RADIOIMMUNOHISTOCHEMICAL QUANTITATION OF EGFR AND HER2 EXPRESSION IN PREINVASIVE COMPARED TO INVASIVE BREAST CANCER AND THE RELATIONSHIP OF HER2 EXPRESSION IN PRIMARY INVASIVE BREAST CANCER TO OUTCOME.

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1. Page 50 Para 2 Line 9
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ABSTRACT OF FINDINGS

Background: Ductal carcinoma in situ (DCIS) of the breast is being diagnosed with increasing frequency, partly due to the increased use of mammography in mass screening programs. The precise natural history of DCIS is not clear but is thought to represent a stage in the progression to frank malignancy. This group has previously described a radioimmunohistochemical technique which allows quantitation of the EGFR and HER2 in all cases of tumour examined (1;2). Application of this quantitative radioimmunohistochemistry to pure DCIS, DCIS in association with invasive disease and primary invasive breast cancers may clarify the role, if any, of EGFR and HER2 in the pathogenesis of invasive breast cancer. If EGFR downregulation or HER2 overexpression are required for progression to invasion we might expect to see more lesions with normal levels of expression in pure DCIS, and an invasive or intermediate pattern in DCIS adjacent to invasive areas. In this way, the quantitative information available from radio-immunohistochemistry may allow us to predict the biological role of EGFR and HER2 in breast carcinogenesis and progression.

HER2 gene amplification or protein overexpression identifies 20-30% of breast cancer patients with a poor outlook. Current methods of HER2 analysis reveal nothing about the remaining 70 to 80% of patients. This may compromise the prognostic or predictive value of HER2.

Methods: In frozen sections of pure DCIS tumours (n=37 for EGFR, n=36 for HER2) and in DCIS lesions in association with invasive cancers, (n=50 for EGFR, n=47 for HER2) receptor levels were assayed quantitatively using a radiolabelled antibody method. EGFR was quantified in 193 and HER2 in 177 frozen primary

breast tumours using radioimmunohistochemistry. The results of HER2 expression were related to patient survival and major pathological variables.

Results: EGFR and HER2 expression each varied by a factor of several thousand. Levels of EGFR and HER2 expression in pure DCIS, in DCIS associated with invasion and in a larger group of invasive tumours (n=193 for EGFR, n=177 for HER2) were compared. The frequency distributions for expression of both factors were comparable in pure DCIS and in DCIS associated with invasive tumours (Mann-Whitney U test, EGFR p=0.17, HER2 p=0.16). Similarly, frequency distributions for expression of both factors were comparable in DCIS associated with invasive tumours and in purely invasive tumours (Mann-Whitney U test, EGFR p=0.16, HER2 p=0.91). Comparing pure DCIS with invasive tumours , there was a trend to significance difference in the frequency distributions of these 2 growth factor receptors. Within each tumour that had both DCIS and invasion, there was no significant difference in expression of EGFR or HER2 in the DCIS and invasive components (Wilcoxon Signed Rank Test, EGFR p=0.39, HER2 p=0.087). However, on reviewing individual cases, those tumours that exhibit the largest change in expression of growth factor receptor between the DCIS and invasive components appeared to have very aggressive invasive tumours.

Fifteen percent of primary invasive cancers expressed less HER2 than normal breast parenchyma, 62% had between 1 and 15 times normal and 23% between 15 and 400 fold overexpression. We have shown previously that this last group corresponds to those with HER2 gene amplification. Survival analysis indicated that prognosis was best for tumours with intermediate levels of HER2, becoming worse for both lower and higher expressing cases. The best outcome was

associated with a 5.7 times the normal HER2 level. The relative hazard was 3.5 times higher for tumours with 0.1x normal expression, and 4 times higher for tumours with 100x normal expression. HER2 remained a significant predictor of survival when the pathological variables were introduced into the analysis.

Conclusions: These data suggest that alterations in type I growth factor receptors occur before progression of *in-situ* disease to invasive cancer. High levels of HER2 overexpression in both *in-situ* and invasive areas suggest that the HER2 product is a potential therapeutic target for the treatment of breast cancer at an early stage. In addition, these results indicate that the role of HER2 in breast cancer is complex, as tumours with down regulation as well as those with gene amplification exhibit more aggressive behaviour. These findings are of clinical importance given the developing role of HER2 as a prognostic and predictive factor, and as a target for therapy.

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I would like to acknowledge the work done by Mr. G. Smith[3] who did most of the laboratory and radioimmunohistochemical work and image analysis on the initial series of EGFR and HER2 expression in invasive breast cancer which was extended from 118 tumours for EGFR expression to 193 and from 81 tumours for HER2 expression to 177 tumours in this work.

Without the guidance and tutoring of Dr. James J. Going[4] and especially Dr. E.A. Mallon[5] in identifying those tumours that contained DCIS and those that also contained invasive areas this work would not have been possible.

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ABBREVIATIONS

A431	Cell line with known EGFR density used in quantifying EGFR
DCIS	Ductal carcinoma in situ
EAM	Dr. E.A. Mallon, Consultant Breast Pathologist
EGFR	Epidermal growth factor receptor
EGFR1	Monoclonal antibody to the epidermal growth factor receptor
EORTC	The European Organisation for Research and Treatment of Cancer
GRI	Glasgow Royal Infirmary
ICR12	Monoclonal antibody to HER2
JJG	Dr. J.J. Going, Consultant Breast Pathologist
LCIS	Lobular carcinoma in situ
NPI	Nottingham Prognostic Index
NSABP	National Surgical Adjuvant Breast and Bowel Project
VNPI	Van Nuys Prognostic Index
VNCS	Van Nuys DCIS Classification system
WGI	Western General Infirmary, Glasgow
ZR75, MDA-MB-453, MDA-MB-361, BT474, SKBR3	Cell lines used in Scatchard analysis for quantifying HER2

LIST OF TABLES

LIST OF FIGURES

DECLARATION OF WORK PUBLISHED AND PRESENTED

The following papers have been presented in person at scientific meetings:

1) **D. Chong, T. Cooke, J. Reeves, W.D. George, E. Mallon, B. Ozanne and P. Stanton.**

   Quantitation of HER2 in primary breast cancer allows identification of a further poor prognostic group.

   European Conference on Clinical Oncology, Vienna, September 1999.

2) **D. Chong, T. Cooke, J. Reeves, W.D. George, E. Mallon, B. Ozanne and P. Stanton.**

   Quantitation of EGFR and HER2 expression in preinvasive compared to invasive breast cancer.

   European Conference on Clinical Oncology, Vienna, September 1999.

3) **D. Chong, J. Reeves, T. Cooke, W.D. George, E. Mallon, B. Ozanne and P. Stanton.**

   EGFR and c-*erb*b-2 expression in breast cancer progression.

   Joint meeting of the British Oncological Association, the Association of Cancer Physicians and the Royal College of Radiologists, Nottingham, July 1998.

4) **D. Chong, J. Reeves, T. Cooke, W.D. George, E. Mallon, B. Ozanne and P. Stanton.**

   Quantitation of c-*erb*B-2 in primary breast cancer: gene amplification is not the whole picture.

   Joint meeting of the British Oncological Association, the Association of Cancer Physicians and the Royal College of Radiologists, Nottingham, July 1998.

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   Quantitation of c-*erb*b-2 in primary breast cancer allows identification of a further poor prognostic group.

   Joint British and Irish Association Cancer Research Meeting, Dublin, June 1998.

6) **D. Chong, J. Reeves, T. Cooke, W.D. George, E. Mallon, B. Ozanne and P. Stanton.**

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7) **D. Chong, J. Reeves, T. Cooke, W.D. George, E. Mallon, B. Ozanne and P. Stanton.**

Quantitation of c-*erb*B-2 in primary breast cancer: gene amplification is not the whole picture.

British Association of Surgical Oncologists 56th Scientific Meeting, June 1998.

8) **D. Chong, J. Reeves, T. Cooke, W.D. George, E. Mallon, B. Ozanne and P. Stanton.**

Quantitation of EGFR and c-*erb*b-2 expression in preinvasive compared to invasive breast cancer.

British Association of Surgical Oncologists 56th Scientific Meeting, June 1998.

9) **D. Chong, J. Reeves, T. Cooke, W.D. George, E. Mallon, B. Ozanne and P. Stanton.**

EGFR and c-*erb*b-2 expression in breast cancer progression.

Surgical Research Society of Great Britain and Ireland, Dublin, July 1998.

10) **D. Chong, J. Reeves, T. Cooke, W.D. George, E. Mallon, B. Ozanne and P. Stanton.**

EGFR and c-*erb*b-2 expression in ductal carcinoma in situ.

Scottish Society for Experimental Medicine, Glasgow, May 1998.

PUBLISHED ABSTRACTS:

The following papers have been published in abstract form:

1) **D. Chong, J. Reeves, T. Cooke, W.D. George, E. Mallon, B. Ozanne and P. Stanton.**

   Quantitation of c-*erb*b-2 in primary breast cancer allows identification of a further poor prognostic group.

   Breast Cancer Research and Treatment 1999, Vol 57;1;103.

2) **D. Chong, J. Reeves, T. Cooke, W.D. George, E. Mallon, B. Ozanne and P. Stanton.**

   EGFR and c-*erb*b-2 expression in breast cancer progression.

   British Journal of Cancer Supp 2, 1998; 78; 24

3) **D. Chong, J. Reeves, T. Cooke, W.D. George, E. Mallon, B. Ozanne and P. Stanton.**

   Quantitation of c-*erb*B-2 in primary breast cancer: gene amplification is not the whole picture.

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4) **D. Chong, J. Reeves, T. Cooke, W.D. George, E. Mallon, B. Ozanne and P. Stanton.**

   Quantitation of c-*erb*b-2 in primary breast cancer allows identification of a further poor prognostic group.

   British Journal of Cancer Supp 1, 1998; 78; 69

5) **D. Chong, J. Reeves, T. Cooke, W.D. George, E. Mallon, B. Ozanne and P. Stanton.**

   Quantitation of EGFR and c-*erb*b-2 expression in preinvasive compared to invasive breast cancer.

   British Journal of Cancer Supp 1, 1998; 78; 68

SECTION 1 INTRODUCTION

1.1 OVERVIEW

Ductal carcinoma in situ (DCIS) of the breast is being diagnosed with increasing frequency, partly due to the increased use of mammography in mass screening programs. The precise natural history of DCIS is not clear but is thought to represent a stage in the progression to frank malignancy.

EGFR and HER2 have been extensively studied in breast cancer. This group has previously described a radioimmunohistochemical technique which allows quantitation of the EGFR and HER2 in all cases (1;2).

This work has applied this method to DCIS lesions and to primary invasive breast cancers in two ways:

1. application of quantitative radioimmunohistochemistry to pure DCIS, DCIS in association with invasive disease and primary invasive breast cancers to clarify the role, if any, of EGFR and HER2 in the pathogenesis of invasive breast cancer.
2. to expand the number of tumours examined in the previous studies from 118 to 193 for EGFR and from 81 to 177 for HER2. In addition the long term follow up of patients for whom quantitative HER2 measurements were made is presented.

1.2 In situ carcinoma of the breast

During the past decade, as mammography has become more widely used and technically advanced, the prevalence of lesions that appear to be in the early stages of tumour development has been emphasized. In situ carcinoma of the breast is an example of such an early lesion. As in other parts of the body these in situ lesions show some of the cytological properties of infiltrative tumours, but have not yet begun to infiltrate themselves. The implication is that they might do so if left for long enough although the length of time is unknown. Intense research is now being directed at determining how far these lesions have evolved from normality in terms of time and biological events and whether any of these events are reversible. In situ carcinoma of the breast may then be a potential candidate for targetted anti-cancer therapy allowing early treatment before progression to frank invasion.

1.2.1 Types of in situ breast carcinoma

Carcinoma in situ was originally classified as ductal or lobular based on the resemblance of involved spaces to ducts and lobules. These descriptive terms are still in use although all carcinomas are now thought to arise from the terminal duct lobular unit and the terms no longer imply a site or cell type of origin (3).

The 2 types are:

- lobular carcinoma in situ (LCIS)
- ductal carcinoma in situ (DCIS)

1.3 Lobular carcinoma in situ (LCIS)

LCIS is much less common than ductal carcinoma in situ (DCIS) and is usually an incidental finding in breast tissue removed for other reasons. In contrast with DCIS, LCIS is often multifocal and bilateral in up to 30% of cases. The term lobular carcinoma in situ (LCIS) is misleading and is more appropriately termed "lobular neoplasia." Strictly speaking, it is not known to be a premalignant lesion, but rather a marker that identifies women at an increased risk for subsequent development of invasive breast cancer. This risk remains elevated even beyond 2 decades, and most of the subsequent cancers are ductal in nature rather than lobular. In a large prospective series from the National Surgical Adjuvant Breast and Bowel Project (NSABP) with 5-year follow-up of 182 women with LCIS managed with excisional biopsy alone, only 8 women developed ipsilateral breast cancer (4 of them invasive) (4). In addition, 3 developed contralateral breast tumors (2 of them invasive).

LCIS will not be considered further in this work as it forms no part of this study.

1.4 Ductal carcinoma in situ (DCIS)

DCIS is a proliferation of malignant epithelial cells within the ductolobular system of the breast that show no light microscopic evidence of invasion through the basement membrane into the surrounding stroma.

1.4.1 Incidence of DCIS

Until the late 1970's, DCIS was an uncommon disease, representing only about 1% of all newly diagnosed cases of breast cancer (5). Then, most patients presenting with DCIS had symptoms – a palpable mass or discharge from the nipple. The incidence of DCIS has increased in recent years, partly because screening mammography is more refined and more widely used (6-9). During 1997, more than 36 000 new cases of DCIS, representing 17% of all new breast cancers, were diagnosed in the United States (10). The rate of increase in incidence has been higher for DCIS than for any other type of breast cancer (11). The reported incidence in women 50 years of age or older increased 235% from 1979 to 1986; in contrast, the incidence of invasive cancer increased 50% (12). Wider use and greater acceptance of screening policies as well as technological advances in mammographic techniques do not seem to fully explain the increasing incidence of DCIS. Data from the metropolitan Atlanta Surveillance, Epidemiology, and End Results (SEER) study (1979 to 1986) showed that asymptomatic tumors (those detected on screening mammography alone) accounted for only 25% to 40% of the increase in incidence (13). These reports suggest that increased detection accounts for some but not all of the increase in incidence. The National Cancer Institute SEER data for the entire United States confirmed this trend (14). Overall, the total number of cases of DCIS in 1992 was

200% greater (23 368 cases) than expected for that year as calculated from the trends between 1973 and 1983. Apart from increased detection, it is at present unclear why the incidence of DCIS has increased so dramatically. With this vast increase in diagnosis, our understanding of DCIS as a single disease has evaporated. It is now well recognised as a heterogeneous group of lesions with a diverse malignant potential.

The exact incidence of DCIS in the general population is unknown. In an autopsy study of 519 women of various ethnic backgrounds, only one case (0.2%) of DCIS was found (15). In this study of 519 women, only 117 were older than 55 years of age. Alpers and Wellings (16) performed autopsies on 185 patients, of whom 9 (4.9%; 11 breasts) had evidence of the tumor on subgross sampling (17). The findings of autopsy studies suggest that incidental DCIS of uncertain clinical relevance is not common. Variations in incidence seen in published autopsy studies is likely to reflect differences in sampling techniques and diagnostic criteria. Ductal carcinoma in situ of the male breast represents 3.5% to 7% of all cases of male breast cancer (18).

1.4.2 Pathology of DCIS

DCIS is characterized by a proliferation of malignant looking epithelial cells within the mammary ductal-lobular system that show no light microscopic evidence of invasion into the surrounding stroma and therefore, an intact basement membrane.

Traditional methods of classifying DCIS were based on the architectural pattern of the lesion. The most common types are designated comedo, cribriform, micropapillary, papillary, clinging and solid. Examples of these are shown in Figures 1 - 4. This method was initially recommended for use in the UK National Breast Screening Programme (19).

These initial methods paid little attention to cytologic features and proved unsatisfactory for a number of reasons:

- Larger lesions showed considerable variations in architectural pattern even within the same slide and occasionally within a single terminal ductal lobular unit.
- The criteria are subjective and ill defined, allowing for a high level of interobserver variation. In a study assessing the consistency of histopathological reporting in the UK National Breast Screening Programme there was very low agreement in classifying DCIS when doing so by predominant architecture alone (20).

These factors help explain the varying incidence of the different subtypes in reported series of DCIS (21) and the differences in the reported incidence of subsequent invasive carcinoma following the various subtypes of DCIS

undergoing treatment with breast conservation therapy. As the incidence of DCIS increases it becomes more important that the biology of the disease is understood. Many pathologists believe it is useful to subdivide DCIS into two groups – the comedo and the noncomedo type, although this is controversial.

This preference is based on the observation that comedo DCIS

- appears more malignant cytologically (22;23),
- is more often associated with microinvasion (24-26),
- more often exhibits biologic markers indicative of high-grade malignant lesions than do the other types,
- more frequently lacks oestrogen receptors (27),
- has a high proliferative rate (28),
- more frequently exhibits aneuploidy (29),
- more frequently overexpresses the HER2 oncogene (30-32),
- shows mutations of the p53 tumour suppressor gene with accumulation of its protein product (33;34),
- more frequently exhibits angiogenesis in the surrounding stroma (35),
- has been shown to have a higher risk of local recurrence (36).

The comedo necrosis type of DCIS is diagnosed when at least one duct in the breast is filled and expanded by large, markedly atypical cells and has abundant central luminal necrosis.

All other forms of DCIS are of the noncomedo necrosis type and include the cribriform, micropapillary, and solid types. Many examples of the noncomedo necrosis type consist of a combination of the various histologic patterns. Although

necrosis may be present, it is less prominent than in the comedo necrosis type and is not as prone to calcification.

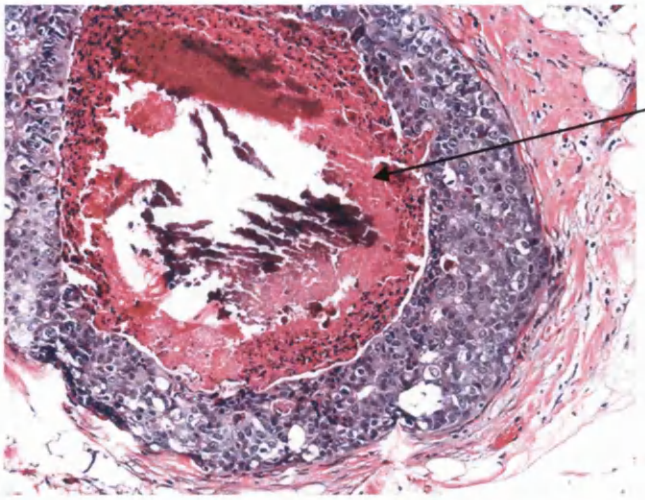


Figure 1. *Comedo type DCIS. Medium power. Haematoxylin and eosin stain. Solid sheets of malignant cells fill the dilated ducts. The centre of the involved ducts undergoes necrosis and calcification.*

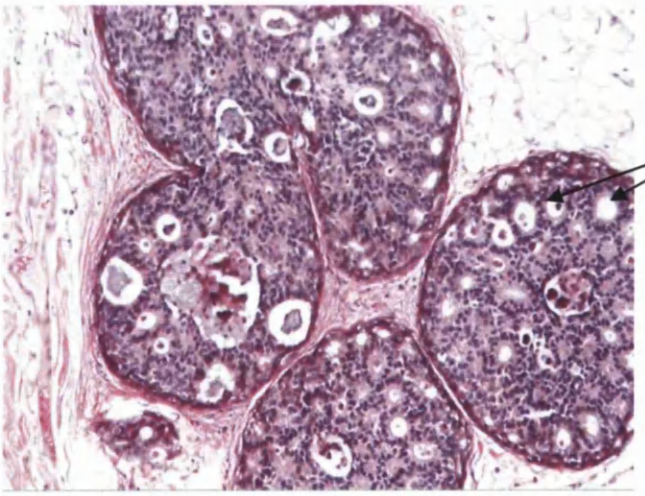
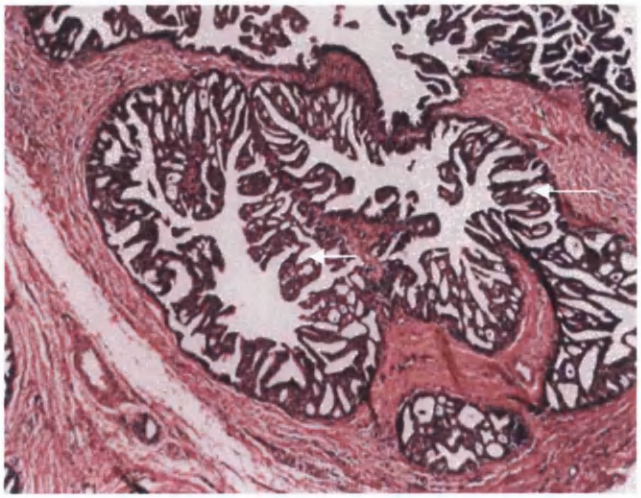
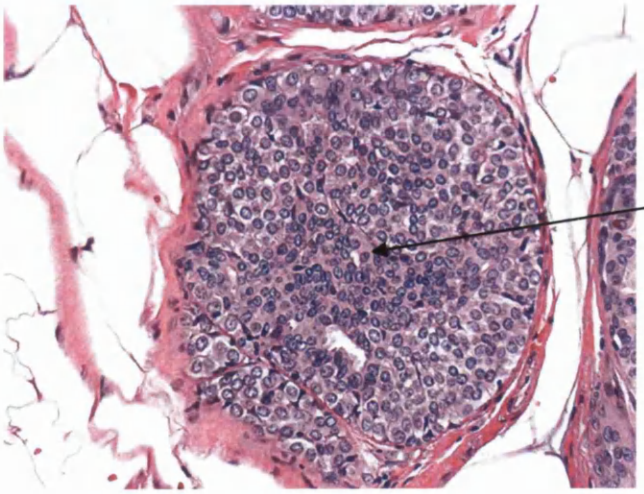


Figure 2. *Cribriform type DCIS. Low power. Haematoxylin and eosin stain. The growth pattern, called cribriform, has multiple round spaces within each duct. Some of these spaces may contain microcalcifications.*



The dilated ducts are lined by multiple finger-like projections (white arrows)

Figure 3. *Micropapillary type DCIS. Medium power. Haematoxylin and eosin stain.*



Solid filling of the ducts by malignant cells without necrosis.

Figure 4. *Solid type DCIS. Medium power. Haematoxylin and eosin stain.*

In the 1980's Lagios drew attention to the value of nuclear grade and necrosis as predictors of disease recurrence following wide excision of DCIS (37). This has subsequently been supported by several studies (38-40). Various classification systems have now been proposed, based on one or more of the following features: nuclear differentiation, the presence or absence of necrosis, growth patterns, tumour size and architectural differentiation. The optimal classification scheme for DCIS remains controversial.

Recently revised guidelines for pathology reporting in the UK NHS breast screening programme recommend a classification of DCIS based entirely on nuclear grading because all the major classifications reflect nuclear grade. DCIS is divided into high, intermediate and low grades. The defining nuclear features in this system were originally defined by a European group of pathologists (38). High grade DCIS is composed of cells with markedly pleomorphic nuclei, coarse clumped chromatin, prominent nucleoli and frequent mitoses. Intermediate grade lesions have pleomorphic cells, which do not show such variation in size as in the high grade group, chromatin is fine to coarse, nucleoli are present but not prominent and mitoses are occasionally present. In the low grade group, nuclei are monomorphic with fine chromatin, insignificant nucleoli and few mitoses.

At a recent consensus conference, it was suggested that, while categorizing DCIS according to nuclear grade, other features, in particular the presence or absence of necrosis and of cellular depolarisation and the architectural growth pattern, should also be recorded. In addition, margins, lesion size, microcalcification associated with DCIS, correlation of DCIS with specimen x-ray and mammographic

findings, and the presence of any microinvasion should all be documented in the pathology report (41).

The introduction of breast conservation therapy for invasive breast cancer in the early 1980's led to pressure for the use of this form of therapy for carcinoma in situ. In the USA, between 1983 and 1992, there was a marked decline in the proportion of cases treated by mastectomy (from 71% to 43.8%) and an increase in those treated by lumpectomy (from 25.6% to 53.3%) (14). The need for assessment of the risk of both recurrence and, more importantly, subsequent development of invasive disease has, therefore, been emphasised. This has highlighted not only the importance of histology in the diagnosis of DCIS but also in the morphological classification of the lesion. The importance of nuclear grade in DCIS has been recognised and the presence or absence of necrosis has also been considered of importance (37). Nuclear grade has been shown to correlate with subsequent development of invasive carcinoma (36;42;43). In addition, the role of certain biological markers is being explored with respect to their association with high grade lesions and conferring increased risk of recurrence. Such markers include HER2, p53 and hormone expression and ploidy.

Such studies have emphasised the heterogeneity of DCIS by suggesting that some types of noncomedo DCIS must be seen as high grade either because of cytologic criteria or because they express some other markers with the same significance, such as immunoreactivity for HER2 and aneuploidy (44).

1.4.2.1 The Van Nuys classification system of DCIS

Nuclear grade, comedo-type necrosis, tumour size, and the width of the tumour margin are all important predictors of the probability of local recurrence after breast conservation treatment for DCIS (36;37;39;43;45-47). Two of these factors, nuclear grade and necrosis, were used to develop a simple, reproducible classification called the Van Nuys classification (39). This classification yields three different subgroups of patients with DCIS, with different rates of local recurrence after breast conservation (39). The presence of any amount of high nuclear grade (nuclear grade 3) defines the most aggressive group. The remaining non-high-grade lesions (nuclear grades 1 or 2) are then divided by the presence (group 2) or absence (group 1) of comedo-type necrosis (Figure 5).

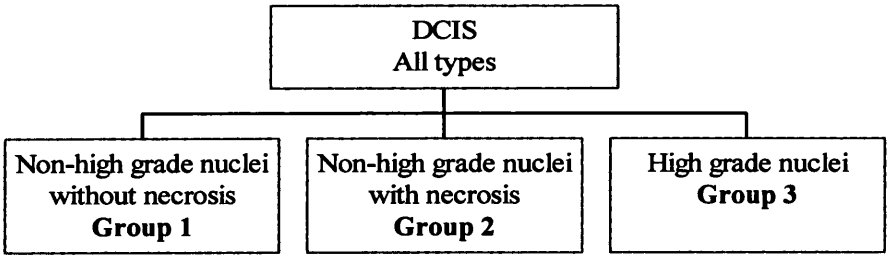


Figure 5. The Van Nuys DCIS Classification system

A statistically significant risk of local recurrence was found; 7% for Group 1, 16% for Group 2 and 39% for Group 3.

Pathologic classification alone is insufficient for selecting appropriate treatment for patients with DCIS because it fails to take into account 2 additional significant predictors of local recurrence: tumour size and the histologic margin width after excision. The Van Nuys Prognostic Index was therefore developed to overcome this problem (see section 1.4.7.3).

1.4.2.2 The Nottingham Prognostic Index (NPI) for invasive breast cancer

In 1992 the NPI was first published as an attempt at using some fairly objective parameters to determine the odds that a newly diagnosed case of primary invasive breast cancer would benefit from adjuvant chemotherapy (48). The NPI uses a combination of size, nuclear grade and lymph node status to divide patients into 3 groups, each of which have differing prognoses. The equation used to stratify patients is:

Nottingham prognostic index = (0.2 x size)+lymph node stage + grade

where size is measured in centimeters; lymph node stage 1 is lymph node–negative, stage 2 is one to three positive lymph nodes, stage 3 is >= four positive lymph nodes; and the scoring of histologic grade is 1 to 3.

The histologic grade used in this index was evaluated according to Elston and Ellis (49). The grading procedure consisted of judgment of tubule formation, nuclear pleomorphism, and mitotic count. The number of mitoses was counted in 10 high-power fields, and the results were adjusted to the area of the microscopic field. Each of the three morphologic features; tubules, nuclear pleomorphism, and number of mitoses, was given a score of 1 to 3 points. The overall histologic grade was obtained by adding the score of each characteristic, giving a possible

total score of 3 to 9 points. The histologic grade allocation was as follows: grade 1, 3 to 5 points; grade 2, 6 to 7 points; and grade 3, 8 to 9 points.

The overall score then determines which group patients fall into as shown in Table 1.

	NPI Group 1 (Score < 3.4)	**NPI Group 2 (Score 3.4-5.4)**	**NPI group 3 (Score >5.4)**
Need for chemotherapy	Need is doubtful	May benefit from chemotherapy	Chemotherapy required

Table 1. *The Nottingham Prognostic Index.*

This index is widely though not universally used, as it requires the status of the axillary lymph nodes to be determined which is not routine practice in some centers. It is used in Glasgow to aid in management decisions regarding chemotherapy.

1.4.3 Clinical and radiological presentation of DCIS

Prior to the advent of mass population screening, the commonest presentation of DCIS was a palpable mass. With mass screening programs and improvements in mammographic techniques, DCIS is most commonly picked up as a result of abnormalities seen on mammography (50). DCIS now accounts for up to 30% of newly diagnosed cases of breast cancer in centres offering screening mammography (51).

Microcalcifications are the most common mammographic appearance associated with DCIS. However, benign lesions also present as microcalcifications. Accurate characterisation and visualisation of calcifications is therefore essential to the correct diagnosis and usually requires magnification of mammographic imaging. The morphology of calcifications is generally considered to be the most important factor in distinguishing benign forms of calcification from malignant ones. Malignant calcifications are usually in the intraductal spaces of the lesion and appear faint, irregular, linear and often have branching distribution patterns. Heterogeneous clustered calcifications, fine linear branching calcifications, or calcifications in a segmental distribution are suggestive of malignancy. Figure 6 shows the type of calcification seen in a case of comedo DCIS.

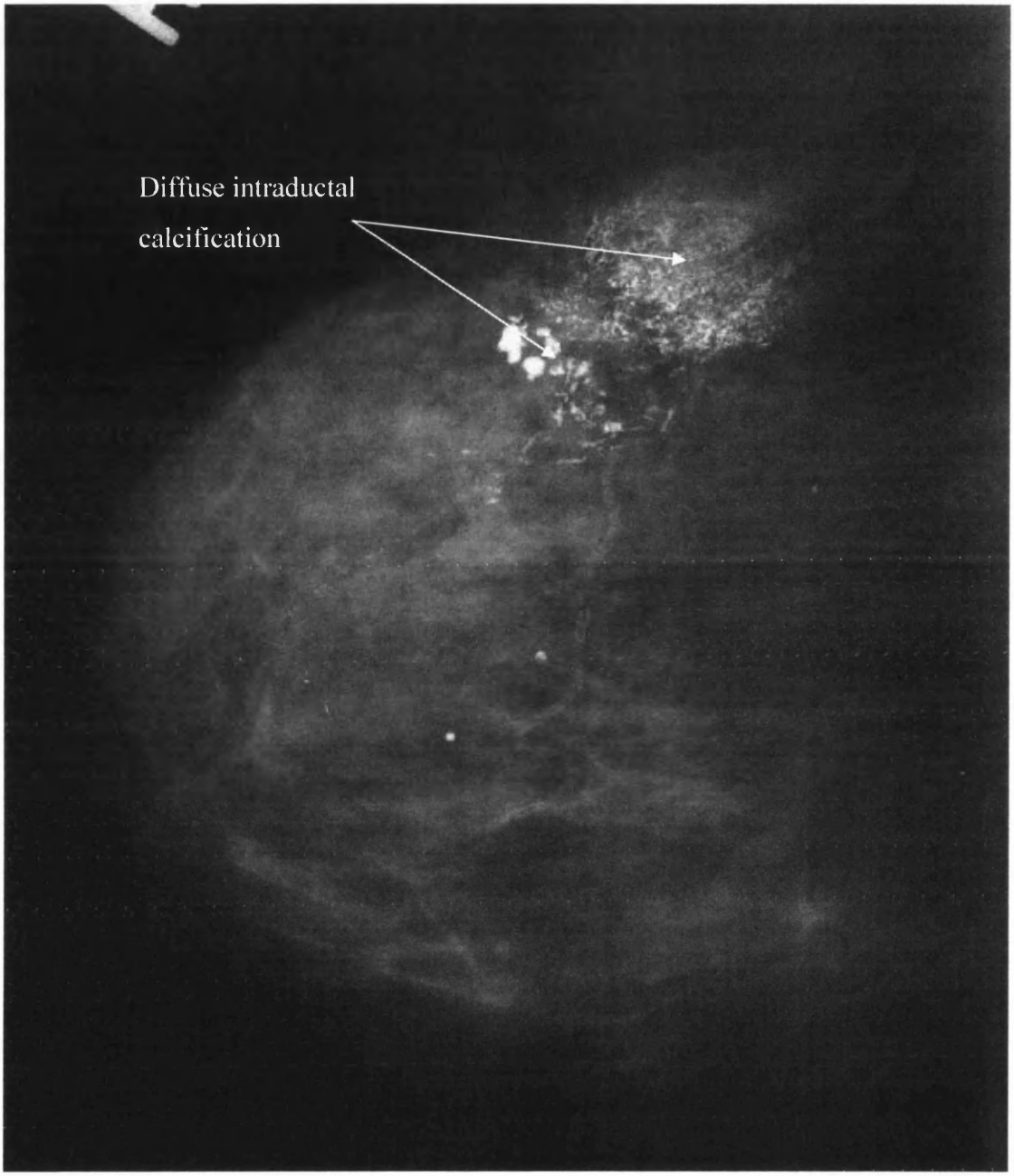


Figure 6. *Calcification on mammography of a case of comedo type DCIS.*

Benign calcifications often appear as random calcifications scattered throughout large volumes of breast tissue. Calcium sediment layering in cysts (milk of calcium), lucent centered calcifications of fat necrosis, dermal calcifications, and solid, large rod-shaped secretory calcifications are indicative of a benign lesion.

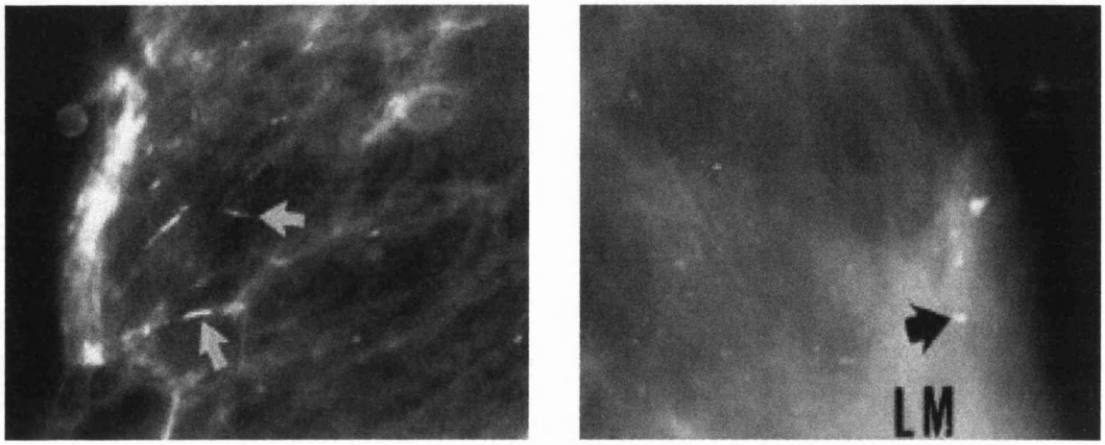


Figure 7. *Examples of mammographic appearance of benign calcifications. On the left are rod-like calcifications (identified by arrows) indicating benign secretory disease. On the right are "milk of calcium" type calcifications.*

Despite these differences and careful mammographic examination, calcifications frequently have an indeterminate appearance. In these circumstances tissue sampling using image directed procedures such as stereostactic needle core biopsy may be necessary.

