the 64 in invasive ductal carcinomas, for which a histological grade had been reported. (See Table 24, Figure 25.)

TABLE 24. COMPARISON OF CYTOLOGICAL GRADE WITH HISTOLOGICAL GRADE.

HISTOLOGICAL GRADE	CYTOLOGICAL GRADE			TOTAL
	I	II	III	
GRADE I	0	4	0	4
GRADE II	1	22	3	26
GRADE III	1	31	2	34
TOTAL	2	57	5	64

$X^2 = 1.24$ four degrees of freedom, $p < 0.5$

However, the cytological grade identifies two small groups of high and low grade tumours, and a broad intermediate group, whereas histological grading results in most of the tumours being grade II or III. (See Figure 26). The most important difference was the case, described as cytological grade one when graded histologically three. (See Figure 25). Histologically this tumour exhibited little duct formation, showed areas of necrosis, extensive pleomorphism and a high mitotic activity and was given grade three. The cytological preparation had been given the lowest number of points for nucleoli and pleomormphism by both observers. The cell nuclei were small but there was a difference between the values counted by the two observers (a mean of 5.0μm and 6.35μm). In retrospect, when the slide was reviewed, cytological pleomorphism was greater than described above. Counting a larger number of cell nuclei may have highlighted this problem at an earlier date.

5.4.5. Correlation of cytological grading for breast tumours with oestrogen receptor analysis.

There were 103 patients with positive and negative oestrogen receptor analysis (ER-ICA), for comparison with the cytological grade of breast tumours, see Table 25, Figure 27.

TABLE 25. COMPARISON OF CYTOLOGICAL GRADE WITH ER-ICA, IN SYMPTOMATIC WOMEN.

CYTOLOGICAL GRADE	ER-ICA POSITIVE	ER-ICA NEGATIVE	TOTAL
GRADE I	10	5	15
GRADE II	45	29	74
GRADE III	2	12	14
TOTAL	57	46	103

X^2 with 2 degree of freedom = 11.27 $p < 0.005$

This shows a statistical correlation between cytological grade and ER-ICA status, stronger than that found between histological grade and ER-ICA analysis, see chapter 2, Figure 7.

The most striking finding was that 86% of grade III tumours were ER negative compared with 32% of grade I and II combined. A similar percentage of grade I and grade II tumours were ER negative, (33% and 39% respectively).

5.5 DISCUSSION.

Histological grading of breast tumours is a well established technique, providing valuable information, contributing to the assessment of prognosis and thus the clinical treatment regimes to be used in each individual patient. The object of devising a cytological grading system was to ascertain whether a cytological grade could be similarly useful in the assessment of breast carcinomas and whether it might predict parameters such as ER status and proliferative rate.

Technically the evaluation of nuclear size was the most difficult parameter to assess successfully. Comparing nuclear size to groups of 2 or 4 or more red cells has its advantages: although it is much quicker to examine a number of cell nuclei in this way, it can never be as accurate making a proper measurement of each cell. Therefore measurement of cell nuclei with a microscope graticule was used as the method of choice to produce a more accurate result and to provide a more objective method of assessing pleomorphism.

The assessment of cell cohesion proved to be the most subjective of the parameters to analyse, and did not help to discriminate between grade I, II and III. Cohesion also relies on architectural elements to hold the cell together in strands and groups and is not a true cellular phenomenon. Thomas et al (1989) examined separate variables to ascertain whether any one variable might stand on its own, being able to separate breast carcinomas into three different grades. Nuclear size was able to achieve this goal but nuclear pleomorphism and cell to cell cohesion did not, highlighting the necessity for a number of parameters to be included in the cytological grading systems.

Other studies show no association between lymph node status and cytological morphometry. (Kuenen-Boumeester et al 1984 and Thomas et al 1989). Mossler et al (1981) using a similar morphometric technique to that of Thomas et al (1984) found a correlation between nuclear area and oestrogen receptor status and histological grade. The correlation has been upheld in this study, ER-ICA correlating well with cytological grade, $p < 0.005$.

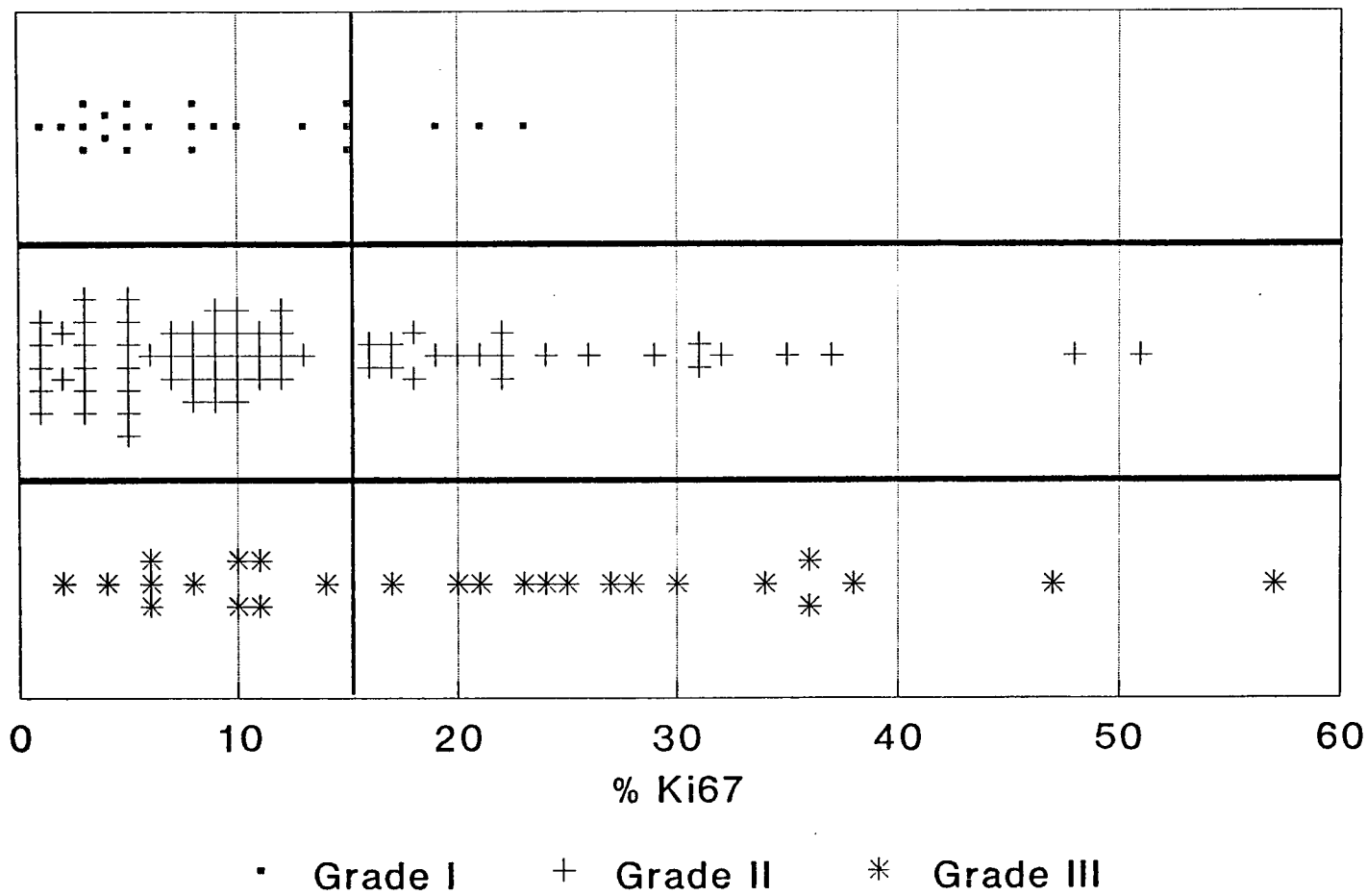
There was no association in this study between cytological grade and histological grade, $p < 0.5$. This may be accounted for by discussing the way in which cases were divided into the three separate grades. The cytological grading system identifies a small group of well differentiated carcinomas, and a similarly small group of poorly differentiated tumours, leaving the majority of the breast carcinomas in the middle,

grade two group. If however, the histological grading system is studied carefully a far larger proportion of carcinomas fall into the grade III category than grade I which would account for the discrepancy with cytological grading.

Grade I tumours were shown to have a high proportion of low proliferative rate tumours, with Ki67 immunostaining, (87%, compared with 60% for grade II and III). Grade III tumours had a higher proportion of ER-ICA negative tumours, (86% compared with 32% for grade I and II).

