Is the biology of breast cancer changing? A study of hormone receptor status 1984-86 and 1996-1997

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Abstract

Introduction: There has been a significant improvement in breast cancer survival in the UK in recent decades. Changes in the molecular epidemiology of breast cancers may have contributed to this, but the existing evidence may be confounded by heterogeneity of laboratory protocols. Methods: Archived tumour samples from all breast cancer surgery patients at two Glasgow hospitals from 1984-86 and 1996-97 were sought, and linked to clinicopathologic, screening, demographic and survival data. Samples were placed in tissue microarrays and underwent immunohistochemistry for ER, PR and Her-2 with strict standardisation. H&E sections were constructed to assess tumour grade. Results: In 1984-86 8% of tumours were grade 1 and 42.9% grade 3 but in 1996-97 14.9% were grade 1 and 36.8% grade 3 (p=0.009). In 1984-86 64.2% of tumours were ER positive and in 1996-97 71.5% were ER positive (p=0.042). This did not appear to be a function of breast screening as there was a significant rise in ER positivity in symptomatic patients between the two cohorts. 44.9% of tumours in 1984-86 and 49.9% of tumours in 1996-97 were PR positive. 21.5% of tumours in 1984-86 and 20.6% of tumours in 1996-97 were Her-2 positive (p=0.772). 5-year survival in 1984-1986 patients was significantly lower than in 1996-1997 patients (p<0.001). When the effect of cohort on survival was adjusted for these changes in ER status and grade, cohort remained a significant independent factor. Conclusions: This study suggests a small but significant rise has occurred in the incidence of ER positive tumours in women in Glasgow. There has also been a shift in grade distribution of tumours, The changes do not fully explain improvements in breast cancer survival but should

be borne in mind when applying the results of clinical trials performed in the past to the women of today.

Breast cancer is the commonest cancer in women in the UK, with over 40,000 cases diagnosed annually. The epidemiology of the disease is unusual as survival rates are increasing in the face of increasing incidence rates (Coleman, 2000). Reasons for increasing survival undoubtedly include advancements in hormonal and chemotherapeutic management, in addition to a trend towards multidisciplinary management and specialist surgeons. The UK's nationwide mammographic screening programme was designed to reduce mortality. In addition, it has been suggested that over time the epidemiology of breast cancer may have been changing, with a greater percentage of tumours being oestrogen receptor (ER) positive than in the past (Bradburn 1998; Glass 2007; Pujol 1994; Li 2003; Henley 2005). However, the disadvantage of studying retrospective data on ER status is that the recommended assays used to establish ER status, and hence their sensitivity and specificity, have changed over time. The following study was therefore undertaken to demonstrate trends in the molecular biology of breast cancers in patients from two large centres in Glasgow by performing immunohistochemistry on archived tumour samples, thereby avoiding artefactual changes in receptor status over time. A secondary aim was to look retrospectively at the survival of the patients in the study.

Materials and Methods

Patient Selection

The current study aimed to compare the molecular phenotype of stored tissue samples from two separate cohorts of patients, defined by the period in which they had their surgery. All female patients who had surgery for operable breast cancer at two teaching units in Glasgow in the pre-defined periods 1984-1986 and 1996-1997 were identified retrospectively from the Scottish Cancer Registry database, sample size calculation having revealed that at least 712 patients were required to detect a rise in the percentage of ER positive tumours from 60% to 70% with a power of 80%. The study had local ethics committee approval. Full pathological, demographic (including whether tumours

were detected at screening mammography) and 5-year survival data (deaths from breast cancer or unrelated causes) was either available from the Scottish Cancer Registry or obtained from the patient's case record or pathology records. Deprivation status was ascertained using established postcode Carstairs deprivation categories (1-7) derived from 1981 or 1991 census data. For each patient an archived paraffin-embedded tumour block was searched for within the relevant pathology department.