1.4.4 Natural history of DCIS

The natural history of any disease forms the basis for recommended treatments and assessments of outcome. Breast cancer is a heterogeneous group of tumours. The various pathological types are listed in Table 2. The natural history of breast cancer is normally characterized by long duration, but shows extreme variation between individual patients. In contrast to the well-defined successive progression of colorectal carcinoma from adenomas, there is no generally accepted natural history of breast cancer (52;53).

	Approximate incidence (%)
In situ carcinoma	15-30
DCIS	80
LCIS	20
Invasive carcinoma	70-85
Ductal carcinoma (no special type)	79
Lobular carcinoma	10
Tubular / Cribriform carcinoma	6
Mucinous carcinoma	2
Medullary carcinoma	2
Papillary carcinoma	1

Table 2. *Histologic types of breast cancer* (54).

The best measures of the course of DCIS have been found in autopsy studies and in retrospective reviews of biopsies for what was originally thought to be benign disease and was later found to be DCIS.

There have been 7 major autopsy studies of women not known to have had breast cancer. Six of these found an incidence of DCIS of 4% to 18% (55). The largest of these studies showed a much lower incidence of DCIS at 0.2% (One case out of 519) (15). However, this paper included a significant number of women from ethnic subgroups (Hispanic and American Indian) known to have a lower risk of developing breast cancer. This study also found that proliferative disease without atypia, atypical ductal hyperplasia and DCIS are progressively less frequent in the breasts of women dying from causes other than breast cancer (15). Only 1 case out of more than 1000 patients in these 7 studies was found to have invasive breast cancer.

The largest retrospective review, with adequate follow up, of breast biopsies which were originally thought to be benign and subsequently found to be malignant has come from Vanderbilt (56;57). On histologic review of slides from 11 760 women in whom benign lesions had originally been diagnosed between 1959 and 1968, 28 patients were identified as having DCIS lesions. These tumours were predominantly small, low grade, non-comedo type DCIS with uncertain surgical margins. Follow up was for an average of almost 30 years. Nine of these 28 women have gone on to develop invasive breast cancer in the same quadrant from which DCIS was originally biopsied, giving a rate of 32%. The first of these cases did not present until 15 years after the initial surgery. A further patient developed metastatic breast cancer over 30 years after the original biopsy.

It is important to note that this series did not contain any cases of comedo type DCIS and that this may have been a factor in the long delay in the progression to invasive disease.

Other similar studies have yielded concurring results. Rosen and colleagues (58) reported on the long term follow up (average of 21.6 years) of 15 women in whom benign lesions were originally diagnosed. After a median of 9.7 years, 10 women had developed clinically apparent carcinoma in the ipsilateral breast with 4 of these having developed metastatic breast carcinoma. As with the Vanderbilt experience, these DCIS lesions were of the low grade type.

Eusibe and colleagues (59) also reported on 80 patients with a mean follow up of 17.5 years with an original diagnosis of benign disease. They found that DCIS recurred in 5 patients (6%) and invasive carcinoma developed in 11 women (14%). The recurrence was ipsilateral in the majority of patients (11 of 16 patients).

A striking feature of these studies is that the natural history of DCIS may take over 20 years to develop and that a patient not undergoing definitive treatment of even low grade lesions may still be at significant risk.

Our understanding of the preclinical phases of breast cancer is mainly based on indirect observations of the morphological changes from normal epithelium through a series of increasingly abnormal changes ranging from hyperplasia (proliferative disease without atypia), to atypical hyperplasia, to non-invasive carcinoma, to primary invasive carcinoma, and finally to metastatic carcinoma (60). Such observations include studies which show progressively increasing relative risks of later developing invasive breast cancer in women with previously

excised proliferative disease without atypia (relative risk = 1.2-2.0), atypical ductal hyperplasia (relative risk = 4.0-6.0) and DCIS (relative risk = 10.0-12.0) (61-63). Other studies have observed that lesions such as proliferative disease without atypia, atypical ductal hyperplasia, and DCIS are concurrently observed in 50-90% of breasts containing invasive breast cancer (16;64). Progression through these stages is not inevitable and, in theory, stages may be missed out. It is also possible that a given stage may be permanent or may even regress towards normality. There are several reasons why our understanding of the natural history of DCIS is inconclusive:

1. It is likely that palpable DCIS detected before mass screening programmes had a different natural history to mammographically detected DCIS. Palpable lesions were more likely to have had larger areas of DCIS and more likely to have had undetected areas of invasion (65) compared to small impalpable DCIS detected as an area of microcalcification on mammography.
2. The traditional treatment of DCIS was mastectomy and therefore the natural history could not be studied.
3. Autopsy studies suggest that some but not all cases of DCIS progress to invasive disease.
4. Many treatment reports include data on patients with microinvasion which is therefore not true DCIS.

The obvious challenge in the study of DCIS and in managing patients with this condition lies in reliably identifying those at risk of a poor outcome and applying aggressive initial management while not subjecting those with a good outcome to the same, perhaps unnecessary, treatments.

1.4.5 Multicentricity of DCIS

DCIS has in the past been thought to be a multicentric condition, i.e. it arises separately in different quadrants of the breast. This has been the rational for treating the disease by mastectomy thereby eliminating the risk to other parts of the breast and obtaining a "cure". Studies supporting the theory of multicentricity have reported an incidence of multicentricity as high as 32% (65-70). Most of these studies were done in mastectomy specimens from patients who had previously had a biopsy for DCIS. Where cases had cancer cells remaining, these were considered as separate foci, which is clearly incorrect, and these lesions were therefore labelled as being multicentric.

Current theory is that most DCIS cases are not multicentric. Holland and colleagues found that of 82 mastectomy specimens from patients with DCIS, using subgross sectioning and extensive sampling, only one lesion was truly multicentric (71). They found that 23% of lesions extended over more than one breast quadrant and that mammographic estimations frequently underestimated the size of the lesions. This may be a factor in the finding that DCIS often recurs at or near the site of the original lesion (72). This is a crucial factor in determining the feasibility of breast conserving therapy because clearly resection of the affected area must involve a complete resection with clear margins.

1.4.6 Treatment of DCIS

As would be expected with a lesion which is at worst preinvasive, a diagnosis of DCIS carries an excellent prognosis regardless of the surgical treatment chosen. A wide range of treatment plans have been developed to treat DCIS and this is partly the result of uncertainty regarding the natural history of the lesion and the lack of pathological standardisation between centres. The treatment and management of DCIS is today extremely controversial. The treatment options include:

1. local excision only
2. local excision plus radiotherapy
3. mastectomy
4. axillary staging
5. hormonal therapy

Current treatments for DCIS range from simple tumour excision, to various forms of wider excision such as quandrantectomy to mastectomy with or without reconstruction. All forms of treatment less than mastectomy may be followed by radiotherapy. As DCIS is a heterogeneous group of lesions and all patients are different with varying requirements and expectations, no single treatment approach is correct for all forms of the disease.

1.4.6.1 Mastectomy

In the past, mastectomy was the standard treatment for DCIS in all its forms. Although this is clearly overtreatment for a large number of cases, it results in an extremely low local recurrence rate (0-5%) and mortality (0-2%) from breast cancer (5;45). The rationale for mastectomy included a 30% incidence of multicentric disease, a 40% prevalence of residual tumour at mastectomy following wide excision alone, and a 25-50% incidence of breast recurrence following limited surgery for a palpable tumour, with half of those recurrences being invasive carcinoma (65;73). Some of these reasons have now been disproved, such as multicentricity (see section 1.4.5).

The success of breast-conserving surgery for invasive carcinoma resulted in this approach being used in DCIS although no randomised trials comparing mastectomy to breast-conserving surgery with radiotherapy have been carried out. Many of the series evaluating mastectomy for the treatment of DCIS were carried out before mammography was widely used and were performed on patients with palpable disease or nipple discharge. Comparisons with more recent series of breast-conserving surgery containing patients with impalpable disease detected on mammography is therefore difficult.

The advantage of mastectomy is that it is an effective treatment which negates the need for close follow up of the ipsilateral breast. However, it will represent over-treatment for the majority of patients given that many DCIS lesions will not progress to invasive cancer or cause death.

For selected patients with invasive breast carcinoma, breast-conserving surgery has proved to be as effective as mastectomy (74-77). It is therefore inconsistent

that a non-invasive condition should be treated by more aggressive methods than an invasive breast cancer. However, mastectomy for DCIS almost invariably results in cure and it is a tragic situation where a woman develops metastatic disease from an invasive recurrence of a lesion that was non-invasive at the time of detection. Mastectomy remains the gold standard for treatment of DCIS against which other treatment modalities should be compared.

1.4.6.2 Breast-conserving surgery with radiotherapy

Reported series of breast-conserving surgery and radiotherapy show a reduction in the rate of local recurrence by the addition of radiotherapy (Table 3) (78-95). Local recurrence rates are reported at between 5% and 10%, with approximately 50% of recurrences being invasive.

Time period	Authors	Number of patients	Median length of follow up (months)	Recurrence		Invasive recurrence (%)
				No	%	
1955-1980	Montague et al(80)	34	NR	1	3	100
1958-1987	Stotter et al(95)	44	92	4	9	100
1967-1985	Fourquet et al(91)	67	104	7	10	71
1968-1990	Cataliotti et al(78)	34	50	3	9	100
1974-1988	Baird et al(84)	8	43*	2	25	50
1975-1987	Cutuli et al(79)	34	56	3	9	33
1975-1985	Kurtz et al(93)	43	61	3	7	100
1976-1978	Fisher et al(85)	27	83*	2	7	50
1976-1990	Ray et al(88)	58	61	5	9	20
1976-1990	Hiramatsu et al(83)	76	74	7	9	57
1977-1988	McCormick et al(94)	54	36	10	19	30
1978-1985	Solin et al(89)	51	68	5	10	40
1978-1985	Fowble(92)	46	35	2	4	NR
1979-1990	Silverstein et al(82)	103	63	10	10	50
1979-1987	Kuske et al(86)	70	48	3	4	100
1979-1991	Bullock et al(87)	43	62	3	7	33
1982-1988	White et al(90)	52	68	3	6	33
1985-1990	Fisher et al(81)	399	43*	28	7	29
Total number of patients:		1243		101		47.5 of recurrences

Table 3. *Published studies of the outcome of treatment of DCIS with breast-conserving surgery and radiotherapy. * mean; NR, not reported.*

A further collaborative study was published in 1995 involving 10 European and North American centres reporting follow up on 268 patients treated with breast-conserving surgery and radiotherapy with a median follow up of 10.3 years (96). The actuarial rate of local recurrence at 15 years was 20% and survival was 96%. This study suggests that, as is the case with invasive breast cancer, the rate of local recurrence of DCIS increases with time, either because new disease develops or residual DCIS progresses (36). A complicating factor of this study is that the surgical resection margins were not known for almost 50% of patients. As described in section 1.4.6.2, resections margins are an important factor determining the risk of local recurrence. With this fact now generally accepted, it is likely that future studies will show a further decrease in local recurrence for patients treated by breast-conserving surgery and radiotherapy.

In addition to the single institution studies described above, in the mid-1980's two randomised controlled trials were started to find out whether breast-conserving surgery was a reasonable approach to the management of DCIS. These are described in the following sections.

1.4.6.2a National Surgical Adjuvant Breast and Bowel Project (NSABP) study Protocol B-17 (97;98)

The results of the National Surgical Adjuvant Breast and Bowel Project (NSABP) study Protocol B-17 were published in 1993 (97) and updated in 1995 (98) and 1998 (97). This study randomly assigned 818 women with localised DCIS and negative surgical margins following excisional biopsy to breast irradiation with 50 Gy or to no further therapy. In this study, 80% of patients were diagnosed by mammography and 70% had small lesions (1cm or less). Events were defined as the presence of new ipsilateral disease, contralateral disease, metastases, a second primary tumour or death from any cause. After 8 years of follow-up, the event-free survival for the irradiated patients was 75% compared to 62% for the non-irradiated patients (p=0.00003). A significant decrease in local recurrence of DCIS and of invasive breast cancer was seen in patients treated with radiotherapy. The overall local recurrence rate for patients treated by excision alone was 27% at 8 years. For patients treated with excision plus irradiation, it was 12% at 8 years (p=0.000005). Specifically, the occurrence of invasive breast cancer decreased from 13.4% to 3.9% with the addition of radiotherapy (p=0.001), and recurrent DCIS was reduced from 13.4% to 8.2% (p=0.007). The recommendations of the study were therefore for all patients with DCIS treated by breast-conserving surgery to have post-operative radiotherapy. These recommendations are thought to be too wide by many authors (99) and the study itself has been criticised for a number of reasons discussed below.

1.4.6.2b The European Organisation for Research and Treatment of Cancer (EORTC) study (100)

The EORTC trial began and concluded after the NSABP trial and essentially supported the findings of the earlier study. The aim of the study was to investigate the role of radiotherapy after complete excision of DCIS. Between 1986 and 1996, 1008 women with DCIS lesions measuring 5cm or less were randomly assigned to either no further treatment or to radiotherapy and followed up for a median of 4.25 years. The 4-year local relapse-free was 84% in the group treated with local excision alone compared with 91% in the women treated by local excision plus radiotherapy (log rank p=0.005; hazard ratio 0.62). When the local relapses were classified as either DCIS or invasive carcinomas, there were approximately equal reductions by radiotherapy; a reduction of DCIS recurrence from 8% to 5% (hazard ratio = 0.65 [95% CI 0.41-1.03]) and a reduction from 8% to 4% (hazard ratio = 0.60 [95% CI 0.37-0.97]) of invasive carcinomas. Similar reductions in the risk of invasive (40%, p=0.04) and non-invasive (35%, p=0.06) local recurrence were therefore seen.

They conclude that the addition of radiotherapy after complete excision of DCIS reduced the overall number of both invasive and non-invasive recurrences in the ipsilateral breast at a median follow up of 4.25 years. They also stress the point that patients with an unacceptably high risk of local recurrence despite breast-conserving surgery plus radiotherapy need to be identified so that they can be offered mastectomy. Equally, patients with a very low risk of local recurrence and potentially very small benefit from radiotherapy should be identifiable so that unnecessary irradiation can be avoided.

1.4.6.2c Criticism of NSABP Protocol B-17 and the EORTC study

The EORTC study was published in 2000, 2 years after the outcome data for the NSABP B-17 were published. The EORTC study design and definition of clear margin were essentially the same as the NSABP B-17 study. The overall reductions in local recurrence of DCIS were similar for the 2 trials (approximately 50%) but there were differences in the rates of invasive local recurrence and contralateral breast cancer as discussed below (section 1.4.6.3).

The main criticism of the NSABP B-17 study was a lack of analysis of different subsets of DCIS and the lack of size measurements in more than 40% of cases in the initial report (101;102). Other problems were the lack of requirement for mammographic-pathological correlation or of specimen radiography; the lack of uniform guidelines for tissue processing or of estimation of size of the lesions; possible inclusion of patients with atypical ductal hyperplasia; and the authors' definition of what constitutes a clear margin. Margins were defined as clear when a tumour was not transected. Most investigators would now agree that local recurrences after lumpectomy for DCIS most likely reflect residual disease, (103-105) and that the acceptance of minimally clear margins of 1 mm. or less, is inadequate for local control.

The EORTC study is open to similar criticisms as the NSABP study (102;106). There was no subset analysis giving comparative rates of local recurrence for various subgroups, such as high grade versus low grade; wide excision versus narrow margins; or presence of comedo type necrosis versus absence of this. At the time of publication there were too few recurrences in the EORTC trial for such subset analyses.

In defence of these 2 studies, they were designed at a time when an important question in the treatment of DCIS was whether radiotherapy was beneficial to patients with DCIS treated by breast-conserving surgery. The studies did exactly what they were designed to do; i.e. to prove that radiotherapy was effective for patients with DCIS treated by breast-conserving surgery. However, by the time of publication of their results the important questions that needed answered and the understanding of DCIS as a disease had changed considerably. The questions asked now are more complex. We now want to know exactly which subgroups will benefit from radiotherapy and by how much. If the benefit is known to be small then the cost and complications of radiotherapy may deem it inappropriate.

1.4.6.2d Is routine use of radiotherapy for DCIS treated by local excision justified?

The findings of the NSABP B-17 and the EORTC trials have encouraged clinicians to recommend radiotherapy for all patients with DCIS undergoing breast conserving surgery. Despite their data, there continues to be vigorous debate regarding the broadness of the recommendation that all such patients should undergo post-excisional radiotherapy.

A unique finding of the NSABP B-17 study was the 3.5 fold reduction (from 13.4% to 3.9%) in invasive local recurrences after radiotherapy. Neither the EORTC nor any other study of breast conservation followed by radiotherapy has been able to support this finding. In the EORTC trial the reductions for DCIS and invasive recurrences were similar. The NSABP B-17 investigators have used the marked decrease in invasive local recurrence seen in their study as the main justification for recommending that all patients who have breast conservation surgery also have post-excisional radiotherapy. Although radiotherapy reduced the rate of local recurrence, neither of these 2 trials showed that post-operative radiotherapy had a beneficial effect on what should be considered the most important outcome variables: distant recurrences and breast cancer mortality. In both trials the rates for these variables were the same whether or not patients received radiotherapy.

The EORTC study found a potentially important increased rate of contralateral breast cancer in irradiated patients which reached statistical significance ($p=0.01$). The method of administering radiotherapy in this trial was to use a wedge or filter, which may have caused increased scatter of radiation to the contralateral breast. If

this increased scatter proves to be the cause of the increased rate of contralateral breast cancer, it will decrease the relative value of a policy of indiscriminate radiotherapy in favour of a policy only for those patients who will benefit substantially from post-excisional radiotherapy.

Radiotherapy administration can cause significant side effects as well as having a substantial financial cost. Much of the information on the side effects of radiotherapy is confounded by treatment received decades ago with outmoded delivery techniques. Current delivery methods administer lower daily doses with field arrangements that limit exposure of normal tissue. These side effects include cardiac toxicity, development of second cancers, fatigue and radiation fibrosis of the breast, which can make mammographic follow up more difficult. While most of these effects such as cardiac toxicity are thankfully now very uncommon, it must be borne in mind that patients with DCIS are likely to live for a fairly long time. The effects of treatment are therefore with them for a long time and some of these may not as yet be apparent.

1.4.6.3 Breast-conserving surgery alone

The potential benefits of breast-conserving surgery are that it allows preservation of the breast and it avoids the costs, inconvenience and potential toxicity of radiotherapy. The main disadvantage compared to mastectomy or breast-conserving surgery with radiotherapy is an increased risk of local recurrence, which if invasive has potential to progress to metastatic disease and death.

Published series of DCIS treated by breast-conserving surgery alone are presented in Table 4 (37;43;69;78;81;82;84-86;107-114). Despite careful patient selection, recurrence rates approach 20% after relatively short follow up. An equal proportion of invasive and DCIS recurrences occur. Lagios et al (37) reported that DCIS lesions less than 2.5cm with clear margins had a local recurrence rate of 10% after a mean follow up of 44 months. As with invasive breast cancer, this figure is likely to increase with longer follow up. In a similar study, Bellamy et al (43) reported a recurrence rate of 30% for 31 patients at 60 months follow up where the re-excision margins were clear.

Survival rates after treatment of DCIS with breast-conserving surgery alone of 98-100% albeit after short follow up is similar to those reported for mastectomy and for breast-conserving surgery with radiotherapy. The disadvantage lies in the significantly higher rates (approaching 20%) of local recurrence which puts patients at a higher risk of potentially incurable invasive recurrence of 10% and an equal 10% risk of recurrent DCIS. Recurrent DCIS can potentially be cured by salvage mastectomy but invasive recurrence is potentially incurable and in a proportion of patients will result in metastatic disease. The precise indications for

breast-conserving surgery alone will be best determined by prospective randomised clinical trials.

Time period	Authors	Number of patients	Median length of follow up (months)	Recurrence		Invasive recurrence (%)
				No	%	
1944-1981	Gallagher et al (113)	13	100	5	38	60
1948-1968	Millis and Thynne (111)	8	24-180§	2	25	0
1949-1967	Farrow (110)	25	NR	5	20	0
1953-1984	Temple et al (107)	17	72	2	12	50
1968-1990	Cataliotti et al (78)	46	70	5	11	100
1972-1982	Price et al (112)	35	108	22	63	55
1972-1987	Lagios et al (37)	79	44	8	10	50
1974-1988	Baird et al (84)	30	43*	4	13	25
1974-1989	Bellamy et al (43)	31	60	10	32	50
1976-1978	Fisher et al (85)	21	83*	9	43	56
1978-1984	Arnesson et al(109)	38	60	5	13	20
1978-1990	Schwartz et al (69)	72	49*	11	15	27
1979-1986	Carpenter et al (108)	28	38	5	18	20
1979-1987	Kuske et al (86)	7	48	3	43	33
1979-1990	Silverstein et al(82)	26	19	2	8	50
1982-1987	Ottesen et al (114)	112	53	25	22	20
1985-1990	Fisher et al (81)	391	43*	64	16	50
Total number of patients:		979		187		42.7% of recurrences

Table 4. *Published studies of the outcome of treatment of DCIS with breast-conserving surgery alone. * mean; NR, not reported. § range*

1.4.6.4 The axilla

There is now uniform agreement that for true DCIS there is no indication for treatment to the axilla (115;116). The incidence of lymph node metastases in DCIS is negligible (<1%) (117-119). In a study published in 1994, Silverstein and colleagues examined 200 axillary lymph node dissections for patients with DCIS without evidence of microinvasion (120). None of the cases examined had involved lymph nodes. This is perhaps not surprising in true in situ disease, however it can prove impossible to identify a small focus of invasion in a large area of DCIS. The challenge for pathologists lies in identifying those cases of true DCIS without evidence of microinvasion and therefore no potential for metastasising. The risk of having foci of microinvasion in DCIS appears to be directly proportional to the size of the lesion. In a study published in 1989 Lagios and colleagues (37) analysed 115 specimens for microinvasion by using intraoperative radiography and careful sequential sectioning at 2-3mm. intervals. The overall rate of microinvasion was 14.7% (Table 5). This evidence is even more convincing as all 4 lesions less than 35mm which showed evidence of microinvasion were thought to have inadequate localisation biopsies and that an adequate biopsy would have revealed invasive disease and the patients therefore excluded from this study.

Size of DCIS lesion (mm)	Incidence of microinvasion	
	No/Total	%
< 25	1/60	1.6%
26-35	3/15	20%
36-45	0/9	0%
46-55	1/6	16.7%
>55	12/25	48%

Table 5. *Incidence of microinvasion in relation to the size of the DCIS lesion. The rate of microinvasion appears to have a direct relationship to the size of the DCIS lesion.*

1.4.6.5 The role of Tamoxifen

Tamoxifen has been shown in animal studies to have anti-initiator and anti-promoter properties (121;122). It has also been shown to prevent tumour recurrences in the ipsilateral breast and second primary tumours in the contralateral breast of women who have undergone lumpectomy and radiotherapy for primary invasive breast cancer (123;124). This suggests that tamoxifen can interfere with development of primary invasive breast cancer from the start or with the progression of DCIS to invasive cancer. The NSABP B-24 trial (125) was set up to investigate whether or not tamoxifen was effective in reducing recurrences of DCIS or invasive cancer when used in conjunction with breast-conserving surgery followed by radiotherapy. In this study, following breast-conserving surgery and radiotherapy for DCIS or DCIS with LCIS, 1804 women

were randomised to receive either placebo or tamoxifen 20mg per day for 5 years. Follow up was for a median of 74 months. Patients with positive resection margins were included. The addition of tamoxifen resulted in a statistically significant 44% decrease in development of invasive breast cancer in the ipsilateral breast ($p = 0.004$) and a 37% decrease in the contralateral breast which was not statistically significant ($p = 0.22$). The rate of ipsilateral non-invasive tumours was not significantly lower in the tamoxifen group (18% reduction, $p = 0.43$). The rate of contralateral non-invasive tumours in the tamoxifen group was significant although the numbers were small (13 vs. 3, 78% reduction, $p = 0.02$). These findings suggest that the addition of tamoxifen is beneficial to women treated by breast-conserving surgery and radiotherapy. The advantage appears mainly to be in a decrease in the rate of invasive cancer especially in the ipsilateral breast. When events occurring in the contralateral breast and at regional and distant sites are included, there is a significantly lower rate and cumulative incidence of all breast cancer related events than in the no tamoxifen group.

In addition to this study there is a United Kingdom DCIS trial (UKCCCR-DCIS Phase III-90001) evaluating tamoxifen in DCIS which is as yet unpublished.

While tamoxifen appears to be effective at preventing contralateral breast cancer and reducing local recurrence in patients with DCIS undergoing breast conservation treatment it does have significant side effects. In premenopausal patients it may cause menopausal symptoms including hot flushes, vaginal discharge and irregular menses. In this group of patients it may also cause an increase in bone loss due to its oestrogen antagonist activity (126). In postmenopausal patients, tamoxifen therapy is associated with an approximate

doubling of the risk of endometrial cancer (127-129), especially in women over the age of 50. There is also a slightly increased risk of deep vein thrombosis and pulmonary embolism (127;128). Tamoxifen does preserve bone mineral density in postmenopausal women (130) but whether it reduces the risk of vertebral or hip fractures is uncertain. Obviously, the risks and benefits of tamoxifen therapy should be discussed with each patient on an individual basis.

1.4.7 Risk factors for recurrence of DCIS.

1.4.7.1 Nuclear grade

In the 1980's Lagios drew attention to the value of nuclear grade and necrosis as predictors of disease recurrence following wide excision of DCIS (37;42). In earlier studies (37;42), Lagios reported a scheme for classifying DCIS based on architectural features, nuclear grade and tumour necrosis. Nuclear grade was found to be the strongest predictor of recurrence with 9 of 36 (25%) high nuclear grade lesions recurring compared to 1 of 43 (0.02%) of intermediate or low nuclear grade lesions. In a subsequent study (131) in which the Lagios system was used high grade DCIS was associated with a local recurrence rate of 32% compared to 10% for intermediate grade and 0% for low grade. This has subsequently been supported by several studies (38-40;43;132).

Nuclear grade is now a component of most classification systems for DCIS, including the European Pathologists' Working Group (38) (nuclear grade and cell polarisation), the Nottingham Classification (40) (nuclear grade, necrosis and morphology) and the Van Nuys Classification System (39) (nuclear grade and necrosis). The European Breast Screening Working Groups (19) uses a system which has been adopted by the UK National Coordinating Group for Breast Screening Pathology and the European Working Group for Breast Screening Pathology and consequently is the classification recommended in the pathology guidelines for the NHS Breast Screening Programme and the EC Breast Screening Network. This system is based solely on nuclear grade.

1.4.7.2 Resection margins

There is now general agreement that tumour margins should be assessed adequately before a treatment option is confirmed. However, there is no consensus on what constitutes an adequate margin following breast-conserving surgery. Most recurrences in patients treated by breast-conserving surgery or breast-conserving surgery and radiotherapy occur in the immediate area of the original excision site which suggests that the recurrences arise from incomplete surgical removal rather than from separate areas of DCIS (82;88;89;91;93;133-135).

In those retrospective series where an attempt has been made at assessing margins, a clear margin has been accepted as the absence of tumour cells at the inked resection margin. These series include those reported by Gallagher et al (113) (recurrence rate 38.5%) and Lagios et al (37) (recurrence rate 10%). However, NSABP (B-06) trial showed this to be unacceptable for treating patients by lumpectomy alone: patients treated by lumpectomy alone with 'clear' margins had a local recurrence rate of 43% (85). The pathological assessment of the NSABP (B-17) trial showed that the most significant predictor for local recurrence was the presence of a margin that was either involved or uncertain (98). A further study by Arnesson et al (109) reported recurrence rates of 38% when margins were <5mm and 6% when margins were >5mm. The Nottingham group have adopted a policy of resecting a cylinder of tissue from skin to the pectoral fascia with a minimum margin of 10mm. Since adopting this policy, 1 of 48 patients has had a recurrence of DCIS after follow up of 45 months (136). Histopathologic mapping studies of DCIS lesions have shown that gaps of normal duct epithelium exist between foci of DCIS. In a study by Faverley et al (137), they reported on a 3-dimensional

study of DCIS in 60 mastectomy specimens. Continuous and multifocal growth patterns were usual, and that multicentric tumours (defined as a gap of 4cm or more) are rare, occurring in only 1 case. Poorly differentiated tumours more often had continuous growth, whereas well differentiated tumours had multifocal distribution. Low grade lesions have more gaps than high grade lesions but only 8% of all gaps were more than 10mm and 7% were in well differentiated tumours. This implies that leaving a 10mm margin is likely to result in lower rates of local recurrence, as DCIS is a contiguous process in the majority of cases (138).

1.4.7.3 Van Nuys Prognostic Index (VNPI) (139)

The VNPI was devised by combining 3 statistically significant predictors of local tumour recurrence in patients with DCIS: tumour size, margin width and pathological classification. A score ranging from 1 for lesions with the best prognosis to 3 for the worst prognosis was assigned for each of the 3 predictors as described in Table 6 below.

Size score:	1	15mm or less
	2	16-40mm
	3	41mm or more
Margin score:	1	10mm or more
	2	1-9mm
	3	Less than 1mm
Pathological classification score:	1	Group 1 (non high grade lesions without necrosis)
	2	Group 2 (non high grade lesions with necrosis)
	3	Group 3 (all high grade lesions)

Table 6. *Scoring system for the Van Nuys Prognostic System.*

When these 3 scores are summed a prognostic value ranging from 3 to 9 is obtained (the Van Nuys Prognostic Index). The best possible score is 3 and the worst is 9. The VNPI was tested on 333 patients treated with breast-conserving surgery alone or breast-conserving surgery and radiotherapy. Table 7 summarises the results.

VNPI score	3 or 4	5, 6 or 7	8 or 9
8-year actuarial local recurrence free survival rate (%)	97	77	20
8-year actuarial breast cancer specific survival rate (%)	100	97	100
Treatment recommendation	Breast-conserving surgery alone	Breast-conserving surgery & radiotherapy	Mastectomy

Table 7. *Local recurrence rates and treatment recommendations based on the VNPI.*

Patients with VNPI scores of 3 or 4 did not show a disease-free survival benefit from radiotherapy (100% *vs.* 97%). Patients with intermediate VNPI scores of 5, 6 or 7 benefited from radiotherapy (85% *vs.* 68%; $p = 0.017$). Patients with a VNPI of 8 or 9 also benefited from radiotherapy (36% *vs.* 0%); $p = 0.026$), although the risk of recurrence is so high with or without radiotherapy that mastectomy should be considered.

The VNPI is an attempt to quantify known important prognostic factors in DCIS, making them clinically useful in the treatment decision making process. Although there is an obvious treatment choice for each group, the VNPI is a guideline which acts as a starting place for discussion with patients.

1.4.8 Summary of treatment of DCIS

As with most forms of cancer treatment each case should be considered individually and treatment tailored to the individual's tumour characteristics, general health and overall expectations. A reasonable management plan is given below:

Mastectomy may be appropriate if:

- the lesion is extensive (more than 5cm diameter of calcification on mammogram)
- margins still show DCIS after 2 attempts at wide local excision
- the initial lesion did not have calcification on mammography
- personal choice for any size or type of lesion

Breast-conserving surgery and radiotherapy may be appropriate if the lesion:

- is 1-5cm in diameter
- is high grade
- has a <10mm clear margin

Breast-conserving surgery alone may be appropriate if the lesion:

- is <15mm diameter
- is low grade
- has normal ducts between the lesion and the inked margins
- has a >10mm clear margin

Although the treatment of DCIS remains a controversial area, this is clearly because our goals and the expectations of patients have become higher. The days when mastectomy was routine for all types of DCIS, including its most benign

forms, are thankfully over. The challenge now lies in providing appropriate treatment tailored to each lesion and to each patient. Adequate diagnosis and treatment continue to require a multi-disciplinary approach involving surgeons, radiologists, pathologists, radiation oncologists and medical oncologists. Our understanding of the disease and our ability to better subclassify this disease will be helped by a better understanding of the role of various markers, including HER2 and other members of the Type 1 growth factor receptor family as well as p53 and hormone expression, DNA-ploidy and S-phase fraction, and perhaps other markers as yet undiscovered.

SECTION 2 CELL CYCLE CONTROL

2.1 Oncogenes

Oncogenes are genes that have been implicated in the formation of tumours. They are derived from protooncogenes, which are cellular genes that control normal growth and differentiation. These cellular genes were first discovered within the genome of acute transforming retroviruses, by the Nobel Laureates Varmus and Bishop (134), but are now known to be viral (*v-onc*) or cellular (*c-onc*). These protooncogenes can become oncogenic by retroviral transduction or by influences that alter their behaviour in situ, thereby converting them into cellular oncogenes. Oncogenes encode proteins (oncoproteins) which resemble the normal products of protooncogenes, with the exception that oncoproteins are devoid of important regulatory elements and their production in the transformed cells does not depend on growth factors or other external signals. The result is, therefore, excessive cell multiplication which may lead to malignant transformation. The discovery that certain oncogene products showed high amino acid sequence homology to components of growth factor signal transduction pathways triggered intense efforts to further define the role of growth factors in carcinogenesis. Although viruses are primarily associated with tumourigenesis in animals, some human examples are shown in Table 8.

Disease	Virus
Cervical cancer	Papilloma virus (HPV 16,18,31)
Nasopharyngeal cancer	Epstein-Barr virus
T-cell leukaemia	HTLV
Kaposi sarcoma	Human Herpes Virus (HHV 8)

Table 8. *Examples of viruses associated with tumourigenesis in humans*

2.2 The Cell Cycle

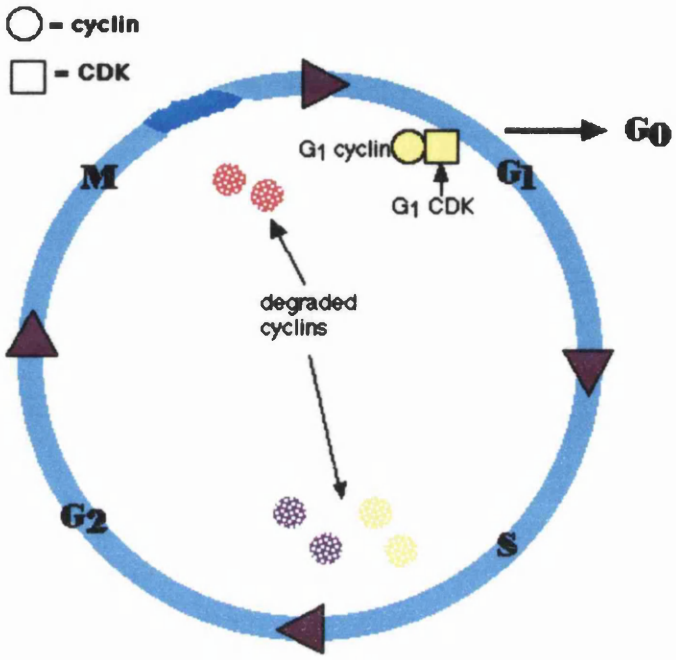


Figure 8. *The cell cycle*
G_1 = growth and preparation of the chromosomes for replication

S = synthesis of DNA (and centrioles)

G_2 = preparation for

M = Mitosis

G_0 = Resting phase

CDK = Cyclin dependent kinase

A eukaryotic cell cannot divide unless two processes alternate: doubling of its genome (DNA) in S phase (synthesis phase) of the cell cycle and halving of that genome during mitosis (M phase). The period between M and S is called G_1; that between S and M is G_2.