Most importantly we must not forget that any cytological grading system is designed to complement morphological diagnosis. As there is a considerable degree of overlap between some benign breast conditions and the well differentiated carcinomas. (Thomas et al 1989), it is not suggested that the cytological grading system would supplant conventional assessment of breast FNAC but that it should act as an additional parameter.

Figure 23. Cytological Grade Compared with Ki67



Chi-squared = 12.82, $p < 0.025$

Figure 24. Percentage of Tumours with Low Proliferation Rate Compared with Cytological Grade.

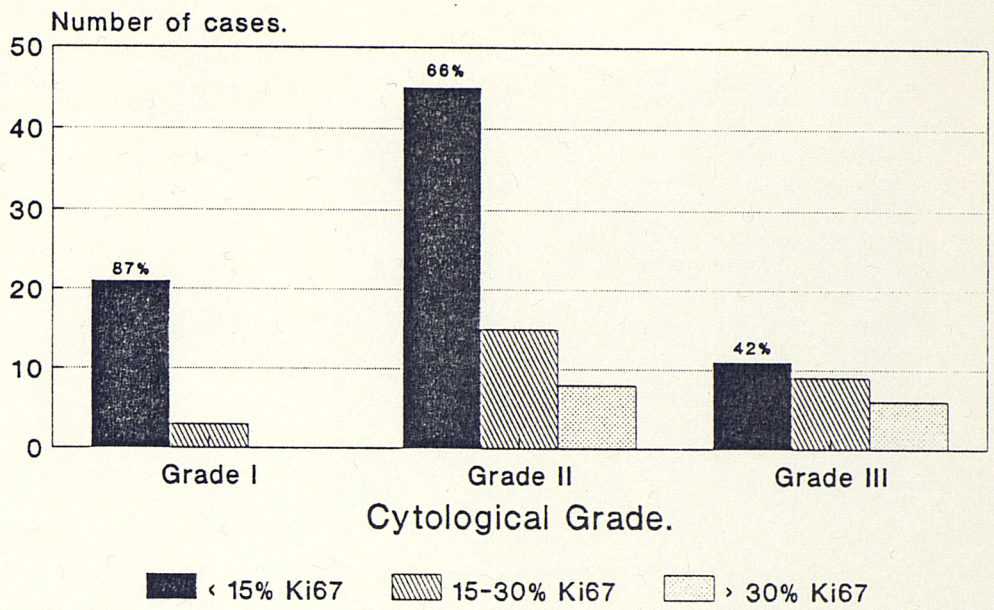
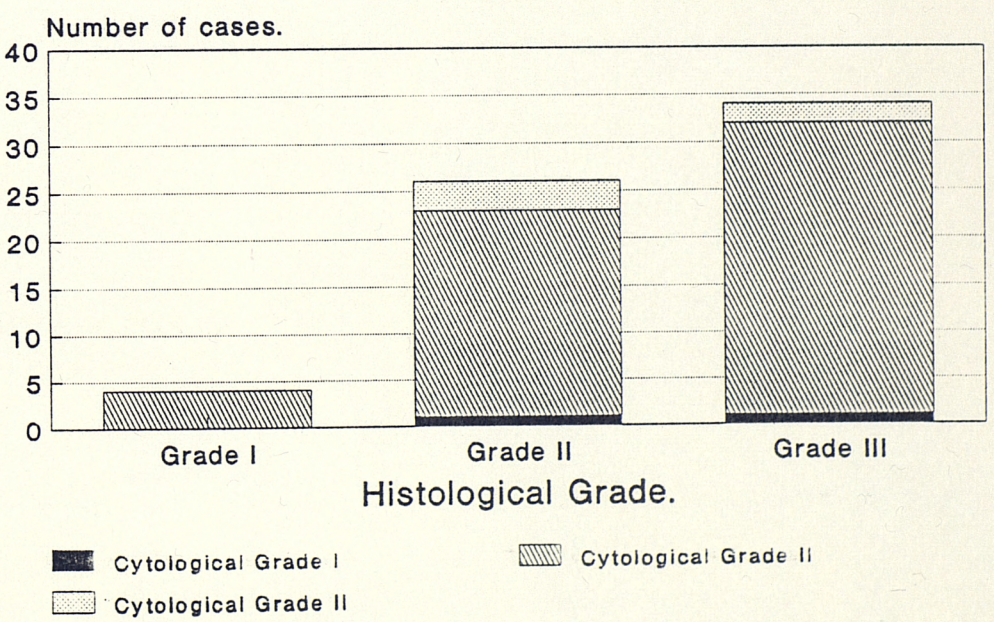


Figure 25. Distribution of Cytological Grade within the Histological Grade.



Chi-squared = 1.24, P ‹ 0.5

Figure 26. Histological Grade
Compared with Cytological Grade

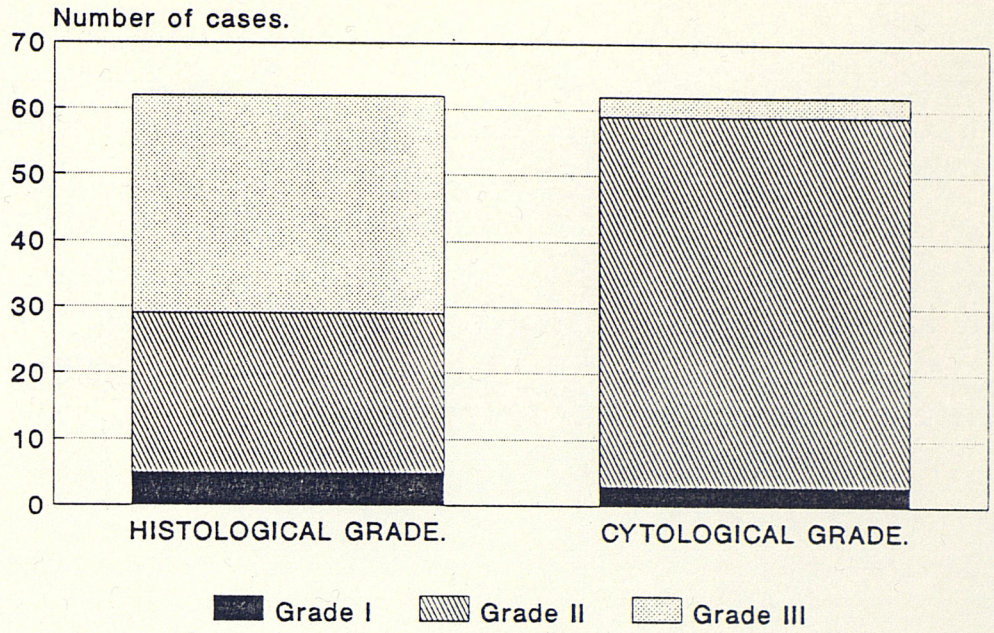
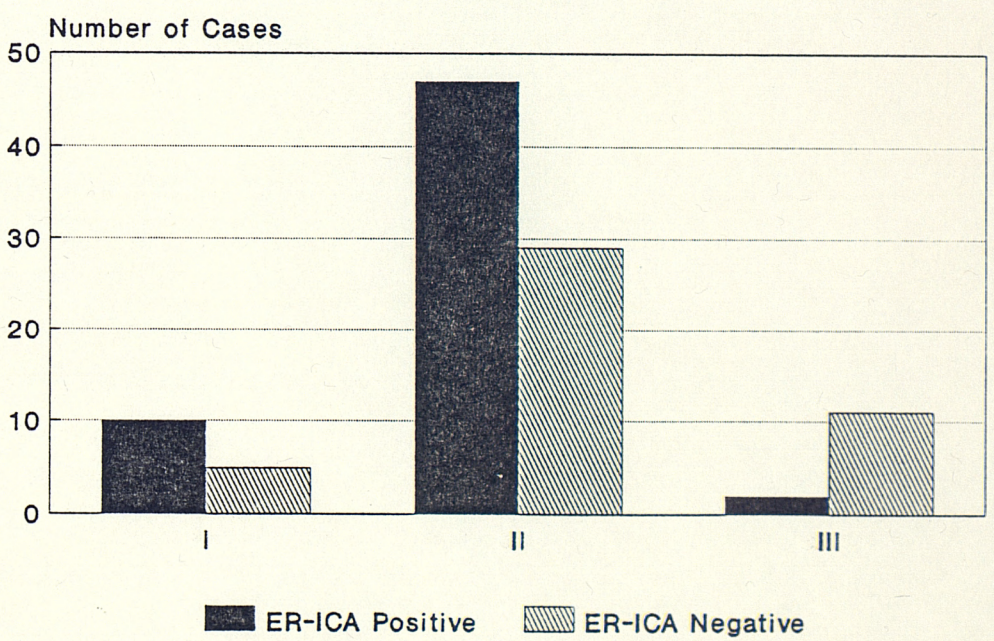


Figure 27. ER-ICA Compared with
Cytological Grade



Chi-squared = 11.27, $p < 0.005$

6.0 DISCUSSION.

This study into the cytology of breast carcinoma has been designed to use the fine needle aspiration technique to develop prognostic indicators in breast cancer. The use of FNAC immunocytochemistry has been proven to be extremely useful in the pre-operative assessment of prognostic indicators in breast cancer and also in inoperable and recurrent disease.

