Association between CD8+ T-cell infiltration and breast cancer survival in 12 439 patients


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Background: T-cell infiltration in estrogen receptor (ER)-negative breast tumours has been associated with longer survival. To investigate this association and the potential of tumour T-cell infiltration as a prognostic and predictive marker, we have conducted the largest study of T cells in breast cancer to date.

Patients and methods: Four studies totalling 12 439 patients were used for this work. Cytotoxic (CD8+) and regulatory (forkhead box protein 3, FOXP3+) T cells were quantified using immunohistochemistry (IHC). IHC for CD8 was conducted using available material from all four studies (8978 samples) and for FOXP3 from three studies (5239 samples)—multiple imputation was used to resolve missing data from the remaining patients. Cox regression was used to test for associations with breast cancer-specific survival.

Results: In ER-negative tumours [triple-negative breast cancer and human epidermal growth factor receptor 2 (HER2) positive], presence of CD8+ T cells within the tumour was associated with a 28% [95% confidence interval (CI) 16% to 38%] reduction in the hazard of breast cancer-specific mortality, and CD8+ T cells within the stroma with a 21% (95% CI 7% to 33%) reduction in hazard. In ER-positive HER2-positive tumours, CD8+ T cells within the tumour were associated with a 27% (95% CI 4% to 44%) reduction in hazard. In ER-negative disease, there was evidence for greater benefit from anthracyclines in the National Epirubicin Adjuvant Trial in patients with CD8+ tumours [hazard ratio (HR) = 0.54; 95% CI 0.37–0.79] versus CD8− tumours (HR = 0.87; 95% CI 0.55–1.38). The difference in effect between these subgroups was significant when limited to cases with complete data (P heterogeneity = 0.04) and approached significance in imputed data (P heterogeneity = 0.1).

Conclusions: The presence of CD8+ T cells in breast cancer is associated with a significant reduction in the relative risk of death from disease in both the ER-negative [supplementary Figure S1, available at Annals of Oncology online] and the ER-positive HER2-positive subtypes. Tumour lymphocytic infiltration may improve risk stratification in breast cancer patients classified into these subtypes.

NEAT ClinicalTrials.gov: NCT00003577.

Key words: breast cancer, lymphocytes, inflammation, chemotherapy, molecular subtypes

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**introduction**

The importance of lymphocytic infiltration in predicting disease progression has been shown in different types of solid tumour but most impressively in colorectal and ovarian cancer where the presence of tumour-infiltrating T cells is associated with reduced recurrence rates and longer survival [1, 2]. Moreover, modulation of the T-cell response has shown clinical efficacy in solid tumours [3] and the tumouricidal effect of trastuzumab has been shown to depend upon the immune response in breast cancer [4]. As a major component of the adaptive immune system, cytotoxic (CD8+) T cells represent a candidate biomarker of the tumour-associated immune response. Most previous studies of CD8+ T lymphocytes in breast cancer have reported an association with favourable outcome [5–7] but others have not [8]. Unlike CD8+ T lymphocytes, T-regulatory lymphocytes (T-regs) exert an immunosuppressive effect by diminishing the response to self-antigens. Therefore, tumours may hijack this function of T-regs to create an immune-privileged niche to facilitate unimpeded tumour growth [9]. Nuclear expression of forkhead box protein 3 (FOXP3) characterises T-regs. The presence of FOXP3+ T lymphocytes in breast tumours has been associated with both reduced survival [10] and improved survival [11]. In addition to their association with survival, some reports have also found a link between the presence of immune cells and the effect of chemotherapy [12, 13].

We have investigated the importance of cytotoxic (CD8+) and regulatory (FOXP3+) T cells in breast tumours by conducting a study of over 12,000 patients from the UK and Canada. Our aims were to characterise the effect of these subsets of T lymphocytes on survival, to determine whether this effect is modified by the molecular subtype of the primary tumour and to establish whether lymphocytic infiltration influenced the effect of chemotherapy on breast cancer mortality.