Progression through each of the four distinct phases (G_1, S, G_2 and M) is carefully controlled by the sequential formation, activation and subsequent degradation or modification of a series of cyclins and their partners, the cyclin-dependent kinases (CDKs). In addition, a further group of proteins, the cyclin-dependent kinase inhibitors (CDKIs) are important for coordination of each stage. The transition from one stage to the next is regulated at a number of checkpoints which prevent premature entry into the next phase of the cycle. The degradation of various cyclins occurs at each checkpoint and it is this mechanism together with interaction of the CDKIs and CDKs which allows the cell to enter the next phase.

2.3 Protooncogenes

The roles of many cellular protooncogenes have now been elucidated. There are 4 broad classes: -

a) Secreted growth factors

These exist as polypeptides, oligopeptides or steroid hormones. They bind to their own specific receptors or, occasionally cross-react with several receptors, to stimulate or inhibit cell growth acting via a number of pathways leading to alterations in gene expression. Some stimulate growth by advancing the cell from G_0 to G_1, for example epidermal growth factor (EGF), platelet derived growth factor (PDGF) and fibroblast growth factor (FGF), or by aiding progression through G_1, for example insulin like growth factor. In contrast transforming growth factor β (TGFβ) acts as an anti-mitogen by reversibly inhibiting cells at G_1.

b) Cell surface receptors

These bind a specific growth factor outside the cell. The receptor recognises the growth factor binding and sends a signal to within the cell. The transmembrane receptor tyrosine kinases are the most important of the growth factor receptors with respect to malignant transformation. As described below, these include the Type 1 Growth Factor Receptor family.

c) Components of intracellular signal transduction

Signal transducers act as proteins which transmit signals from a receptor to their cellular target. This is the largest class of oncogenes.

d) DNA-binding nuclear proteins, including transcription factors

This is the final site of action for messages sent from the growth factors and is the level at which control of growth and proliferation ultimately operates. The

proteins either bind to specific DNA sequences exerting an immediate effect or form complexes which in turn bind to DNA. The activity of these transcription factors is regulated by phosphorylation.

2.4 Mechanisms of oncogene activation

a) Structural alteration

Point mutations have been described in several protooncogenes, especially in genes of the *ras* family of signal transducer proteins. Amino acid substitutions have been detected particularly at positions 12, 13 and 61. This results in the protein having reduced GTPase activity preventing deactivation of the active *ras*-GTP complex. This in turn leads to prolonged stimulation of signals from growth factor receptors.

Chromosomal translocation can create novel fusion proteins. The best known rearrangement produces the Philadelphia chromosome in chronic myeloid leukaemia (CML) where part of chromosome 9 is translocated to chromosome 22. This places the *ABL* oncogene on chromosome 9 next to the breakpoint cluster region (BCR) of the Philadelphia gene on chromosome 22 creating a novel fusion gene. The novel gene product has enhanced tyrosine kinase activity resulting in transforming properties.

b) Amplification

Many cancer cells contain multiple copies of structurally normal protooncogenes, for example, some breast cancers amplify HER2. Hundreds of extra copies may be present and can exist as small separate chromosomes (double minutes) or as insertions within normal chromosomes (homogeneously staining regions, HSRs) (135). This may lead to a very large overexpression of the gene product.

Loss of appropriate control mechanisms.

Chromosomal translocation. Cellular oncogenes may show loss of normal transcription control through chromosomal translocation, for example, in Burkitt's Lymphoma. Following the translocation of the *myc* protooncogene on chromosome 8 to one of the immunoglobulin heavy chain loci on chromosome 14 (75-80% of patients), chromosome 2 or 22, there is constitutive overexpression of the transposed *myc* gene. This leads to over-production of the gene product resulting in a continuous stimulus for cell proliferation.

Insertional mutagenesis. Retroviruses can activate protooncogenes by insertional mutagenesis. The insertion of a DNA copy of the retrovirus into the cellular genome close to a protooncogene can cause abnormal activation of that gene by stimulation of gene expression via the promoter action of the retrovirus. The gene is then dysregulated from normal control mechanisms.

A specific oncogene may be activated by a variety of mechanisms and there is no single consistent mechanism of activation of a specific oncogene. In addition, multiple oncogenic mutations may occur within each specific tumour.

SECTION 3 GROWTH FACTOR RECEPTORS IN BREAST CANCER

3.1 Growth factors and growth factor receptors

Growth factors are small polypeptides that are involved in the stimulation and also inhibition of cellular proliferation. Polypeptide growth factors mediate their actions by binding to cell surface proteins (receptors), which are thereby activated and 'transduce' the signal within the cell cytoplasm to the cell nucleus. Growth factor-induced changes in cellular status are multistage processes. Induction of growth factor synthesis, delivery to the target cell and the response of the cell itself all play a role in growth factor action. In many transformed tissues, particularly hormonally responsive tumours (breast, ovarian and endometrial cancers), steroid or other hormones may regulate tumour growth factor production. Once synthesized, growth factors act by endocrine, paracrine and autocrine mechanisms to affect cellular function. In some cases externalization and release of growth factors may not be required for signaling to take place. Interactions between growth factors and receptors, either while both are anchored in the cell membrane (juxtacrine regulation), or even while both are still within the cell cytoplasm (intracrine regulation) have been postulated to explain some of the interactions seen between growth factors with transmembrane domains (juxtacrine) or those lacking sequences required for cellular release (intracrine). A feature of many transformed cells is the acquisition of autocrine growth control, mediated by tumour cells producing their own growth factors, which releases them from normal physiological regulators of proliferation.

Similarities in structure or function of many of the growth factors and their receptors have led to a classification of the majority of receptors into families and

subclasses. Three major divisions of growth factor receptors have been identified, based on the primary mechanisms by which they mediate their effects. Growth factors attach to the ligand binding sites of growth factor receptors and the signal is mediated either by the intracellular domain or proteins associated with the receptor within the cell. This is the start of 'signal transduction' (140-142). Signal transduction mechanisms involve many diverse biochemical changes, but the initial event is usually clearly identifiable for any particular receptor family. Thus, this event is used to classify receptors into subclasses. For many receptors the initial signalling event that occurs following ligand binding is the activation of a kinase. Kinases induce phosphorylation of specific sites in target proteins. This phosphorylation causes marked changes in the conformation and activity of such targets often initiating a cascade of events leading to modification of cellular proteins and activation of nuclear transcription factors which initiate the synthesis of new proteins.

- **Type I growth factor receptors (Tyrosine kinase receptors)**

  The epidermal growth factor receptor (EGFR) and the HER2 receptor are part of this family of receptors. A detailed account of this family of receptors is given in section 3.2. As their name implies these receptors initiate signaling by phosphorylation of tyrosine residues within the receptor.

- **Type II growth factor receptors (Serine-threonine kinase receptors)**

  As with tyrosine kinase receptors the signalling event mediated by this class of receptors involves phosphorylation of serine and threonine residues within the receptor. Included in this group is the *ras* superfamily of signal transducer proteins. The active *ras* form activates a cascade of serine/threonine protein

kinases controlling cell growth and differentiation. *ras* mutations are the commonest oncogenic abnormalities found in tumours, being present in ~20% of tumours.

- **Type III growth factor receptors (G-protein-linked receptors or seven-transmembrane receptors)**

These are the most common type of growth factor receptor. These receptors mediate their action by association with a family of adenyl cyclase and kinase regulating proteins via an intermediary – the G protein (guanine nucleotide binding protein). All G-protein coupled receptors have 7 transmembrane spanning regions. Activation of the receptor leads to inactivation of these regulatory proteins and subsequent inactivation of intracellular signal pathways (140).

3.2 Type I growth factor receptors

3.2.1 Characteristics of type I growth factor receptors

It has been known for over 30 years that EGFR is expressed in breast tissue where it plays a role in mitogenesis and differentiation. EGFR was the first type I growth factor receptor to be cloned (143). It showed considerable homology to the avian erythroblastosis virus transforming protein, v-*erb*B. Following the pioneering work of Cohen and colleagues, this receptor has served as a prototype for the understanding of tyrosine kinase signalling, receptor dimerization, and signal transduction cascades. The mutation or amplification of this gene can convert its cognate protein to a form that confers increased cancer risk on experimental animals and humans. The EGF family (i.e. ligands binding to the EGFR) now includes transforming growth factor alpha, amphiregulin, heparin-binding EGF, β-cellulin, the heregulins, cripto-1 and certain viral-encoded proteins.

To date, three other family members of this growth factor family have been identified: HER2 (144-146), c-*erb*B-3 (147;148) and c-*erb*B-4 (149). These three receptors are also known as HER2, HER3 and HER4 (***H***uman ***E***pidermal Growth Factor ***R***eceptor). (The last 2 members of this family will not be considered further in this work as they do not form any part of this study).

These receptor proteins have a common structure consisting of an extracellular domain, a transmembrane region, a short juxtamembrane sequence and a cytoplasmic domain. The extracellular domain is the ligand-binding area and contains two cysteine rich regions. The cytoplasmic domain consists of a tyrosine kinase domain and a carboxyl-terminal tail that contains the mapped

autophosphorylation sites (150;151). A schematic diagram of this receptor family is shown in Figure 9.

The generally accepted mechanism of receptor activation is as follows: binding of ligand promotes receptor dimerization which increases the activity of the tyrosine kinase. This leads to specific tyrosine residues in the C-terminal tail becoming phosphorylated which then appear to serve as docking sites for molecules involved in signal transduction. Such molecules may be either substrates for the receptor tyrosine kinase or adaptor proteins involved in recruiting further proteins to the activated complex (152).

Study of this receptor family has resulted in critical insights into regulation of the normal mammary gland, in identification of one of the most commonly activated oncogenes in breast cancer (HER2), in recognition of critical mechanisms of breast tumour production, in identification of mechanisms of immune surveillance of breast cancer and in new ideas for drug therapy of the disease.

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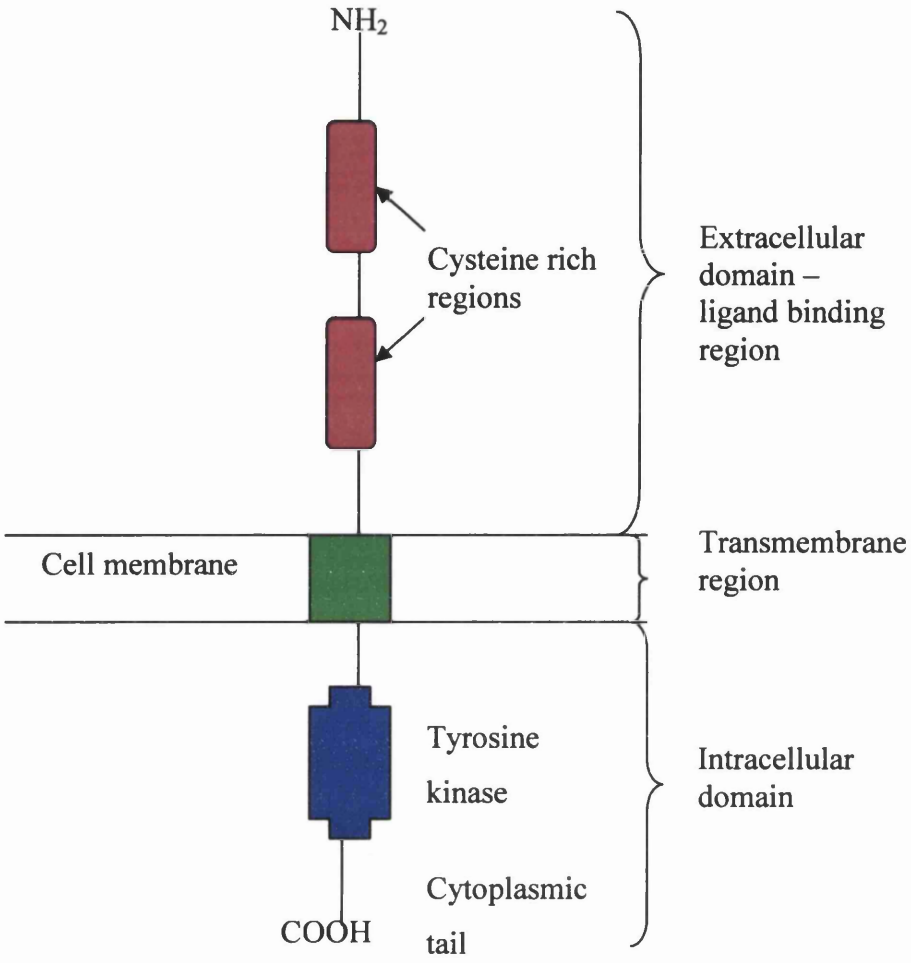

Figure 9. *A schematic diagram of the structure of the Type 1 growth factor receptor family.*

3.2.2 Conventional methods for measuring type 1 growth factor receptors in breast cancer

Previous examinations of the relationship between type 1 growth factor receptors and breast cancer have primarily used two types of methodology: immunohistochemistry on tissue sections and ligand binding or ELISA based assays on tumour homogenates.

The ligand binding assay is the most widely applied method used to measure EGFR expression (153). This method requires a relatively large amount of fresh frozen tissue, is cumbersome to perform and cannot be applied to archival material. Other methods which have been used include enzyme immunoassay (154) which also requires frozen tissue, mRNA detection methods (155), autoradiography (156) and EGFR associated phosphotyrosine kinase activity (157). Initial studies of EGFR expression using immunohistochemistry (158;159) were significantly hampered by the need to use frozen tissue. This is because the majority of antibodies recognise an epitope on the internal domain of this transmembrane molecule which does not survive routine formalin fixation.

Although ligand binding assay is generally accepted as the 'gold standard' in EGFR measurements, even here there is disagreement about the cut-off level used to define positivity. (160). Several studies using immunohistochemical (161-163) and ligand binding (164) methods have shown EGFR expression more frequently in non neoplastic than in malignant tissue. These assays are performed on membrane preparations derived from tumour biopsies which include, besides malignant cells, nontumour elements, including normal breast, in situ disease, connective tissue and lymphoid cells. As such the technique is inherently flawed

since receptor measurement reflects the level of expression by the entire tumour mass rather than by the carcinoma cells alone. The inclusion of even small quantities of non-malignant tissues may cause a severe overestimation of the EGFR level. This is especially relevant with regards to this particular receptor protein as several studies have shown it to be preferentially expressed in stromal and myoepthelial cells and not in epithelial cells (165).

Newby et al (166) have recently developed an immunohistochemical assay which appears to work on paraffin embedded sections; using an IgG antibody produced by Biogenex, USA and which may have potential for use on archival material.

No definite ligand for the HER2 receptor has yet been identified and so ligand binding techniques are not currently applicable. Most studies of HER2 expression in breast cancer have used immunohistochemical analysis of formalin-fixed, paraffin-embedded tissues. Most antibodies used recognise an epitope on the internal domain of this transmembrane molecule which, unlike epidermal growth factor receptor, survives routine fixation and allows paraffin sections to be used. This type of assay suffers from a number of limitations and may not be sufficiently accurate to thoroughly assess the relationship between the levels of HER2 expression and the biology of breast cancer for the following reasons:

- First, routinely processed material may be fixed for times ranging from a few hours to several days, which is likely to result in variable antigenicity, and tissue in the centre of a large block will have a lesser degree of fixation compared to tissue on the periphery, which may explain the variation in staining commonly seen across an individual section.

- Second, the many methods for amplifying the signal generated by binding of the primary antibody result in a range of sensitivities.
- Third, methods for scoring immunohistochemical sections are subjective, with a high degree of interobserver variability.

These factors are likely to lead to significant interstudy variation and may account for the wide range of values (9-39%) for the proportion of breast tumours reported to overexpress HER2 (167).

Studies of HER2 in breast cancer using Southern, Northern and Western blots use tissue homogenates and suffer from the same drawbacks as the ligand-binding assay for EGFR.

3.3 Biology of EGFR

The EGFR is a 170 kDa transmembrane glycoprotein translated from mRNAs of 6 and 10 kb encoded by a gene on chromosome 7q21. The protein comprises a 1186 amino acid polypeptide chain (168) and, like the other members of the family, is composed of 3 domains: an extracellular ligand-binding domain, a transmembrane lipophilic region, and an intracellular protein tyrosine kinase domain. The EGFR signal transduction pathways have been correlated with various processes that contribute to the development of malignancies, such as effects on cell cycle progression, inhibition of apoptosis, angiogenesis, tumour cell motility, and metastases (169-172).

EGFR is expressed in many normal cells and organs. Hepatocytes, pancreatic ducts, oropharynx, salivary glands, uterus, prostate and the epididymis all show strong immunohistochemical staining, whilst the brain, spleen, lymph nodes and stomach are essentially negative.

3.3.1 EGFR in Normal Breast Tissue

There have been a limited number of studies investigating the expression of EGFR in normal breast tissue. However, it has been found in general that EGFR is expressed more frequently in non-malignant than malignant breast tissue (164;173;174). In addition some reports have shown that the level of EGFR expression is higher in normal tissue than malignant (164;175). There seems to be a lack of the inverse relationship between EGFR and oestrogen receptor expression, which is found in malignant tissue, in normal breast tissue (164;176). It is not known if the two receptors are co-expressed in the same cells, though Berthon et al (177) found that when human mammary epithelial cells are

propagated from breast tissue obtained by mammoplasty, the cells are exclusively positive for EGFR and negative for oestrogen receptor.

Epidermal growth factor (i.e. the ligand for EGFR) is an important growth factor in the mammary gland of mice and is abundant in human breast milk. It is also essential for the growth of normal epithelial breast cells in culture (178). There is *in vitro* evidence of an interaction between EGFR and its ligands in normal breast cells (179). In breast cancer cells, it has been shown that TGF-α is an essential autocrine factor secreted under oestradiol control and that its level of expression, as well as that of EGFR mRNA, is under the control of EGF (180). Thus, EGFR and its ligands interact to regulate the growth of epithelial cells, at least *in vitro*, and can be influenced by oestradiol, a mitogen for breast cells. However, the relevance of these different pathways *in vivo* is unclear.

Immunohistochemical studies of EGFR in normal breast tissue are rare, and the few available studies give conflicting results (165;181-183). Damjanov et al (183) described positive staining of epithelial and myoepithelial cells coexisting with negative staining of stromal cells. Tsutsumi et al (181) described predominant staining in myoepithelial cells, occasional staining in fibroblasts and negative staining in epithelial cells. Gompel et al (165) report predominant staining in stromal and basal cells of large ducts.

3.3.2 EGFR and DCIS

There is a distinct lack of studies investigating EGFR expression in DCIS, perhaps because of the added difficulty of having to use frozen tissue.

3.3.3 EGFR and Invasive Breast Cancer

In vitro, EGFR and its ligands have been implicated in malignant transformation via autocrine and paracrine growth factor pathways (184). *In vivo*, EGFR is expressed in a number of human tissues, both benign and malignant. Among cancers, EGFR is most strongly expressed in squamous cell carcinomas, but it is also found in a variety of other tumours. The incidence of expression in breast carcinomas (using ligand based studies) averages 49% with an interstudy range of 16-91% (160). Similarly, immunohistochemical studies show that about 40% of breast cancers express EGFR with an interstudy variation of 14-65% (160). A number of studies have shown it to be an adverse prognostic factor in breast cancer (173;185-193), though this is not confirmed in all such studies (155;160;193-195). These conflicting results are, at least in part, likely to be due to differences and the lack of standardisation between the assays used. In contrast, the literature is consistent in reporting an inverse relationship between EGFR and ER (160). EGFR positivity has also been shown to be an indicator of a poor chance of response to endocrine therapy (187;196;197).

Sainsbury et al (198) first described EGFR as a prognostic factor in human breast cancer in 1985 (using a ligand binding assay method). Since then there have been a large number of studies which have measured EGFR in breast cancers by a variety of methods and with differing definitions and proportions of positivity.

3.4 Biology of HER2

The HER2 receptor gene is present on chromosome 17q21 and is transcribed into a 4.5 kb mRNA which is translated into a 185 kDa glycoprotein with tyrosine kinase activity (199). Structurally the HER2 receptor shares significant homology with, but is distinct from, the EGFR (146;200-202). Low levels of HER2 expression are detectable immunohistochemically in a variety of epithelial cells throughout the gastrointestinal, respiratory and genitourinary tracts (203). Amplification of the HER2 protooncogene or overexpression of the receptor protein has been identified in gastrointestinal, pulmonary, and genitourinary tumours as well as in breast cancers (204-207). Laboratory studies suggest that overexpressed HER2 plays a direct role in the pathogenesis and aggressiveness of tumours through several lines of experimental evidence:

- transfection of HER2 into non-neoplastic cells effects malignant transformation (208;209)
- transgenic mice expressing HER2 develop mammary tumours (210)
- the presence of HER2 overexpression may be associated with the development of metastatic disease (210).

The mechanism by which overexpressed HER2 leads to a neoplastic phenotype is associated with at least 2 factors:

- its ability to induce resistance to tumour necrosis factor-α, which is a protein that has been shown to play an important role in tumour cell killing *in vitro* as well as *in vivo* via cytotoxicity mediated by macrophages and natural killer cells (209)

- its activation of several different signalling pathways that lead to gene activation and, ultimately, cell proliferation (205)

The mechanism of activation of HER2 is thought to involve the formation of spontaneous homodimers or heterodimers with other members of the same family of growth factor receptors (211-214). As previously stated, no definite ligand has yet been identified for HER2. However, a family of ligand peptides named "neu differentiation factors" or "heregulins" have been identified that bind to the c-*erb*B-3 and c-*erb*B-4 receptors. This induces heterodimerisation with the HER2 receptor, thereby inducing phosphorylation and activation of the HER2 receptor . It has also been shown that on binding of ligands such as EGF or TGFα to the EGFR, heterodimers between the EGFR and HER2 can also form (214).

3.4.1 HER2 in Normal Breast Tissue

The precise physiological actions and regulation of HER2 in normal breast tissue are unknown. HER2 is expressed in many cell types including those outside the haematological system; expression has been found in neural tissue, bone, muscle, skin, heart, lungs and intestinal epithelium (144). Expression levels are higher in fetal tissues than in corresponding adult tissues (203), suggesting a role in cell differentiation. The activity of the HER2 receptor in tissues is affected by the variety of ligands and other HER receptors that are present. For example, EGFR and HER2 are often co-expressed and heregulins and EGF-like ligands are known to be able to activate HER2 via both autocrine and paracrine mechanisms (215;216). Studies have demonstrated that EGF-like ligands and neuregulins can inhibit or stimulate cell proliferation depending on the cell line tested (217;218). Neuregulins induce both growth and differentiation in a rodent cell model of mammary cell differentiation (219). *In vivo* treatment of mammary glands with EGF results in alveolar and ductal epithelial differentiation (220).

Studies of HER2 immunohistochemical staining in frozen tissue sections of normal human breast tissue have shown exclusive localisation to the epithelial compartment (165;179;203). Double labelling studies of EGFR and HER2 immunohistochemical staining show that the proteins can coexist in mature epithelial cells in the normal breast, albeit with differential distribution: EGFR is preferentially present in the stroma and myoepithelial cells whereas HER2 is exclusively expressed in epithelial cells of lobules and ducts (165).

3.4.2 HER2 and DCIS

At the molecular level, the stepwise enhancement of the histological grade of cancer cells within individual cancers is generally accompanied by the accumulation of alterations in oncogenes and tumour suppression genes. Gene alterations have been shown to accumulate in breast cancers with high grade atypia (221-224) and studies have shown that these alterations occur during the preinvasive stages (225-228). A much higher proportion of cases of pure DCIS express HER2 than do invasive carcinomas: between 45% and 60% of DCIS staining positively compared to only 20-25% of invasive carcinomas (30). The exact significance of this is unclear but there have been attempts to explain it (see section 3.6).

Many antibodies have been raised against HER2 and used in several studies to evaluate the relationship of HER2 to various histological features. A study by van de Vijver et al (using the 3B5 monoclonal antibody) was one of the first to show that nearly all DCIS with HER2 overexpression had a large cell comedo type histological appearance (30). In the same study, none of 16 cases of DCIS with small cell papillary or cribriform pattern stained positively for HER2. In a study of 74 cases of DCIS, Ramachandra et al (using the 21N antibody) (229) found a significant correlation between HER2 positivity and large cell size, periductal lymphoid infiltrate, marked nuclear pleomorphism, multinucleation and a high mitotic rate. Using the same antibody, Bartkova et al (230) found that all cases of large cell comedo type DCIS were positive for HER2 while no cases of small cell DCIS, usually with a cribriform or micropapillary pattern were positive. Lodato et al (231) found that in cases of DCIS with more than one type of histological

pattern, positive staining was only present in the comedo areas. Studies by Bobrow et al (232) and Zafrani et al (233) have found an inverse relationship between the degree of differentiation in DCIS and the proportion of cases staining positive for HER2. Both studies also showed a direct relationship between the degree of necrosis and HER2 staining. The importance of nuclear grade in DCIS and the presence or absence of necrosis has been recognised (37). Nuclear grade, in particular, has been shown to correlate with subsequent development of invasive carcinoma (36;42;43). High levels of HER2 are found in comedo DCIS, and in general low or absent levels of expression are seen with other subtypes such as papillary and cribriform in situ tumours (234;235). The high levels of staining and high incidence of HER2 positivity in DCIS, together with the absence of expression in premalignant breast epithelial cells such as atypical ductal hyperplasia, indicate that HER2 expression may be an early event in the progression of tumorigenesis (236).

These studies show that HER2 positivity is associated with markers of more aggressive forms of DCIS. It follows that HER2 positivity may help identify more aggressive lesions which may be more likely to develop into invasive carcinoma.

3.4.3 HER2 and Invasive Breast Cancer

Amongst the invasive tumours, ductal carcinoma is associated with HER2 overexpression whereas lobular carcinoma is in general negative (235).

3.4.3.1 HER2 as a prognostic factor

A prognostic factor is one that provides information regarding patient outcome at the time of diagnosis. In 1987, Slamon and colleagues were the first to report a relationship between HER2 amplification and prognosis in breast cancer (221). In this study, 28% of the primary breast cancers studied showed a 2-20 fold gene amplification as assessed by Southern blot analysis. On multivariate analysis, HER2 amplification retained its independent prognostic significance when compared with size of tumour, hormone receptor status and number of lymph nodes involved (221;237). A subsequent study performed by the same group (238) confirmed the independent prognostic significance of HER2 amplification in predicting disease free survival and overall survival in a subgroup of node positive patients but not in a subgroup of node negative patients.

The methods used for assessing gene overexpression have included Southern blot analysis, slot blot testing, polymerase chain reaction, fluorescence in situ hybridisation and chromagenic in situ hybridisation. For assessing protein overexpression, Western blot analysis, immunohistochemistry and enzyme linked immunosorbent assays have been used (200). The majority of studies suggest gene amplification in approximately 20% (234) of breast tumours and the highest rate of protein overexpression recorded is 39% (239) (Range 9-39%, reviewed by Gullick (167)). Gene amplification is always accompanied by mRNA and protein overexpression (221), however overexpression can occur independently of

amplification (240) and thus deregulated transcription may also be an important factor influencing HER2 expression. It appears likely that increased transcription precedes amplification in the natural history of breast cancer and that selection of cells expressing progressively higher levels of the receptor occurs.

Since these initial studies, at least 52 publications evaluating approximately 16,975 patients have explored the prognostic significance of HER2 gene amplification and protein overexpression (reviewed by Kaptain et al (241), Ross and Fletcher (242)). Overall, 46 of the 52 studies (88%) have shown that HER2 amplification or overexpression predicted a worse outcome in either univariate or multivariate analysis. Six of the 52 studies (12%) showed no correlation between HER2 status and outcome. Thirty-nine of the 46 studies showing a positive correlation between HER2 status and outcome included multivariate analysis. Of these 39, 33 showed that HER2 was an independent prognostic indicator while in 6 of the studies, HER2 status was an independent prognostic indicator in univariate analysis only. The prognostic significance of HER2 status has been analysed in the context of subgroups of patients with positive and negative lymph nodes.

3.4.3.1a HER2 in lymph node positive breast cancer

The majority of studies that have looked at the prognostic significance of HER2 in patients with positive axillary lymph nodes have shown an adverse outcome associated with HER2 amplification or overexpression in univariate or multivariate analysis (221;238;243-257). In addition, many of these studies have shown that it maintains independent prognostic significance in determining shorter disease free survival and / or overall survival in multivariate analysis

(221;238;245;247;249-257). Only a very few studies have not shown a statistically significant correlation between HER2 status and adverse outcome (208;258).

3.4.3.1b HER2 in lymph node negative breast cancer

The prognostic significance of HER2 in node negative breast cancer is less clear than in that of node positive disease. Many studies have shown that HER2 amplification or protein overexpression is associated with an adverse outcome (245;250;251;256;257;259-263) or in certain subsets of patients with node negative disease (264-266). However, an equal number of studies have shown no significant difference in outcome associated with HER2 amplification or protein overexpression (208;238;243;244;247-249;252;253;255;267-269). In addition, only a few studies that have shown a prognostic importance for HER2 status in patients with negative lymph nodes have shown that it maintains an independent significance for disease free survival and/or overall survival in multivariate analysis (245;250;251;256;259;262). The reasons for these conflicting results include small patient numbers in many of the studies, patient selection, variations in treatment, length of follow up, statistical analysis, and in methodology used to assess HER2 status. Examination of studies that have involved more than 100 patients with more than 3 years follow up (237) shows equally conflicting results. Thus, although it is generally accepted that patients with positive lymph nodes and HER2 amplification or overexpression have a worse prognosis, the prognostic significance of HER2 status in patients with negative lymph nodes is unresolved.

3.4.3.1c HER2 and other prognostic factors in breast cancer

Controversial results have also plagued attempts to correlate HER2 amplification or overexpression with other prognostic factors in breast cancer. Many studies have shown an association between HER2 amplification or overexpression and the absence of oestrogen receptors or progesterone receptors (221;243-246;249;251;253;254;257;259;270-273), while others have not (208;260;262;269;274-278). Many studies have correlated HER2 amplification or overexpression with poor histologic or nuclear grade of the primary tumour (221;244-246;254;255;257;259;260;266;275;277;279;280), while others have not (249;258;259;262;265;269;272;274;276). Similarly, the association with positive lymph node status found in some studies (221;243;246;248;251;253;254;270;278) has not been confirmed by others (208;243;245;249;250;255;257;258;272;274-277). Most studies have not found an association with tumour size (221;248-251;253-255;257;259;260;262;265;270;271;276;277) although a few have found an association with tumours greater than 2cm in size (243;244;258;281). Other studies have associated HER2 amplification or overexpression with a higher tumour cell proliferation rate as shown by mitoses (255;272)147 or a higher S-phase fraction (244;259)(Reviewed by Kaptain et al (241)).

As is the case with EGFR, there is a tendency in some studies to view any expression as overexpression. Clearly this can be misleading as normal breast tissue has a degree of expression of both of these growth factors (162;164;173;175;282). The correlation between HER2 expression and prognostic factors in some of these studies may therefore be irrelevant.

3.4.3.2 HER2 as a predictive factor

A predictive factor is one that provides information regarding the likelihood of response to a given therapeutic modality. There has recently been great interest in the interactions between HER2 overexpression and various forms of systemic therapy in patients with breast cancer as this could potentially provide information that helps in determining the most suitable treatment for any given patient.

3.4.3.2a HER2 and anthracyclines

The most convincing evidence of such an interaction has been seen in patients with chemotherapeutic regimens containing anthracyclines. These studies have generally shown that HER2 overexpression is associated with increased sensitivity to anthracyclines (283-285). *In vitro* studies have shown that amplification or overexpression of HER2 in breast cancer cells is frequently correlated with coamplification/expression of topoisomerase IIα (211;283;286;287). Topoisomerase IIα is an enzyme involved in DNA replication that acts by generating and resealing double stranded DNA breaks (286;287). Topoisomerase IIα is a target for several anticancer drugs, including doxorubicin (211;283;286;287), which inactivate this enzyme, leading to the accumulation of double-stranded DNA breaks, which are lethal to the cell (286). Sensitivity to these chemotherapeutic agents is correlated with the level of expression of topoisomerase IIα (287). This may explain the sensitivity of HER2 amplified or overexpressing tumours to anthracycline containing chemotherapeutic regimens (211;287).

3.4.3.2b HER2 and CMF regimens

Various studies have evaluated the effectiveness of chemotherapeutic regimes containing cyclophosphamide, methotrexate and fluorouracil (CMF) in patients with or without HER2 amplification or overexpression (288). Initial studies (245;264), published in 1992, looking at HER2 status in response to CMF treatment regimens suggested HER2 positive tumours showed a decreased response to CMF therapy. However, both these studies had small numbers of patients in the HER2 positive subgroups and small numbers of relapse events in these subgroups. They may, therefore, have been underpowered and a beneficial effect of treatment may have been missed (289). Several other studies have also shown a relative resistance of HER2 positive tumours to CMF treatment (207;211;245;254;264;289). However, a recent study (273) has found that both HER2 positive and negative tumours, as detected by immunohistochemistry, benefit from CMF treatment.

3.4.3.2c HER2 and taxanes

In a study performed at Memorial Sloan-Kettering Cancer Center (290), sensitivity to taxanes was studied in a group with metastatic breast cancer. The taxane used was Paclitaxel, which has been found to be active against many cancers which have been refractory to conventional chemotherapy. They found an overall response rate to Paclitaxel of 47%. Patients with HER2 positive tumours (37% overall) had a response rate of 65% while patients with HER2 negative tumours had a significantly lower response rate of 36%. Paclitaxel is thought to act via activation of the HER2 signal transduction pathway, which is thought to lead to drug-induced apoptosis (290). Nonetheless, these clinical trials are in

conflict with experimental data that suggests that HER2 overexpression in transfected mammary cells leads to resistance to taxanes *in vitro* (291-293).

3.4.3.2d HER2 and tamoxifen

The predictive effect of HER2 status has also been studied in relation to tamoxifen therapy. Expression of hormone receptors is associated with increased response (70-80%) to tamoxifen therapy among patients with advanced breast cancer, although up to 50% of patients with receptor positive tumours will not benefit from such treatment and approximately 10% of those with receptor negative tumours will respond (294). Most studies addressing this question conclude that patients with tumours with amplification or overexpression of HER2 do not respond or may even do worse with tamoxifen therapy (207;211;294-299). In contrast a study published in 1998 by Elledge et al (300) found no significant association between resistance to tamoxifen or worse outcome on treatment with tamoxifen in patients with metastatic tumours that were positive for both oestrogen receptors and HER2.

3.4.3.3 Summary

There is fairly consistent evidence that HER2 overexpression is predictive of sensitivity to anthracyclines. Although patients with HER2 overexpressing tumours may be less responsive to CMF treatment and to tamoxifen than those with tumours that do not show HER2 overexpression, current data are insufficient to deny patients treatment with either CMF or tamoxifen on the basis of the HER2 status of the tumour.