In this project, the areas under investigation have been; one, oestrogen receptor analysis; two, proliferating cell markers and three, cytological grading techniques.

Oestrogen receptor (ER) status already plays an important role in the protocol for the management of breast cancer at the Royal South Hants. Hospital, Southampton, particularly in women who opt conservative surgery rather than mastectomy. Patients with a carcinoma have the choice between mastectomy with axillary clearance or a wide excision with bed biopsies and axillary clearance, as long as the tumour is not central in the breast, is less than 4 cm. in diameter and there is no skin invasion. A variety of post-operative treatments are available, largely depending on lymph node and ER status. Pre-menopausal women with ER negative tumours and positive lymph nodes, are most likely to benefit from adjuvent chemotherapy. Post-menopausal women, over the age of fifty years, who are lymph node negative and ER positive, are most likely to benefit from Tamoxifen (20 mg. daily for two years). Most importantly, many elderly women over 70 years of age have been treated successfully with Tamoxifen as their primary treatment, thus avoiding surgery in many cases. (Allen et al 1985).

When this project began to investigate the feasibility of assessing ER status from FNA specimens and immunocytochemistry, the Southampton surgical unit was already sending tissue from breast carcinomas to the Tenovus Institute, Cardiff, for the biochemical assay of ER. However, these were retrospective studies, produced on a three monthly basis on the post operative tissue sent to Cardiff in liquid nitrogen. The present project began with the aim of producing a reliable preoperative assay, using Abbott's monoclonal antibody to the oestrogen receptor site. The results from Chapter 2 show how successful this has been. Both methods were run in tandem whilst the ER immunocytochemistry was evaluated, but the ER-ICA immunocytochemistry has become the method of choice for the assessment of the ER status of the patient, and has been incorporated into the breast cancer management policies in Southampton. A

number of difficulties had to be overcome in order to provide an efficient service for the breast cancer patients from the symptomatic clinic.

At first ER-ICA staining was assessed by comparison with the biochemical assay although it was recognised that the latter had disadvantages, making it potentially less accurate than the ER-ICA assay. The apparently high "predictive value" of ER-ICA positivity for a biochemical value of more than 10 fento/mol., is reflected by an apparent "low sensitivity" of ER-ICA staining. Most of the ER-ICA negative cases showed low levels of ER measured by the biochemical assay. Since tumours with low levels were already known to respond less well to Tamoxifen than those with high levels (Campbell et al 1981), the ER-ICA negativity of such tumours might make the ER-ICA test a better prognostic indicator.

The high predicative value of a positive ER-ICA result may well be due to its low sensitivity for a positive biochemical result. In this particular group of women the ER-ICA positive result of greater than 25% of the malignant nuclei staining positive with the ER-ICA assay, corresponds with a biochemical assay result of approximately 30 fmol./mg. (Davies et al 1991), which was relatively high considering <10 fmol./mg. and in some cases <5 fmol./mg. of oestrogen receptor per milligram of cytosolic protein, is considered a positive result. (Hawkins et al 1988). Tumours rich in ER are known to respond better to endocrine treatments than those with relatively low ER content. (Campbell et al 1981).

Technical problems with the immunocytochemical method for the demonstration of ER status were not insignificant, however they proved to be surmountable. It is important to remember that the ER antigen is fragile and may not be demonstrable unless the correct protocol is followed for the collection, storage, fixation and immunostaining of the breast tumour cells. Cells removed from the tumour by FNA or alternative methods must be placed into liquid nitrogen within the first few minutes because immunoreactivity is lost if the cells are allowed to air dry or are kept at room temperature. However, after the frozen aspirate has been fixed in buffered formaldehyde and post fixed in the cold acetone and methanol, the antigen is stable and may be stained by a standard immunocytochemical technique. In Southampton, the laboratory staff attend the symptomatic breast clinic and breast screening unit to prepare the FNAC slides for routine morphological examination. It is therefore possible for additional slides to be frozen in liquid nitrogen and retained for ER immunocytochemistry.

The assessment of the immunocytochemical staining required careful examination, identifying the positive and negative nuclei and formulating the cut-off point for a positive and negative result. During this process of assessing ER status, the heterogeneity of breast tumours was also taken into account. Some breast tumours consist of a mixture of two or more distinct types of breast carcinoma, different types of carcinoma may express ER to varying degrees. (Leo et al 1991). Even if the tumour is composed of one histological type of breast cancer, more than one clone of breast tumour cells may be present in the same preparation expressing ER to varying degrees. This has been clearly demonstrated by the photograph in Chapter Two, showing heterogeneity. Long term studies will be required to assess the relevance of mixed populations of both ER positive and negative cells in the same tumour.

One of the most significant findings in the study was the high number of younger women, under fifty years of age, with ER-ICA negative results. It had been thought this might be due to the endogenous circulating oestrogens in the blood stream masking oestrogen receptor sites which may otherwise be used to attach the anti-oestrogen drug Tamoxifen. However, this is not the case, since ER-ICA analysis demonstrates a moiety adjacent to the area of oestrogen and Tamoxifen uptake on the oestrogen receptor site.

Clinically, it is the elderly patients who have most to gain from analysis of their ER status. The protocol in Southampton recommends that women over the age of 70 years should be considered for Tamoxifen only therapy. This procedure does not compromise survival and is successful in 60-70% of patients. (Hawkins et al 1980). However, clinical assessment of the treatment may take between 12 and 20 weeks, which can be disturbing to the failed Tamoxifen patient who must subsequently undergo more invasive therapy, such as a mastectomy. In a few patients where disease progression has occurred this may compromise effective surgical treatment. (Davies et al 1991). ER assessment using the ER-ICA assay helps predict those ER positive patients most likely to benefit from the Tamoxifen therapy. ER-ICA negative patients, those most likely to have progressive or a relapsed disease on the Tamoxifen only therapy, may be offered alternative treatment at the appropriate time. The principle behind Tamoxifen only therapy is to provide adequate disease control for the remainder of the patient's life: the ER-ICA test was found to be a reliable in finding those women whom would benefit from this coarse of action.

The use of ER status in the management of breast screened patients has also been presented. It might be expected to find an altered distribution in the ER status in

screen detected cancers, since the histological types of breast carcinoma also have an altered distribution. For example, tubular carcinoma and maybe some mucoid carcinomas, may evolve into ordinary ductal carcinomas. There tends to be a higher percentage of "special" types of carcinoma and in-situ lesions, presumably due to the early stage in which the tumours are detected. Screen detected carcinomas may not have had the opportunity to grow and diversify into the non-specific ductal carcinomas. However, this was not the case with the ER-ICA analysis, there was no alteration in the distribution of ER-ICA positive and negative cases. In the breast screening group, patients were equally divided between positive and negative ER-ICA results, as were the symptomatic patients between 50 and 64 years, making ER assessment particularly relevant in this age group.

Analysis of the methods of collection of the specimen in the breast screening group revealed problems with delay in performing the FNAC and with the 'touch' imprints, made after the sliced histological specimen had undergone further X-ray. Delay in obtaining the specimen produced a reduction in the ER-ICA positivity. These results only reiterates the need for prompt action concerning the fixation of the specimens for ER-ICA analysis, any significant delay in placing the specimen in liquid nitrogen prior to the fixation procedure, will result in the loss of positive immunostaining.

Breast cancer cell proliferation is affected by a number of other growth factors as well as circulating oestrogens and oestrogen receptor sites. In this project proliferation has been examined by immunocytochemical staining of proliferation cell antigen, from the tumour cell clone, Ki67 and with epidermal growth factor receptor (EGFR). Ki67 identifies all cells in the proliferating stages of the cell cycle, other than the resting phase G0. Ki67 was applicable to FNAC and provided a quantitative assessment of the proliferative state of the tumour. There was a strong inverse correlation between Ki67 and ER-ICA status, $p < 0.001$. 56% of ER negative tumours had Ki67 scores of more than 15%, whilst 83% of ER positive tumours had Ki67 scores of less than 15%. EGFR proved more difficult to identify in cytological specimens, but with time and effort this too may be included among the antigens used to assess breast tumours.