**methods**

**ethics statement and study populations**

We used data from three observational studies of newly diagnosed breast cancer [Study of Epidemiology and Risk Factors in Cancer Heredity (SEARCH) [14], N = 4079; the British Columbia Cancer Agency (BCCA) [5], N = 4520; the Nottingham Tenovus Primary Breast Cancer Series (NBCS) [6], N = 1842] and one randomised, controlled trial [the National Epirubicin Adjuvant Trial (NEAT) [15], N = 1998 composed of both NEAT (n = 1684) and BR9/601 (n = 314)] of breast cancer. Analyses of T-cell data from two of these studies have been published previously [5, 6, 10]. All participating studies were approved by the relevant research ethics committee. SEARCH is a prospective population-based study of women diagnosed with breast cancer in East Anglia, England. The BCCA study comprised women diagnosed with breast cancer between 1986 and 1992 in British Columbia and referred to BCCA for consideration of adjuvant therapy. The NBCS comprises patients diagnosed and treated at Nottingham City Hospital between 1987 and 1998. Details of the National Epirubicin Adjuvant Trial and BR9601 trial (here referred to collectively as NEAT) have been published previously [15]. Briefly, this was a phase III trial in which patients were randomised on a 1 : 1 basis to receive cyclophosphamide, methotrexate and fluorouracil (CMF) or epirubicin in addition to CMF (E-CMF). Results of this trial were first published in 2006. Additional details are provided in the supplementary Methods and in Tables S1 and S2, available at *Annals of Oncology* online.

**immunohistochemistry and scoring**

Immunohistochemistry (IHC) was conducted for CD8 and FOXP3 proteins at host institutions. Details of scoring systems and cut points for positivity are provided in supplementary Table S3, available at *Annals of Oncology* online. Tissue microarrays (TMAs) were used to analyse large numbers of tumour samples simultaneously, each represented by a single 0.6-mm tissue core. Additional details of IHC scoring are provided in supplementary Methods, available at *Annals of Oncology* online. Absolute numbers of immunoreactive tumour-infiltrating lymphocytes were counted and classified as ‘intratumoral’ (IT) if seen in direct contact with tumour cells and ‘stromal’ (S) if they were not in direct contact with tumour cells. Tumours were classified into different molecular subtypes as previously described [16] (supplementary Table S4, available at *Annals of Oncology* online). T-cell counts were dichotomised for statistical analyses using a pre-specified cut point of zero versus any more than zero immunoreactive lymphocytes. This cut point was chosen because discrimination between tissue completely devoid of positive lymphocytes and tissue containing any positive lymphocytes is likely to be reliable. Information on FOXP3+ T lymphocytes was available for the SEARCH, NBCS and NEAT studies only.

**statistical analyses**

Cox regression models stratified by study were used to test for associations with breast cancer-specific survival (BCSS). Follow-up time was truncated at 10 years. Women with estrogen receptor (ER)-positive and ER-negative breast cancer were analysed separately because of differences in their patterns of short- and long-term survival [16]. Late entry for the SEARCH study was accounted for by left truncation of survival time data. Variables that showed a time-dependent association with survival, and therefore violated the Cox proportional hazards assumption, were modelled by using an extended Cox model to include a coefficient (T) which varied linearly as a function of the logarithm of time. Variables significantly associated with BCSS on univariate analyses were also evaluated in multivariate analysis. Data on hormone therapy was not available for the NEAT study hence multivariate models excluding this study and including hormone therapy as a covariate are presented in the supplementary material, available at *Annals of Oncology* online. Cochran’s Q-test was used to test for heterogeneity of the prognostic effect of T-cell status according to different patient and tumour subgroups and of differential benefit of anthracyclines according to T-cell status in the NEAT trial. To determine whether cytotoxic and regulatory T lymphocytes contributed complementary prognostic value and, therefore, whether their prognostic accuracy could be improved by accounting for it, an interaction term between the variables was included in exploratory Cox regression analyses. Multiple imputation was used to adjust for the bias of missing data. This is a statistical technique which resolves missing values by predicting their probable value based on the complete data using a multivariate regression model. The variability between imputed (predicted) values is accounted for by producing multiple datasets. We imputed 50 datasets including all 12 439 patients. Survival estimates based on these data were computed per dataset and combined to account for between- and within-dataset variation (additional details are provided in supplementary Methods, available at *Annals of Oncology* online). The distributions of imputed versus predicted values is accounted for by producing multiple datasets. We imputed 50 datasets including all 12 439 patients. Survival estimates based on these data were computed per dataset and combined to account for between- and within-dataset variation (additional details are provided in supplementary Methods, available at *Annals of Oncology* online).
Statistical methods are further detailed in the supplementary material, available at Annals of Oncology online. Analyses are reported in accordance with REMARK guidelines [17]. All analyses were conducted using Intercooled Stata version 11.2 (Stata Corp., College Station, TX). Data analysis was conducted by Ali. The Stata code used for all survival analyses can be made available upon request from the corresponding author.