3.5 Targeting type 1 growth factor receptors in the treatment of breast cancer

Systemic treatments for breast cancer have improved significantly over the last 25 years. New insights into cancer biology, refinements in biotechnology, and bioengineering of macromolecules may provide even greater reductions in breast and other cancer mortality through the use of "biologicals". Biologicals are defined by the World Health Organisation as being molecules of complex structure resulting from genetic expression in living organisms. As molecules with mechanisms of antitumour actions that are different from those of existing therapeutics, they have potential to further improve clinical outcomes through combination with other modalities. New biological therapies have been evaluated clinically and confirmed to have curative and palliative potential, starting with the introduction of recombinant DNA-produced interferons into clinical trials about 20 years ago. Part of the improved outcome with biologicals for cancer therapy has resulted from either augmentation of host inflammatory immune responses by manipulation of cytokines or through antigen specific therapies such as monoclonal antibodies. Monoclonal antibodies, targeted to the CD20 antigen on B-cell lymphomas or HER2 on breast carcinoma or other adenocarcinoma cells, represent the first antigen-specific biological therapies for cancer. The clinical results with the monoclonal antibody to HER2 have demonstrated that biological therapies for breast cancer hold significant promise. A phase II study reported by Baselga et al (301) of 45 women with HER2 overexpressing metastatic breast cancer, resistant to multiple previous therapies, showed that 11.6% and 37% of patients, respectively, developed objective response and disease stabilization

when given monoclonal antibody rhuMAB HER2 [Herceptin, (trastuzumab)] directed against HER2. Herceptin has a favourable toxicity profile, and has achieved a significant overall response rate of 15% when given alone in metastatic breast cancer to patients whose tumours have progressed after chemotherapy (302).

The results of a randomised multinational controlled phase III clinical trial using Herceptin have now been reported (303). In this trial, the effect of Herceptin given in addition to first-line chemotherapy for metastatic disease was assessed in 469 women whose tumours overexpressed HER2. Patients who had not received anthracycline-based adjuvant chemotherapy (n=281) were treated with doxorubicin and cyclophosphamide and randomly allocated Herceptin or no additional treatment. Patients who had received anthracycline-based regimens as adjuvant therapy (n=188) received paclitaxel with or without Herceptin. Addition of antibody to chemotherapy increased the response rate and the time to progression, and was associated with a survival advantage of 5 months, although 65% of patients who were not allocated Herceptin initially received it on relapse.

These results suggest that use of anti-HER2 antibody therapy has potential as a new method of breast cancer treatment, whether given alone or in combination with other treatment methods. Additional approaches to anti-HER2 therapy under investigation include the use of antisense gene therapy probes and anti-HER2 vaccination.

3.6 Expression of HER2 in DCIS compared to that in invasive carcinoma

An interesting issue with regards to HER2 expression in both DCIS and invasive carcinoma is the reason why more DCIS lesions express HER2 (45-60%) than invasive lesions (20-25%). In a paper published in 1992, Barnes et al (159) attempt to explain this. They suggest that, with regards to HER2 expression in invasive carcinomas there are 3 groups of tumour:

- those composed of cells with small nuclei, which have arisen from small cell DCIS. These have a low rate of proliferation and of HER2 overexpression
- those composed of large cells which have arisen from large cell DCIS. These have a high rate of proliferation and of HER2 overexpression
- those composed of cells with variable nuclear sizes, but including some with large nuclei, over half of which have a high rate of proliferation but none of which overexpress HER2

They hypothesise that this last group of tumours only have a transient in situ stage and quickly become invasive, explaining the lower rate of overall HER2 positivity seen in studies of invasive carcinomas when compared to pure in situ lesions.

SECTION 4 AIMS OF THIS STUDY

The breast cancer research group at the Department of Surgery, Glasgow Royal Infirmary, has previously reported the development of radio-immunohistochemistry for quantifying epidermal growth factor receptor (EGFR) and HER2 in frozen tissue sections and applied this method to breast tumours (1). This radioimmunohistochemistry method is highly sensitive and allows the quantitation of receptor levels throughout the range of expression in breast carcinomas. The quantitative, objective data produced allow a more thorough evaluation of the relationship between receptor expression and tumour biology. The aims of this study are to answer the following questions using this quantitative assay:

1. *Are the levels of expression of these type 1 growth factor receptors seen in invasive lesions the same as in situ breast cancer, and might these be a factor in determining progression to invasive disease?*

   If EGFR downregulation or HER2 overexpression are required for progression to invasion we might expect to see more lesions with normal levels of expression in pure DCIS, and an invasive or intermediate pattern in DCIS adjacent to invasive areas. In this way, the quantitative information available from radio-immunohistochemistry may allow us to predict the biological role of EGFR and HER2 in breast carcinogenesis and progression.

2. *What is the relationship between the expression of these type 1 growth factor receptors and morphological features and histologic subtype of DCIS?*

   Studies of DCIS correlating HER2 expression to morphological features such as cell size and nuclear differentiation have found expression exclusively within

DCIS composed of large cells containing poorly differentiated nuclei. Studies correlating histologic subtype of DCIS have found a significantly higher prevalence of HER2 protein expression in the comedo subtype than the cribriform-micropapillary subtype. Very few studies have looked at EGFR expression in DCIS.

The quantitative information available from radio-immunohistochemistry will allow us to characterise further the relationship between these type 1 growth factor receptors and morphological features known to be associated with more aggressive forms of DCIS.

3. *What is the relationship between* HER2 *expression, particularly sub-amplification levels, and pathological variables and outcome as measured in a large series of primary breast cancers?*

   As previously stated the breast cancer research group at Glasgow Royal Infirmary has applied this radio-immunohistochemical method to a series of primary breast cancers (1) (n = 118 for EGFR, n = 81 for HER2, n = 75 for both receptor types). In this small number of tumours studied, there is a suggestion that HER2 correlates better with pathological predictors of outcome when cut-offs below the very high levels seen with amplification are used. As part of this work, using tumours from the frozen tumour bank at Glasgow Royal Infirmary, this series has now been extended to 193 tumours for EGFR and 177 for HER2. The question as to the biological importance of HER2 expression at these lower levels is explored using patient outcome as the indicator of biological activity, studied against the exact level of expression as measured by radio-immunohistochemistry.

SECTION 5 MATERIALS AND METHODS

All specimens in this study were frozen tissue samples. All clinical follow-up was carried out in a specialist breast cancer unit (either WIG or GRI). All specimens were examined by a dedicated breast pathologist (EAM or JJG). A diagram of the methodology is shown in Figure 10 below.

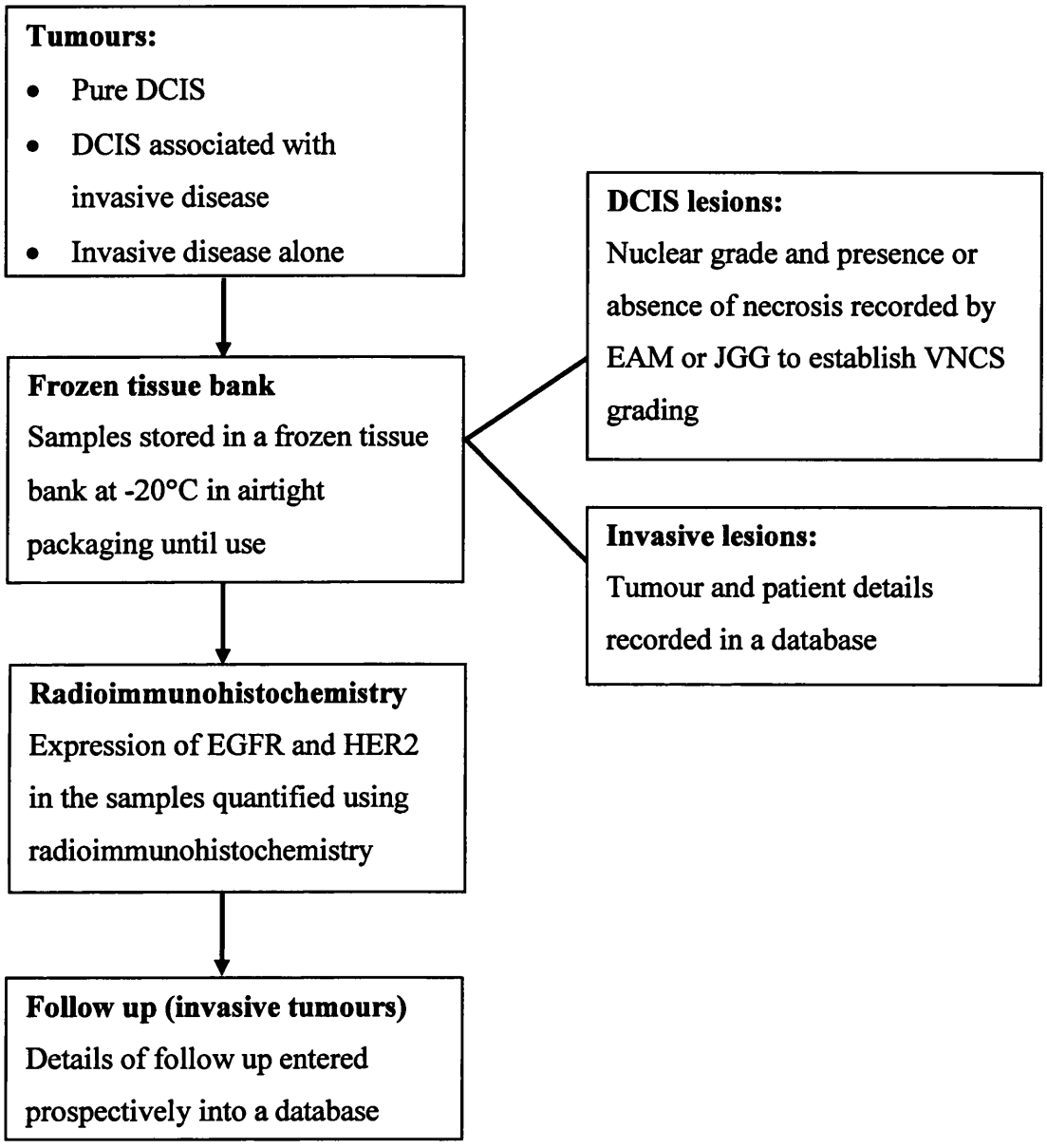

Figure 10. *A schematic diagram of the methodology used.*

5.1 Tumour populations

Three tumour populations were studied and 1 population of normal breast tissue.

5.1.1 Normal breast.

This study has used specimens of histologically normal breast which were obtained from 9 reduction mammoplasty procedures, in a manner similar to that for tumour biopsies. Blocks macroscopically containing breast parenchyma, subsequently confirmed on H&E sections, were dissected from the surgical specimen. The median age of the patients from whom these specimens were obtained was 35.5 years, with a range from 17-49 years. Counts were carried out over terminal ducts and lobules, and not over major ducts. These specimens are the same ones which have formed the basis of "normal" receptor expression in previous work by this group (1;2;304).

5.1.2 Pure ductal carcinoma in situ (DCIS) with no evidence of invasion.

A frozen tissue bank of pure in-situ carcinoma was collected prospectively. Cases likely to contain DCIS were highlighted prior to surgery. The majority of these were screen detected lesions. A single dedicated breast pathologist (EAM) processed the tissue in the usual manner and if it contained only DCIS, cryostat sections (5 μm) were thawed onto silanized slides and stored at -20°C in airtight packaging until use. The diagnosis of pure DCIS was later confirmed by checking the final pathology report. Forty samples of pure DCIS were obtained in this way. EGFR receptor levels were measured in 37 cases and HER2 receptor levels were measured in 36 cases. Both receptor types were quantified in 33 tumours. There were 7 cases in which receptor measurement was only possible for one or other receptor as the sections obtained for these research purposes either did not contain

tumour or the fixation process had altered the histology such that areas of tumour could not be reliably identified.

5.1.3 Ductal carcinoma in situ (DCIS) adjacent to invasive disease.

The same pathologist examined sections of invasive tumours to identify those tumours which also contained areas of in situ disease. The expression of EGFR and HER2 receptors within these areas of in-situ carcinoma were quantified. Levels for EGFR were measured in 50 tumours and HER2 receptors in 47. Both receptor types were quantified in 40 tumours. Once again, there were 10 cases in which receptor measurement was only possible for one or other receptor as the sections obtained either did not contain tumour or the fixation process had altered the histology such that areas of tumour could not be reliably identified.

5.1.4 Invasive tumours.

Unilateral, primary, operable breast cancer biopsies taken during lumpectomy or mastectomy procedures in Glasgow Royal Infirmary between 1984 and 1994 formed the study material for the project. Biopsies were frozen and stored in liquid nitrogen. One hundred and ninety-three specimens were stained for EGFR and 177 for HER2. (The breast cancer research group at Glasgow Royal Infirmary have previously reported on the application of radioimmunohistochemistry to primary breast tumours. This work has expanded the number of tumours and examined long term follow up of these patients with regard to HER2). Pathological data including macroscopic tumour size and axillary lymph node status were recorded by the pathologist reporting on that specimen. Tumours were graded by a dedicated pathologist using Elston's modification of the Bloom and Richardson system (49). Slides of the sections were reviewed by a dedicated

breast pathologist (EAM) to ensure they did not contain DCIS. Follow-up and survival information was obtained from unit records and confirmed with data from the cancer deaths registry of the West of Scotland Cancer Surveillance Unit, from whose records cause of death was taken. Median follow up for surviving patients at the time of analysis was 69 months (5.8 years).

This series of tumours do not represent consecutive tumours. Our results could therefore be criticised as it could be claimed these tumours were selected. The frozen tissue bank of pure DCIS was collected prospectively. All patients who were suitable i.e. had only DCIS and no invasive disease and had sufficient tissue available so as not to affect the histological examination of the case were used.

Cases of DCIS in association with invasive disease were selected only for those that were shown to contain DCIS as well as invasive disease and for no other features.

The invasive tumours were those that were contained in the frozen tissue bank at Glasgow Royal Infirmary. The characteristics of the examined group were compared with those of other unilateral, primary operable tumours treated in the same unit during the time period that the frozen tissue bank was established. This data is shown in Table 21. The characteristics of the examined group and the unselected group are clearly comparable.

It should be mentioned that these 3 tumour populations involve different patients. Ideally, a population of patients who had progressed from DCIS to develop invasive disease would have been best to investigate whether a marker is a true test for disease progression. From what we know of the natural history of the

disease, this is a process that can take many years. This population of tumours would have been very small, making it very difficult to draw any firm conclusions from the data.

5.2 Statistical analysis for HER2 in invasive tumours

Survival analysis was performed for HER2 expression in invasive tumours. This was performed for disease-specific survival, patients being censored at last follow-up or at the date of death from causes other than breast cancer.

Two types of analyses were performed.

a) Univariate and multivariate analyses using Kaplan-Meier estimates

The distribution of HER2 expression in invasive disease appears to be bimodal, with a nadir at about 15 times normal expression levels which is a level we have previously shown is due to gene amplification (2). This appears to give a natural division of HER2 expression into 3 groups:

Group A where expression is less than that of normal breast tissue.

Group B where expression is between normal and 15 times normal expression.

Group C where expression is greater than 15 times normal expression.

Univariate analysis comparing disease free survival in these 3 groups was carried out.

Multivariate analysis was carried out comparing disease free survival in these 3 groups against known pathological variables and the Nottingham Prognostic Index.

Univariate and multivariate analysis alone was felt to be inadequate as it ignores the quantitative continuous nature of the data, and gives arbitrary user defined categories. (Which is essentially what conventional immunohistochemistry does, except the categories are methodologically defined).

b) Cox's proportional hazards model

Cox Proportional Hazard regression models are fitted to the data using the logarithm of HER2 as the quantitative diagnostic variable of interest. Linear, quadratic and cubic terms in log(HER2+1) are fitted. The fits are made both ignoring, and adjusting for, the status of standard pathological indicators (number of nodes, size and grade of tumours). The models are fitted both for the total sample, and within the two groups defined by Oestrogen receptor status.

The gain from adding terms to the model is examined using standard likelihood ratio tests. Where appropriate, stepwise fitting is employed using the forward likelihood ratio as the basis for selection of variables. Acceptability of the Proportional hazards component of the model is checked by visual examination of derived graphs.

5.3 Radio-immunohistochemistry.

For EGFR and HER2 quantification the radio-immunohistochemical method used was that which has been previously described and validated by this group (1;305). A diagram of the radioimmunohistochemical method used is shown in Figure 11.

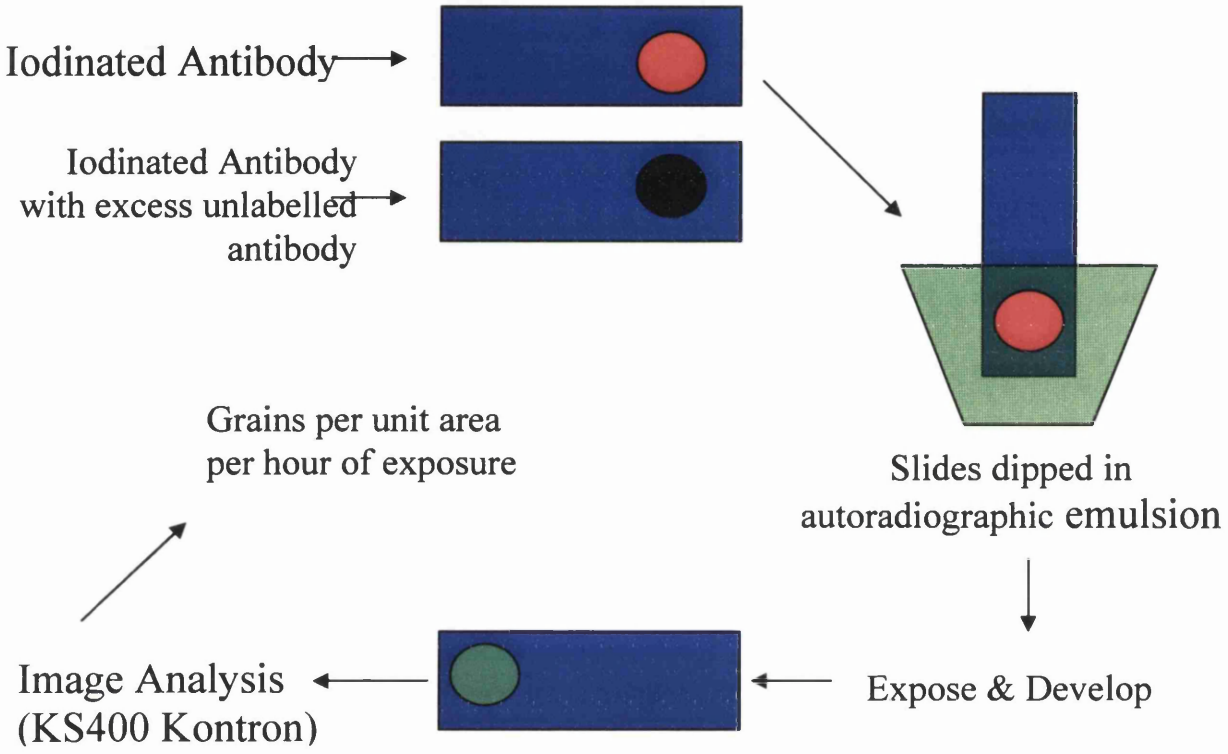

Figure 11. *Schematic diagram of the radioimmunohistochemical method.*

Briefly, this method involves incubation of frozen tissue sections with radioiodinated anti receptor monoclonal antibody to label the receptors. The slides are then coated with autoradiographic emulsion and exposed and developed. This results in silver grains directly over the section. The density of grains developed directly over the tumour cells is counted with an image analysis system. The grain density is converted to receptor density by comparison with a standard curve for each run, created by including sections of pellets of a panel of cultured cells of known receptor expression.

The following protocols were developed for each stage of this technique:

5.3.1 Iodogen catalysed antibody iodination

The EGFR1 monoclonal antibody is of the IgG2 class and recognises an epitope on the external domain of the EGF receptor (306). The ICR12 rat IgG2a monoclonal antibody was raised to the external domain of the HER2 receptor of BT474 cells (307) (a gift from C. Dean, Institute of Cancer Research, Sutton, London). Both antibodies are iodinated by the Iodogen method to a specific activity of 0.5MBq/μg. This method is detailed in Harrow and Lane, Antibodies: A laboratory manual, 1988 (308).

5.3.2 Antibody application

i. Cryostat sections (5μm) are thawed onto silane coated slides and stored at -20°C in airtight packaging until use.

ii. When ready for use the slides are warmed to room temperature before the airtight packaging is removed to prevent the formation of condensation.

iii. To prevent drops of solution running off the tissue sections, the sections are ringed with a Dako pen (Dakopatts Ltd.).

iv. The sections are pre-fixed in 100 % acetone for 5 minutes.

v. Sections are then put through 2 washes of phosphate buffered saline (PBS = 10mM sodium phosphate and 140mM sodium chloride, pH 7.4)

vi. Incubate sections with 100μl of blocking solution for 10 minutes to minimise non-specific binding. For test slides this blocking solution is 50% normal rabbit serum in PBS. For the control sections this blocking solution is 50% normal rabbit serum with unlabelled antibody.

vii. Add 10μl of the radiolabelled antibody. The specific activity of the iodinated antibody was adjusted with unlabelled antibody so that 50ng (4KBq) I^{125}EGFR1 or (50ng 1KBq) I^{125}ICR12 were added to each section. Duplicate test sections and a single control section (which is incubated with a 100-fold excess of unlabelled antibody) were processed for each specimen.

viii. Incubate sections for 3 hours in humidified chambers at 22°C.

ix. The sections are then washed through three 10-minute changes of PBS and fixed for 10 minutes in 2% formaldehyde.

x. Finally, the sections are washed through 3 changes of distilled water before being air dried.

5.3.3 Autoradiography

5.3.3a Determining exposure time.

i. One test slide from each case and cell line used is secured into an X-ray cassette and its position noted.

ii. In a dark room using a Wratten red filtered safe light, the slides are overlaid with X-ray film (Dupont Cronex) and exposed for approximately 72 hours.

iii. This film is then developed and is used as a necessary guide to the optimum length of exposure for the final stage of the preparation, where the slides are dipped in autoradiographic emulsion.

iv. Separate the slides in racks according to the length of exposure. Racks of slides requiring the same exposure are then stored together in containers.

5.3.3b Dipping in autoradiographic emulsion.

This part of the procedure must be carried out in a dark room using a Wratten red filtered safe light. It is essential that neither the emulsion nor the dipped slides are exposed to light at any time. All materials must therefore be brought into the dark room before starting the dipping process. The emulsion used is Kodak NTB-2 diluted 1:1 with distilled water. This is stored in the dark at 4°C.

i. The emulsion is placed in a water bath at 42°C and allowed to liquefy for 1 hour.
ii. Dip several plain slides to ensure that the emulsion is smooth, even and bubble free.
iii. Dip slides in the emulsion and drain excess emulsion from the bottom edge of the slide. Stand slides upright and allow to dry for approximately 30 minutes.
iv. When slides are dry, place them into their appropriate racks, with slides requiring the same exposure times in each rack.
v. Place racks in a container with silica gel as a dehumidifier.
vi. Seal the container, place inside a black bin liner and store at 4°C.
vii. The slides are then exposed for the appropriate time: the highly expressing tumours and cell lines being exposed for 4 hours, the intermediate tissues for 24 and 48 hours and the weakly expressing specimens for 4 to 7 days. The correct exposure results in an optimum density of silver grains for counting. Overexposed sections where large numbers of grains fuse together were not used.

5.3.3c Development of autoradiographs

Once again, this part of the procedure must be carried out in a dark room using a Wratten red filtered safe light. The slides should be allowed to reach room temperature before the container is opened.

i. Remove the slides from the container in the dark room.

ii. The emulsion is developed with Kodak D 19 developer solution (1:1 in distilled water). The developer solution is kept at 10°C by placing it on ice. Place the slides in the developer solution for 4 minutes.

iii. Rinse the slides in distilled water for 1 minute at room temperature.

iv. Place the slides in Kodak Unifix for 5 minutes at room temperature. After this stage the slides may be exposed to light.

v. The slides are then washed for 20 minutes through 4 changes of distilled water and then tap water.

5.3.3d Safranin counterstaining

At this stage the slides contain sections of tissue with silver grains on them. In order to accurately count the grains over the areas of interest, the sections are then counterstained lightly with Safranin O to allow the histology to be viewed.

i. From tap water, place the slides in Scots Tap Water Solution for 0.5-1 minute.

ii. Rinse the slides in tap water.

iii. Place the slides in Safranin O solution for 1-2 minutes.

iv. Rinse the slides in tap water.

Steps i – iv can be repeated at this stage if the staining is not deep enough.

v. The slides are then dehydrated and mounted through standard solutions.

Figures 12 -14 show examples of developed sections.

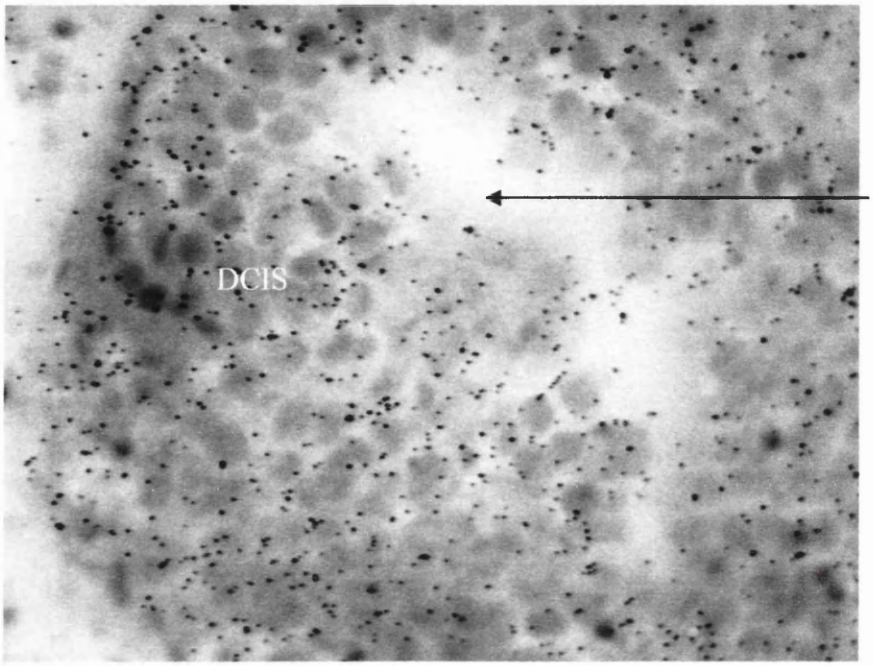

Figure 12.

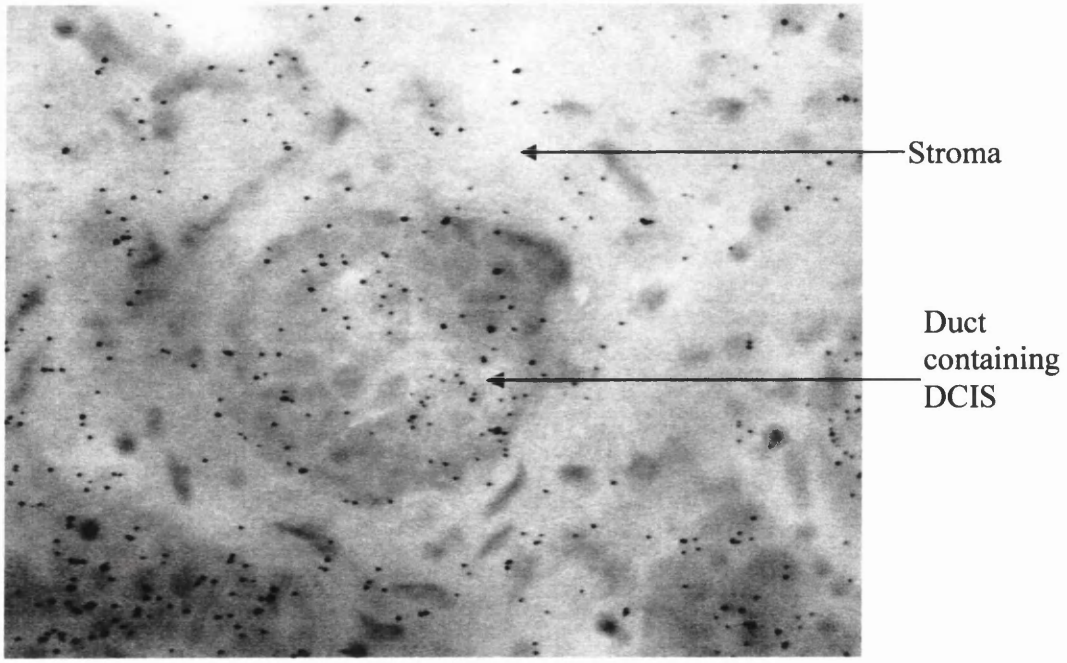

Figure 13.

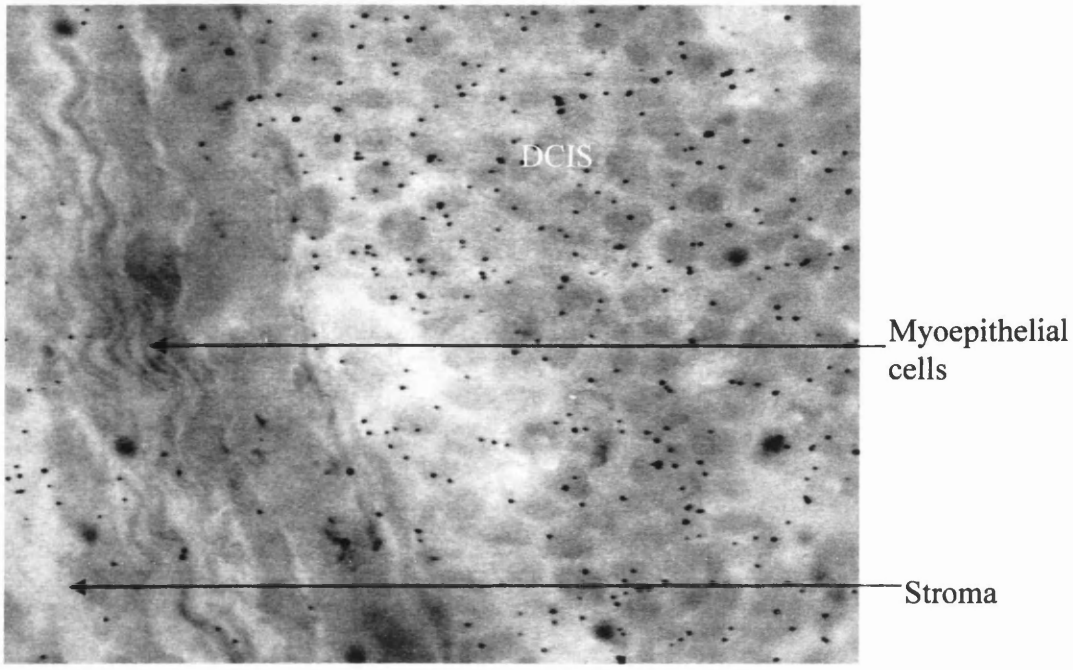

Figure 14.

Figures 12, 13 and 14. *Developed sections at X40 magnification. The number of grains per unit area is then calculated as discussed below.*

5.4 Image Analysis

In the previously reported study applying this method to invasive breast cancers a Joyce-Loebl MiniMagiScan image analysis system was used (2). In this study, expanding the number of invasive cancers and applying the method to DCIS, a Kontron KS-400 system was used. This was attached to an Olympus OM-2 microscope for quantification of silver grain density. The operator defines areas of tumour and the system counts the silver grains per unit area. Counts are made over an average of 10 full or partial fields, with the histology being confirmed by the Safranin O counterstain in conjunction with serial H&E sections. Results were

expressed as grains per unit area per hour of exposure after subtraction of the counts for the control slides.

Figures 15-17 show how the image analysis program quantifies the silver grains directly over the area of interest.

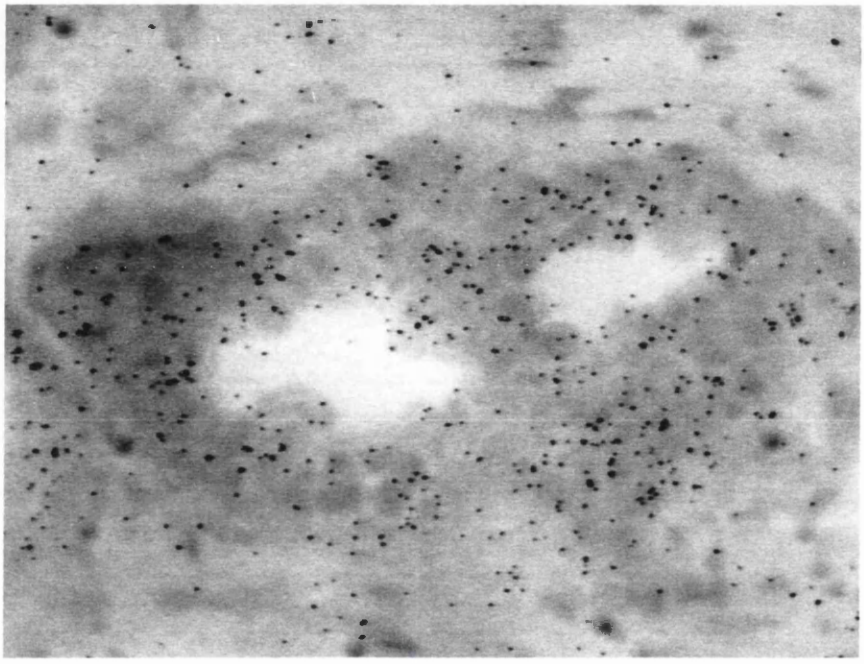

Figure 15. *A developed section at X40 magnification showing the terminal ductolobular unit containing DCIS.*

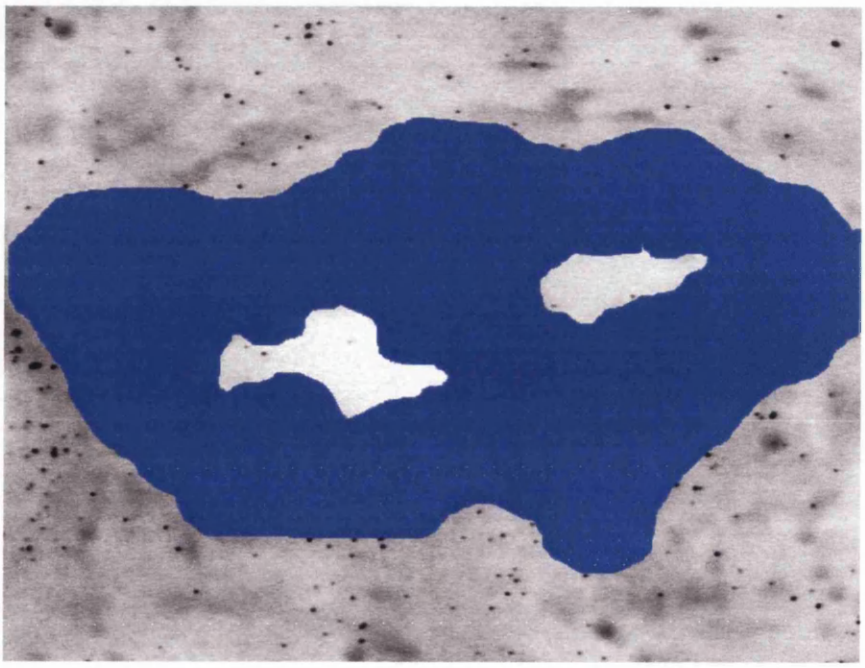

Figure 16. *The operator then draws around the area containing DCIS, excluding stroma and the spaces within the duct.*

Figure 17. *Image analysis software then counts the number of silver grains per unit area.*

5.5 Data Interpretation

Standardisation of results for each set of test sections is achieved by the concomitant running of sections of normal breast and sections of cell pellets. The latter are formed from cell lines with known receptor densities of EGFR and HER2. Pellets are formed by scraping sub confluent cell colonies, which are then centrifuged prior to snap freezing in liquid Nitrogen.