Construction of tissue microarrays

Most of the tumours had been originally fixed in formalin, but tumours from 1984 to 1986 at one hospital had been fixed in mercuric chloride. To allow grading of tumours and marking of tumour areas a 4 μm-thick section was taken from each block. Sections were prepared according to routine pathological techniques for paraffin-embedded sections, stained using haematoxylin and eosin and then sent to a pathologist for determination of tumour grade using the modified Scarff-Bloom-Richardson scale and marking of suitable tumour areas. Three 0.6mm circular cores were then taken from the marked areas in each tumour block and placed into paraffin blocks in tissue microarray format, with 198 cores per block. Sections from each block were taken to allow immunohistochemistry for oestrogen receptor (ER), progesterone receptor (PR) and Her-2 receptor to be performed; each full set of sections underwent ER, PR or HER-2 immunohistochemistry at the same time to ensure standardisation of laboratory conditions.

Immunohistochemistry

Positive and negative control slides were used in each protocol. ER immunohistochemistry was carried out using Novocastra 6F11 mouse anti-human ER with secondary antibody and visualisation reagents supplied by Dako; optimisation steps revealed optimal staining to be achieved with a manual protocol at a dilution of 1:50, with epitope retrieval carried out using EDTA at pH 8.0 with a microwave pressure cooker technique for 5 minutes. After the primary antibody step, slides were refrigerated overnight, with the rest of the steps carried out at room temperature. PR immunohistochemistry was carried out using Dako 636 mouse anti-human progesterone receptor with secondary antibody and visualisation reagents also supplied by Dako; optimisation steps revealed optimal staining to be achieved using a dilution of 1:50 and

epitope retrieval using citrate pH 6.0 and a microwave pressure cooker technique for 5 minutes, with the final protocol being carried out at room temperature using a Dako Autostainer. Her-2 immunohistochemistry was carried out in a Dako Autostainer at room temperature using the standard Dako Herceptest protocol.

Analysis

Once immunohistochemistry had been carried out, each core was assessed by light microscopy and scored by an experienced scorer using a weighted histoscore ([% of tumour cells scoring at intensity 1] + [2x % scoring at intensity 2] + [3 x % scoring at intensity 3] (Fraser, 2003). As each tumour had been cored in triplicate a mean histoscore for each core was calculated. For ER and PR, 'positive' was taken as a histoscore of 10 or over and for Her-2 positivity was taken as a histoscore of 90 or over. A second experienced scorer examined 10% of the cores, (Kirkegaard, 2006). Statistical analysis, performed using SPSS statistics software, version 14.0, included chi-squared analysis to compare percentage positivity, t-test to compare means and Kaplan-Meier survival analysis with log-rank test and Cox's proportional hazard regression. Cox's proportional hazard regression was performed in a stepwise fashion. Cohort, ER status, Her-2 status, grade, nodal status, tumour size, age, screen-detected status, and deprivation category were inserted into the model. The final model excluded those variables in which a difference in survival could be explained by one or more other variables, and generated hazard ratios for death from breast cancer for each variable after adjusting for the others

Results

The original sample size was 1076 patients (423 in 1984-86 ['cohort 1'] and 653 in 1996-97 ['cohort 2']) from which 900 tumour blocks were available for analysis (323 in cohort 1, 577 in cohort 2). The demographics of the patients whose tumour blocks were available are seen in Table 1. Mean age of diagnosis was 56.9 in the first cohort and 58.4 in the second cohort, ($p = 0.049$) with median age of diagnosis in the first cohort being 59 and in the second 58. All tumours in the first cohort had been detected symptomatically rather than by screening, the screening programme having yet to be introduced in Scotland. There was no statistically significant difference in the cohorts as regards socioeconomic status.

Table 1: Patient demographics

	1984-1986	**1996-1997**
Mean age at diagnosis	56.9	58.4
Median age at diagnosis	59	58
Age range	23-74	24-93
%detected at screening	0	29
Affluent patients %	12.1	16.5
Intermediate patients %	40.9	47.2
Deprived patients %	47	36.3
Node positive %	59.3	42.4

862 of the 900 samples (95%) underwent grade analysis. 20% of the tumours in cohort 1 and 19% of tumours in cohort 2 did not undergo ER immunohistochemistry due to fragmented cores or absence of tumour in the core. For the same reasons 14% of tumours in the first cohort and 10% in the second cohort did not undergo PR immunohistochemistry, and 15% of tumours in the first cohort and 18% of tumours in the second cohort did not undergo Her-2 staining.