Having analysed ER status and proliferative rate, we attempted to relate this to cytological grade to see how much of the prognostic data could be assessed from the morphology alone. In the past, the only grading system available to make a comparison was the post-operative histological grading system. This has been suitable for the assessment of histological tissue, but since the specialist immunocytochemical stains were performed on cytological material and subsequent histological material was

not always available for assessment, it seemed only appropriate to devise a system of grading for cytological specimens. Cytological FNA specimens may also retain, a degree of association between cells. However, in Chapter 5, it was shown that cytological architectural features added little to the accuracy of the cytological grading and was not included in the final assessment. The grading system devised here in this study, confined itself to nuclear detail. The comparison of cytological grade with ER-ICA status showed a good statistical correlation, $p < 0.005$. 86% of Grade III tumours were ER negative, whilst 72% of Grade I and II tumours were ER positive, (33% and 39% respectively). Correlation of cytological grade with Ki67 staining showed a less strong statistical comparison, $p < 0.025$.

It is necessary to take into account the artificial processes brought about by the different ways in which the specimens are collected, fixed, processed and stained. Perhaps the two most fundamental aspects which stand in the way of a direct comparison between the two techniques are; firstly, cytological specimens are observing whole intact cells, whilst histological specimens take a 4 μm. section through the tissue thus displaying the cut surface of cells. Secondly, nuclear features such as nucleoli nuclear pleomorphism may be assessed more easily in intact cells.
In the long term the only way to assess the predictive value of these techniques accurately, is to study the statistical analysis for the survival data accumulated on these patients. It was possible to obtain a five year survival analysis for the breast cancer patients, for comparison with the ER-ICA and Ki67 analysis. Patients were grouped into those above and below 50 years of age, post and pre-menopausal women. The breast screening age group, 50-65 years, were analysed separately as were the elderly women over 70 years of age, however, there were insufficient events, deaths in the survival data to show any statistical significance. When the data was re-examined using recurrences as the event, it was possible to demonstrate statistical significance with the ER-ICA analysis. This showed women under the age of fifty years were one and a half times more likely to suffer a recurrence of their breast disease if they were ER negative as opposed to ER positive. Davies et al (1991), indicated the importance of being ER positive in the over 70 age group, with regard to their response to the drug Tamoxifen. In the over 70 years of age group, significant statistical information was not obtained from the five year recurrence analysis, however, when considering all of the breast cancer patients, a trend in the data was demonstrated, suggesting ER positive had a longer disease free interval than those who were found to be ER negative. Therefore, it may be postulated with a longer follow-up, the theories suggested by Davies et al (1991), will be upheld. Recurrence times were also considered in the Ki67 analysis group but there were insufficient numbers of patients

and events to prove any statistical significance in the theory of a poor prognosis for tumours with a high Ki67 percentage and a good prognosis for those with the lowest Ki67 percentage score and therefore the slowest turn over of cells within the breast tumour. Again both the ER-ICA and Ki67 survival analysis would benefit from a longer period of follow-up and therefore an increased number of events for analysis.

7.0 CONCLUSIONS.

Fine needle aspiration cytology is a rapid reliable technique which provides a cost effective assessment of the breast tumour preoperatively, in recurrent and inoperable disease.

Cells collected from the tumour in this way may be used for purposes other than the morphological diagnosis of the tumour. When sufficient material is obtained, this may be used to demonstrate a variety of antibodies, which may not be suitable to be used on paraffin sections but which will be useful in assessing the behaviour of the tumour and potential response to treatment.

The study showed that ER status measured from FNAC was more accurate than the biochemical assay previously used. ER-ICA positivity and negativity was likely to be clinically relevant in all age groups, particularly those included in the breast screening programme.

The proliferating cell antigen Ki67 correlated well with ER-ICA status, showing a strong inverse correlation between the two parameters.

Cytological grading of the FNAC specimens showed correlation with ER-ICA analysis and a weak correlation with the proliferating cell antigen Ki67 but no significant correlation with histological grading.

8.0 APPENDICES.

8.1 APPENDIX A.

PAPANICOLOAU STAINING METHOD FOR ALCOHOL FIXED CYTOLOGICAL SPECIMENS.

1. Remove Carbowax fixative or 95% formol alcohol with a rinse in water, 2 minutes.

2. Stain in Gill's haematoxylin, 2 minutes.

3. Rinse in water, 2 minutes.

4. Differentiate in 0.2% aqueous hydrochloric acid, 30 seconds.

5. Rinse in tap water or Scott's tap water substitute, 2 minutes.

6. Rinse in 99% alcohol, 2 minutes.

7. Stain in Orange G6 (OG6), 2 minutes.

8. Rinse in 99% alcohol, 2 minutes.

9. Stain in EA50 solution, 2 minutes.

10. Rinse in 99% alcohol.

11. Place into a xyelene bath, 5 minutes until ready to mount.

RESULTS.

Nuclei	blue/black
Non-keratinised cytoplasm	green
Keratinised cytoplasm	pink
Red blood cells	orange

8.2 APPENDIX B.

MAY-GRUNWALD GEIMSA STAINING METHOD FOR AIR DRIED CYTOLOGICAL SPECIMENS.

1. Fix air dried smears in methanol, 3 minutes.

2. Stain May-Gruwald diluted 1:10 in Sorenson's buffer pH 6.8, 6 minutes.

3. Stain Geimsa diluted 1:15 in Sorenson's buffer pH 6.8, 10 minutes.

4. Rinse in Sorenson's buffer pH 6.8, 5 minutes.

5. Air dry and mount.

RESULTS.

Nuclei	purple
Degenerate nuclei	pink
Cytoplasm	pink or blue
Red blood cells and eosinophils	pink/red

8.3 APPENDIX C.

INDIRECT IMMUNOCYTOCHEMICAL METHOD.

1. Fix smears and controls in dry acetone, 20 minutes.

2. Rinse TRIS buffered saline, 2 x 3 minutes.

3. Apply primary antibody at current dilution, 30 minutes.

4. Rinse TRIS buffered saline, 3 x 3 minutes.

5. Apply secondary antibody at current dilution, 30 minutes.

6. Rinse TRIS buffered saline, 3 x 3 minutes.

7. Stain with cromagen substrate 3'3 diaminobenzedine tetrahydrochloride (DAB), 6 minutes.

8. Rinse TRIS buffered saline, wash in running tap water, 5 minutes.

9. Counterstain the nuclei, 1% Harris's Haematoxylin, 10 minutes.

10. Dehydrate, clear and mount specimens.

RESULTS.

Antigen-antibody reaction	dark brown with the DAB product.
Nuclei	pale blue with 1% Harris's Hx., for use with nuclear antigens.

8.4 APPENDIX D.

PEROXIDASE ANTI-PEROXIDASE IMMUNOCYTOCHEMICAL METHOD.

1. Fix smears and controls in dry acetone, 20 minutes.

2. Rinse TRIS buffered saline, 2 x 3 minutes.

3. Apply primary antibody at current dilution, 30 minutes.

4. Rinse TRIS buffered saline, 3 x 3 minutes.

5. Apply secondary antibody at current dilution, 30 minutes.

6. Rinse TRIS buffered saline, 3 x 3 minutes.

7. Apply peroxidase anti-peroxidase complex at current dilution, 30 minutes.

8. Rinse TRIS buffered saline, 3 x 3 minutes.

9. Stain with cromagen substrate 3'3 diaminobenzedine tetrahydrochloride (DAB), 6 minutes.

10. Rinse TRIS buffered saline, wash in running tap water, 5 minutes.

11. Counterstain the nuclei, 1% Harris's Haematoxylin, 10 minutes.

12. Dehydrate, clear and mount specimens.

RESULTS.

Antigen-antibody reaction	dark brown with the DAB product.
Nuclei	pale blue with 1% Harris's Hx., for use with nuclear antigens.

8.5 APPENDIX E.

AVIDIN BIOTIN COMPLEX IMMUNOCYTOCHEMICAL METHOD.

1. Fix smears and controls in dry acetone, 20 minutes.
2. Rinse TRIS buffered saline, 2 x 3 minutes.
3. Apply primary antibody at current dilution, 30 minutes.
4. Rinse TRIS buffered saline, 3 x 3 minutes.
5. Apply biotinylated secondary antibody at current dilution, 30 minutes.
6. Rinse TRIS buffered saline, 3 x 3 minutes.
7. Apply Strep Avidin Biotin complex at current dilution, 30 minutes.
8. Rinse TRIS buffered saline, 3 x 3 minutes.
9. Stain with cromagen substrate 3'3 diaminobenzedine tetrahydrochloride (DAB), 6 minutes.
10. Rinse TRIS buffered saline, wash in running tap water, 5 minutes.
11. Counterstain the nuclei, 1% Harris's Haematoxylin, 10 minutes.
12. Dehydrate, clear and mount specimens.