5.5.1 EGFR

For the EGFR assay the A431 cell line is used as a point standard. EGFR levels in the normal specimens was expressed as a percentage of the receptor density of the A431 cell line (which are known to have 2 X 10^6 EGFR/cell) (304;305).

Table 9 below shows the grain counts per unit area for the A431 cell line over the 16 radioimmunohistochemistry runs that formed the basis of this work. This clearly shows that the values did not vary greatly during the various passages.

Date of radioimmunohisto chemistry run	A431 GRAINS		Date of radioimmunohisto chemistry run	A431 GRAINS
06/04/1993	166.0		15/04/1994	160.0
13/04/1993	144.1		18/04/1994	190.0
04/05/1993	140.0		29/04/1994	148.1
06/05/1993	135.3		05/06/1995	121.3
11/05/1993	121.0		06/06/1995	131.6
08/06/1993	206.0		07/06/1995	160.6
10/06/1993	212.2		08/06/1995	152.1
14/04/1994	170.0		17/12/1997	147.1

Table 9. *Grain counts per unit area for the A431 cell line for the 16 radioimmunohistochemistry runs.*

5.5.2 HER2

HER2 expression spans a greater range and thus a number of lines are chosen to provide a similar range of standardisation points. In ascending order, these are the ZR75, MDA-MB-453, MDA-MB-361, BT474 and SKBR3 lines. Using the ICR12 antibody as a radiolabelled ligand, Scatchard analysis of receptor number on sections of cell pellets was performed using the LIGAND program. Ratios of receptor number by this method over grain counts per unit area by radio-immunohistochemistry produce a linear scale. Few tumours fall outwith this scale. By incorporating this scale in each tumour batch, and with the receptor number in each cell line known from the Scatchard analysis, it is possible to define the number of receptors in terms of silver grains per unit area per hour of emulsion exposure. A conversion factor for receptor number per unit area is calculated by averaging the ratios for cell pellets. This, when applied to grain counts for the tumour biopsies in the same batch, allows calculation of receptor numbers.

Cell line	Grain counts per unit area (RIHC)	Receptors by Scatchard analysis (mm^2x10^6)	Ratio
ZR75	0.49	10.9	22.20
MDA-MB-453	1.25	49.3	39.44
MDA-MB-361	3.94	131	33.20
BT474	11.49	581	50.60
SKBR3	21.05	687	32.60

Table 10. *The grain counts per unit area for each of the cell lines used and the corresponding receptors by Scatchard analysis. RIHC = radioimmunohistochemistry.*

Above SKBR3 levels of HER2 expression, the scale loses linearity and receptor expression is underestimated. This applies to a few HER2 amplified tumours in this series but is unlikely to significantly influence the statistical analyses (Rank correlations will still apply). In all cases the quantity of receptor protein in the areas measured by image analysis is then compared with expression in normal breast tissue.

5.6 Patient follow up

The patient population comprised both screen detected patients and symptomatic patients. Symptomatic patients were initially seen at a one-stop breast clinic where the diagnosis was made. All pathology and management decisions were reviewed at a multidisciplinary meeting attended by surgeons, oncologists and the breast pathologist (EAM).

After surgery patients were followed up at 3 monthly intervals for the first 2 years then 6 monthly for the next 3 years and annually thereafter. If breast-conserving surgery had been performed, mammograms were performed on the affected breast annually and the contralateral breast every 2 years. If treatment was by mastectomy a mammogram was performed on the contralateral breast every 2 years.

After each clinic visit, details of follow up were entered into a database prospectively. The database management conformed to the data protection act.

5.7 Ethics

The study received ethical approval from the local ethics committee. In the late 1980's and early 1990's it was not deemed necessary to obtain individual consent to keep discarded tissue following surgery. In the latter part of collection, generic consent was obtained to use this tissue for research purposes.

SECTION 6 RESULTS

6.1 Tumour characteristics

6.1.1 Pure DCIS

Forty samples of pure DCIS were obtained as described in the Materials and Methods section. These were classified according to the Van Nuys DCIS Classification system as shown below in Figure 18 and Table 11.

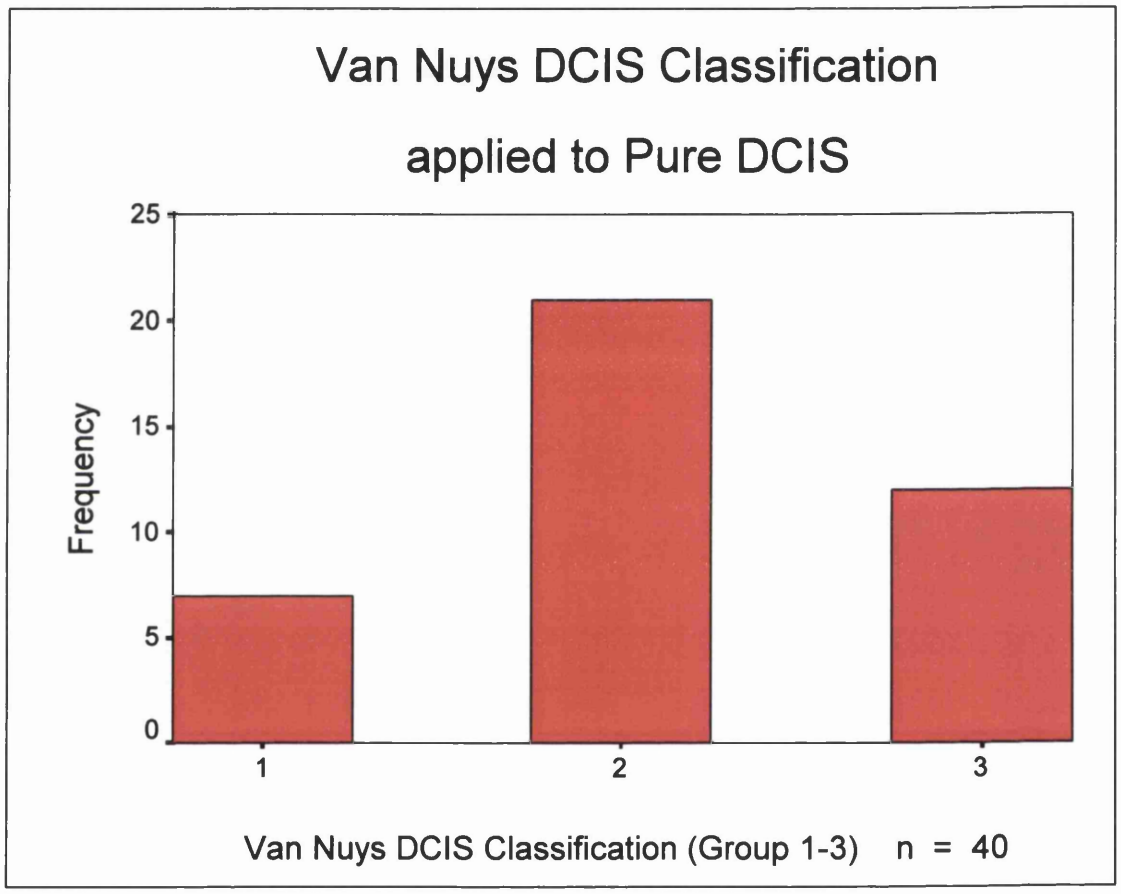

Figure 18. *Pure DCIS cases stratified according to the Van Nuys DCIS Classification system vs. Frequency. n = 40*

VNCS* Group	N	%
1	7	17.5
2	21	52.5
3	12	30.0
Total	40	100.0%

Table 11. *Pure DCIS according to Van Nuys DCIS Classification system groups 1-3. *VNCS = Van Nuys DCIS Classification system.*

6.1.2 DCIS associated with invasive disease

Fifty cases of DCIS in association with invasive disease were obtained and classified according to the Van Nuys DCIS Classification system as shown below in Figure 19 and Table 12.

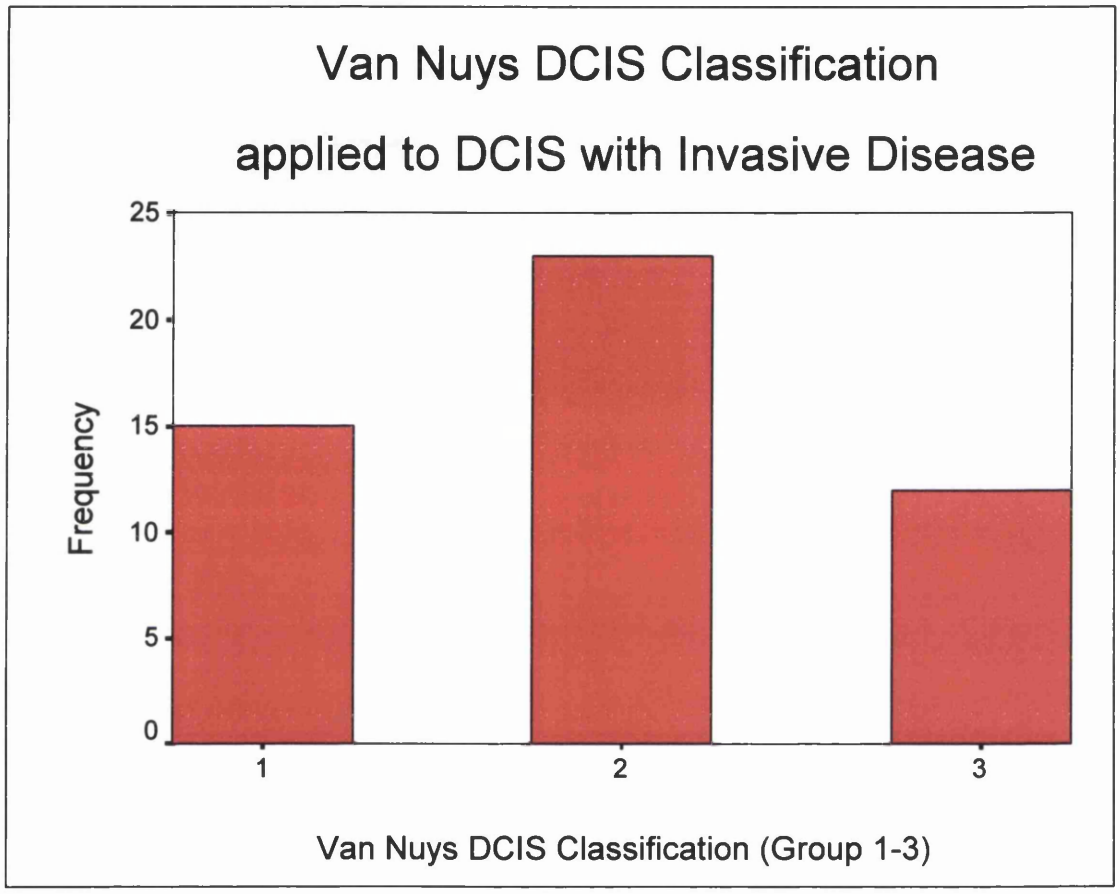

Figure 19. *Cases of DCIS associated with invasive disease stratified according to the Van Nuys DCIS Classification system vs. Frequency.* $n = 50$

VNCS* Group	N	%
1	15	30.0
2	23	46.0
3	12	24.0
Total	50	100%

Table 12. *DCIS associated with invasive disease according to Van Nuys DCIS Classification system groups 1-3. *VNCS = Van Nuys DCIS Classification system.*

6.2 Results for EGFR

6.2.1 EGFR expression in normal breast tissue.

EGFR levels in the normal specimens are expressed as a percentage of the receptor density of a cell line standard, A431. A431 cells are known to have 2 X 10^6 EGFR/cell (305;309). As described in the Materials and methods section, standardisation of results for each set of test sections is achieved by the concomitant running of sections of normal breast and sections of cell pellets. The latter are formed from cell lines with known receptor densities of EGFR and HER2. As shown in Table 13, normal levels of EGFR expression ranged from 5.2 to 10%, with a mean of 7.6% of A431 levels of expression.

Number	Range	Minimum	Maximum	Mean
9	4.79	5.21	10.0	7.61

Table 13. *EGFR expression in normal breast tissue expressed as a percentage of A431 levels of expression.*

In the analyses below, the level of expression of EGFR is expressed relative to the mean level of expression in these normal samples. Levels of EGFR expression are expressed on a log scale as they vary by a factor of several thousand. The reference line on the graphs is at the zero point and represents the level of expression in normal breast tissue.

6.2.2 EGFR expression in pure DCIS.

The frequency distribution of EGFR in pure DCIS is shown in Figure 20. All tumours show lower levels of expression than control breast reduction specimens.

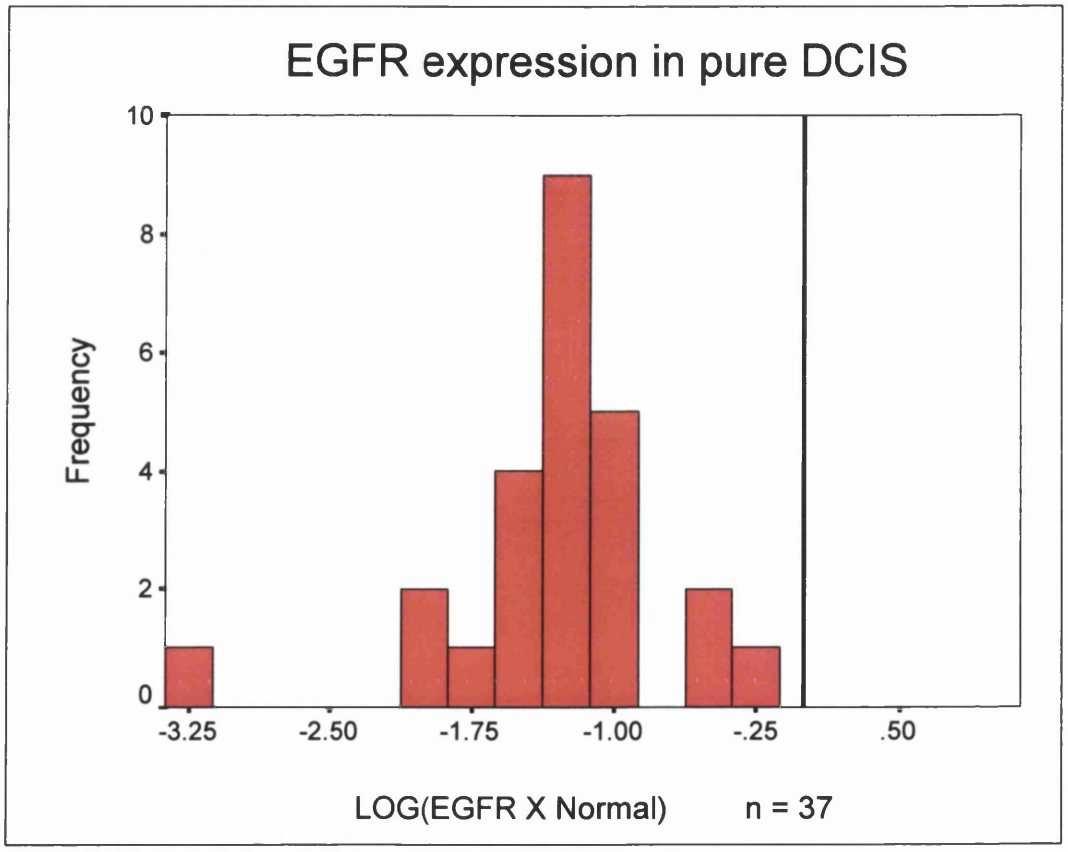

Figure 20. *Log_{10}(EGFR X Normal) vs. Frequency for pure DCIS.*

6.2.3 EGFR expression in pure DCIS as classified by the Van Nuys DCIS Classification system.

Levels of EGFR expression in pure DCIS groups 1, 2 and 3 of the Van Nuys DCIS Classification system were analysed using the Kruskal-Wallis test. There was no difference in EGFR expression between the 3 groups ($p = 0.585$). The descriptive statistics for expression of EGFR in these groups are shown in Table 14.

VNCS* Group	N	Median	Interquartile range
1	7	0.031	0 – 0.062
2	20	0.033	0 – 0.068
3	10	0.043	0 – 0.17
Total	37		

Table 14. *Descriptive statistics for EGFR expression in pure DCIS according to Van Nuys DCIS Classification system groups 1-3. *VNCS = Van Nuys DCIS Classification system.*

This implies that the level of EGFR expression is the same in each pure DCIS group as classified by the Van Nuys DCIS Classification system.

6.2.4 EGFR expression in DCIS associated with invasive disease

6.2.4.1 EGFR expression in DCIS associated with invasive disease – the DCIS component

The frequency distribution of EGFR in the DCIS component of DCIS associated with invasive disease is shown in Figure 21. All but 1 tumours show lower than control levels of expression.

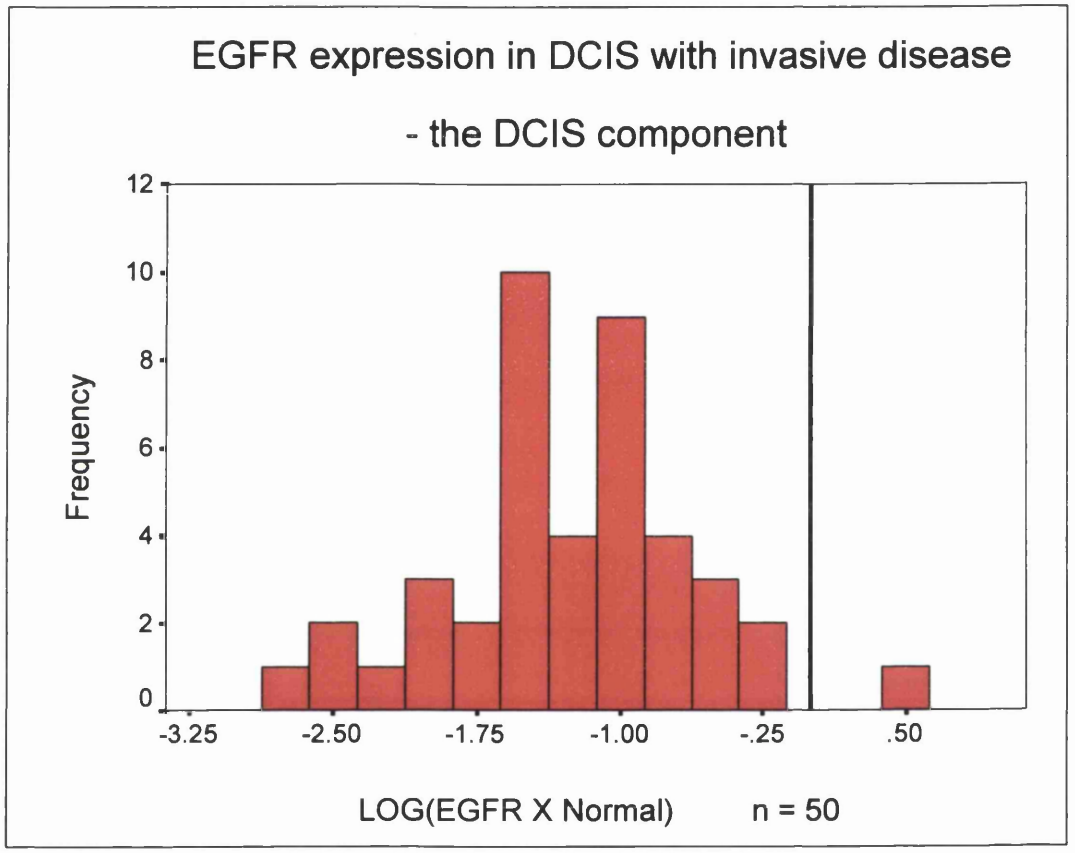

Figure 21. *Log_{10}(EGFR X Normal) vs. Frequency for the DCIS component of DCIS associated with invasive disease.*

6.2.4.2 EGFR expression in DCIS associated with invasive disease – the invasive component

The expression of EGFR of the invasive component for these same tumours is shown in Figure 22. All but 1 tumours downregulate EGFR in relation to normal breast. The tumour which had very high expression of EGFR in the DCIS component also had very high expression in the invasive component.

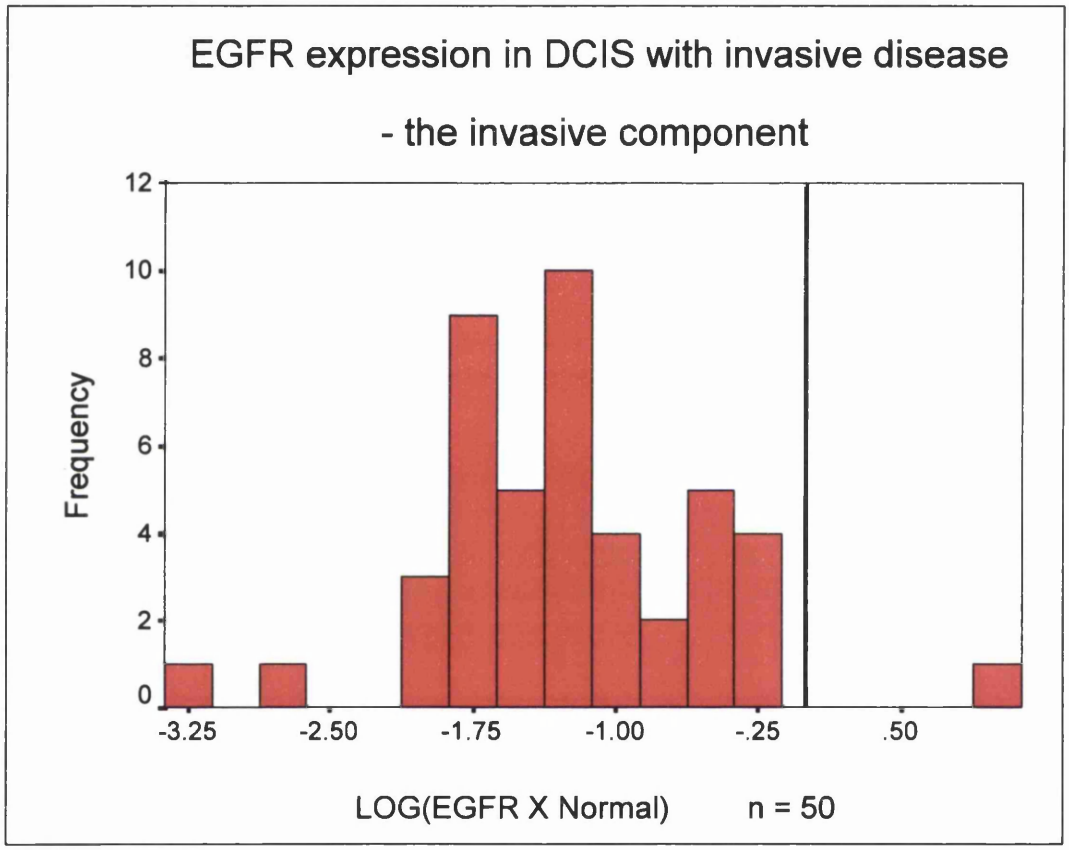

Figure 22. *Log_{10}(EGFR X Normal) vs. Frequency for the invasive component of DCIS associated with invasive disease.*

The expression of EGFR in the DCIS and invasive components of each tumour is similar. Indeed, when analysed using the Wilcoxon Signed Rank test, there is no significant difference in the expression of EGFR ($p = 0.39$) in each component of the tumours. This is shown in figure 23 below.

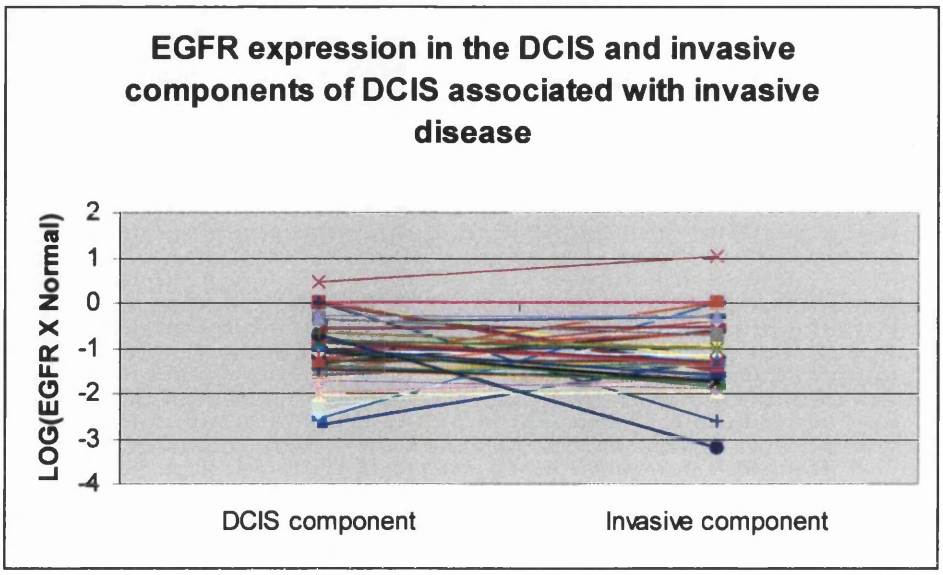

Figure 23. *EGFR expression in the DCIS and invasive components of each tumour.*

Although overall there appears to be no statistically significant difference between these 2 components of the tumours, some interesting findings can be found comparing the level of EGFR expression in the in situ versus the invasive components of the tumours of individual patients. In the graph above there are clearly some individuals whose expression of EGFR varies considerably in the in situ and invasive components. This is shown graphically in figure 24 below.

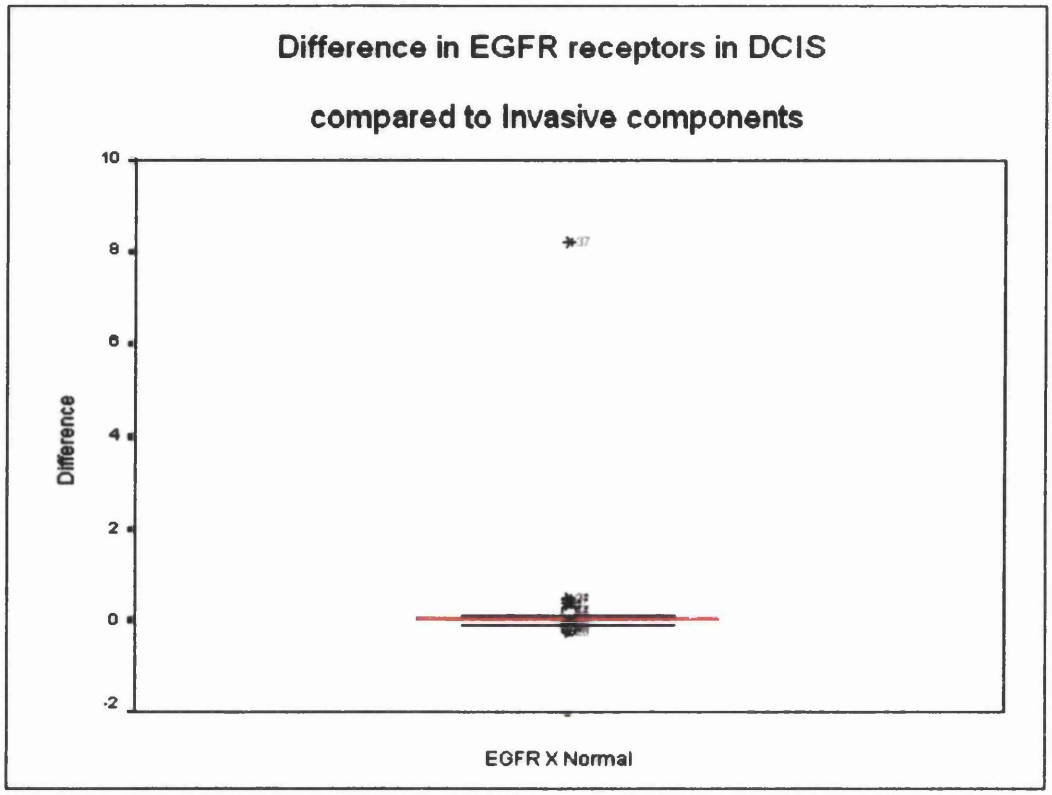

Figure 24. *The size of the difference in EGFR expression for those tumours with both DCIS and invasive components.*

The 6 tumours with the largest change in expression from moving to in situ to invasive disease are presented in Table 15 below. The Nottingham Prognostic Index for the invasive components of these tumours are also listed. Unfortunately only 3 of these 6 scores were available as one tumour was diffuse and the size of the other 2 tumours was not recorded.

Patient ID	EGFR receptors in DCIS	EGFR receptors in invasive component	Difference	NPI
DCIV37	2.77	10.98	8.21	N/A
DCIV24	0.02	0.51	0.49	N/A
DCIV27	0.00	0.46	0.45	5.4
DCIV4	0.06	0.41	0.35	5.5
DCIV47	0.03	0.24	0.20	4.6
DCIV26	0.45	0.19	-0.26	N/A

Table 15. *The 6 tumours with the greatest change in EGFR expression between the in situ and invasive components. A positive value implies an increase in EGFR expression and a negative value a decrease. NPI = Nottingham Prognostic Index. N/A = Not available.*

The NPI for 37 of the 50 invasive tumours was available, with a median of 4.40 (interquartile range = 2.02). As only 3 of the 6 tumours with the largest change in EGFR expression had an NPI available the median here is 5.4. Using the Mann-Whitney U test there was no statistically significant difference in the NPI between those tumours that had the largest difference in EGFR expression in the in situ compared to the invasive components of the tumour and the other invasive tumours that had associated DCIS (p=0.13). There does however appear to be a trend towards significance and certainly those tumours that had a large change in

EGFR expression appeared to do badly.

Patient DCIV37 with the largest such increase in EGFR expression between the in situ and invasive components in this series had a very aggressive tumour with 6 out of 6 lymph nodes tested being positive. The tumour was multifocal and there was widespread lymphatic permeation and soft tissue metastases in the axilla.

Patient DCIV24 had a poorly differentiated tumour with a positive lymph node and developed liver metastases.

Patient DCIV27 had a lobular carcinoma with an NPI of 5.4 and 19 out of 19 positive lymph nodes.

Patient DCIV4 also had a ductal carcinoma with an NPI of 5.5 and 4 out of 7 lymph nodes involved with tumour.

Patient DCIV47 also had a node positive breast cancer which recurred in the chest wall post mastectomy and later recurred in the axilla.

Patient DCIV26 had the largest fall in EGFR expression between the DCIS and invasive components of the tumour. This was also an aggressive lesion with 5 out of 9 lymph nodes positive and went on to develop pulmonary metastases.

While this data is not scientific and the change in EGFR expression between the DCIS and invasive components of the tumours does not reach statistical significance, it is interesting to note these individual cases of clearly aggressive tumours where the change in expression from DCIS to invasive disease was greatest.

6.2.4.3 EGFR expression in DCIS associated with invasive disease as classified by the Van Nuys DCIS Classification system.

Levels of EGFR expression in DCIS associated with invasive disease were stratified into groups 1, 2 and 3 of the Van Nuys DCIS Classification system and then analysed using the Kruskal-Wallis test. There was no difference in EGFR expression between the 3 groups ($p = 0.053$). Although this was just above the level required for statistical significance, after Bonferroni's correction (which adjusts for the fact that multiple comparisons are being made), this p value becomes even less significant ($p_{corr} = 0.156$). The descriptive statistics for expression of EGFR in these groups are shown in Table 16.

VNCS* Group	N	Median	Interquartile range
1	12	0.02	0.0032 – 0.037
2	21	0.051	0.02 – 0.164
3	14	0.097	0.019 – 0.315
Total	47		

Table 16. *Descriptive statistics for EGFR expression in DCIS associated with invasive disease according to Van Nuys DCIS Classification system groups 1-3.*

**VNCS = Van Nuys DCIS Classification system.*

6.2.5 EGFR expression in invasive disease

The frequency distribution for the levels of EGFR expression in the 193 cases studied is shown in Figure 25. Only 5 of these lesions had EGFR expression above that of the normal breast controls. 97.4% or 188 lesions had a lower than normal expression of EGFR.

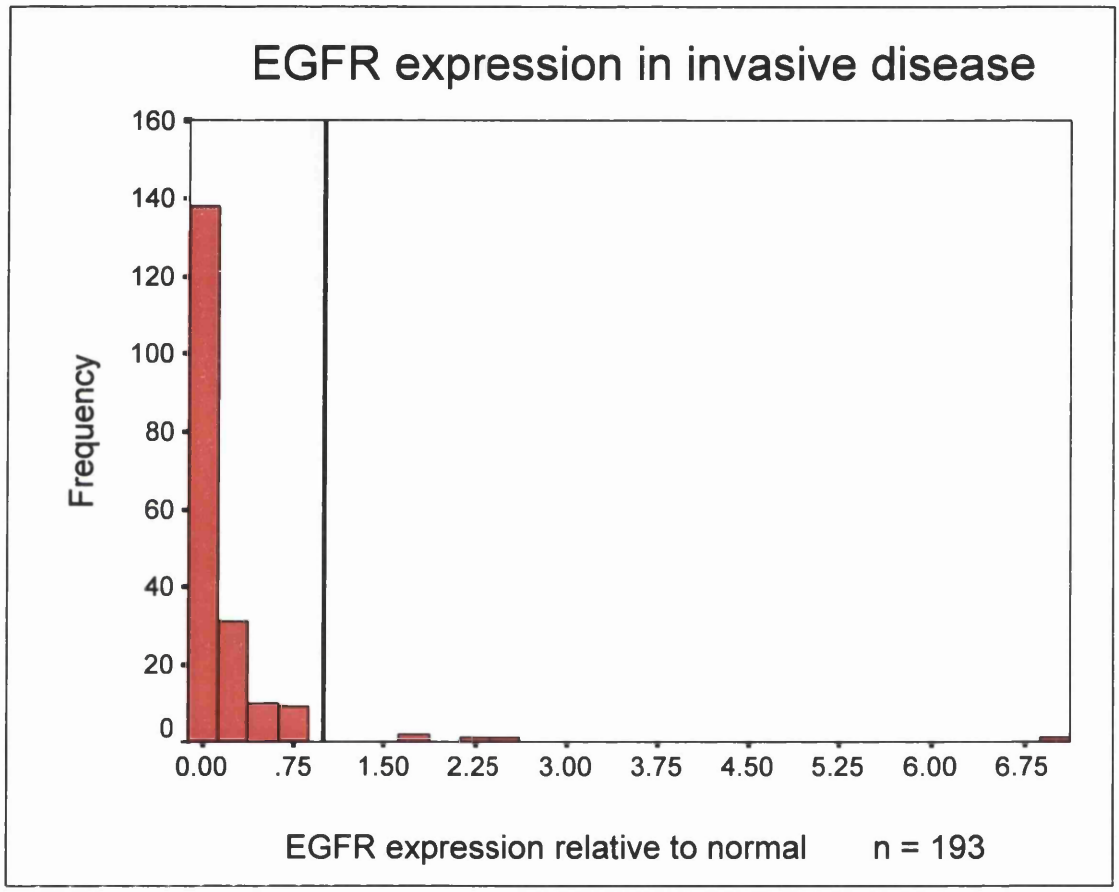

Figure 25. *EGFR X Normal vs. Frequency for all invasive tumours. Most of these lesions downregulate EGFR.*

6.2.6 Frequency distributions of EGFR expression within the spectrum of breast cancer

The frequency distributions for EGFR for pure DCIS, DCIS associated with invasive disease and invasive disease alone, were compared using the Mann-Whitney U test. This is shown pictorially in Figure 26. There was found to be no significant difference between the frequency distributions of:

pure DCIS as compared to DCIS with invasion (Mann Whitney U test, $p = 0.17$),

or

DCIS with invasion compared to pure invasive lesions (Mann Whitney U test, $p = 0.16$).