Grade:

In cohort 1, 8% were of tumours were graded overall as grade 1, 49.2% were of grade 2, and 42.9% were grade 3. In cohort 2, 14.9% of tumours were grade 1, 48.3% grade 2 and 36.8% grade 3 (p for difference between cohorts=0.009). The frequencies of grade distribution in the two cohorts are represented in histogram format in figures 1 and 2.

Figure 1: Histogram of grade distribution in 1984-1986 cohort

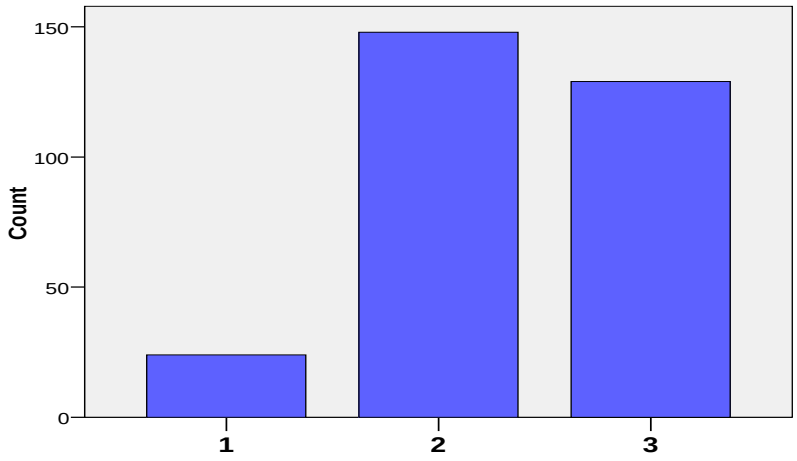
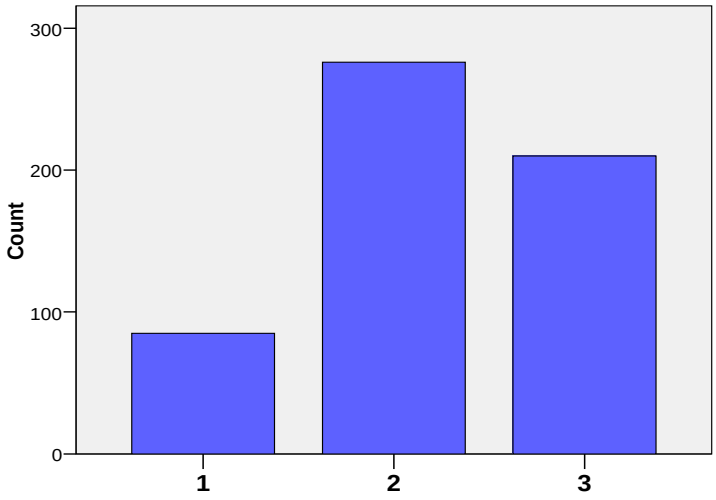


Figure 2: Histogram of grade distribution in 1996-1997 cohort



Analysis of grade distribution within symptomatic and screen-detected patients

Further analysis of tumour grade distribution revealed that there was no significant difference in grade distribution between symptomatically-detected patients in the two cohorts (p=2).

A direct comparison of screen-detected versus symptomatic tumours in cohort 2 showed that within screen-detected tumours, 22.3% were grade 1, 53% were grade 2 and 24.7% were grade 3. Within symptomatic tumours, 12.2% of tumours were grade 1, 46.8% were grade 2 and 41% were grade 3. This difference in grade distribution was significant ($p<0.001$) suggesting that the difference in grade distribution between the two cohorts appears to be being exerted by the screen-detected tumours in cohort 2 having fewer grade 3 and more grade 1 tumours.