RESULTS.

Antigen-antibody reaction	dark brown with the DAB product.
Nuclei	pale blue with 1% Harris's Hx., for use with nuclear antigens.

8.6 APPENDIX F.

METHANOLIC HYDROGEN PEROXIDE BLOCKING FOR NON-SPECIFIC BINDING OF IMMUNOLOGICAL REAGENTS.

1. Add 100 μl. hydrogen peroxide to 5.9 mls. methanol, mix well.

2. Incubate all test and control slides in the methanolic hydrogen peroxide, 30 minutes, prior to immunostaining with the primary antibody.

3. Rinse in TRIS buffered saline, 3 x 3 minutes.

8.7 APPENDIX G.

SODIUM AZIDE BLOCKING FOR NON-SPECIFIC BINDING OF IMMUNOLOGICAL REAGENTS.

1. 0.5 mg. Sodium Azide added to the aqueous chromogen substrate, 3'3 diaminobenzidine tetrahydrochloride. (DAB).

8.8 APPENDIX H.

PREPARATION OF THE DAB CHROMOGEN SUBSTRATE.

1. Weigh out 5 mg. diaminobenzidine tetrahydrochloride (DAB).

2. Add DAB to 10 mls. TRIS buffered saline. See appendix I for the preparation of the TRIS buffer.

3. Immediately prior to use add 100 μl. of 0.1% hydrogen peroxide.

8.9 APPENDIX I.

PREPARATION OF TRIS-BUFFERED SALINE.

1. Weigh out 8.1 gms. sodium chloride and 0.6 gms. TRIS hydrooxymethylmethylamine.

2. Dissolve the two salts in 100 mls. distiled water.

3. Add 3.8 cm 1N HCl.

4. Check pH 7.6, adjust if necessary.

5. Dilute to 1000 mls. in distiled water, check pH 7.6 again.

8.10 APPENDIX J.

PRODUCT-LIMIT SURVIVAL (RECURRENCE) ESTIMATES.
SYMPTOMATIC AND BREAST SCREENING PATIENTS.

* censored observation.

ER-ICA NEGATIVE PATIENTS.

TIME	SURVIVAL	FAILURE	SURVIVAL STANDARD ERROR	NUMBER FAILED	NUMBER LEFT
0	1.0000	0	0	0	156
0	0.9936	0.00641	0.00639	1	155
0*	.	.	.	1	154
15	0.9871	0.0129	0.00904	2	153
27*	.	.	.	2	152
41*	.	.	.	2	151
43	0.9806	0.0194	0.0111	3	150
62*	.	.	.	3	149
76*	.	.	.	3	148
78*	.	.	.	3	147
83*	.	.	.	3	147
91	0.9739	0.0261	0.0129	4	145
101*	.	.	.	4	144
106*	.	.	.	4	143
107	0.9671	0.0329	0.0145	5	142
119*	.	.	.	5	141
119*	.	.	.	5	140
126*	.	.	.	5	139
130*	.	.	.	5	138
134*	.	.	.	5	137
141	0.9600	0.0400	0.0160	6	136
143	0.9530	0.0470	0.0174	7	135
153	0.9459	0.0541	0.0186	8	134
160*	.	.	.	8	133
160*	.	.	.	8	132
168*	.	.	.	8	131
173*	.	.	.	8	130
176*	.	.	.	8	129
177*	.	.	.	8	128
180*	.	.	.	8	127
189	0.9384	0.0616	0.0199	9	126
204	0.9310	0.0690	0.0211	10	125
207	0.9236	0.0764	0.0222	11	124
211*	.	.	.	11	123
211*	.	.	.	11	122
221*	.	.	.	11	121
237*	.	.	.	11	120
238*	.	.	.	11	119
239	0.9158	0.0842	0.0233	12	118
239*	.	.	.	12	117
251	0.9080	0.0920	0.0244	13	116
258*	.	.	.	13	115
260*	.	.	.	13	114
260*	.	.	.	13	113

TIME	SURVIVAL	FAILURE	SURVIVAL STANDARD ERROR	NUMBER FAILED	NUMBER LEFT
265*	.	.	.	13	112
266*	.	.	.	13	111
268	0.8998	0.1002	0.0255	14	110
273*	.	.	.	14	109
273	.	.	.	14	108
277	0.8915	0.1085	0.0266	15	107
278	0.8831	0.1169	0.0276	16	106
286*	.	.	.	16	105
288*	.	.	.	16	104
301*	.	.	.	16	103
303*	.	.	.	16	102
308	.	.	.	16	101
324*	.	.	.	16	100
336*	.	.	.	16	99
338*	.	.	.	16	98
343	0.8741	0.1259	0.0288	17	97
344*	.	.	.	17	96
346	0.8650	0.1350	0.0299	18	95
347*	.	.	.	18	94
358	0.8558	0.1442	0.0310	19	93
373*	.	.	.	19	92
384*	.	.	.	19	91
391*	.	.	.	19	90
407*	.	.	.	19	89
409*	.	.	.	19	88
412	0.8461	0.1539	0.0321	20	87
442*	.	.	.	20	86
446*	.	.	.	20	85
449*	.	.	.	20	84
445*	.	.	.	20	83
457	0.8359	0.1641	0.0333	21	82
468*	.	.	.	21	81
473*	.	.	.	21	80
476*	.	.	.	21	79
485*	.	.	.	21	78
503*	.	.	.	21	77
512	0.8250	0.1750	0.0346	22	76
520*	.	.	.	22	75
524	.	.	.	22	74
534	.	.	.	23	73
534	0.8027	0.1973	0.0371	24	72
538	0.7916	0.2084	0.0382	25	71
538*	.	.	.	25	70
541	0.7803	0.2197	0.0393	26	69
546*	.	.	.	26	68
548	0.7688	0.2312	0.0404	27	67
554*	.	.	.	27	66
566*	.	.	.	27	65
568	0.7570	0.2430	0.0414	28	64
575	0.7451	0.2549	0.0424	29	63
596*	.	.	.	29	62
597*	.	.	.	29	61
604*	.	.	.	29	60
616*	.	.	.	29	59

TIME	SURVIVAL	FAILURE	SURVIVAL STANDARD ERROR	NUMBER FAILED	NUMBER LEFT
617	0.7325	0.2675	0.0436	30	58
624*	.	.	.	30	57
645*	.	.	.	30	56
665*	.	.	.	30	55
671*	.	.	.	30	54
673*	.	.	.	30	53
693*	.	.	.	30	52
709*	.	.	.	30	51
737*	.	.	.	30	50
756	0.7179	0.2821	0.0451	31	49
782*	.	.	.	31	48
785*	.	.	.	31	47
792	0.7026	0.2974	0.0466	32	46
803	.	.	.	32	45
881*	.	.	.	32	44
888*	.	.	.	32	43
919*	.	.	.	32	42
923*	.	.	.	32	41
947*	.	.	.	32	40
951	0.6850	0.3150	0.0487	33	39
964*	.	.	.	33	38
972*	.	.	.	33	37
994*	.	.	.	33	36
1009*	.	.	.	33	35
1009*	.	.	.	33	34
1021*	.	.	.	33	33
1045*	.	.	.	33	32
1056*	.	.	.	33	31
1058*	.	.	.	33	30
1069*	.	.	.	33	29
1074	0.6614	0.3386	0.0524	34	28
1099*	.	.	.	34	27
1107*	.	.	.	34	26
1112*	.	.	.	34	25
1126*	.	.	.	34	24
1147*	.	.	.	34	23
1149*	.	.	.	34	22
1169*	.	.	.	34	21
1170*	.	.	.	34	20
1205*	.	.	.	34	19
1212*	.	.	.	34	18
1245*	.	.	.	34	17
1278*	.	.	.	34	16
1294*	.	.	.	34	15
1343*	.	.	.	34	14
1350*	.	.	.	34	13
1352*	.	.	.	34	12
1373*	.	.	.	34	11
1420*	.	.	.	34	10
1434*	.	.	.	34	9
1435*	.	.	.	34	8
1441*	.	.	.	34	7
1450*	.	.	.	34	6
1457*	.	.	.	34	5

TIME	SURVIVAL	FAILURE	SURVIVAL STANDARD ERROR	NUMBER FAILED	NUMBER LEFT
1520*	.	.	.	34	4
1677	0.4961	0.5039	0.1485	35	3
1728*	.	.	.	35	2
2997*	.	.	.	35	1
3391*	.	.	.	35	0

ER-ICA POSITIVE PATIENTS.

