Original article

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

H. R. Ali [1,2,4], E. Provenzano[1,4], S-J. Dawson[1,3,4], F. M. Blows[3,], B. Liu[1,3,4], M Shah[3,4,5], H. M. Earl[3,4], C. J. Poole[6], L. Hiller[6], J. A. Dunn[6], S. J. Bowden[7], C. Twelves[8], J. M. S. Bartlett[9], S. M. A. Mahmoud[10, 11], E. Rakha[10], I. O. Ellis[10], S. Liu[12], D. Gao[12], T. O. Nielsen[12], P. D. P. Pharoah[3,4,5], C. Caldas[1,3,4]

Affiliations

1. Cancer Research UK Cambridge Institute, University of Cambridge Cambridge, UK
2. Department of Pathology, University of Cambridge, Cambridge, UK
3. Department of Oncology, University of Cambridge, Cambridge, UK
4. Cambridge Experimental Cancer Medicine Centre and NIHR Cambridge Biomedical Research Centre, Cambridge, UK
5. Strangeways Research Laboratory, Cambridge, UK
6. Warwick Clinical Trials Unit, University of Warwick, Coventry, UK
7. Cancer Research UK Clinical Trials Unit, Institute for Cancer Studies, The University of Birmingham, Birmingham, UK
8. University of Leeds and Leeds Cancer Research UK Centre, St James' Institute of Oncology, Leeds, UK
9. Ontario Institute for Cancer Research, Toronto, Canada and Biomarker and Companion Diagnostics, Edinburgh Cancer Research Centre, Edinburgh, UK.
10. Department of Histopathology and School of Molecular Medical Sciences, The University of Nottingham and Nottingham University Hospitals NHS Trust, Nottingham, UK

11. Current affiliation: Department of Pathology and Laboratory Medicine, University of British Columbia; Integrative Oncology Department, British Columbia Cancer Agency, Vancouver, Canada
12. Genetic Pathology Evaluation Centre, Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver, Canada

Corresponding author:
Professor Carlos Caldas
Cancer Research UK Cambridge Institute,
University of Cambridge,
Li Ka Shing Centre,
Robinson Way,
Cambridge,
CB2 0RE,
UK
Tel: +44 1223 769650
carlos.caldas@cruk.cam.ac.uk

Abstract

Background

T-cell infiltration in 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 ($FOXP3^+$) T-cells were quantified using immunohistochemistry (IHC). IHC for CD8 was conducted using available material from all four studies (8,978 samples) and for FOXP3 from three studies (5,239 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 HER2-positive), presence of $CD8^+$ T-cells within the tumour was associated with a 28% (95% CI, 16% - 38%) reduction in the hazard of breast cancer-specific mortality, and $CD8^+$ T-cells within the stroma with a 21% (95% CI, 7% - 33%) reduction in hazard. In ER-positive HER2-positive tumours, $CD8^+$ T-cells within the tumour were associated with a 27% (95% CI, 4% - 44%) reduction in hazard. In ER-negative disease there was evidence for greater benefit from anthracyclines in the NEAT trial in patients with $CD8^+$-positive tumours (hazard ratio = 0.54; 95% CI, 0.37-0.79) versus $CD8^+$-negative tumours (hazard ratio = 0.87; 95% CI, 0.55-1.38). The difference in effect between these subgroups was significant when limited to cases with complete data ($P_{\text{heterogeneity}}$=0.04) and approached significance in imputed data ($P_{\text{heterogeneity}}$=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 and the ER-positive HER2-positive subtypes.

Tumour lymphocytic infiltration may improve risk-stratification in breast cancer patients classified into these subtypes.

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

NEAT trial ClinicalTrials.gov number, NCT00003577

Key message

We have conducted the largest study of the prognostic and predictive value of tumour infiltrating T-cells in breast cancer including over 12,000 patients. $CD8^+$ cytotoxic T-cells in breast tumours are associated with a reduced risk of death from breast cancer in ER-negative (HER2-positive and negative) and ER-positive HER2-positive breast cancer but not in ER-positive HER2-negative breast cancer.

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 U.K. 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 (SEARCH[14], *N*=4,079; BCCA[5], *N*=4,520; NBCS[6], *N*=1,842) and one randomised controlled trial (NEAT[15], *N*=1,998 composed of both NEAT (*N*=1,684) 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 British Columbia Cancer Agency (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 Nottingham Tenovus Primary Breast Cancer Series (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 collectively referred to 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 Supplementary Tables 1 and 2.