**results**

Details of the participating studies and patient characteristics are provided in supplementary Tables S1–S2 and Figure S3, available at Annals of Oncology online. In total, there were 12,439 patients of which 2,674 (21%) died of breast cancer within 10 years of diagnosis. The median survival was 9.57 years (range 0.05–20.6 years). Supplementary Figure S4, available at Annals of Oncology online illustrates the distribution of lymphocyte counts by study, tumour morphology and molecular subtype.

**prognostic value of T cells**

In ER-negative tumours, the presence of S- and iT-CD8+ lymphocytes was independently associated with a reduced relative risk of death from breast cancer (Table 1 and Figure 1). Supplementary Tables S5 and S6, available at Annals of Oncology online detail univariate Cox regression analyses for all variables. Table 1 contains the multivariate Cox regression models (supplementary Tables S7–S9, available at Annals of Oncology online detail multivariate models based on complete data and including hormone therapy as a covariate). The adjusted hazard ratio (HR) for iT-CD8+ tumours was 0.72 [95% confidence interval (CI) 0.62–0.84, \( P = 0.00003 \)] and, for S-CD8+, the HR was 0.79 [95% CI 0.67–0.93, \( P = 0.004 \)]. For women with ER-negative breast cancer, absolute survival estimates (Kaplan–Meier survival function) for tumours positive for both iT-CD8+ and S-CD8+ lymphocytes compared with tumours negative for both were 77% (95% CI 74% to 79%) versus 66% (95% CI 62% to 70%) at 5 years and 71% (95% CI 68% to 74%) versus 58% (95% CI 54% to 63%) at 10 years (Figure 1). The presence of CD8+ lymphocytes was not associated with BCSS in ER-positive breast tumours (supplementary Table S5, available at Annals of Oncology online). The presence of FOXP3+ lymphocytes was not associated with BCSS after adjustment for known prognostic factors (supplementary Tables S10 and S11, available at Annals of Oncology online) irrespective of ER status. Unadjusted Cox regression analyses including an interaction term between cytotoxic and regulatory T-cell variables did not reveal a significant interaction between iT or S T-cell types irrespective of ER status (supplementary Table S12, available at Annals of Oncology online).

**subgroup analysis and chemotherapy**

Significant heterogeneity of the prognostic effect of T cells was observed for different patient and tumour subgroups. Figure 2 shows HRs and 95% CIs from univariate Cox regression

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*Sample size varied between imputed datasets. Reported sizes are the smallest of 50 imputations.

<sup>1</sup>Variables which violated the proportional hazards assumption were accounted for by an extended Cox model where the \( \beta \) coefficient varies linearly with the natural logarithm of time. \( T \) represents the exponent of the extended coefficient where a value >1 implies increasing hazard over time whereas a value <1 implies decreasing hazard over time.