TIME	SURVIVAL	FAILURE	SURVIVAL STANDARD ERROR	NUMBER FAILED	NUMBER LEFT
0	1.0000	0	0	0	140
0*	.	.	.	0	139
12*	.	.	.	0	138
22*	.	.	.	0	137
29*	.	.	.	0	136
48	0.9926	0.00735	0.00733	1	135
91*	.	.	.	1	134
111*	.	.	.	1	133
141	0.9852	0.0148	0.0104	2	132
154*	.	.	.	2	131
154*	.	.	.	2	130
182	0.9776	0.0224	0.0128	3	129
186	0.9700	0.0300	0.0148	4	128
190*	.	.	.	4	127
193*	.	.	.	4	126
204*	.	.	.	4	125
205*	.	.	.	4	124
210*	.	.	.	4	123
217*	.	.	.	4	122
251	0.9621	0.0379	0.0166	5	121
255*	.	.	.	5	120
267	0.9541	0.0459	0.0183	6	119
269	0.9460	0.0540	0.0199	7	118
273	0.9380	0.0620	0.0212	8	117
273*	.	.	.	8	116
287*	.	.	.	8	115
299*	.	.	.	8	114
308*	.	.	.	8	113
330*	.	.	.	8	112
330*	.	.	.	8	111
330*	.	.	.	8	110
337*	.	.	.	8	109
340*	.	.	.	8	108
350*	.	.	.	8	107
351	0.9293	0.0707	0.0228	9	106
365*	.	.	.	9	105

TIME	SURVIVAL	FAILURE	SURVIVAL STANDARD ERROR	NUMBER FAILED	NUMBER LEFT
370*	.	.	.	9	104
378*	.	.	.	9	103
401*	.	.	.	9	102
402*	.	.	.	9	101
410*	.	.	.	9	100
413*	.	.	.	9	99
414*	.	.	.	9	98
420*	.	.	.	9	97
420*	.	.	.	9	96
429*	.	.	.	9	95
447*	.	.	.	9	94
459*	.	.	.	9	93
462	0.9193	0.0807	0.0264	10	92
470*	.	.	.	10	91
490*	.	.	.	10	90
491*	.	.	.	10	89
501	0.9089	0.0911	0.0264	11	88
502*	.	.	.	11	87
524*	.	.	.	11	86
524*	.	.	.	11	85
525*	.	.	.	11	84
545	0.8981	0.1019	0.0282	12	83
546	0.8873	0.1127	0.0299	13	82
546*	.	.	.	13	81
546*	.	.	.	13	80
559*	.	.	.	13	79
601*	.	.	.	13	78
613*	.	.	.	13	77
630*	.	.	.	13	76
639	0.8756	0.1244	0.0317	14	75
641*	.	.	.	14	74
645*	.	.	.	14	73
666*	.	.	.	14	72
672*	.	.	.	14	71
672*	.	.	.	14	70
676*	.	.	.	14	69
685*	.	.	.	14	68
692*	.	.	.	14	67
693*	.	.	.	14	66
700*	.	.	.	14	65
719	0.8621	0.1379	0.0340	15	64
730*	.	.	.	15	63
731	0.8485	0.1515	0.0361	16	62
742*	.	.	.	16	61
743*	.	.	.	16	60
746*	.	.	.	16	59
747	0.8341	0.1659	0.0382	17	58
760*	.	.	.	17	57
761*	.	.	.	17	56
762*	.	.	.	17	55
799*	.	.	.	17	54
803	0.8186	0.1814	0.0405	18	53
819*	.	.	.	18	52
834*	.	.	.	18	51
861*	.	.	.	18	50

TIME	SURVIVAL	FAILURE	SURVIVAL STANDARD ERROR	NUMBER FAILED	NUMBER LEFT
875*	.	.	.	18	49
895*	.	.	.	18	48
902*	.	.	.	18	47
904	0.8012	0.1988	0.0432	19	46
912	0.7838	0.2162	0.0457	20	45
933*	.	.	.	20	44
959*	.	.	.	20	43
961*	.	.	.	20	42
982*	.	.	.	20	41
989*	.	.	.	20	40
1000*	.	.	.	20	39
1007*	.	.	.	20	38
1016*	.	.	.	20	37
1021*	.	.	.	20	36
1023*	.	.	.	20	35
1029*	.	.	.	20	34
1042*	.	.	.	20	33
1056*	.	.	.	20	32
1067*	.	.	.	20	31
1070*	.	.	.	20	30
1081*	.	.	.	20	29
1085*	.	.	.	20	28
1092*	.	.	.	20	27
1094*	.	.	.	20	26
1106*	.	.	.	20	25
1121*	.	.	.	20	24
1128*	.	.	.	20	23
1137*	.	.	.	20	22
1140*	.	.	.	20	21
1141*	.	.	.	20	20
1147*	.	.	.	20	19
1154*	.	.	.	20	18
1162*	.	.	.	20	17
1167*	.	.	.	20	16
1168*	.	.	.	20	15
1217*	.	.	.	20	14
1224*	.	.	.	20	13
1226*	.	.	.	20	12
1238*	.	.	.	20	11
1245*	.	.	.	20	10
1273*	.	.	.	20	9
1287*	.	.	.	20	8
1301*	.	.	.	20	7
1337*	.	.	.	20	6
1361*	.	.	.	20	5
1378*	.	.	.	20	4
1401*	.	.	.	20	3
1473*	.	.	.	20	2
1560*	.	.	.	20	1
1561*	.	.	.	20	0

8.11 APPENDIX K.

DETAILS FROM THE CASES OF SYMPTOMATIC PATIENTS.

KEY:-

- AGE = Age at diagnosis.
- TNM = Tumour, Node, metastases.
- ER = ER-ICA analysis.
- DCC = Radioligand Binding Dextran Coated Charcoal Assay.
- NEG* = Negative result but with less than 25% of tumour nuclei staining positive.
- POS* = Positive result but with a mixed population of negative and positive staining nuclei.
- TAM = Intial therapy, Tamoxifen only.
- CHE = Initial therapy, chemotherapy only.
- UNS = Unsatisfactory result

Patient Number	Age	TNM	ER	DCC	%Ki67	Histology Grade	Cytology Grade
1	70	210	UNS	244	-	3	-
2	81	310	UNS	-	-	3	-
3	56	200	NEG	-	-	2	-
4	52	100	NEG	19	24	3	2
5	69	200	POS	503	8	3	2
6	47	200	NEG	NEG	18	3	2
7	66	210	NEG	NEG	51	3	2
8	52	210	NEG	-	10	2	2
9	69	100	POS	483	-	2	-
10	66	200	NEG	35	-	Intraduct	-
11	40	210	UNS	NEG	-	-	-
12	65	200	NEG	-	-	3	-
13	64	310	UNS	277	21	3	2
14	33	310	POS	-	5	Lobular	-

Patient Number	Age	TNM	ER	DCC	%Ki67	Histology Grade	Cytology Grade
15	63	210	UNS	-	13	3	-
16	70	210	POS	369	-	2	-
17	66	210	POS	1079	-	3	-
18	71	210	UNS	63	-	3	-
19	51	100	NEG	228	-	2	-
20	25	210	NEG	-	21	-	-
21	65	100	NEG	NEG	-	3	-
22	40	210	UNS	-	-	2	-
23	75	310	POS	348	8	2	-
24	40	300	NEG	-	-	2	-
25	66	210	POS	96	-	2	-
26	68	210	NEG	-	27	3	2
27	67	200	POS	-	3	2	2
28	47	200	NEG	NEG	-	2	-
29	75	-	NEG	-	-	2	-
30	69	210	UNS	-	-	2	-
31	51	200	NEG	NEG	57	3	2
32	53	100	POS	-	17	2	2
33	51	210	NEG	55	31	3	2
34	50	210	NEG	-	36	-	3
35	68	210	NEG	-	47	-	3
36	54	210	POS	-	10	2	2
37	69	200	UNS	56	-		-
38	75	100	NEG	-	-	3	-
39	68	200	NEG	272	2	3	2
40	72	200	NEG	NEG	-	3	-
41	59	210	POS	870	-	3	-