Comparing pure DCIS with pure invasive lesions there is a difference between the frequency distributions (Mann Whitney U test, $p = 0.009$). This may reflect a subtle change in EGFR expression as the in situ lesions evolve into invasive cancers or it may simply reflect a type 2 statistical error in view of the small number of pure in situ tumours involved.

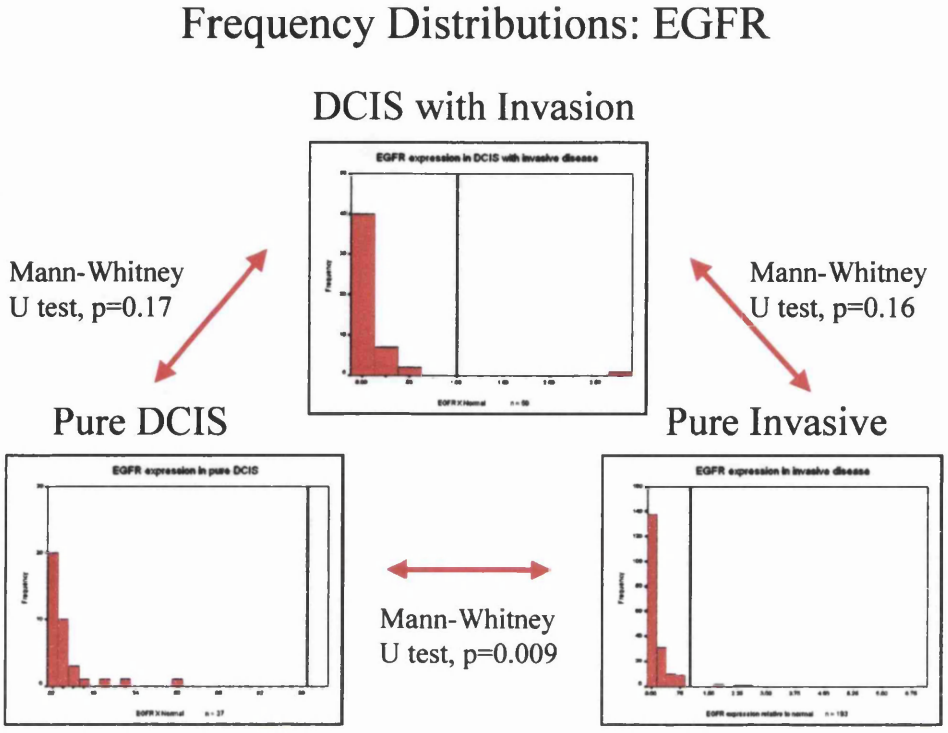

Figure 26. *The frequency distributions of growth factor expression for EGFR within the spectrum of breast cancer.*

The components of the Van Nuys DCIS Classification system i.e., nuclear grade and necrosis, have been shown to be important predictors of the probability of local recurrence after breast conservation treatment for DCIS (36;37;39;43;45-47). These data show that EGFR is not overexpressed in pure DCIS when compared with samples of normal breast tissue. Furthermore, they suggest that EGFR expression is at the same low level irrespective of the aggressiveness of the DCIS. The level of expression of EGFR is not significantly different throughout its range, as shown by the non-significant p value obtained from performing the Kruskal-Wallis one-way analysis of variance. The more aggressive forms of DCIS in group 3 of the Van Nuys DCIS Classification system had a similar overall level of EGFR expression compared to the less aggressive forms in groups 1 and 2. This was true in both the pure DCIS tumours and DCIS associated with invasive disease, which has an already proven aggressive phenotype. This would also suggest that EGFR is not a major factor in determining the progression of DCIS to invasive disease.

6.3 Results for HER2

6.3.1 HER2 expression in normal breast tissue

HER2 expression was calculated as described in the Materials and methods section. The expression in samples obtained from breast reduction specimens is shown below in Table 17. The mean expression is 8.56 grains per $(mm)^2$.

Number	Range	Minimum	Maximum	Mean
8	8.52	4.42	12.9	8.56

Table 17. *HER2 expression in normal breast tissue.*

While there were 9 reduction mammoplasty results for EGFR there were only 8 for HER2 as, following processing for HER2, there was no identifiable breast tissue in one of the specimens.

As was done for EGFR, in the analyses below, the level of expression of HER2 is expressed relative to the mean level of expression in these normal samples.

6.3.2 HER2 expression in pure DCIS

The frequency distribution of HER2 in pure DCIS is shown in Figure 27. As with EGFR expression, levels of HER2 expression are expressed on a log scale as they varied by a factor of several thousand. As shown in figure 26, HER2 expression appears to be bimodal. Only 3 tumours have levels lower than normal tissue. The first peak, 21 tumours (68%) includes these 3 tumours and has a range of expression from 0.07 to 6.8 times normal. The second frequency peak contains 15 tumours (42%) within which expression ranges from 24 to 180 times normal. In total, 92% of tumours overexpress HER2.

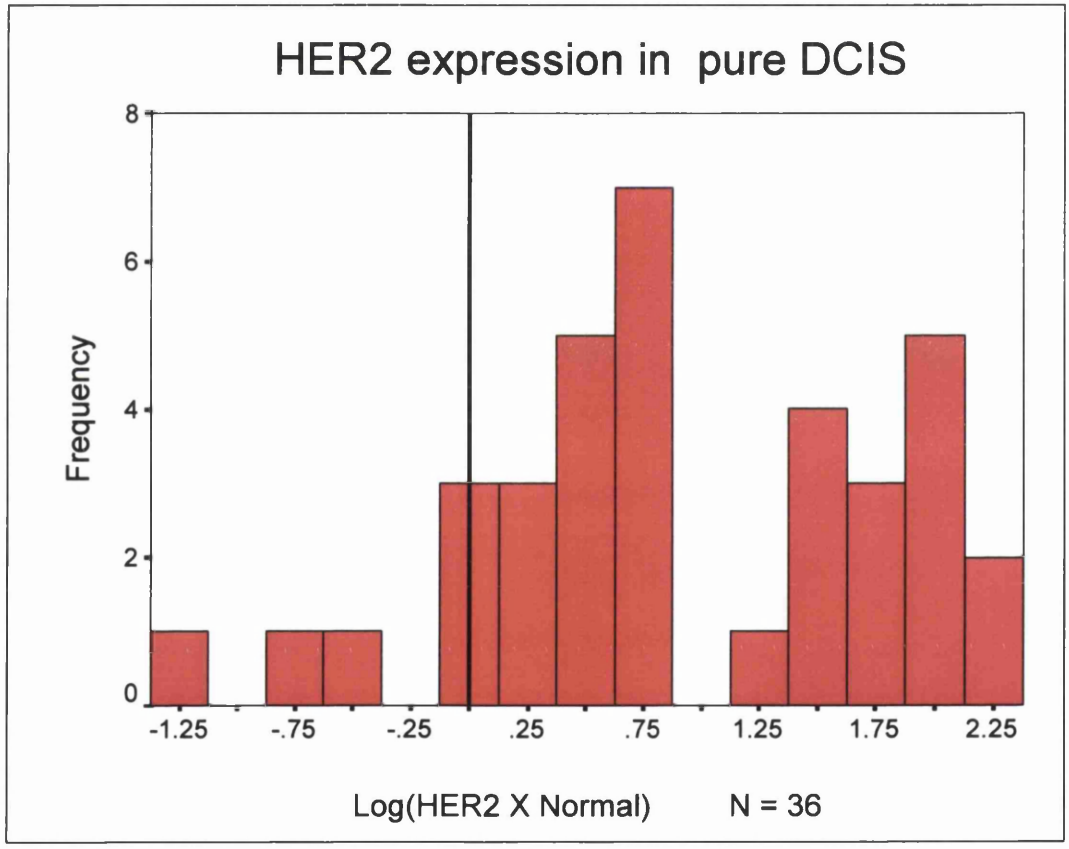

Figure 27. *Log_{10}(HER2 X Normal) in pure DCIS vs. Frequency.*

6.3.3 HER2 expression in pure DCIS as classified by the Van Nuys DCIS Classification system

Levels of HER2 expression in pure DCIS were stratified into groups 1, 2 and 3 of the Van Nuys DCIS Classification system and then analysed using the Kruskal-Wallis one-way analysis of variance. The descriptive statistics for expression of HER2 in these groups are shown in Table 18.

VNCS* Group	N	Median	Interquartile range
1	6	1.96	0.28 – 5.05
2	19	3.64	1.6 – 6.8
3	11	55.31	39.1 – 120.5
Total	36		

Table 18. *Descriptive statistics for HER2 expression in pure DCIS according to Van Nuys DCIS Classification system groups 1-3. *VNCS = Van Nuys DCIS Classification system.*

There was a significant difference in HER2 expression between the 3 groups ($p < 0.0001$) by the Kruskal-Wallis test. The Mann-Whitney test was then carried out to further explore this highly statistically significant difference:

- No difference was found between groups 1 and 2 ($p = 0.16$).
- A highly significant difference was found between groups 1 and 3 ($p = 0.001$).
- A highly significant difference was found between groups 2 and 3 ($p < 0.0001$).

The expression of HER2 in these groups is further presented below in the form of a boxplot graph (Figure 28). This clearly shows that although the interquartile range for HER2 expression within group 3 of the Van Nuys DCIS Classification system is very wide (81.42) there is little overlap with expression in group 2.

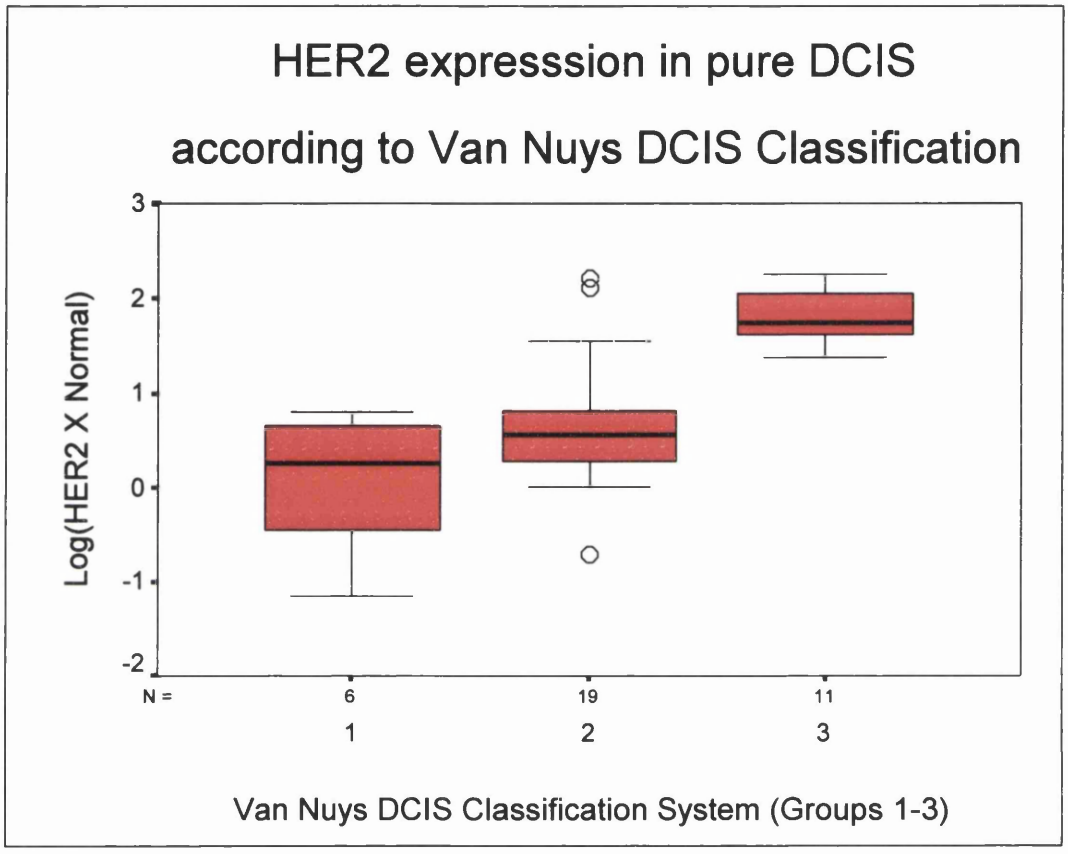

Figure 28. *Expression of HER2 in pure DCIS according to groups 1-3 of the Van Nuys DCIS Classification system.* ***O*** *denotes outliers within group 2.*

6.3.4 HER2 expression in DCIS associated with invasive disease

The frequency distribution of HER2 in DCIS associated with invasive disease is shown in Figure 29. All but 4 of these tumours overexpress HER2. As with pure DCIS, expression of HER2 in these lesions is bimodal. The first peak, 36 tumours (76.6%), has a range of expression from 0.13 to 11.3 times normal, and the second has 11 tumours (23.4%) with a range of expression from 29.5 to 324 times normal expression.

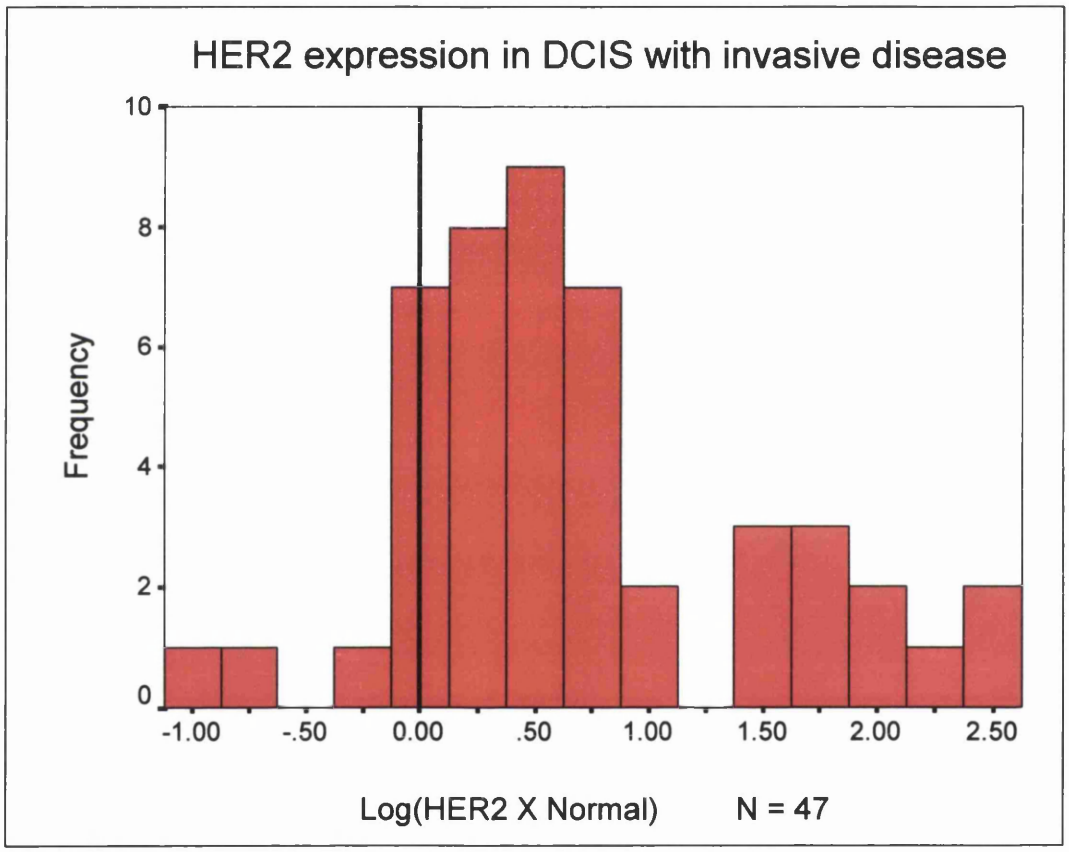

Figure 29. *HER2 expression in the DCIS component of DCIS associated with invasive disease vs. Frequency.*

The expression of HER2 in the DCIS component was compared with the expression in the invasive component of these same tumours using the Wilcoxon Signed Rank test with no significant difference in the expression of HER2 in each

component of the tumours (p = 0.087). This is shown in figure 30 below.

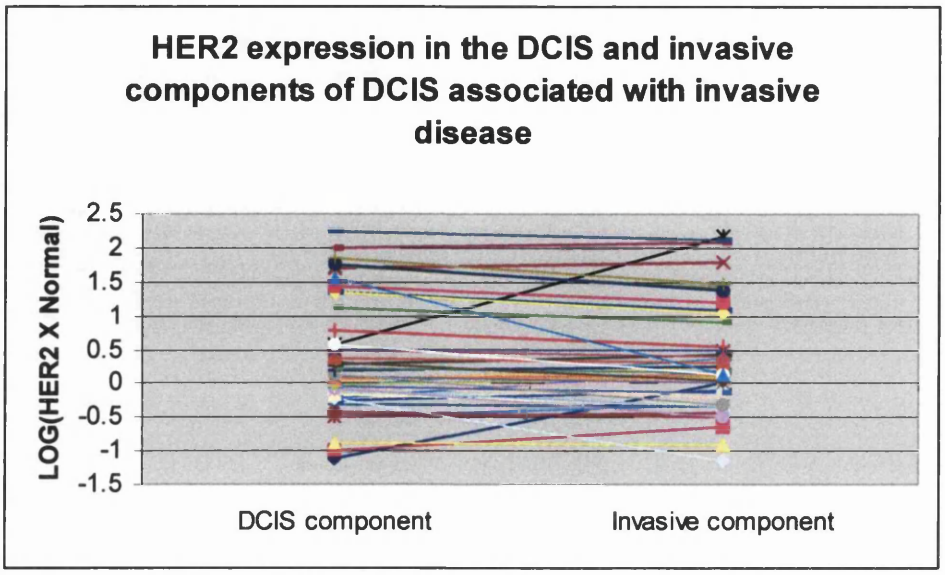

Figure 30. *Expression of HER2 in the DCIS and invasive components of each tumour.*

Although overall there appears to be no statistically significant difference between these 2 components of the tumours, as is the case with EGFR expression, some interesting findings can be found comparing the level of HER2 expression in the in situ versus the invasive components of the tumours of individual patients. In the graph above there are clearly some individuals whose expression of HER2 varies considerably in the in situ and invasive components. This is shown graphically in figure 31 below.

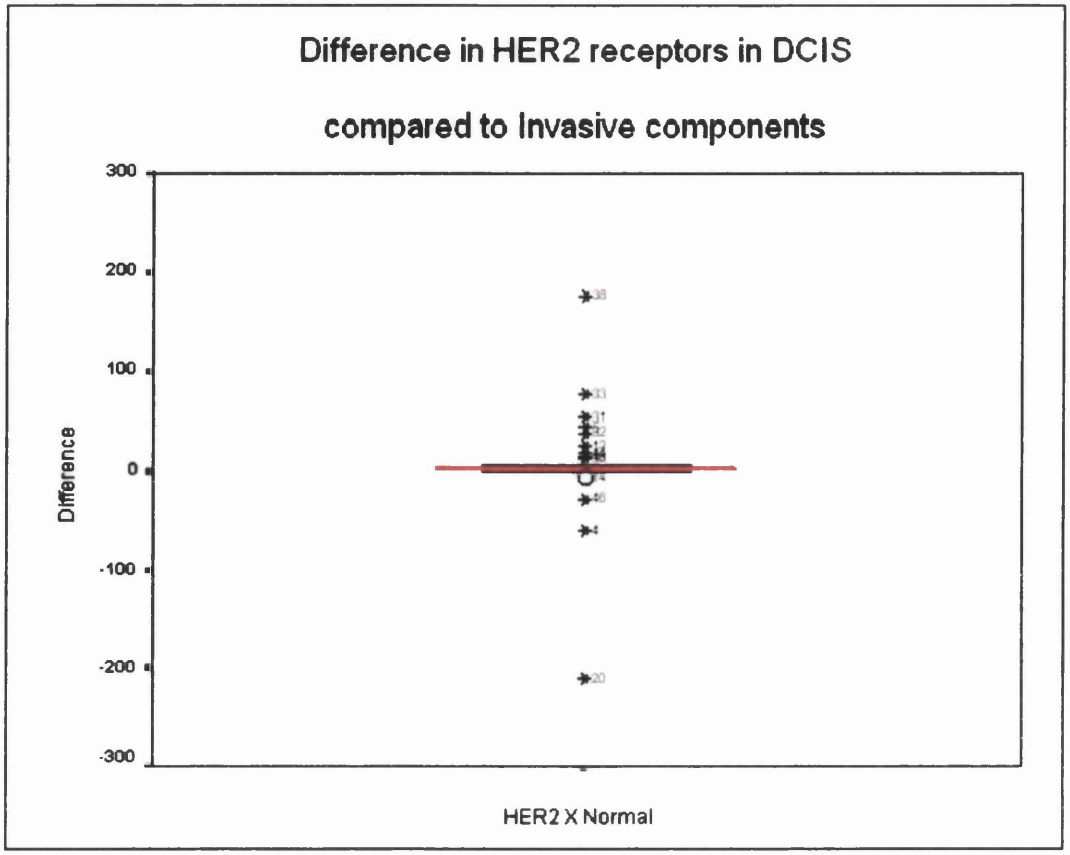

Figure 31. *The size of the difference in HER2 expression for those tumours with both DCIS and invasive components.*

The 8 tumours with the largest change in HER2 expression from moving to in situ to invasive disease are presented in Table 19 below. The Nottingham Prognostic Index for the invasive components of these tumours are also listed. Unfortunately only 6 of these 8 scores were available as one tumour was diffuse and the size of the other tumour was not recorded.

Patient ID	EGFR receptors in DCIS	EGFR receptors in invasive component	Difference	NPI
DCIV39	5.39	217.11	211.72	4.80
DCIV5	264.20	324.22	60.02	6.00
DCIV47	126.02	154.61	28.59	4.60
DCIV52	70.17	31.99	-38.17	4.70
DCIV29	72.52	29.35	-43.18	2.40
DCIV30	90.93	36.68	-54.25	N/A
DCIV37	324.19	246.57	-77.62	N/A
DCIV14	183.27	6.85	-176.42	4.70

Table 19. *The 8 tumours with the greatest change in HER2 expression between the in situ and invasive components. A positive value implies an increase in HER2 expression and a negative value a decrease. NPI = Nottingham Prognostic Index. N/A = Not available.*

The NPI for 34 of the 47 invasive tumours was available, with a median of 4.40 (interquartile range = 1.64). For the 6 tumours with the largest change in HER2 expression which had an NPI available the median here is 4.70 (interquartile range = 1.05). Using the Mann-Whitney U test there was no statistically significant difference in the NPI between those tumours that had the largest difference in HER2 expression in the in situ compared to the invasive components of the tumour and the other invasive tumours that had associated DCIS (p=0.31).

However, as with EGFR, those tumours that had a large change in HER2 expression between the in situ and invasive components did appear to do badly.

Patient DCIV39 with the largest such increase in HER2 expression between the in situ and invasive components in this series had a very aggressive tumour with 2 of 2 lymph nodes tested being positive. A year later she unfortunately developed a local recurrence treated by mastectomy but developed liver metastases the following year.

Patient DCIV5 had a large 50mm tumour with a positive lymph node (1 of 4) and died from metastatic breast cancer less than 2 years from the time of diagnosis.

Patient DCIV47 had a node positive breast cancer which recurred in the chest wall post mastectomy and later recurred in the axilla.

Patient DCIV52 had a ductal carcinoma with an NPI of 4.7 which was node negative (0 out of 6sampled). Unfortunately she died 7 years after diagnosis from metastatic breast cancer.

Patient DCIV29 had a node negative breast cancer with an NPI of 2.4 and was alive and well at last follow up (June 1997, 9 years after diagnosis).

Patient DCIV30 had a large 120mm tumour excised which was Grade 3 but did not have any lymph nodes found on sampling. She unfortunately died from metastatic breast cancer within a year of diagnosis.

Patient DCIV37 is the same patient with the largest increase in EGFR expression between the in situ and invasive components (page 108). She had a very aggressive tumour with 6 out of 6 lymph nodes tested being positive. The tumour was multifocal and there was widespread lymphatic permeation and soft tissue metastases in the axilla. She also died just 2 years after diagnosis of breast cancer.

Patient DCIV14 had a 35mm, node negative breast cancer treated by mastectomy and axillary node clearance with an NPI of 4.70. Unfortunately she also succumbed to metastatic breast cancer 5 years after diagnosis.

As highlighted earlier, these do not represent consecutive cases and are small numbers. Nevertheless, it is interesting to note these individual cases of clearly aggressive tumours where the change in expression from DCIS to invasive disease was greatest. This perhaps indicates an area for future work as a means of identifying aggressive forms of breast cancer where there is an identifiable in situ component.

6.3.5 HER2 expression in DCIS associated with invasive disease as classified by the Van Nuys DCIS Classification system

Levels of HER2 expression in DCIS associated with invasive disease were stratified into groups 1, 2 and 3 of the Van Nuys DCIS Classification system and then analysed using the Kruskal-Wallis test. The descriptive statistics for expression of HER2 in these groups are shown in Table 20.

VNCS* Group	N	Median	Interquartile range
1	13	2.88	1.42 – 5.55
2	18	4.45	2.5 – 29.7
3	16	3.10	1.2 – 71.9
Total	47		

Table 20. *Descriptive statistics for HER2 expression in pure DCIS according to Van Nuys DCIS Classification system groups 1-3. *VNCS = Van Nuys DCIS Classification system.*

There was no difference in HER2 expression between the 3 groups ($p = 0.4$) using the Kruskal-Wallis test.

The expression of HER2 in these groups is further presented below in the form of a boxplot graph (Figure 32).

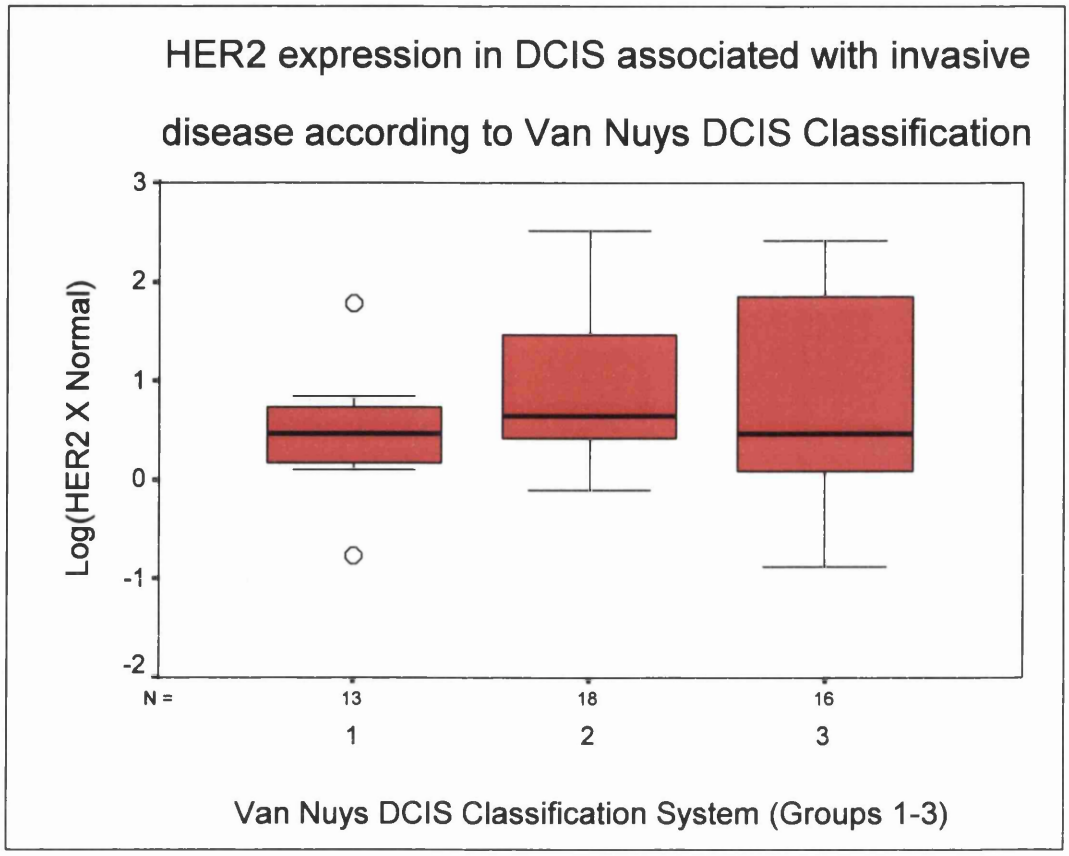

Figure 32. *Expression of HER2 in DCIS associated with invasive disease according to groups 1-3 of the Van Nuys DCIS Classification system.* ○ *denotes outliers within the groups.*

6.3.6 HER2 expression in invasive disease

The frequency distribution for the levels of HER2 expression in the 177 cases studied is shown in Figure 33. As with the expression of HER2 in pure DCIS and in DCIS associated with invasive disease, this distribution is again bimodal, with a nadir at about 15 times normal expression level. Twenty-six tumours (15%) had levels lower than those in the normal breast controls, of which only 2 had no detectable expression of HER2. Forty-one cases (23%) had levels more than 15 times normal. Previous studies by this group have shown that a level of 15 times normal expression corresponds to gene amplification (1). The remaining 110 tumours (62%) showed levels of HER2 expression between 1 and 15 times normal.

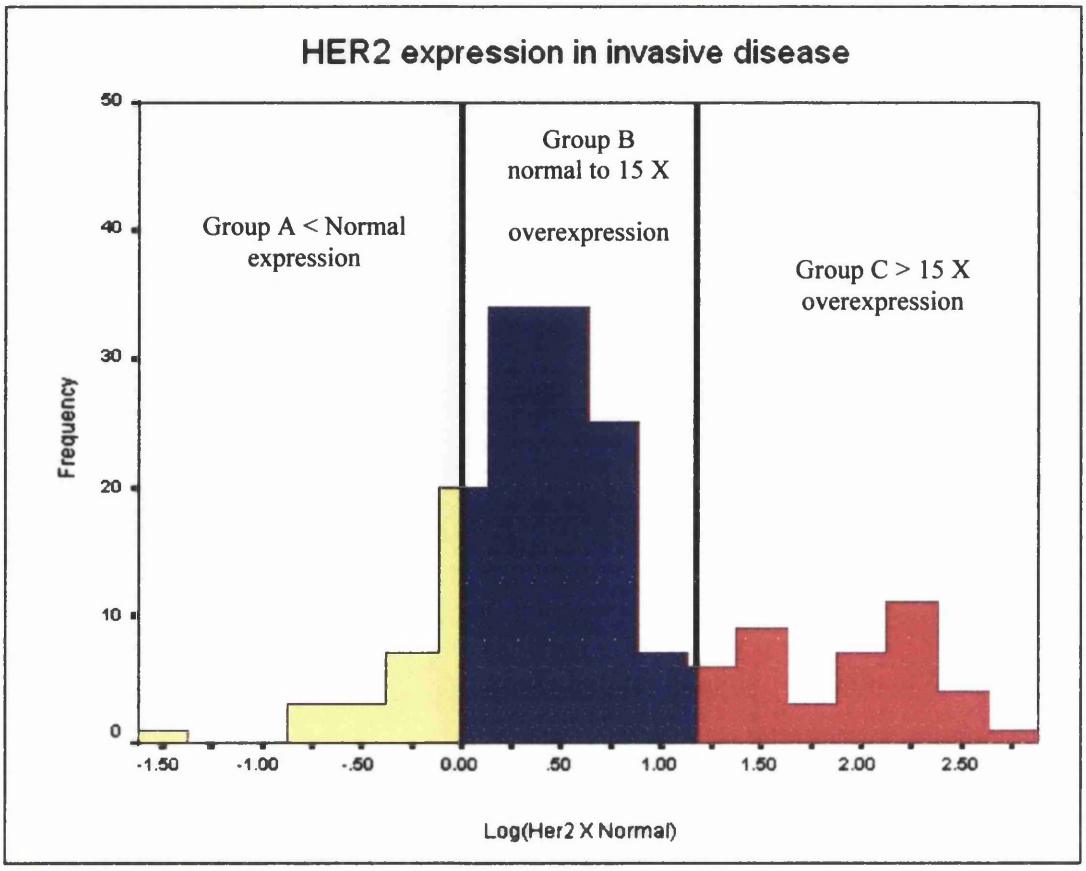

Figure 33. *Log_{10}(HER2 X Normal) vs. Frequency for all invasive tumours.*

This shows the 3 groups of HER2 expression in invasive disease:

Group A where expression is less than that of normal breast tissue.

Group B where expression is between normal and 15 times normal expression.

Group C where expression is greater than 15 times normal expression.

6.3.7 Frequency distributions of HER2 within the spectrum of breast cancer

The frequency distributions for HER2 in pure DCIS, DCIS associated with invasive disease and invasive disease alone, were compared using the Mann-Whitney U test. This is shown pictorially in Figure 34. There was found to be no significant difference between the frequency distributions of:

- pure DCIS as compared to DCIS with invasion (Mann Whitney U test, p = 0.16),

or

- DCIS with invasion compared to pure invasive lesions (Mann Whitney U test, p = 0.91).

- pure DCIS compared to pure invasive lesions (Mann Whitney U test, p = 0.07).