A further analysis was performed in order to estimate the effects of screen-detection on grade in women having a subsequent or 'incident' screen in cohort 2 as compared to symptomatic women in cohort 2; eliminating prevalence-screened women should eliminate the postulated effect of length bias on tumour grade. Data on whether tumours were detected in a woman having her first screen or a subsequent screen had not been recorded; however any women aged 50 to 53 with a screen detected tumour is likely to have been having a first screen as these are the ages at which women are invited for a first screen. Therefore analysis was made of the grade distribution within screen-detected tumours in women aged over 53 as compared with younger women. A potential confounding factor in the use of the age cut-off for this analysis is that some women over 53 may be having a first screen having not attended screening previously and some 53-year old women may have previously had a screen aged 50 and be having a repeat screen.

For symptomatic tumours in cohort 2: 12.2% of tumours were grade 1, 46.8% were grade 2 and 41% were grade 3. For tumours presumed detected at subsequent screen in cohort 2: 21.9% of tumours were grade 1, 52.3% were grade 2 and 25.8% were grade 3. p value was <0.001 showing that there was a significant difference in grade distribution between women likely to have been having an 'incident' screen and those with symptomatic tumours.

ER status:

In the 1984-86 cohort, 64.2% of tumours undergoing ER immunohistochemistry were ER positive. In the 1996-97 cohort, 71.5% of tumours were positive (p=0.042). There was no difference in the mean ER score of the two groups; mean ER score of cohort 1 was 97.1 and mean ER score of cohort 2 was 102 (p=0.454). In cohort 1 the median ER score was 104.2; the interquartile range was 190; in cohort 2 the median ER score was 120 and the interquartile range 180.

ER status and screening:

Within the tumours that successfully underwent ER analysis, 66.8% of symptomatically-detected tumours and 78.4% of screen-detected tumours were ER positive (p=0.009). In the 1996-97 cohort there remained a higher percentage of ER positive patients in the screen-detected patients than in the symptomatic patients (78.4% of screen-detected and 68.8% of symptomatic patients, p=0.024). In addition there was a significant difference in ER status between the symptomatic patients in cohort 1 and cohort 2, with 64.2% of patients in cohort 1 and 68.8% of symptomatic patients in cohort 2 being ER positive.

PR status:

44.9% of the 1984-86 cohort tumours that underwent immunohistochemistry and 49.9% of the 1996-97 cohort were PR positive; this rise was not statistically significant (p=0.181). The mean PR score in cohort 1 was 41.2 and the mean score in cohort 2 was 37.9 (p=0.418). The median PR score in cohort 1 was 0 and the interquartile range 79.6; the median PR score in cohort 2 was 8.3 and the interquartile range 61.3.

ER/PR status:

The combined ER/PR status of the tumours in each cohort that underwent both ER and PR immunohistochemistry was also assessed: + represents positivity and – negativity (see table 2)

Table 2: Joint ER and PR distribution

Status	% of cohort 1	% of cohort 2
ER+/PR-	42.4	46.7
ER+/PR-	21.8	24.8
ER-/PR-	33.3	23.5
ER-/PR+	2.5	5

χ^2 analysis revealed a significant difference in distribution between the cohorts. Of particular note is the marked decrease over time in the percentage of tumours that were ER and PR negative from 33.3% to 23.5%.

Her-2 status

In the tumours that underwent Her-2 analysis, 21.5% of tumours in 1984-86 were Her-2 positive and 20.6% of tumours in 1996-97 were Her-2 positive (p=0.772). The mean Her-2 score in the 1996-97 was lower than it had been in 1984-86 (43.1 vs 52.2) but this did not reach statistical significance (p=0.170). The median Her 2 score in cohort 1 was 0 and the interquartile range 50; the median Her2 score in cohort 2 was 0 and the interquartile range 66.7.