Patient Number	Age	TNM	ER	DCC	%Ki67	Histology Grade	Cytology Grade
42	68	210	POS	196	-	2	-
43	69	210	POS	214	-	2	-
44	39	210	NEG	14	2	Lobular	-
45	76	210	POS	-	-	2	-
46	87	TAM	NEG	-	-	-	-
47	40	210	NEG	336	-	3	-
48	68	210	POS	47	-	3	-
49	64	310	POS	-	-	2	-
50	69	200	NEG	NEG	-	3	-
51	76	200	POS	-	-	3	-
52	67	310	NEG	NEG	-	3	-
53	57	100	NEG	-	-	-	-
54	79	TAM	NEG	-	-	-	-
55	58	200	NEG	-	-	2	-
56	64	200	UNS	NEG	-	3	-
57	84	TAM	POS	-	-	-	-
58	73	210	POS	-	-	1	-
59	68	200	POS	43	-	-	-
60	57	200	POS	-	1	3	2
61	56	200	POS	NEG	-	2	-
62	44	210	UNS	NEG	-	2	-
63	88	TAM	POS	-	-	-	-
64	64	100	NEG	677	-	Carcinoid	-
65	51	3-0	NEG	NEG	-	2	-
66	65	200	UNS	-	-	2	-
67	51	210	POS	480	-	3	-
68	57	310	NEG	NEG	-	3	-

Patient Number	Age	TNM	ER	DCC	%Ki67	Histology Grade	Cytology Grade
69	65	210	POS	168	-	2	-
70	57	100	POS	-	-	2	-
71	72	210	UNS	31	-	3	-
72	50	200	POS	NEG	-	Lobular	-
73	67	200	POS	87	-	-	-
74	58	200	POS	110	-	3	-
75	66	200	POS	96	-	3	-
76	77	100	POS	-	-	2	-
77	67	200	UNS	18	-	3	-
78	62	210	POS	367	-	3	-
79	64	100	POS	70	-	3	-
80	51	210	NEG	NEG	-	3	-
81	69	200	UNS	-	-	-	-
82	62	210	NEG	115	-	2	-
83	60	110	POS	656	-	-	-
84	73	-	UNS	-	-	-	-
85	64	210	UNS	NEG	-	3	-
86	80	TAM	POS	-	-	-	-
87	82	TAM	POS	-	-	-	-
88	44	200	NEG	NEG	-	3	-
89	74	200	UNS	-	-	2	-
90	70	20	NEG	-	-	3	-
91	47	TAM	NEG	-	-	-	-
92	82	TAM	UNS	-	-	-	-
93	78	TAM	UNS	-	-	-	-
94	82	TAM	POS	-	-	-	-
95	79	TAM	POS*	-	-	-	-

Patient Number	Age	TNM	ER	DCC	%Ki67	Histology Grade	Cytology Grade
96	52	CHE	NEG	-	-	-	-
97	62	110	NEG	NEG	-	2	-
98	70	200	POS	-	-	2	-
99	68	200	POS	14	-	3	-
100	68	200	POS	345	-	3	-
101	78	TAM	POS	-	-	-	-
102	57	200	NEG	-	-	-	-
103	58	200	POS	260	-	3	-
104	69	210	NEG	43	10	2	-
105	75	TAM	UNS	-	-	-	-
106	67	100	UNS	20	35	3	2
107	52	210	UNS	144	-	3	-
108	41	200	NEG	12	-	3	-
109	68	300	POS	NEG	8	3	2
110	62	210	UNS	-	-	-	-
111	79	TAM	POS	-	-	-	-
112	69	200	UNS	NEG	-	3	-
113	61	210	UNS	NEG	-	-	-
114	74	-	POS	-	28	-	-
115	43	100	NEG	NEG	25	1	2
116	75	210	POS	-	-	-	-
117	80	210	POS	-	6	2	-
118	70	200	NEG	23	30	-	3
119	71	TAM	POS	-	10	-	2
120	63	210	POS	251	8	2	2
121	41	210	NEG	-	6	3	2
122	60	200	POS	407	4	Papillary	2

Patient Number	Age	TNM	ER	DCC	%Ki67	Histology Grade	Cytology Grade
123	84	TAM	POS	-	-	-	-
124	61	310	POS	425	20	2	2
125	84	200	POS	-	-	2	-
126	69	200	UNS	111	-	2	-
127	77	210	POS	-	2	2	2
128	75	200	POS	-	3	2	2
129	50	-	NEG	17	28	3	3
130	55	210	NEG	-	35	3	-
131	46	-	NEG	59	-	3	2
132	44	-	NEG	NEG	48	Medullary	-
133	65	-	NEG	NEG	-	Intraduct	-
134	48	-	NEG	-	-	-	-
135	57	210	NEG	-	-	3	-
136	57	100	UNS	NEG	9	Intraduct	2
137	47	-	POS	-	-	-	-
138	65	-	POS	410	-	2	-
139	40	210	NEG	NEG	-	2	-
140	78	-	POS	-	-	-	-
141	84	-	NEG	-	-	-	-
142	79	-	POS	-	-	-	-
143	60	110	UNS	-	-	-	-
144	58	110	UNS	NEG	-	-	-
145	69	110	NEG	-	-	3	3
146	50	210	NEG	-	-	3	-
147	79	-	NEG	-	-	-	-
148	56	200	POS	-	-	3	-
149	55	200	UNS	-	-	-	-

Patient Number	Age	TNM	ER	DCC	%Ki67	Histology Grade	Cytology Grade
150	41	-	POS	-	-	-	-
151	44	110	POS	-	-	2	-
152	54	-	POS	-	9	3	2
153	68	-	POS	-	1	2	2
154	64	200	POS	28	-	3	-
155	77	-	POS	-	-	-	-
156	72	310	NEG	-	-	3	-
157	74	-	POS	-	17	3	2
158	50	-	UNS	-	-	2	-
159	63	110	POS	-	-	1	-
160	84	-	NEG	-	8	-	-
161	77	-	POS	-	22	-	2
162	45	310	POS	65	-	3	-
163	72	-	NEG	-	-	Intraduct	3
164	62	-	POS	244	-	-	-
165	64	-	POS	249	-	-	-
166	29	-	NEG	13	20	2	2
167	44	-	POS	33	14	3	2
168	31	-	NEG	-	36	3	2
169	78	210	POS	-	5	2	2
170	77	200	POS	-	9	2	2
171	86	-	POS	-	4	-	-
172	57	200	UNS	-	34	3	2
173	59	-	POS*	68	-	-	-
174	41	-	UNS	14	-	-	-
175	42	-	POS	34	2	3	2
176	75	-	UNS	-	16	-	2

Patient Number	Age	TNM	ER	DCC	%Ki67	Histology Grade	Cytology Grade
177	87	-	POS	-	-	-	-
178	56	110	POS*	82	-	2	-
179	59	210	POS*	151	22	3	2
180	67	-	POS	47	-	3	-
181	63	200	POS	252	6	2	2
182	60	-	NEG	-	-	-	-
183	70	200	POS	598	5	2	2
184	69	210	POS	114	15	3	2
185	66	-	POS*	253	-	3	-
186	44	200	UNS	NEG	-	3	-
187	46	100	POS	117	-	-	-
188	61	210	NEG	NEG	24	3	2
189	76	-	POS	-	12	-	2
190	74	-	NEG	-	5	Colloid	1
191	73	-	POS	-	5	-	1
192	73	-	NEG	-	8	2	2
193	72	200	POS	-	11	-	2
194	49	200	NEG	-	17	-	3
195	47	-	POS*	-	3	1	2
196	75	-	UNS	-	9	-	2
197	79	-	POS	-	6	Mucoid	2
198	75	-	NEG	-	16	-	2
199	32	-	NEG	-	9	-	2
200	77	-	POS	-	-	-	-
201	39	-	NEG	137	-	3	-
202	85	-	POS*	-	-	-	-
203	46	-	NEG	31	-	Lobular	-

Patient Number	Age	TNM	ER	DCC	%Ki67	Histology Grade	Cytology Grade
204	77	-	POS	-	-	-	-
205	65	200	NEG	NEG	-	-	-
206	73	310	NEG*	-	13	-	2
207	47	210	NEG	53	-	-	-
208	65	-	POS	-	-	-	-
209	56	-	NEG	-	-	-	-
210	68	200	NEG*	NEG	-	-	-
211	94	-	POS	-	3	-	1
212	44	-	UNS	-	-	-	-
213	48	-	NEG	16	-	-	-
214	84	-	POS	-	-	-	-
215	67	-	NEG	-	-	2	-
216	82	-	POS	-	-	-	-
217	55	300	NEG	-	21	-	3
218	68	-	UNS	-	-	-	-
219	71	-	POS	-	-	-	-
220	32	-	NEG	-	6	3	2
221	53	310	POS	824	10	-	2
222	83	-	POS	-	-	-	-
223	80	-	POS	-	-	-	-
224	63	210	POS	-	-	-	-
225	66	-	POS	-	-	-	-
226	82	-	POS	-	-	-	-
227	75	200	UNS	-	-	2	-
228	68	-	POS	-	3	-	2
229	64	-	NEG	-	-	-	-
230	60	-	NEG	-	35	-	2