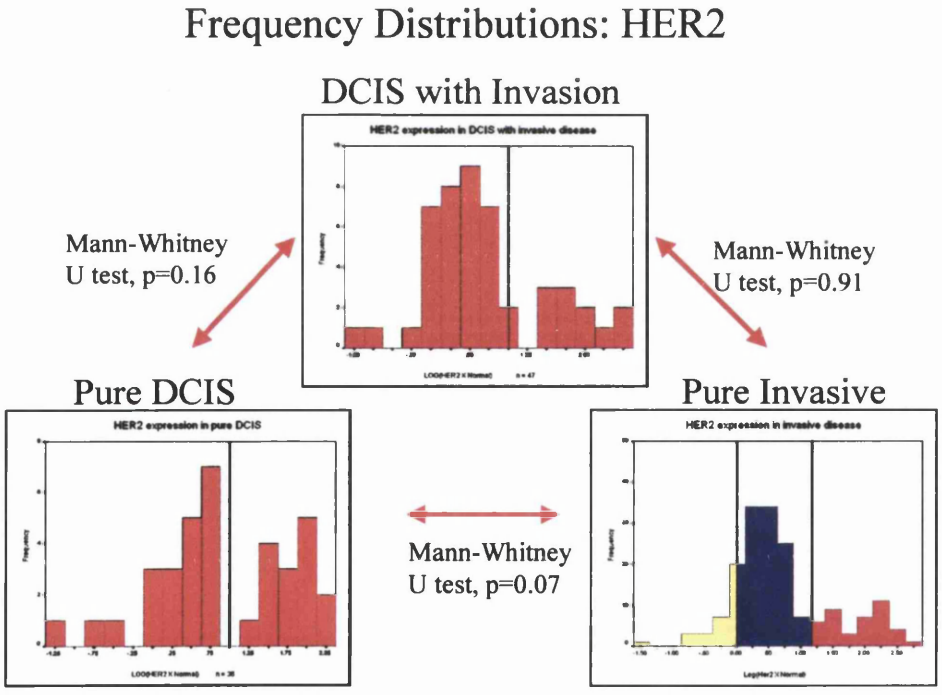

Figure 34. *The frequency distributions of growth factor expression for HER2 within the spectrum of breast cancer.*

6.3.8 Patient and tumour characteristics for invasive tumours in which HER2 was measured

The patient and tumour characteristics for the invasive tumours where HER2 was measured have been compared to those of the unselected tumour population. These characteristics are presented in Table 21.

The comparative group is comprised of cases treated in the same surgical unit as the selected group. These were not included in the study because frozen tissue was not available for these tumours. As can be seen, there is no significant difference between the 2 groups in terms of patient age, grade or size of tumour, involved nodes or oestrogen receptor status.

	Dataset used in this study: 1984 to August 1993	Unselected comparative group: 1984 to 1994
Overall cases	n =177	n = 218
Patient age		
Median	62.28	61.24
Grade	(%)	(%)
1	25 (14)	15 (21)
2	64 (36)	24 (33)
3	87 (50)	34 (46)
Total	176	73
Tumour Size		
<21 mm	70 (40)	94 (48)
21-50 mm	81 (47)	91 (47)
>50 mm	22 (13)	9 (5)
Total	173	194
Involved		
0	74 (48)	97 (52)
1-3	53 (35)	62 (33)
>3	26 (17)	28 (15)
Total	153	187
ER Status		
Negative	55 (44)	59 (40)
Positive	71 (56)	87 (60)
Total	126	146

Table 21. *Patient and tumour characteristics for the 177 cases in this study and the characteristics of the unselected data set for comparison.*

6.3.9 Survival analysis

6.3.9.1 Analysis with HER2 expression considered as a single variable

Initial survival analysis was carried out examining HER2 expression alone as a prognostic variable. Univariate analysis of disease specific survival (Figure 35) showed prognosis to be significantly worse in groups A and C compared to group B (overall Chi square = 21.27, df = 2, $p < 0.00005$). There was no significant difference between outcome in groups A (below normal) and C (amplified) (Chi square = 0.13, df = 1, p = 0.72). The significantly poorer outcome in groups A and C applied to both overall survival (global Chi square = 20.74, df = 2, $p < 0.00005$) and recurrence free survival (global Chi square = 19.65, df = 2, p = 0.0001). This relationship applied in both node negative and node positive tumours.

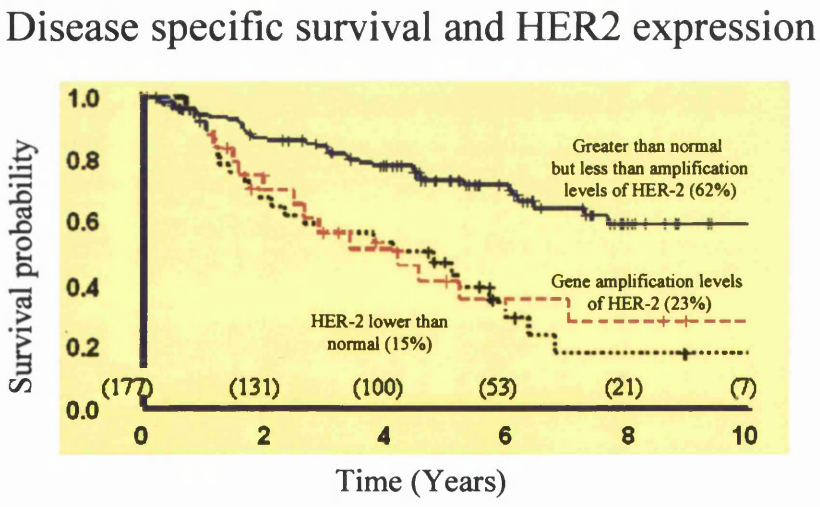

Figure 35. *Disease specific survival and HER2 expression.*

6.3.9.2 Multivariate analysis comparing disease free survival in these 3 groups against known pathological variable and the Nottingham Prognostic Index.

Multivariate analysis showed that the survival effect of HER2 expression was independent of pathological variables and the Nottingham Prognostic Index (see section 1.4.2.2). Dividing patients according to the Nottingham prognostic index, those in NPI group 1 with between normal and amplified levels of HER2 expression had a 5-year survival of 92%. But only 79% of patients with amplified or downregulated HER2 levels survived 5 years. This is shown below in Figures 36-38 and Table 22.

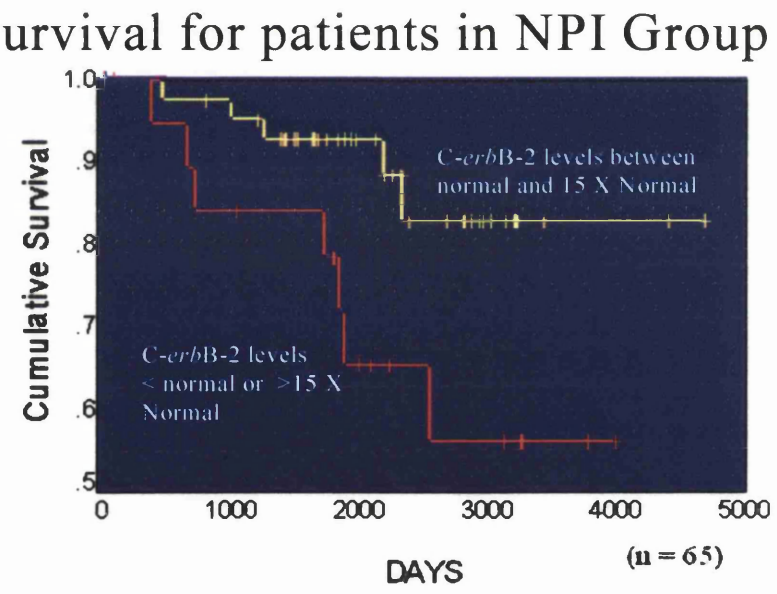

Figure 36. *Survival for patients in NPI group 1 according to HER2 expression.*

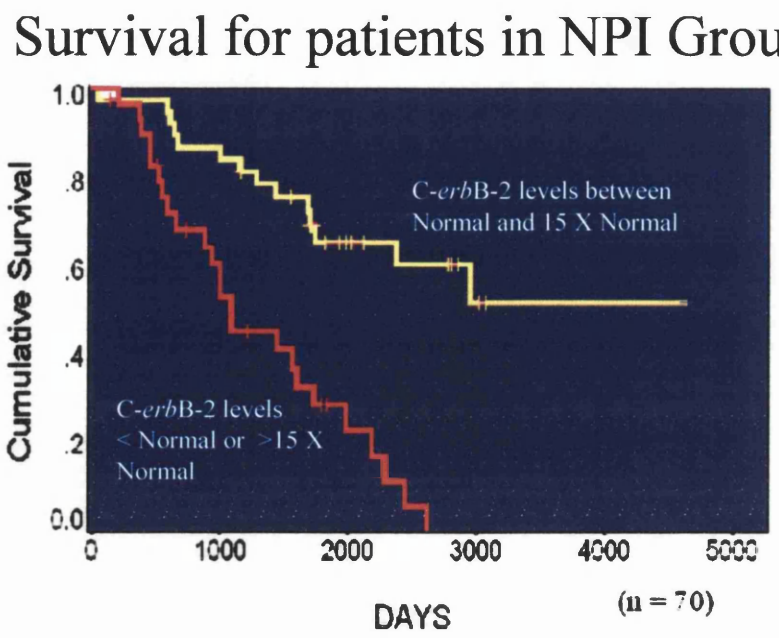

Figure 37. *Survival for patients in NPI group 2 according to HER2 expression.*

Similarly, for patients in NPI group 2, 66% of those with HER2 levels of expression between normal and amplified levels survived 5 years while only 29% of those with either very low or very high levels of HER2 expression survived 5 years.

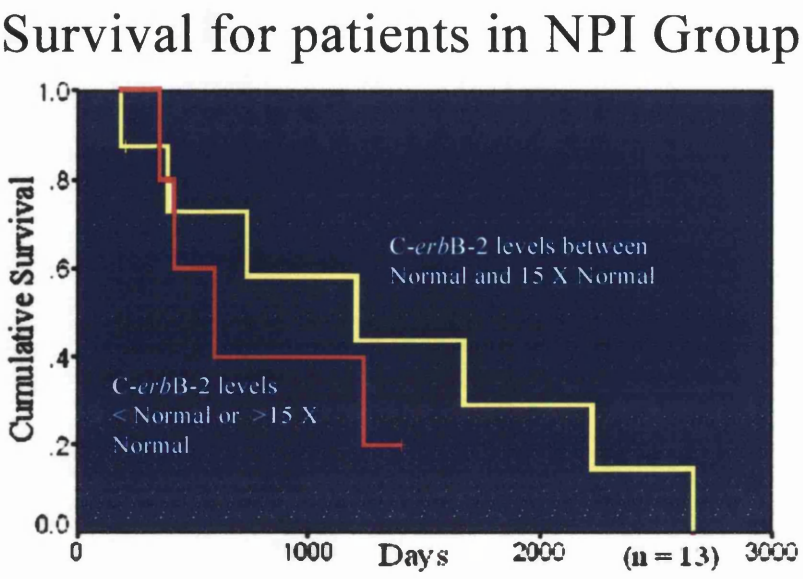

Figure 38. *Survival in NPI group 3 according to HER2 expression.*

	HER2 expression	
	Group B	**Group A & C**
NPI Group 1	**92%**	**79%**
NPI Group 2	**66%**	**29%**
NPI Group 3	**29%**	**20%**

Table 22. *Five year survival for patients in NPI Groups 1-3 according to level of HER2 expression.*

Group A (26 tumours, 15%) – Less than normal levels of expression.

Group B (110 tumours, 62%) – Between normal and 15 X Normal.

Group C (41 tumours, 23%) – More than 15 X Normal levels of expression.

6.3.9.3 Cox's proportional hazards model

The statistical analyses above could be criticised as the division of HER2 expression into 3 groups is artificial. For this reason, more complicated statistics were then carried out. The inclusion of quadratic and cubic terms of HER2 was done to make full use of the quantitative information available from radioimmunohistochemistry without making any assumptions as to the nature of the relationship between HER2 and outcome. The results of this analysis demonstrate a continuous relationship, whereby the best prognosis was associated with intermediate levels of HER2 expression. Expression levels at both the lower and upper ends of the distribution are associated with poorer outcome.

The model which fits the data includes a quadratic term to allow for the fact that relative hazard increases with either decreasing or increasing levels of HER2 from an optimal level which is at a HER2 value of about 5.7. The inclusion of a cubic term allows for asymmetry in change, e.g. halving the HER2 level does not have the same effect as doubling the HER2 level as the level moves away from the optimal. The best fitting hazard function is:

$h(t) = h(t_0) \exp(-1.8790*\log(b2+1) + 0.7159*[\log(b2+1)]^2 - 0.0673*[\log(b2+1)]^3).$

Where b2 = the HER2 level expressed relative to normal breast. The function is shown in Table 23.

Component*	LR	d.f.	p-value
linear	8.2	1	0.001
add quadratic	7.0	1	0.001
add cubic	6.2	1	0.001

Table 23. *Model based only on the quantitative level of HER2.*

*in respect of the quadratic and cubic terms, terms were added to a model which included terms in previous rows and the p-value records the gain from each addition.

The prognostic effect of HER2 when considered in isolation is complex, in that the best outcome is associated with an intermediate level of expression. Prognosis then becomes worse at both higher and lower levels of expression, the relative risk being about 3.5 for a tumour with a level of 0.1x normal, and about 4 for a tumour with a value of 100x normal. So, although amplified tumours (as identified by expression level above 15x normal) overall do worse than unamplified ones, tumours with very low levels of expression do very nearly as poorly as those with amplification. This relationship does not suggest the presence of expression thresholds, for instance associated with HER2 amplification, but rather that outcome is associated with HER2 expression in a continuous fashion. This approach does not specifically test the possibility that there are thresholds at which the outcome changes, but we have preferred not to do so in order to avoid both generating and testing hypotheses within the same dataset.

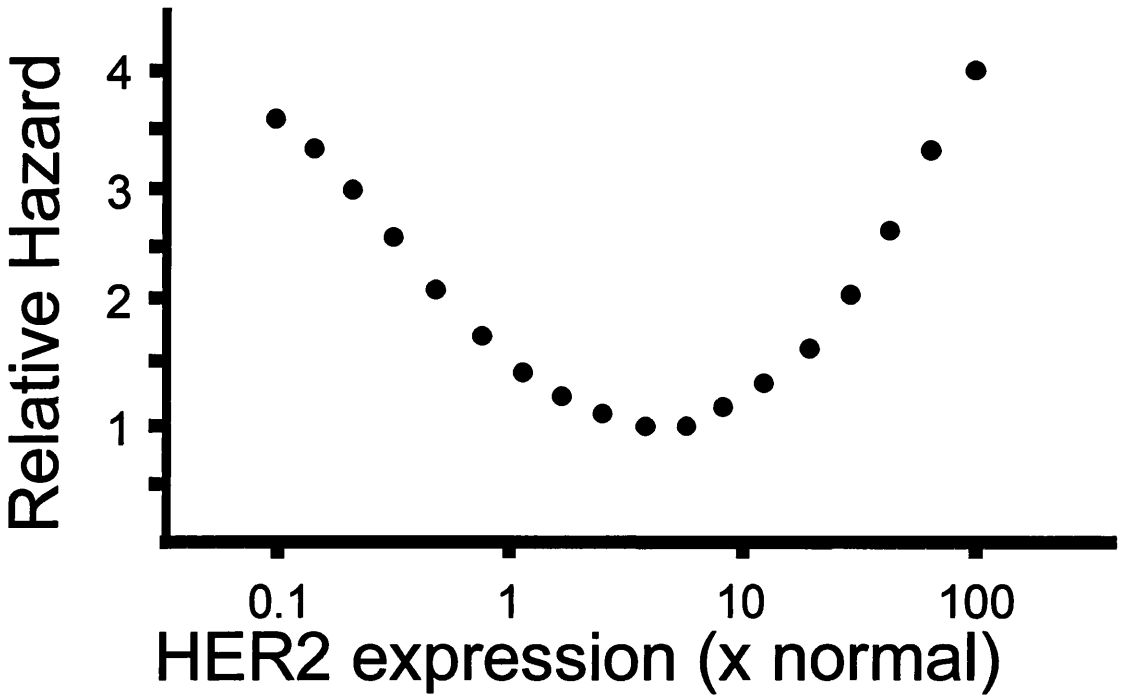

Figure 39. *Survival function for HER2 as an isolated variable. HER2 expression is given as a multiple of mean expression in normal breast. Relative hazard is expressed with respect to that at the best prognosis level of 5.7 times normal HER2 expression. Note that prognosis is worse in tumours with both higher and lower levels of HER2 than this.*

6.3.9.4 Analysis with size, nodes and grade included with HER2 expression.

Subsequent analysis was carried out to see if controlling for the standard pathological variables of tumour grade, tumour size and nodal status altered the relationship to prognosis, which we have just described for HER2. This analysis indicates that the basic nature of the relationship between HER2 and prognosis was not altered by the addition of the other variables.

Stepwise fitting of a model which includes histological grade, macroscopic tumour size and number of nodes involved indicated that all three variables are required. If the linear, quadratic and cubic terms in HER2 are also included, Grade is the only variable which is *not* included in a stepwise fit (p=0.2). The best fitting hazard function is

$h(t) = h(t_0)\ \exp(0.1023*\text{nodes} + 0.0276*\text{size} - 2.806*[\log(b2+1] + 1.0656*[\log(b2+1)]^2 - 0.1008*[\log(b2+1)]^3).$

The effect of varying size and the number of nodes on the hazard function for HER2 is illustrated in Figure 38. Thus, there is evidence that there is information about the risk of dying in the quantitative level of HER2 over and above that contained in the standard pathological variables. Furthermore, with the inclusion of the HER2 terms, tumour grade becomes redundant, i.e. the information contained in the 'grade' variable is contained in the level of HER2.

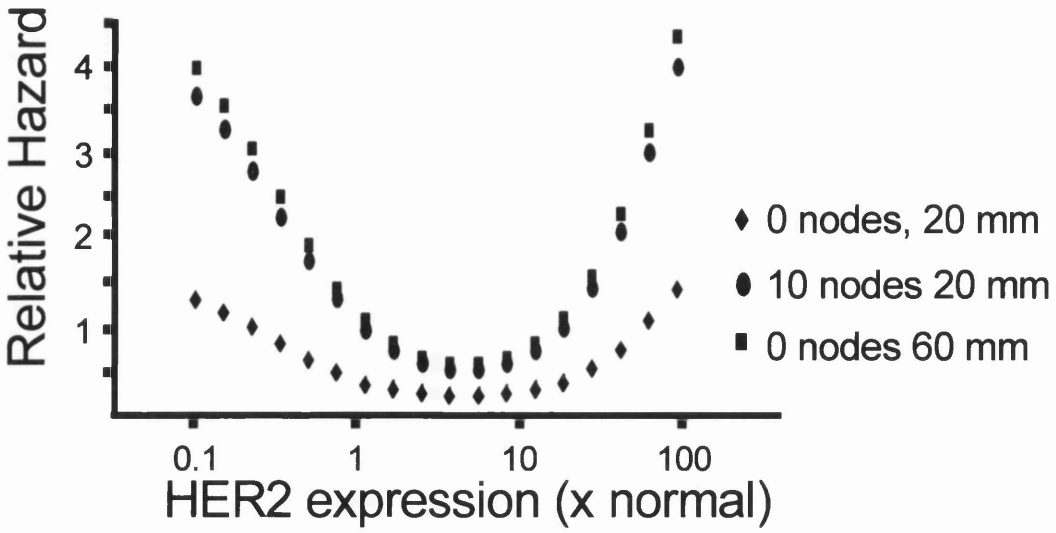

Figure 40. *Example of survival function for HER2 when allowance is made for pathological variables, in this case nodal status. As in Figure 33, HER2 expression is given as a multiple of mean expression in normal breast. Relative hazard, where HER2 of 5.7 times normal has hazard of 1, is shown for patients with no axillary nodes involved, and for those with 10 nodes involved. Although these two groups have differing overall prognoses, the relationship between HER2 expression and outcome persists within each group.*

6.3.9.5 Analysis with above variables as well as ER

Within this series, oestrogen receptor status is the single most powerful prognostic factor in its own right. This is clearly indicated by the size of the gain from its inclusion in a hazard function. (LR gain=33.6 (1 d.f.) $p<0.001$). It is also clear that there is a strong linkage between ER and HER2, with the ER negative tumours being clustered in the low and high expression tails of the HER2 frequency distribution (Figure 39). Fitting of ER status and the standard pathological variables (number of involved nodes, macroscopic tumour size and histological grade) in a survival analysis results in ER status and size being the only predictors required. The relationship between ER status and HER2 displayed in Figure 29 indicates that separate hazard functions are required for ER positive and negative groups. Adding HER2 as a variable in the ER positive group produces no significant effect ($p=0.6$). A similar finding is obtained for the ER negative group ($p = 0.2$). These results are unchanged if adjustment for size differences of tumours is made. Thus, the models which are suggested above as having prognostic power when the ER status of patients is ignored, lose that power when fitted separately to the ER positive and ER negative groups.

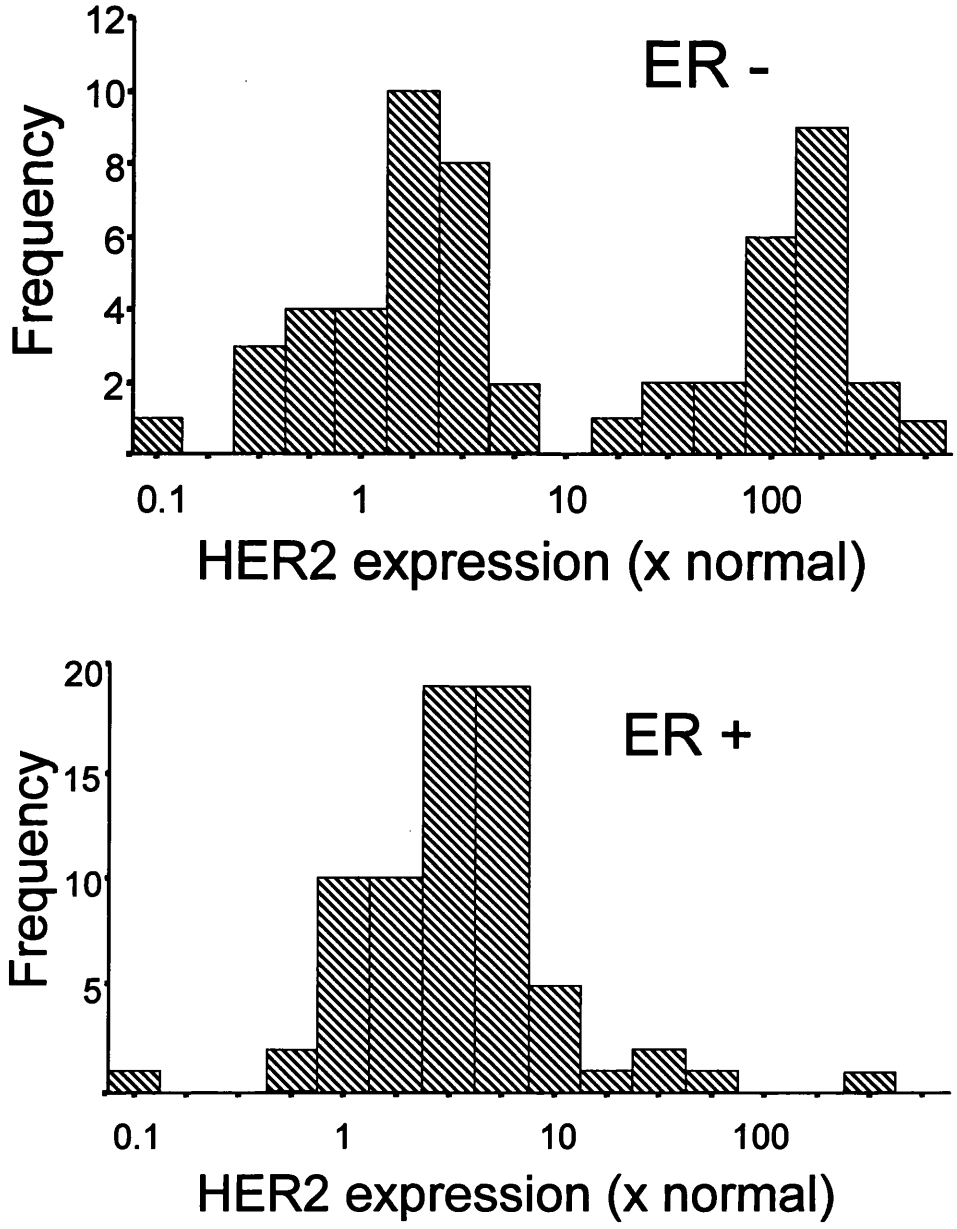

Figure 41. *HER2 expression in oestrogen receptor positive and negative subgroups. n = 55 for the ER- group and n = 71 for the ER+ group. Note that the ER- cases congregate at the low and high end of the HER2 expression spectrum (poor prognosis) whilst the ER+ cases tend to have intermediate HER2 levels and a correspondingly good prognosis.*

SECTION 7 DISCUSSION

7.1 Growth factor expression in "normal" breast tissue

This study has used specimens of histologically normal breast which were obtained from 9 reduction mammoplasty procedures. It can be reasonably argued that tissue from reduction mammoplasty specimens is not the ideal control for breast cancer specimens. The indication for the operation, i.e. very large breasts, may be a reflection of deregulation of growth factor receptors. There is to our knowledge no evidence to suggest that this is so. Reduction mammoplasty patients are also younger than the majority of cancer patients but it is very rare for completely normal breast tissue to be removed from middle aged or older women. In an attempt to use other "normal" breast tissue, areas of normal breast tissue within the tumour tissue studied were measured for these growth factors. These areas showed similar receptor expression to that of the control specimens; however, these areas may be subject to some of the genetic changes that have resulted in the adjacent pathology.

As can be appreciated, it is extremely difficult to obtain normal breast tissue for use as controls. It can even be difficult to define what constitutes normal control tissue for breast cancer tissue. Accepting these arguments we have used tissue removed for purposes other than cancer treatment which histologically shows no sign of disease. Since the consensus origin of breast carcinoma is at the level of the terminal ductolobular unit, lobular tissue from the reduction mammoplasty specimens was used for our control measurements.

There has been a tendency in most studies of growth factor expression to regard any expression as overexpression. We believe that it is crucial, especially in these

sensitive and quantitative analyses such as has been used here, to attempt to assess expression relative to that in normal tissues.

7.2 Radioimmunohistochemistry and growth factor expression in DCIS

This study represents one of a very few investigations into EGFR expression in DCIS. As described in section 3.2.2, the need to use frozen tissue does make it more difficult and may explain the lack of studies into EGFR expression in DCIS. Radioimmunohistochemistry combines the objective quantification of ligand binding studies with the tissue specificity of conventional immunohistochemistry. Using this technique, we have previously shown that the vast majority of primary breast cancers overexpress HER2 and downregulate EGFR, relative to normal breast tissue (2). We have shown that in the 3 groups of tumours studied here (pure DCIS, DCIS associated with invasive disease and invasive disease alone), there is a high level of HER2 overexpression and EGFR downregulation when compared to normal breast tissue.

There was no significant difference in expression of either of these growth factor receptors in the pure DCIS group compared to the group with DCIS in association with invasive disease, nor was there a significant difference between the group with DCIS in association with invasive disease and the purely invasive group. There was, however, a significant difference between the expression of EGFR in the pure DCIS group compared to the invasive group ($p = 0.009$). There was also a trend to significance in the expression of HER2 between the pure DCIS group and the invasive group ($p = 0.07$). This may represent a subtle change in the expression of these growth factor receptors as in situ lesions become invasive. This may imply that even this sensitive radioimmunohistochemical technique is

unable to detect and display a very subtle change in expression of these growth factor receptors in these 2 tumour populations. While this finding of a trend to significance in these 2 tumour populations may simply be a statistical error due to the small numbers involved, this may represent an area for future work into the intricate workings of this family of growth factor receptors in breast cancer.

Comparing the 2 groups of DCIS, the lesions found adjacent to invasive cancers have a proven progressive genotype whereas the pure DCIS group may or may not subsequently progress. The similarities in expression between these 2 groups suggest that these changes in growth factor expression are not a predictor of progression of DCIS to frank malignancy. Indeed, by the time of development of in situ breast cancer, HER2 upregulation and EGFR downregulation are almost universally present. It is interesting, however, that those tumours that undergo a large change in EGFR and also HER2 expression between the in situ and invasive components had very aggressive invasive tumours. The intricacies involved in receptor binding and interactions between these 2 members of the Type 1 growth factor receptor family, and indeed the other members of the family, are enormously complex and at present not well understood. Larger studies would be required to show whether a large change in expression of these receptors between in situ disease in association with invasive disease and the invasive components of the same tumours could indeed act as a prognostic indicator and so allow those tumours to be treated more aggressively from the beginning.

We know from autopsy studies on women dying from causes other than breast cancer that proliferative disease without atypia, atypical ductal hyperplasia and ductal carcinoma in situ (DCIS) are progressively less frequent (15). Accurate

quantification of earlier lesions such as proliferative disease without atypia and atypical hyperplasia may help determine which lesions are the specific precursors of in situ carcinoma.

On the other hand, numerous studies, including our own (2), have consistently shown HER2 to be overexpressed in a large proportion of invasive breast cancers and conferring a poorer prognosis. We have shown here that in the earlier stages of breast cancer, the same changes in expression of these 2 growth factors can be found. These results imply that the disregulation of these growth factor receptors is an early event in breast carcinogenesis. Members of this growth factor receptor family are known to form heterodimers which can result in an increase in kinase activity. While it is interesting to speculate that receptor interaction or internalisation explains the relative ability to detect these receptors, the precise mechanisms involved in the relative changes in the expression of these growth factor receptors in the spectrum of breast cancer is unclear.

Overexpression of HER2 occurs as a result of increased gene transcription, with or without gene amplification. Conventional methods of measuring HER2 are far less able to detect expression of HER2 at sub-amplification levels of overexpression. This sensitive radioimmunohistochemical method allows very low levels of expression to be detected and allows the cell type expressing the receptor to be identified. Although labour intensive, sensitive methods such as this will in time help to unravel the complex interactions between members of this type 1 growth factor receptor family. Similarly, if it is indeed true that increased transcription precedes gene amplification in the natural history of the disease, then identification of early, small increases in expression may prove important.

7.3 Growth factor expression in DCIS stratified according to the Van Nuys DCIS Classification system

When DCIS is stratified according to the Van Nuys DCIS Classification system there was no difference in growth factor expression according to the different groups apart from that of HER2 in pure DCIS. The more aggressive forms of DCIS according to this classification system had a significantly higher level of expression than the less aggressive forms. This may imply that HER2 expression may play a part in the biology of aggressive DCIS. This difference in expression according to Van Nuys DCIS Classification system groups is then lost when analysing DCIS associated with invasive disease. While the number of lesions analysed by this labour intensive method was small it is interesting to suggest that once progression to invasion occurs, the role played by HER2 is less important. This however would not be consistent with our survival analysis discussed below.

7.4 Molecular biology of breast cancer progression

As is believed to be the case with most other cancers, breast cancer development is thought to have its origin in one cell which through a series of events becomes malignant. The development of breast cancer involves many types of genes that need to be activated or inactivated in order to promote malignancy. The sequential steps in gene alterations with respect to tumour progression are not clear. Indeed the events involved in the progression of breast cancer are far less well understood than the adenoma-carcinoma sequence of colorectal cancer. Genetic changes in presumed precursor stages of breast cancer such as ductal hyperplasia, atypical ductal hyperplasia and DCIS have been studied. Loss of heterozygosity in hyperplastic disease has been detected and this suggests that these lesions are benign neoplasms and that inactivation of tumour suppressor genes play a role in their development (310). Studies of DCIS and more advanced lesions show a complex and poorly understood pattern of genetic alterations. Most genetic defects detected in invasive cancer are already present in DCIS (226;310-314). This is in keeping with our findings presented here. Gene amplifications appear to be a late event in tumour progression as they are mainly found in tumour cells that have acquired genomic instability and tolerate its presence (315). None of the oncogenes located at amplified chromosomal regions in invasive breast cancer are amplified in benign breast disease (316-318). It is generally agreed that oncogene amplification is not an early event in the multistep carcinogenesis of breast cancer but emerges in DCIS (315;319). It is also suggested that oncogene mediated proliferation is predominantly at the intermediate state of breast cancer

development and is not of obvious importance in the progression to metastatic disease (315;319).

Our findings are in keeping with general opinion as suggested by the literature. We find that the changes in EGFR and HER2 expression are already present by the time DCIS has developed. Although a frozen tissue bank of earlier benign lesions would be difficult to establish and would likely require multicentre collaboration, this sensitive and quantitative method may provide further insight into the role that these growth factor receptors play in the progression from benign neoplasms to more advanced disease.

7.5 Radioimmunohistochemistry and growth factor expression in invasive breast cancer

7.5.1 EGFR

This group has previously shown using radioimmunohistochemistry that EGFR overexpression relative to normal breast epithelial cells is rare (2). This is in keeping with work done by Slamon et al (221) who reported that EGFR gene amplification was found in only 4 of 189 breast cancers studied by Southern blotting. It seems unlikely therefore that EGFR abnormalities, at least in expression levels, have an oncogenic influence in breast cancer.

7.5.2 HER2

This work extends our previous study of HER2 expression in invasive breast cancer to a larger population of tumours, and analyses the role of expression in the prediction of patient outcome. This reinforces our earlier suggestions that:

1) 85% of breast cancers overexpress HER2, not only the 25% with gene amplification;

2) expression of HER2 varies by more than three orders of magnitude;

3) the frequency distribution for HER2 expression levels shows two distinct populations.

In addition, we now suggest that:

1) there is a strong, continuous relationship between HER2 expression and prognosis;

2) both very low and very high HER2 expressing tumours have poor outcome;

3) this relationship to prognosis remains significant after the introduction of standard pathological variables into the analysis;

HER2 expression level therefore encodes more information than gene amplification alone.

The most striking finding of this study is that poor survival is experienced by breast cancer patients with low as well as high HER2 expressing tumours. Previous studies have suggested that there is a group of breast cancer patients with low HER2 expression and a poor prognosis. Dittadi and co-workers (320) measured HER2 in an ELISA system on 105 breast cancer specimens and divided the cases into quartiles based on the ranked expression. The upper and lower quartiles were reported to have a significantly shorter relapse free survival than

those cases with intermediate expression (median follow-up of 30 months). In another ELISA based study, Koscielny and co-workers indicated that tumours with low HER2 values (110 out of 1062 cases studied) were more likely to be oestrogen and progesterone receptor negative (321) and hypothesised that this represented a poor prognosis group. A further report focusing on a subset of these patients (322) indicated that the patients with low HER2 expression did indeed have a significantly poorer prognosis. Expression was assessed in 117 primary breast carcinomas and cases were categorised into groups having significantly lower expression than normal (8.5%), within the normal range of expression (77%) or overexpression (14.5%). Four of the 10 patients with low expression developed metastases (median follow-up 38 months) whereas only 8 of the 90 patients with normal expression relapsed over this period.

Recently there has been active debate regarding the relationship of low HER2 expression and poor prognosis, with a report by Ferrero-Pous (323) indicating irreproducibility of Koscielny's findings. Our data are in keeping with the reports of Kosceilny and Dittadi (320-322), but we have been able to assess HER2 as a continuous variable rather than assigning arbitrary cut points to the data. Furthermore, both of the studies mentioned above use an ELISA based approach to assess HER2 expression, requiring tissue homogenization prior to analysis. Using relatively large amounts of tissue these methods potentially average out inconsistency due to tumour heterogeneity, but there is an inherent problem with the disruption of tissue architecture resulting in variable non-tumour contamination in the measured sample.

Attempts in our laboratory using conventional immunohistochemistry to identify the poor prognosis low HER2 expressing group were unsuccessful. Immunohistochemical measurements on paraffin sections, indicated that only 30% of cases with the highest level of expression gave positive signals (data not shown). In frozen sections, immunoperoxidase labelling with a streptavidin biotin system and the ICR12 monoclonal antibody (307) demonstrated positive HER2 labelling in greater than 90% of cases, however, we were unable to demonstrate a significantly poorer prognosis associated with the lowest expressers using this method due to a lack of quantitative accuracy in tumours with very low levels of HER2 expression (data not shown).