HR, hazard ratio; 95% CI, 95% confidence interval; HER2, human epidermal growth factor receptor 2; iT-CD8, intratumoral CD8; S-CD8, stromal CD8.
analyses of iT-CD8+ lymphocytes as separate forest plots for ER-positive and ER-negative disease; equivalent disease. Equivalent plots for S-CD8+, iT-FOXP3+ and S-FOXP3+ status are presented as supplementary Figures S5–S11, available at Annals of Oncology online. In particular, the prognostic effect of iT-CD8+ lymphocytes differed by HER2 status in ER-positive breast cancer (P_{heterogeneity} = 0.006). For ER-positive HER2-negative tumours, the HR was 1.16 (95% CI 1.02–1.32) and, for ER-positive HER2-positive tumours, the HR was 0.76 (95% CI 0.58–1.00). Following adjustment for histological grade, the HR associated with iT-CD8+ status in ER-positive HER2-negative tumours was 1.04 (95% CI 0.91–1.20). Figure 3 shows the absolute differences in survival of iT-CD8+ status within luminal and non-luminal breast tumours. Based on this finding, multivariate analysis of iT-CD8+ lymphocytes was conducted within the ER-positive HER2-positive subgroup as detailed in Table 2 and supplementary Table S13, available at Annals of Oncology online. The HR for iT-CD8+ lymphocytes was 0.75 (95% CI 0.56–0.96, P = 0.022) after adjustment for known prognostic factors.

Supplementary Figures S12 and S13, available at Annals of Oncology online depict the HRs and 95% CIs from Cox regression models adjusted for tumour size, positive lymph nodes and grade according to whether adjuvant chemotherapy was received for different T-cell types. There was no significant heterogeneity of the prognostic effect of T cells according to whether chemotherapy was administered. In order to account for differences in chemotherapeutic regimens between studies, subgroup analyses by study were conducted for iT-CD8+ status (supplementary Figure S14, available at Annals of Oncology online); no significant difference in prognostic effect was observed by whether chemotherapy had been received within each study. supplementary Figures S15 and S16, available at Annals of Oncology online are forest plots of the adjusted HRs and 95% CIs for the benefit of the addition of epirubicin to CMF in the NEAT trial in patient subgroups defined by T-cell status. Although there was no evidence of significant differential benefit of epirubicin in these T-cell-defined subgroups, there was a trend toward increased benefit in ER-positive patients with tumours devoid of S cytotoxic T cells (P_{heterogeneity} = 0.087) and, in ER-negative patients, with tumours positive for iT-CD8+ lymphocytes (P_{heterogeneity} = 0.12) using imputed data. Analysis restricted to cases with complete data only showed that the presence of iT-CD8+ cells was significantly associated with increased relative benefit from epirubicin (HR = 0.60, 95% CI 0.37–0.96) compared with cases negative for iT-CD8+ cells (HR = 1.47, 95% CI 0.72–3.02; P_{heterogeneity} = 0.039).

Further subgroup analyses were conducted to determine whether the prognostic effect of T cells varied according to tumour cell proliferation as suggested by a recent study [18] and according to patient age as a result of the age-related decline of the immune system known as immunosenescence [19]. The results of these analyses are depicted in supplementary Figure S17, available at Annals of Oncology online. No significant heterogeneity was observed by tumour proliferation status or age at diagnosis.

**Figure 1.** Kaplan–Meier survival plot of patient groups defined by the presence of intratumoral (iT)-CD8+ and stromal (S)-CD8+ cells in ER-negative disease. Unadjusted survival estimates at 5 and 10 years for double-positive and double-negative tumours are shown. Note: numbers at risk account for delayed entry of patients enrolled in the SEARCH study.
analyses by using multiple imputation [20]. Supplementary data generally favoured smaller, lower grade, ER-positive breast tumours compared with larger tumours. This means that cases likely that there will be insufficient tissue for analysis in smaller tumours compared with larger tumours. This means that cases with complete data are a biased representation of the overall population. However, unbiased estimates can be computed by using a method to resolve missing values such as multiple imputation [21, 22]. This explains why the distribution of imputed data generally favoured smaller, lower grade, ER-positive tumours for which data are more likely to be missing (supplementary Table S4, available at Annals of Oncology online).

discussion
This study of 12 439 women with breast cancer is the largest evaluation of T cells as a tumour marker in breast cancer to date. It shows that the presence of CD8+ T cells in ER-negative breast tumours is associated with a reduction in the relative hazard of dying from breast cancer of between 57% and 21% depending on their location (iT, S or both) and, for iT-CD8+ T cells, with a 27% reduction in the hazard of dying from breast cancer in ER-positive HER2-positive tumours.