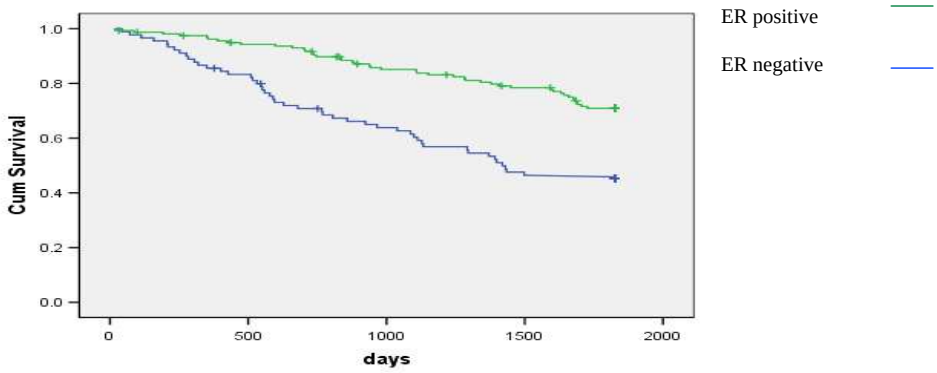
Survival

Kaplan-Meier analysis and log-rank test were performed to assess survival differences (overall and breast-cancer specific survival) between the two cohorts and between subgroups (analysis performed on those patients whose tumours underwent immunohistochemistry). Log-rank test confirmed that cumulative 5-year survival in the 1984-1986 cohort was significantly lower than in the 1996-1997 cohort (0.580 vs 0.834, $p<0.001$); Log-rank test also confirmed that breast cancer-specific 5-year survival in cohort 1 was significantly lower than in cohort 2 (0.620 vs 0.887, $p<0.001$). 5 year

breast-cancer specific survival was significantly higher in ER positive patients than ER negative patients in the study (0.856 vs 0.647, p<0.001).

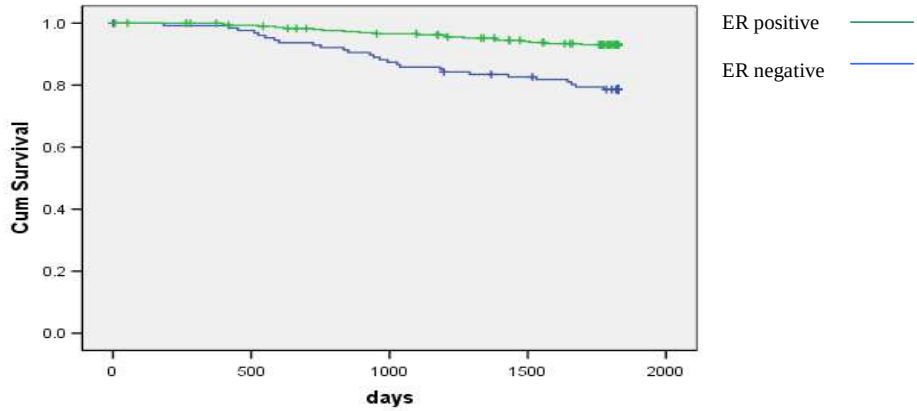
In cohort 1, disease-specific survival was higher in ER positive than ER negative patients (0.709 vs 0.453, p<0.001); this is demonstrated in figure 3.

Figure 3: Breast-cancer specific survival by ER status in 1984-86 cohort



In cohort 2, 5-year survival was again significantly higher in ER positive than ER negative patients (p<0.001); however the survival difference between the two groups appears much narrower (0.930 vs 0.786 - see Figure 4).

Figure 4: Breast cancer-specific survival by ER status in 1996-97 cohort



Survival analysis by screen-detected or symptomatic status

When all the study patients were assessed, disease-specific survival in those women whose tumours were screen-detected was significantly higher than those who had tumours detected symptomatically (0.916 vs 0.753, $p<0.001$). However, when cohort 2 was analysed alone there was no disease-specific survival difference between women whose tumours were screen-detected and those whose tumours were detected symptomatically (0.916 vs 0.874, $p=0.148$). Within symptomatic patients only, there was a significant survival difference between cohorts 1 and 2 (0.614 in cohort 1 and 0.874 in cohort2, $p<0.001$). In both cohort 1 and cohort 2, nodal status was related to survival, with node positive patients having a lower 5-year survival than node-negative patients (0.523 vs 0.799 and 0.801 vs 0.946, with $p<0.0001$ for each). As with ER status, in the second cohort again the survival difference appeared to be narrower than in the first.