Patient Number	Age	TNM	ER	DCC	%Ki67	Histology Grade	Cytology Grade
231	77	200	NEG	-	-	2	-
232	67	-	NEG	-	9	3	2
233	79	-	POS	-	-	-	-
234	64	-	NEG	-	-	3	-
235	51	-	NEG	-	5	-	2
236	89	-	NEG	-	-	-	-
237	82	-	NEG	-	-	-	-
238	54	210	NEG	-	-	2	-
239	80	-	POS	-	-	-	-
240	45	-	POS*	-	31	2	2
241	78	-	POS	-	8	-	2
242	66	-	NEG*	-	-	-	-
243	79	200	NEG	-	37	2	2
244	74	-	NEG	-	-	-	-
245	70	110	POS	85	8	-	2
246	48	210	NEG*	-	11	2	3
247	51	310	NEG	-	15	-	-
248	54	-	UNS	NEG	-	3	-
249	68	200	POS	176	8	-	2
250	36	110	POS	-	-	3	-
251	55	210	NEG	-	21	3	1
252	68	-	POS	-	-	1	-
253	84	-	POS	-	11	-	3
254	61	210	UNS	-	-	3	-
255	78	210	UNS	18	7	-	2
256	72	-	NEG	-	-	-	-
257	57	-	POS	219	<1	2	2

Patient Number	Age	TNM	ER	DCC	%Ki67	Histology Grade	Cytology Grade
258	53	100	NEG	-	-	3	-
259	76	-	POS	-	-	-	-
260	73	-	UNS	-	UNS	-	-
261	55	-	NEG	NEG	UNS	Medullary	3
262	44	-	NEG	NEG	-	2	-
263	61	-	NEG	-	-	-	-
264	34	-	POS*	-	23	-	1
265	67	200	POS	780	12	3	2
266	64	-	NEG	NEG	12	3	2
267	68	110	NEG*	157	11	-	2
268	76	-	POS	-	10	1	2
269	55	210	NEG	423	23	3	2
270	80	-	NEG	-	19	-	2
271	45	-	NEG	-	UNS	-	-
272	66	-	NEG	NEG	-	2	-
273	72	-	UNS	-	-	-	-
274	64	210	POS	940	5	2	1
275	62	-	POS	255	2	2	2
276	30	100	NEG	-	19	3	-
277	63	-	POS	-	1	-	2
278	86	-	POS	-	3	-	2
279	58	210	UNS	-	UNS	3	-
280	69	210	POS	475	5	2	2
281	79	-	POS	-	10	-	2
282	66	-	UNS	-	UNS	-	-
283	84	-	POS	-	11	-	2
284	75	210	NEG	-	5	2	2

Patient Number	Age	TNM	ER	DCC	%Ki67	Histology Grade	Cytology Grade
285	79	-	UNS	-	3	-	1
286	54	-	NEG	-	13	3	2
287	77	200	POS	-	5	2	2
288	95	-	POS	-	38	-	3
289	61	210	POS	-	7	1	2
290	63	-	NEG	-	12	2	2
291	82	-	POS	-	4	-	2
292	76	210	UNS	-	UNS	3	-
293	74	-	NEG	-	29	-	2
294	65	-	POS	-	32	3	2
295	59	200	POS	-	UNS	2	1
296	51	-	NEG	-	18	-	2
297	89	-	UNS	-	UNS	-	-
298	68	-	UNS	-	UNS	2	-
299	26	-	NEG	-	-	2	-
300	45	200	NEG	-	-	3	-
301	50	-	POS	-	-	-	-
302	94	-	POS	-	<1	-	3
303	59	-	UNS	-	26	2	2
304	78	200	POS	-	6	1	1
305	70	-	UNS	-	-	-	-
306	78	-	POS*	-	5	-	2
307	73	-	NEG	-	1	-	3
308	97	-	POS	-	3	-	1
309	81	-	POS	-	22	-	2
310	66	100	POS	-	15	2	2
311	74	-	NEG	-	3	-	2

Patient Number	Age	TNM	ER	DCC	%Ki67	Histology Grade	Cytology Grade
312	78	-	NEG	-	7	-	-
313	46	100	UNS	-	UNS	2	-
314	54	100	NEG	-	-	-	-
315	64	100	POS	-	-	3	-
316	39	-	NEG	-	-	-	-
317	85	-	POS	-	-	-	-
318	57	200	UNS	-	-	3	-
319	72	-	POS	-	-	-	-
320	75	200	NEG	-	-	-	-
321	89	-	NEG	-	-	-	-
322	60	100	POS	-	1	-	-
323	79	-	UNS	-	-	-	-
324	64	300	NEG	-	-	3	-
325	84	-	-	-	-	-	-
326	78	-	NEG*	-	-	-	-
327	38	200	NEG	-	-	-	-
328	81	-	POS	-	-	-	-
329	48	100	NEG	-	-	2	-
330	58	110	NEG	-	-	3	-
331	81	-	POS	-	-	-	-
332	68	-	NEG	-	-	-	-
333	61	-	UNS	-	-	-	-
334		-	POS*	-	-	-	-
335	70	-	NEG	-	-	-	-
336	65	-	POS	-	-	-	-
337	55	-	UNS	-	-	-	-
338	71	-	POS*	-	-	-	-

8.12 APPENDIX L.

DETAILS OF CASES FROM THE BREAST SCREENING GROUP.

PATIENT NUMBER GRADE	ER-ICA	%Ki67	HISTOLOGICAL
1*	NEG	_	3
2*	POS	_	2
3*	NEG	_	2
4*	NEG	5	1
5*	POS	14	LOBULAR
6*	NEG	31	INTRADUCT
7*	NEG	3	3
8*	NEG	_	TUBULAR
9*	POS	10	2
10*	POS	2	1
11*	NEG	_	TUBULAR
12*	NEG	_	INTRADUCT
13*	NEG	3	1
14*	NEG	13	2
15*	POS	14	1
16*	NEG	37	3
17*	NEG	_	3
18*	POS	_	3
19*	NEG	_	3
20*	POS	_	1
21*	POS	4	1
22*	NEG	_	3
23*	NEG	_	2
24*	NEG	_	2

PATIENT NUMBER GRADE	ER-ICA	%Ki67	HISTOLOGICAL
25*	POS	10	3
26*	POS	_	3
27*	NEG	14	3
28*	NEG	_	3
29*	POS	_	2
30*	NEG	6	2
31*	NEG	1	TUBULAR
32*	POS	5	1
33*	NEG	_	1
34*	POS	_	3
35*	POS	_	2
36*	NEG	2	3
37*	NEG	13	3
38*	NEG	3	1
39*	NEG	_	INTRADUCT
40*	POS	6	3
41*	POS	_	INTRADUCT
42*	POS	_	2
43*	POS	_	2
44*	POS	6	2
45*	NEG	10	3
46*	NEG	_	3
47*	POS	1	1
48*	POS	_	2
49*	POS	_	INTRADUCT
50*	POS	1	2
51*	NEG	15	2

PATIENT NUMBER GRADE	ER-ICA	%Ki67	HISTOLOGICAL
52*	POS	_	TUBULAR
53*	NEG	_	INTRADUCT
54*	POS	10	2
55*	NEG	22	2
56*	NEG	9	1
57*	NEG	23	3
58*	NEG	_	LOBULAR
59*	POS	_	3
60*	NEG	_	3
61*	POS	7	1
62*	NEG	_	2
63*	POS	_	1
64*	POS	8	2
65*	NEG	_	1
66*	NEG	_	3
67*	POS	_	TUBULAR
68*	NEG	_	INTRADUCT

8.13 APPENDIX M.

LIST OF SUPPLIERS OF SPECIALIST EQUIPMENT AND REAGENTS.

1. 'CAMECO' Syringe Pistol.
Henleys Medical Supplies Ltd.,
Brownfields,
Welwyn Garden City,
Hertfordshire. AL7 1AN.

2. ER-ICA Kit.
Abbott Laboratories Ltd., Diagnostic Division,
Abbott House,
Moorbridge Road,
Maidenhead,
Berkshire. SL6 8XZ.

3. Proliferating Cell Antigen, Ki67. Biotinylated Secondary Antigen. Strep ABComplex.
Dako Ltd. (formerly DakoPatts Ltd.),
16 Manor Courtyard,
Hughenden Avenue,
High Wycombe,
Buckinghamshire. HP13 5RE.

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