We included a large number of well-characterised patients in this study and, therefore, our conclusions are statistically robust. In addition, we have been able to evaluate breast cancer as a group of related diseases (molecular subtypes) rather than a single entity. We have also adjusted our estimates for the bias of missing data for any of the variables included in multivariate analyses by using multiple imputation [20]. Supplementary Figure S2, available at Annals of Oncology online depicts survival plots of subgroups of patients according to whether data were missing for CD8, FOXP3, ER and HER2. Missing data were significantly associated with improved survival. This is because data were not missing completely at random but were correlated with other variables such as tumour size. For example it is more likely that there will be insufficient tissue for analysis in smaller tumours compared with larger tumours. This means that cases with complete data are a biased representation of the overall population. However, unbiased estimates can be computed by using a method to resolve missing values such as multiple imputation [21, 22]. This explains why the distribution of imputed data generally favoured smaller, lower grade, ER-positive tumours for which data are more likely to be missing (supplementary Figure S1, available at Annals of Oncology online). The sampling error associated with representation of tumours in TMAs can result in reduced power to detect associations.
Figure 3. Kaplan–Meier survival plots of patient groups defined by the presence of iT-CD8+ cells in luminal (left) and non-luminal (right) tumours. Note: numbers at risk account for delayed entry of patients enrolled in the SEARCH study. Definitions of molecular subtypes within ER-positive breast cancer (luminal 1a, luminal 1b, luminal 2) and ER-negative breast cancer (HER2, CBP = core basal phenotype, 5NP = five-marker-negative phenotype) are defined in supplementary Table S4, available at Annals of Oncology online.
However, only by using TMAs has it been feasible to conduct a study of this size, and this has proportionally reduced the likelihood of false-negative findings. Although slight between-institution variation in methods of lymphocyte detection may have introduced some bias, the diversity of studies included in this analysis has also meant that our conclusions are likely to reflect the complete heterogeneity of breast cancer and will therefore be applicable to other populations.

Since the immune response can exert paradoxical effects in cancer, we evaluated two functionally distinct subsets of T cells: cytotoxic and regulatory T cells. Cytotoxic T cells, identifiable by CD8 expression, form a major effector component of the adaptive immune system. Cells that present foreign antigens in association with the major histocompatibility complex class I molecule are recognised by cytotoxic T lymphocytes through a specific interaction between the presented antigen and the T-cell receptor [23]. This interaction causes the activated T cell to release proteins such as perforin and granzyme which kill the cell through membranolysis [23]. These mechanisms can act on tumour cells which, unlike normal cells, can present atypical antigens [24, 25]. Regulatory T cells, which express FOXP3, act to diminish an immune response to self-antigens. The hypothesis that regulatory T cells may be recruited by tumours to evade immune destruction is supported by the observation that their infiltration in ER-negative (both HER2-positive and HER2-negative) and ER-positive/HER2-positive breast cancer, iT- and S-CD8+ lymphocytes were independently associated with a reduced risk of death from breast cancer. In conjunction with clinical parameters, assessment of the immune response may aid risk stratification of patients with these breast cancer subtypes.

acknowledgements

We thank the participants of the SEARCH, BCCA, NEAT and NBCS studies who have permitted the use of their tissue for research and to the many individuals who have made this work possible.

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disclosure

TON reports receiving consultancy fees from Bioclassifier LLC amounting to less than $10 000 and not bearing directly on

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*Sample size varied between imputed datasets. Reported sizes are the smallest of 50 imputations.

*Variables which violated the proportional hazards assumption were accounted for by an extended Cox model where the β coefficient varies linearly with the natural logarithm of time. ‘T’ represents the exponent of the extended coefficient where a value >1 implies increasing hazard over time whereas a value of <1 implies decreasing hazard over time.

HR, hazard ratio; 95% CI, 95% confidence interval; HER2, human epidermal growth factor receptor 2; iT-CD8, intratumoral CD8.
this study. All remaining authors have declared no conflicts of interest.

references