Cox's proportional hazard regression

When the effect of cohort type (1 or 2) on survival was adjusted for differences in ER status alone using Cox's proportional hazard regression, the cohort type was a significant independent factor in survival. After correcting for all the factors in the model, the effect of cohort on survival persisted; patients in cohort 1 had a higher relative risk of death than cohort 2, with hazard ratio of breast cancer death of 3.43. Grade, screen-detected status, Her-2 status and intermediate deprivation status were not independent predictors of survival in the study as a whole and could be explained by differences in other factors. Increasing age, increasing tumour size, node-positive status, ER negative status and deprived status correlated with increased hazard of breast cancer death after adjustment for other factors.

Discussion

This study has a demonstrated a rise over time in the percentage of breast cancers that were ER positive. The rise from 64.2% to 71.5% is statistically significant and clinically significant - in that a rise in the number of breast tumours potentially responsive to hormonal manipulation could contribute to increasing survival rates as a result of the clear prognostic advantage of hormone-treated ER positive disease over ER negative disease (Clarke, 2005). There was also a significant change in combined ER/PR receptor status, most notably a marked decrease in the percentage of tumours that had the poor prognostic ER negative/PR negative status. The percentage of tumours that were PR positive and Her-2 positive did not change over time; notably there was no change in mean score over time for any of the three receptors.

A significant change in grade distribution was seen, particularly a reduction in the frequency of grade 3 tumours and an increase in the frequency of grade 1 tumours. This distribution change appeared to be exerted by the presence of screen-detected tumours in the second cohort, as there was no significant difference in the grade distribution of the symptomatically-detected tumours in both cohorts. The pathological grade of screen-detected tumours and its significance has received much attention in the literature; while

it is accepted that tumours detected at a first screening mammogram are of lower grade than symptomatically detected tumours it is uncertain as to whether this represents an interruption of phenotypic drift or whether the lower-grade tumours are detected at screening because of their longer asymptomatic preclinical phase (Alexander,1997; Crisp, 1993; Duffy, 1991; Tabar, 1999). An attempt was made here to estimate whether women were receiving an incident screen or not, as length bias is a problem with prevalence screen-detected tumours; the results suggested a significant difference in grade between symptomatic patients and patients having a tumour detected at an incident screen. This would appear to support the theory of phenotypic drift but clearly misclassification of screening episode is a clear possible confounding factor.

The study has also demonstrated significant differences in the percentage of tumours that are ER positive within patients with screen-detected compared to symptomatic tumours, with 78.4% of screen-detected and 68.8% of symptomatic patients in the newest cohort being ER positive. Certainly there is evidence to suggest that screen-detected tumours are more likely to be ER positive than negative (Klemi, 1992; Ernst, 2002), probably because the ER positive tumours are more likely to be slower growing with a significant asymptomatic phase. It is notable that the ER positive rate increase over time is not explained by the screened patients alone, with the ER positive rate increase persisting within the symptomatic patients.

Studies in the affluent Marin County area of San Francisco suggested that rates of ER positive disease with incidence rates of ER negative disease remaining fairly constant. (Benz, 2003). A study of women in the large Kaiser Permanente health plan in the US calculated annual incidence rates of ER negative and ER positive breast cancers in the women in the health plan, and found that the incidence rate for ER negative disease had remained relatively constant with an abrupt decline from 1999 onwards, whereas for ER positive disease there was a significant rise in incidence throughout the study period, particularly in the period of 1983-1986 when there was an annual increase in incidence of 18.9% (Glass,2007). A study of 12000 tumours between 1973 and 1992 in the US showed a significant increase in the percentage of tumours that were ER positive (Pujol,1994) after adjustment for the distribution of other clinicopathological factors.

Other smaller studies have also suggested a rise in percentage of ER positivity over time (Bradburn,1998; Henley, 2005; Li,2003).

In most of the studies of trends in ER status over time, the assays and criteria used to determine ER positivity changed several times during the study periods as a result of development of new methods of ER determination. Studies of the concordance of ligand-binding assay and immunohistochemistry have showed that the concordance can be as low as 82% (Allred, 1990). In the current study we used immunohistochemistry on all samples, thereby eliminating the possibility that an increase in ER positivity has been artefactual. Furthermore for each antibody, all the samples underwent immunohistochemistry together to eliminate the potential effect of changing laboratory conditions on staining.