The mechanism behind the aggressive nature of low HER2 expressing tumours is not clear. The majority of these cases were known to be oestrogen receptor negative and thus less likely to respond to hormonal treatment whereas the cases with intermediate expression were more likely to be oestrogen receptor positive receiving the full benefit of adjuvant hormonal therapy. Previously, we have demonstrated an inverse relationship between epidermal growth factor receptor expression and HER2 expression in tumours without amplification of the HER2 gene (2), with the low expressing HER2 tumours more likely to express the epidermal growth factor receptor. This may provide a HER2 independent mechanism for proliferation in response to the stimulation of an epidermal growth factor mitogenic pathway. The clinical significance of low HER2 expression should be addressed with a larger study to assess the potential predictive value of response to hormonal or chemotherapy regimens.

In contrast to the relatively novel finding of poor survival associated with low

HER2 expression, it has been known for more than a decade that the 25 to 30% of patients with the highest levels of HER2, or those with amplification of the HER2 gene, have a poor outlook, although there remains a high level of inter-study variation (324). Our data support this finding and we show that patients with high HER2 expressing tumours have up to a 4 fold greater relative risk of dying from a breast cancer related cause than patients with optimal HER2 expression. While there has been a loss of interest in prognostic factors in breast cancer in recent years, there has been a resurgence in interest in measurement of HER2 as a predictive factor for Tamoxifen and chemotherapy regimens (252;283;296;298;325;326). Furthermore, the application of receptor targeted therapies to appropriate patients is dependent on HER2 measurement. The methods that are currently being used for these indications involve measurement of the HER2 gene copy number or immunohistochemical assessment of HER2 in paraffin sections. Neither method is quantitative, both give results for less than 30% of cases. There have been significant advances in standardization of conventional immunohistochemical methods, particularly with the development of dedicated FDA approved tests (HercepTest® from DAKO). However, conventional immunohistochemical methods remain subjective and dependent on the assignation of arbitrary cutpoints that are determined in part by the sensitivity of the techniques themselves rather than by the biology of the disease. Radioimmunohistochemistry however, gives quantitative results for all cases, and from examination of this relatively small retrospective cohort of breast cancer cases, the data do suggest that the relationship between HER2 expression and survival is a continuous one and can be best defined using quantitative

methodology. As the patients making up this study underwent a variety of treatment regimens, the observed survival effect is likely to be due to a combination of factors with the positive effect of treatment regimens being superimposed upon the pure prognostic component. This may in part explain the very strong apparent prognostic effect of oestrogen receptor in this data set. This strong effect omits not only HER2 as a prognostic factor, but also nodal status and histological grade, when it is included in the model. This finding does not exclude a prognostic role for these factors, but indicates that within this particular patient group they have lower predictive value than ER. In other patient groups, where the effect of ER upon outcome is typically less marked, these other factors might have a greater role in the overall prognostic model. Nor does the powerful effect of ER alter the nature of the relationship between HER2 and prognosis when considered in isolation.

Application of radioimmunohistochemistry to a large cohort of breast cancer patients is warranted in order to assess whether there are continuous relationships between HER2 levels and responses to endocrine therapy, chemotherapy and targeted therapies or whether there are critical thresholds of expression that separate responders and non-responders. Once these relationships have been defined, they can be used as templates to design assays with greater clinical applicability.

7.6 Implications to proposed new treatment modalities

The EGFR is a proposed target for treatment of other cancers such as head and neck cancers. Monoclonal antibodies such as cetuximab (IMC-C225) which have a higher affinity for the EGFR than endogenous ligands have been developed to target the EGFR. This method has been promoted as a means of specifically targeting tumour cells (327). Our results suggest that this approach is unlikely to work in breast cancer as the vast majority of breast cancers downregulate EGFR. Only a small proportion (less than 5%) of tumours studied had a higher level of EGFR expression compared to normal breast tissue. This implies that for breast cancer tissue there would be no such targeting specificity to tumour cells.

The clinical results with the monoclonal antibody to HER2 [Herceptin, (trastuzumab)] hold significant promise. This monoclonal antibody has shown promising results in HER2 overexpressing metastatic breast cancer. Our data suggest that the proportion of tumours overexpressing HER2 is considerably higher than that detected by non-quantitative methods. It is tempting, therefore, to suggest that the potential use for Herceptin may be considerably wider than the 30% or so of currently detectable HER2 overexpressers. Conversely, gene therapy or receptor targeting approaches directed at HER2 pathways may not be desirable as complete abolition of HER2 expression may have an adverse effect on tumour progression.

Detailed counts for pure DCIS - EGFR

Patient ID	Filename	Exposure	Count	Area	Count/mm2/100	Count/mm2/hr/100	Formula	Formula/hr/100	% A431	Normal EGFR	EGFRXN
Pure1	H1	146	4417	0.141	312.156	2.138	25704.275	1.761	3.738	7.614	0.491
	H2	146	6403	0.204	314.427	2.154					
	C	146	534	0.095	56.453	0.387					
Pure2	H1	146	2862	0.143	200.202	1.371	16324.572	1.118	2.374	7.614	0.312
	H2	146	2245	0.096	233.417	1.599					
	C	146	606	0.120	50.316	0.345					
Pure3	H1	48	564	0.110	51.501	1.073	469.314	0.098	0.208	7.614	0.027
	H2	48	472	0.087	54.170	1.129					
	C	48	444	0.093	47.991	1.000					
Pure4	H1	146	655	0.115	56.957	0.390	-412.642	-0.028	-0.060	7.614	0.000
	H2	146	541	0.126	42.835	0.293					
	C	146	800	0.149	53.691	0.368					
Pure5	H1	Slide ruined - no histology apparent					-826.910	-0.057	-0.120	7.614	0.000
	H2	146	430	0.093	46.178	0.316					
	C	146	507	0.093	54.447	0.373					
Pure6	H1	146	667	0.077	86.958	0.596	2623.958	0.180	0.382	7.614	0.050
	H2	146	713	0.079	90.693	0.621					
	C	146	583	0.093	62.609	0.429					
Pure7	H1	146	1973	0.206	95.988	0.657	3259.619	0.223	0.474	7.614	0.062
	H2	146	1154	0.176	65.558	0.449					
	C	146	676	0.137	49.354	0.338					
Pure8	H1	146	326	0.051	64.538	0.442	-16.555	-0.001	-0.002	7.614	0.000
	H2	146	298	0.045	65.604	0.449					
	C	146	681	0.104	65.208	0.447					
Pure9	H1	146	1272	0.150	84.692	0.580	-6470.433	-0.443	-0.941	7.614	0.000
	H2	146	528	0.134	39.538	0.271					
	C	146	1591	0.124	128.144	0.878					
Pure10	H1	146	2479	0.186	133.111	0.912	2528.078	0.173	0.368	7.614	0.048
	H2	146	1598	0.155	102.966	0.705					
	C	146	1753	0.186	94.128	0.645					
Pure11	H1	146	451	0.100	45.032	0.308	871.792	0.060	0.127	7.614	0.017
	H2	146	916	0.119	77.110	0.528					
	C	146	781	0.145	53.719	0.368					
Pure12	H1	146	211	0.124	16.995	0.116	-1232.310	-0.084	-0.179	7.614	0.000
	H2	Slide ruined - no histology apparent									
	C	146	273	0.093	29.318	0.201					
Pure13	H1	146	492	0.026	190.356	1.304	12502.466	0.856	1.818	7.614	0.239
	H2	146	350	0.026	132.863	0.910					

Detailed counts for pure DCIS - EGFR

Patient ID	Filename	Exposure	Count	Area	Count/mm2/100	Count/mm2/hr/100	Formula	Formula/hr/100	% A431	Normal EGFR	EGFRXN
	C	146	140	0.039	36.311	0.249					
Pure14	H1	48	579	0.105	55.331	1.153	-1407.881	-0.293	-0.623	7.614	0.000
	H2	48	466	0.102	45.573	0.949					
	C	48	728	0.113	64.588	1.346					
Pure15	H1	146	1389	0.176	78.963	0.541	-219.268	-0.015	-0.032	7.614	-0.004
	H2	146	541	0.089	60.584	0.415					
	C	146	868	0.116	74.967	0.513					
Pure16	H1	146	1123	0.101	111.705	0.765	-1887.554	-0.129	-0.274	7.614	0.000
	H2	146	783	0.082	95.700	0.655					
	C	146	979	0.079	123.400	0.845					
Pure17	H1	146	665	0.142	46.897	0.321	31.369	0.002	0.005	7.614	0.001
	H2	146	482	0.169	28.532	0.195					
	C	146	568	0.155	36.599	0.251					
Pure18	H1	Slide ruined - no histology apparent					4743.254	0.325	0.690	7.614	0.091
	H2	146	1129	0.135	83.808	0.574					
	C	146	359	0.099	36.376	0.249					
Pure19	H1	146	1284	0.155	82.734	0.567	3622.651	0.248	0.527	7.614	0.069
	H2	146	655	0.124	52.756	0.361					
	C	146	412	0.124	33.184	0.227					
Pure20	H1	Slide ruined - no histology apparent					533.604	0.111	0.236	7.614	0.031
	H2	48	493	0.134	36.832	0.767					
	C	48	329	0.104	31.496	0.656					
Pure21	H1	Slide ruined - no histology apparent					2542.127	0.174	0.370	7.614	0.049
	H2	146	1438	0.205	70.204	0.481					
	C	146	596	0.133	44.783	0.307					
Pure22	H1	146	592	0.093	63.575	0.435	402.578	0.028	0.059	7.614	0.008
	H2	146	645	0.108	59.651	0.409					
	C	146	643	0.112	57.441	0.393					
Pure23	H1	146	914	0.155	58.893	0.403	-2637.788	-0.181	-0.384	7.614	0.000
	H2	146	291	0.093	31.251	0.214					
	C	146	930	0.124	74.905	0.513					
Pure24	H1	146	2497	0.270	92.365	0.633	3364.822	0.230	0.489	7.614	0.064
	H2	146	978	0.116	84.440	0.578					
	C	146	1028	0.182	56.340	0.386					
Pure25	H1	146	1161	0.113	103.121	0.706	4033.561	0.276	0.587	7.614	0.077
	H2	146	626	0.108	57.893	0.397					
	C	146	445	0.110	40.628	0.278					
Pure26	H1	146	660	0.111	59.390	0.407	-1244.778	-0.085	-0.181	7.614	0.000

Detailed counts for pure DCIS - EGFR

Patient ID	Filename	Exposure	Count	Area	Count/mm2/100	Count/mm2/hr/100	Formula	Formula/hr/100	% A431	Normal EGFR	EGFRXN
	H2	146	650	0.112	58.066	0.398					
	C	146	1265	0.178	71.174	0.487					
Pure27	H1	146	455	0.125	36.400	0.249	-69.373	-0.005	-0.010	7.614	-0.001
	H2	146	556	0.146	38.082	0.261					
	C	146	950	0.250	38.000	0.260					
Pure28	H1	146	469	0.115	40.950	0.280	-78.044	-0.005	-0.011	7.614	-0.001
	H2	146	573	0.134	42.842	0.293					
	C	146	980	0.229	42.750	0.293					
Pure29	H1	146	1050	0.105	99.822	0.684	485.480	0.033	0.071	7.614	0.009
	H2	146	884	0.123	71.952	0.493					
	C	146	1682	0.210	79.952	0.548					
Pure30	H1	146	627	0.096	64.979	0.445	1437.869	0.098	0.209	7.614	0.027
	H2	146	649	0.115	56.435	0.387					
	C	146	1011	0.220	45.955	0.315					
Pure31	H1	146	1211	0.089	136.812	0.937	6525.757	0.447	0.949	7.614	0.125
	H2	146	1001	0.105	94.888	0.650					
	C	146	984	0.202	48.758	0.334					
Pure32	H1	146	798	0.081	98.278	0.673	3268.985	0.224	0.475	7.614	0.062
	H2	146	760	0.097	78.535	0.538					
	C	146	1015	0.185	54.853	0.376					
Pure33	H1	146	1251	0.074	167.952	1.016	2063.854	0.141	0.300	7.614	0.039
	H2	146	1098	0.089	112.647	0.772					
	C	146	2093	0.170	123.244	0.844					
Pure34	H1	146	1215	0.068	177.818	1.218	3619.597	0.248	0.526	7.614	0.069
	H2	146	1442	0.081	177.076	1.213					
	C	146	2200	0.156	141.218	0.967					
Pure35	H1	146	1409	0.063	224.793	1.540	3631.782	0.249	0.528	7.614	0.069
	H2	146	1102	0.075	147.519	1.010					
	C	146	2093	0.143	146.457	1.003					
Pure36	H1	146	845	0.057	192.354	1.317	4744.809	0.325	0.690	7.614	0.091
	H2	146	821	0.069	165.959	1.137					
	C	146	1111	0.131	164.764	1.129					
Pure37	H1	146	1306	0.053	247.606	1.696	4990.398	0.342	0.726	7.614	0.095
	H2	146	1208	0.063	192.167	1.316					
	C	146	2015	0.120	167.557	1.148					

Counts for pure DCIS - HER2

Patient ID	Batch Date	Formula/mm2/hr/100	Conversion Factor	Receptors	Normal R	HER2 X N
Pure2	04/02/1997	10.283	19.66	202.172	8.557	23.626
Pure3	04/02/1997	2.203	19.66	43.304	8.557	5.061
Pure5	04/02/1997	0.030	19.66	0.593	8.557	0.069
Pure6	04/02/1997	2.006	19.66	39.439	8.557	4.609
Pure7	04/02/1997	2.797	19.66	54.981	8.557	6.425
Pure8	04/02/1997	2.338	19.66	45.963	8.557	5.371
Pure9	22/12/1997	0.791	69.10	54.677	8.557	6.390
Pure11	22/12/1997	4.031	69.10	278.534	8.557	32.550
Pure13	22/12/1997	6.195	69.10	428.050	8.557	50.023
Pure14	22/12/1997	22.351	69.10	1544.431	8.557	180.485
Pure15	22/12/1997	0.359	69.10	24.798	8.557	2.898
Pure16	23/12/1997	19.905	69.10	1375.469	8.557	160.740
Pure17	22/12/1997	0.549	69.10	37.939	8.557	4.434
Pure18	22/12/1997	0.197	69.10	13.591	8.557	1.588
Pure19	22/12/1997	0.450	69.10	31.125	8.557	3.637
Pure20	22/12/1997	0.327	69.10	22.603	8.557	2.641
Pure21	22/12/1997	0.844	69.10	58.315	8.557	6.815
Pure22	22/12/1997	0.158	69.10	10.933	8.557	1.278
Pure27	22/12/1997	14.919	69.10	1030.882	8.557	120.471
Pure23	22/12/1997	0.182	69.10	12.547	8.557	1.466
Pure24	22/12/1997	4.420	69.10	305.434	8.557	35.694
Pure25	10/06/1998	0.431	64.30	27.726	8.557	3.240
Pure26	10/06/1998	5.197	64.30	334.189	8.557	39.054
Pure28	10/06/1998	3.438	64.30	221.045	8.557	25.832
Pure29	10/06/1998	14.347	64.30	922.502	8.557	107.805
Pure38	10/06/1998	0.047	64.30	3.052	8.557	0.357
Pure39	10/06/1998	7.361	64.30	473.282	8.557	55.309
Pure40	10/06/1998	12.723	64.30	818.110	8.557	95.606
Pure41	10/06/1998	0.306	64.30	19.679	8.557	2.300
Pure43	10/06/1998	16.976	64.30	1091.575	8.557	127.563
Pure44	10/06/1998	0.168	64.30	10.785	8.557	1.260
Pure45	10/06/1998	0.468	64.30	30.109	8.557	3.519
Pure46	10/06/1998	16.697	64.30	1073.610	8.557	125.464
Pure47	10/06/1998	5.944	64.30	382.174	8.557	44.662
Pure48	10/06/1998	0.135	64.30	8.676	8.557	1.014
Pure50	10/06/1998	0.026	64.30	1.653	8.557	0.193

APPENDIX III. Counts for DCIS for DCIS in association with invasive disease - EGFR

Patient ID	Batch Date	Formula	Formula/hr/100	A431 Grains	% A431	Normal EGFR	EGFRXN
DCIV1	13/04/1993	3731.852	0.381	144.1	0.264	7.614	0.035
DCIV2	13/04/1993	15930.709	1.626	144.1	1.128	7.614	0.148
DCIV3	13/04/1993	12038.714	1.228	144.1	0.852	7.614	0.112
DCIV4	13/04/1993	6317.754	0.645	144.1	0.447	7.614	0.059
DCIV5	13/04/1993	28004.800	2.858	144.1	1.983	7.614	0.260
DCIV6	04/05/1993	20416.292	1.047	140.0	0.748	7.614	0.098
DCIV7	04/05/1993	13196.836	5.175	140.0	3.697	7.614	0.486
DCIV8	11/05/1993	6480.716	0.453	121.0	0.375	7.614	0.049
DCIV9	06/05/1993	-2696.698	-0.138	135.3	-0.102	7.614	-0.013
DCIV10	08/06/1993	20431.183	1.419	206.0	0.689	7.614	0.090
DCIV11	04/05/1993	6501.520	0.333	140.0	0.238	7.614	0.031
DCIV12	04/05/1993	2016.477	0.103	140.0	0.074	7.614	0.010
DCIV13	08/06/1993	41706.551	5.713	206.0	2.773	7.614	0.364
DCIV14	13/04/1993	5216.543	0.532	144.1	0.369	7.614	0.049
DCIV15	08/06/1993	3584.330	0.491	206.0	0.238	7.614	0.031
DCIV16	08/06/1993	596.342	0.041	206.0	0.020	7.614	0.003
DCIV17	08/06/1993	28076.556	1.950	206.0	0.946	7.614	0.124
DCIV18	08/06/1993	5556.558	0.386	206.0	0.187	7.614	0.025
DCIV19	04/05/1993	7242.251	0.371	140.0	0.265	7.614	0.035
DCIV20	08/06/1995	1778.372	0.106	152.1	0.070	7.614	0.009
DCIV21	08/06/1995	7930.982	0.475	152.1	0.312	7.614	0.041
DCIV22	08/06/1995	-6116.914	-0.366	152.1	-0.241	7.614	-0.032
DCIV23	05/06/1995	16563.922	1.146	121.3	0.945	7.614	0.124
DCIV24	05/06/1995	446.822	0.172	121.3	0.142	7.614	0.019
DCIV25	06/06/1995	4367.949	0.302	131.6	0.230	7.614	0.030
DCIV26	06/06/1995	11828.076	4.549	131.6	3.457	7.614	0.454
DCIV27	06/06/1995	92.846	0.036	131.6	0.027	7.614	0.004
DCIV28	06/06/1995	7395.406	0.512	131.6	0.389	7.614	0.051
DCIV29	07/06/1995	2094.106	0.125	160.6	0.078	7.614	0.010
DCIV30	07/06/1995	7001.029	2.693	160.6	1.677	7.614	0.220
DCIV31	07/06/1995	1253.147	0.075	160.6	0.047	7.614	0.006
DCIV32	08/06/1995	16144.330	0.967	152.1	0.636	7.614	0.083
DCIV33	08/06/1995	-413.147	-0.025	152.1	-0.016	7.614	-0.002
DCIV34	08/06/1995	-4171.298	-0.250	152.1	-0.164	7.614	-0.022
DCIV35	08/06/1995	-9315.686	-0.558	152.1	-0.367	7.614	-0.048
DCIV36	15/04/1994	2625.271	0.370	160.0	0.231	7.614	0.030
DCIV37	15/04/1994	13485.317	33.713	160.0	21.071	7.614	2.767
DCIV38	29/04/1994	2108.899	1.004	148.1	0.678	7.614	0.089
DCIV39	29/04/1994	7232.441	0.991	148.1	0.669	7.614	0.088
DCIV40	18/04/1994	15057.543	2.121	190.0	1.116	7.614	0.147
DCIV41	18/04/1994	8841.629	5.052	190.0	2.659	7.614	0.349
DCIV42	05/06/1995	271.461	0.019	121.3	0.015	7.614	0.002
DCIV43	05/06/1995	-6355.689	-0.440	121.3	-0.363	7.614	-0.048
DCIV44	05/06/1995	2372.379	0.164	121.3	0.135	7.614	0.018
DCIV45	05/06/1995	26572.021	1.839	121.3	1.516	7.614	0.199
DCIV46	08/06/1995	6701.613	0.401	152.1	0.264	7.614	0.035
DCIV47	07/06/1995	1067.573	0.411	160.6	0.256	7.614	0.034
DCIV48	17/12/1997	-2090.567	-0.143	147.1	-0.304	7.614	-0.040
DCIV49	17/12/1997	4208.251	0.288	147.1	0.612	7.614	0.080
DCIV55	17/12/1997	-1046.281	-0.072	147.1	-0.152	7.614	-0.020

APPENDIX IV. Counts for DCIS for DCIS in association with invasive disease - HER2

Patient ID	Batch Date	Formula	Formula/hr/100	Conversion Factor	Receptors	Normal	ReceptorsXN
DCIV60	04/02/97	6320.071	1.317	19.66	25.886	8.557	3.025
DCIV14	25/01/95	14058.865	35.147	44.62	1568.266	8.557	183.271
DCIV27	16/01/97	6849.720	0.590	11.33	6.690	8.557	0.782
DCIV28	16/01/97	4402.949	0.380	11.33	4.300	8.557	0.503
DCIV26	16/01/97	10434.033	0.899	11.33	10.191	8.557	1.191
DCIV10	23/01/95	4387.053	0.914	37.64	34.402	8.557	4.020
DCIV35	04/02/97	10636.380	26.591	19.66	522.778	8.557	61.093
DCIV17	23/01/95	4705.118	0.490	37.64	18.448	8.557	2.156
DCIV46	04/02/97	12054.224	1.256	19.66	24.686	8.557	2.885
DCIV3	23/01/95	10903.565	2.272	37.64	85.502	8.557	9.992
DCIV29	03/02/97	22669.817	56.675	10.95	620.586	8.557	72.523
DCIV47	03/02/97	39392.694	98.482	10.95	1078.375	8.557	126.021
DCIV1	24/01/95	3789.882	0.790	37.28	29.435	8.557	3.440
DCIV41	28/06/94	9190.809	1.915	8.60	16.467	8.557	1.924
DCIV21	04/02/97	7536.182	3.140	19.66	61.734	8.557	7.214
DCIV30	03/02/97	28422.637	71.057	10.95	778.070	8.557	90.927
DCIV5	25/01/95	20267.126	50.668	44.62	2260.798	8.557	264.201
DCIV63	13/01/97	9790.104	0.844	15.86	13.385	8.557	1.564
DCIV62	03/02/97	21933.723	54.834	10.95	600.436	8.557	70.168
DCIV25	16/01/97	53466.851	22.278	11.33	252.408	8.557	29.497
DCIV18	23/01/95	8767.879	0.913	37.64	34.377	8.557	4.017
DCIV61	24/01/95	6288.592	1.310	37.28	48.841	8.557	5.708
DCIV44	13/01/97	4069.754	1.696	15.86	26.894	8.557	3.143
DCIV34	04/02/97	20047.943	2.088	19.66	41.057	8.557	4.798
DCIV59	04/02/97	8969.050	1.869	19.66	36.736	8.557	4.293
DCIV22	04/02/97	29547.777	3.078	19.66	60.511	8.557	7.071
DCIV31	03/02/97	11604.212	1.209	10.95	13.236	8.557	1.547
DCIV37	04/07/94	70319.596	175.799	15.78	2774.108	8.557	324.187
DCIV40	28/06/94	13228.025	1.102	8.60	9.480	8.557	1.108
DCIV45	13/01/97	7834.803	0.675	15.86	10.712	8.557	1.252
DCIV39	11/07/94	1291.989	3.691	12.49	46.106	8.557	5.388
DCIV24	13/01/97	14685.299	6.119	15.86	97.045	8.557	11.341
DCIV57	16/01/97	1166.642	0.101	11.33	1.139	8.557	0.133
DCIV32	04/02/97	31753.115	13.230	19.66	260.111	8.557	30.397
DCIV58	04/07/94	18364.417	1.583	15.78	24.982	8.557	2.919
DCIV48	04/02/97	5813.934	0.606	19.66	11.906	8.557	1.391
DCIV2	25/01/95	3156.730	0.329	44.62	14.672	8.557	1.715
DCIV23	13/01/97	6659.739	0.574	15.86	9.105	8.557	1.064
DCIV36	04/07/94	7900.864	0.681	15.78	10.748	8.557	1.256
DCIV42	13/01/97	8743.012	21.858	15.86	346.660	8.557	40.511
DCIV43	13/01/97	9103.450	0.785	15.86	12.447	8.557	1.455
DCIV15	23/01/95	5811.677	1.211	37.64	45.573	8.557	5.326
DCIV8	23/01/95	4468.321	0.931	37.64	35.039	8.557	4.095
DCIV20	04/02/97	718.985	0.075	19.66	1.472	8.557	0.172
DCIV55	22/12/97	4748.376	0.325	69.10	22.473	8.557	2.626
DCIV56	22/12/97	3924.062	0.269	69.10	18.572	8.557	2.170
DCIV49	22/12/97	1959.688	0.134	69.10	9.275	8.557	1.084

APPENDIX V

Counts for invasive disease - EGFR

Patient ID	EGFRXN		Patient ID	EGFRXN		Patient ID	EGFRXN
EGFRINV1	0.050		EGFRINV65	0.054		EGFRINV129	0.043
EGFRINV2	0.029		EGFRINV66	0.035		EGFRINV130	0.018
EGFRINV3	0.033		EGFRINV67	0.038		EGFRINV131	0.066
EGFRINV4	0.079		EGFRINV68	0.709		EGFRINV132	0.027
EGFRINV5	0.119		EGFRINV69	0.028		EGFRINV133	0.000
EGFRINV6	0.130		EGFRINV70	0.016		EGFRINV134	0.058
EGFRINV7	0.117		EGFRINV71	0.233		EGFRINV135	0.059
EGFRINV8	0.000		EGFRINV72	0.046		EGFRINV136	0.158
EGFRINV9	0.067		EGFRINV73	0.020		EGFRINV137	0.048
EGFRINV10	0.004		EGFRINV74	0.649		EGFRINV138	0.159
EGFRINV11	0.038		EGFRINV75	0.146		EGFRINV139	0.215
EGFRINV12	0.003		EGFRINV76	0.021		EGFRINV140	0.000
EGFRINV13	0.000		EGFRINV77	0.068		EGFRINV141	0.071
EGFRINV14	0.071		EGFRINV78	0.000		EGFRINV142	0.072
EGFRINV15	0.041		EGFRINV79	0.015		EGFRINV143	0.040
EGFRINV16	0.053		EGFRINV80	0.034		EGFRINV144	0.053
EGFRINV17	0.000		EGFRINV81	0.018		EGFRINV145	0.063
EGFRINV18	0.016		EGFRINV82	0.027		EGFRINV146	0.045
EGFRINV19	0.346		EGFRINV83	0.028		EGFRINV147	0.683
EGFRINV20	0.636		EGFRINV84	0.086		EGFRINV148	0.098
EGFRINV21	0.322		EGFRINV85	0.000		EGFRINV149	0.243
EGFRINV22	0.047		EGFRINV86	0.071		EGFRINV150	0.007
EGFRINV23	1.754		EGFRINV87	0.110		EGFRINV151	0.340
EGFRINV24	0.239		EGFRINV88	0.180		EGFRINV152	0.802
EGFRINV25	0.474		EGFRINV89	0.722		EGFRINV153	0.009
EGFRINV26	0.010		EGFRINV90	0.021		EGFRINV154	0.022
EGFRINV27	0.007		EGFRINV91	0.032		EGFRINV155	0.036
EGFRINV28	0.078		EGFRINV92	0.369		EGFRINV156	0.000
EGFRINV29	0.045		EGFRINV93	0.455		EGFRINV157	0.011
EGFRINV30	0.000		EGFRINV94	0.000		EGFRINV158	0.017
EGFRINV31	0.003		EGFRINV95	0.042		EGFRINV159	0.023
EGFRINV32	0.025		EGFRINV96	0.048		EGFRINV160	0.000
EGFRINV33	0.288		EGFRINV97	0.113		EGFRINV161	1.784
EGFRINV34	0.404		EGFRINV98	0.324		EGFRINV162	0.061
EGFRINV35	0.410		EGFRINV99	0.031		EGFRINV163	0.069
EGFRINV36	0.017		EGFRINV100	0.070		EGFRINV164	0.079
EGFRINV37	0.024		EGFRINV101	0.011		EGFRINV165	0.479
EGFRINV38	0.043		EGFRINV102	0.036		EGFRINV166	0.610
EGFRINV39	0.031		EGFRINV103	0.654		EGFRINV167	2.437
EGFRINV40	0.758		EGFRINV104	0.043		EGFRINV168	0.069
EGFRINV41	0.201		EGFRINV105	0.015		EGFRINV169	7.074
EGFRINV42	0.030		EGFRINV106	0.020		EGFRINV170	0.038
EGFRINV43	0.032		EGFRINV107	2.314		EGFRINV171	0.076
EGFRINV44	0.459		EGFRINV108	0.018		EGFRINV172	0.189
EGFRINV45	0.271		EGFRINV109	0.309		EGFRINV173	0.008
EGFRINV46	0.000		EGFRINV110	0.065		EGFRINV174	0.052
EGFRINV47	0.093		EGFRINV111	0.013		EGFRINV175	0.057
EGFRINV48	0.578		EGFRINV112	0.053		EGFRINV176	0.038
EGFRINV49	0.194		EGFRINV113	0.506		EGFRINV177	0.056
EGFRINV50	0.269		EGFRINV114	0.000		EGFRINV178	0.000
EGFRINV51	0.239		EGFRINV115	0.050		EGFRINV179	0.128
EGFRINV52	0.043		EGFRINV116	0.259		EGFRINV180	0.023
EGFRINV53	0.026		EGFRINV117	0.025		EGFRINV181	0.022
EGFRINV54	0.079		EGFRINV118	0.027		EGFRINV182	0.294
EGFRINV55	0.157		EGFRINV119	0.000		EGFRINV183	0.040
EGFRINV56	0.168		EGFRINV120	0.053		EGFRINV184	0.004
EGFRINV57	0.189		EGFRINV121	0.009		EGFRINV185	0.013
EGFRINV58	0.047		EGFRINV122	0.035		EGFRINV186	0.074
EGFRINV59	0.319		EGFRINV123	0.012		EGFRINV187	0.145
EGFRINV60	0.711		EGFRINV124	0.023		EGFRINV188	0.016
EGFRINV61	0.033		EGFRINV125	0.239		EGFRINV189	0.023
EGFRINV62	0.097		EGFRINV126	0.044		EGFRINV190	0.008
EGFRINV63	0.034		EGFRINV127	0.050		EGFRINV191	0.028
EGFRINV64	0.030		EGFRINV128	0.047		EGFRINV192	0.000
						EGFRINV193	0.470

APPENDIX VI

Counts for invasive disease - HER2

Patient ID	B2INVXN			Patient ID	B2INVXN			Patient ID	B2INVXN
B2INV1	3.16			B2INV60	10.56			B2INV119	31.43
B2INV2	1.96			B2INV61	4.71			B2INV120	1.4
B2INV3	2.61			B2INV62	0.21			B2INV121	4.89
B2INV4	0.84			B2INV63	2.32			B2INV122	1.24
B2INV5	1.52			B2INV64	2.99			B2INV123	5.54
B2INV6	3.4			B2INV65	3.04			B2INV124	1.49
B2INV7	5.75			B2INV66	159.02			B2INV125	94.1
B2INV8	1.19			B2INV67	0.37			B2INV126	7.08
B2INV9	5.05			B2INV68	2.14			B2INV127	157.47
B2INV10	0.96			B2INV69	8.8			B2INV128	3.7
B2INV11	0.31			B2INV70	3.24			B2INV129	1.3
B2INV12	6.2			B2INV71	0.95			B2INV130	2.33
B2INV13	21.44			B2INV72	1.12			B2INV131	0.84
B2INV14	28.05			B2INV73	144.94			B2INV132	1.6
B2INV15	2.7			B2INV74	0.59			B2INV133	5.79
B2INV16	4.59			B2INV75	3.52			B2INV134	5.67
B2INV17	5.89			B2INV76	6.52			B2INV135	0.92
B2INV18	79.41			B2INV77	112.89			B2INV136	3.71
B2INV19	1.05			B2INV78	1.55			B2INV137	2.76
B2INV20	3.81			B2INV79	0.73			B2INV138	4.5
B2INV21	136.37			B2INV80	30.93			B2INV139	154.58
B2INV22	0.83			B2INV81	173.75			B2INV140	5.1
B2INV23	2.05			B2INV82	0.72			B2INV141	1.21
B2INV24	3.56			B2INV83	5.37			B2INV142	0.92
B2INV25	63.21			B2INV84	17.08			B2INV143	3.57
B2INV26	125.79			B2INV85	1.05			B2INV144	2.01
B2INV27	3.46			B2INV86	1.56			B2INV145	2.59
B2INV28	2.01			B2INV87	6.82			B2INV146	2.5
B2INV29	286.67			B2INV88	5.12			B2INV147	4.67
B2INV30	2.67			B2INV89	29.34			B2INV148	6.2
B2INV31	2.34			B2INV90	90.06			B2INV149	36.67
B2INV32	8.24			B2INV91	0.33			B2INV150	0.04
B2INV33	35.24			B2INV92	293.57			B2INV151	1.31
B2INV34	4.37			B2INV93	1.7			B2INV152	1.7
B2INV35	2.23			B2INV94	44.81			B2INV153	3.61
B2INV36	1.63			B2INV95	324.34			B2INV154	2.43
B2INV37	7.67			B2INV96	1.49			B2INV155	192.16
B2INV38	0.14			B2INV97	3.44			B2INV156	1.36
B2INV39	2			B2INV98	10.03			B2INV157	6
B2INV40	1.63			B2INV99	1.35			B2INV158	0.76
B2INV41	43.47			B2INV100	133.31			B2INV159	2.77
B2INV42	2.55			B2INV101	159.97			B2INV160	3.26
B2INV43	3.8			B2INV102	0.94			B2INV161	1.14
B2INV44	213.98			B2INV103	3			B2INV162	2.98
B2INV45	0.14			B2INV104	3.08			B2INV163	3.9
B2INV46	0.44			B2INV105	4.06			B2INV164	214.13
B2INV47	1.88			B2INV106	0.61			B2INV165	18.29
B2INV48	3.76			B2INV107	1.87			B2INV166	1.39
B2INV49	36.94			B2INV108	35.18			B2INV167	10.73
B2INV50	19.22			B2INV109	1.98			B2INV168	5.59
B2INV51	198.07			B2INV110	3.35			B2INV169	1.72
B2INV52	0.64			B2INV111	5.89			B2INV170	90.47
B2INV53	1.22			B2INV112	478.58			B2INV171	1.59
B2INV54	9.08			B2INV113	15.29			B2INV172	0.85
B2INV55	22.8			B2INV114	1.95			B2INV173	6.33
B2INV56	2.94			B2INV115	369.05			B2INV174	6.58
B2INV57	31.99			B2INV116	1.47			B2INV175	1.39
B2INV58	0.74			B2INV117	2.16			B2INV176	2.08
B2INV59	2.63			B2INV118	3.05			B2INV177	5.79

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who used their initiative and imagination

and accepted change

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