The ER content of tumours increases with age (McCarty KS, 1983) with more than 80% of breast cancers in women over 45 being ER positive (Glass,2007). The age range in the second cohort was slightly greater in the second cohort, but the mean and median age of diagnosis in the two cohorts were similar with there being no significant difference in median age at diagnosis.; 85% of women in the 1984-86 cohort were over 45 and 86.6% of the 1996-1997 cohort were over 45. Therefore age has not been a confounding factor in the study.

One explanation for a preferential increase in ER positive tumours could be a population-wide change in the prevalence of certain factors that have been shown to increase the frequency of ER positive disease. Such factors include late age at first pregnancy and postmenopausal obesity (Althuis, 2004; Colditz, 2004; Potter, 1995); use of hormone replacement therapy was linked to ER positive disease in one study only (Potter, 1995). There is evidence that the percentage of all children being born to mothers of age 35 and over is increasing in Scotland, and that mean BMI and prevalence of obesity are increasing (Brown, 2007). In England the estimated prevalence of HRT use in 45-64 year olds increased from 2.2% in 1987 to 22% in 1994 (Townsend,1998); since then there appears to have been little change in prevalence of use (Bromley, 2004), with unpublished work by the authors of the current study suggesting that this is also true for Scotland. Unfortunately data on hormone therapy use by the patients in the current

study had not been routinely recorded and the absence of case notes for many of the women in the 1984-86 cohort precluded the ability to determine this.

The authors acknowledge that study was powered to detect a 10% difference in ER positive prevalence, and is hence underpowered to detect the observed 7% difference. The inability to retrieve tumour block for all patients means that the calculated figures to not represent the entire two cohorts of patients; the cohort is further reduced by the tumours which were not suitable for immunohistochemistry because of sampling error or damage to the core while being inserted into the tissue microarray, a factor common to studies involving tissue microarray immunohistochemistry. Those tumours which underwent analysis should be representative of the cohort as a whole.

Breast cancer-specific and overall 5-year survival in cohort 1 were significantly lower than in cohort 2. 5 year breast-cancer specific survival was significantly higher in ER positive patients than ER negative patients in the study overall and in each cohort independently. In the second cohort, improved survival in ER positive patients may reflect the fact that in 1984-1986, whilst beginning to be used, hormonal therapies may have been relatively under-prescribed by today's standards due to different ER techniques and cut-offs for 'positivity' and different advice on suitability for hormonal therapy; the more marked survival improvement in ER negative patients over this time period is most likely due to improvements in chemotherapy. Unfortunately treatment data is not available for the women in this study, as in 1984-86 the Cancer Registry did not routinely record treatment received and case notes were not available.

In the second cohort there was no disease-specific survival difference between women whose tumours were screen-detected and those whose tumours were detected symptomatically; within the symptomatic patients there was a significant survival difference between cohorts. However, this cannot be directly extrapolated to the conclusion that screening prolongs survival; the effects of screening on survival as opposed to mortality are complex because of the potential for lead-time bias and further analysis of these data is outwith the scope of the current study.

A true change in ER status could have profound implications for the application of data from clinical trials carried out in previous decades to the women of today, as a change in the prevalence of ER positive disease could alter the overall survival benefit seen from

chemotherapy and different hormonal therapies. It is also possible that changes in hormone receptor status are contributing to the observed survival increases.

The patients in cohort 1 had a higher relative risk of death than cohort 2 with a hazard ratio of breast cancer 3.43 after correcting for all tumour and demographic factors; when the effect of cohort on survival was adjusted for ER status alone the cohort remained a significant independent factor in survival. As expected the difference in survival between cohorts is not fully explained by differences in ER status; the fact that cohort remains an independent factor in survival after adjustment for all demographic and tumour factors supports the suggestion that treatment and global management changes have contributed to changes in survival over time (Bradburn, 1998). From the literature, the contribution of screening to this survival improvement remains less clear (Blanks, 2000; Thomson, 2004; Stockton, 1997).

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