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 3. Tissue microarrays (TMAs) were used to analyse large numbers of tumour samples simultaneously, each represented by a single 0.6mm tissue core. Additional details of IHC scoring are provided in Supplementary Methods. 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 4). 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 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 which 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 analysis 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. 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 fifty 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). The distributions of imputed versus observed values for all variables included in the model are illustrated in Supplementary Figure 1. The necessity of adjusting for missing data is illustrated by Supplementary Figure 2 which depicts significant differences in survival in subgroups of patients according to whether data was missing for CD8, FOXP3, ER and HER2. Relative survival estimates derived from imputed data are presented in the main report and estimates from the complete case analysis detailed in the supplementary material. Absolute survival estimates are based on complete data. Statistical methods are further detailed in the supplementary material. Analyses are reported in accordance with REMARK guidelines[17]. All analyses were conducted using Intercooled Stata version 11.2 (Stata Corp, College Station, TX, USA). Data analysis was conducted by H R 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 1-2 and Supplementary Figure 3. 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 4 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 stromal and intratumoral $CD8^+$ lymphocytes were independently associated with a reduced relative risk of death from breast cancer (Table 1 and Figure 1). Supplementary Tables 5 and 6 detail univariate Cox-regression analyses for all variables. Table 1 contains the multivariate Cox-regression models (Supplementary Tables 7-9 detail multivariate models based on complete data and including hormone therapy as a covariate). The adjusted hazard ratio (HR) for iT-$CD8^+$ positivity was 0.72 (95% confidence interval (95% 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 to tumours negative for both were 77% (95% CI, 74%-79%) versus 66% (95% CI, 62%-70%) at five years and 71% (95% CI, 68%-74%) versus 58% (95% CI, 54%-63%) at ten years (Figure 1). The presence of CD8$^+$ lymphocytes was not associated with BCSS in ER-positive breast tumours (Supplementary Table 5). The presence of FOXP3$^+$ lymphocytes was not associated with BCSS after adjustment for known prognostic factors (Supplementary Tables 10 and 11) 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 intratumoral or stromal T-cell types irrespective of ER-status (Supplementary Table 12).

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 analyses of iT-CD8$^+$ lymphocytes as separate forest plots for ER-positive and ER-negative disease; equivalent plots for S-CD8+, iT-FOXP3+ and S-FOXP3+ status are presented as Supplementary Figures 5-11. In particular, the prognostic effect of iT-CD8$^+$ lymphocytes differed by human epidermal growth factor receptor 2 (HER2) status in ER-positive breast cancer ($P_{\text{heterogeneity}}$=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 13. The HR for iT-CD8$^+$ lymphocytes was 0.73 (95% CI, 0.56-0.96, P=0.022) after adjustment for known prognostic factors.

Supplementary Figures 12 and 13 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 14); no significant difference in prognostic effect was observed by whether chemotherapy had been received within each study. Supplementary Figures 15 and 16 are forest plots of the adjusted HRs and 95% CIs for the benefit of the addition of epirubicin to cylcophosphomide, methotrexate and fluorouracil (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 stromal cytotoxic T-cells ($P_{\text{heterogeneity}}$ =0.087) and, in ER-negative patients, with tumours positive for iT-CD8$^+$ lymphocytes ($P_{\text{heterogeneity}}$=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 to cases negative for iT-CD8$^+$ cells (HR = 1.47, 95% CI, 0.72 - 3.02; $P_{\text{heterogeneity}}$=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 17. No significant heterogeneity was observed by tumour proliferation status or age at diagnosis.

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 (intratumoral, stromal or both) and, for intratumoral $CD8^+$ 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 2 depicts survival plots of subgroups of patients according to whether data was missing for CD8, FOXP3, ER and HER2. Missing data was significantly associated with improved survival; this is because data was not missing completely at random but was 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 to 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 is more likely to be missing (Supplementary Figure 1). The sampling error associated with representation of tumours in TMAs can result in reduced power to detect associations. 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 which present foreign antigens in association with the major histocompatibility complex (MHC) class I molecule are recognised by cytotoxic T-lymphocytes through a specific interaction between the presented antigen and the T-cell receptor (TCR)[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 ablation in mice enables an effective anti-tumour immune response[9]. Previous studies evaluating the prognostic value of $FOXP3^+$ T-cells have reported conflicting findings[10, 11, 26].

There have been several studies in breast cancer which find that some readout of the immune response is predictive of chemosensitivity[12, 13, 27]. In the NEAT trial a differential relative benefit of E-CMF over CMF was not observed between patients with tumours positive for T-cells and those with tumours negative for T-cells based on analysis of imputed data (Supplementary Figures 15), however analysis of cases with complete data showed a significant interaction between the relative benefit of epirubicin and iT-$CD8^+$ status in ER-negative tumours (Supplementary Figure 16). Based on these findings it is possible that the efficacy of particular chemotherapies e.g. anthracyclines may be enhanced by cytotoxic T-cells. It has been reported that infiltrating lymphocytes are predictive of response to trastuzumab in HER2-positive breast cancer, but this finding was based on a small sample size and, unlike in our much larger study, no overall association between infiltrating lymphocytes and prognosis in HER2 positive disease was found[28].

This large multi-centre study demonstrates the importance of lymphocytic infiltration in ER-negative (both HER2-positive and HER2-negative) and ER-positive/HER2-positive breast cancer. Intratumoral and stromal $CD8^+$ 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.

Funding

This work was supported by Cancer Research UK (C490/A10119 and C490/A10124) and the NIHR Cambridge Biomedical Research Centre. HRA was supported by a grant from Addenbrooke's Charitable Trust. The BCCA study was supported by an unrestricted educational grant from Sanofi-Aventis Canada. SMAM was supported by a PhD studentship funded by the Government of Egypt. IOE was funded by Breast Cancer Campaign.

Conflicts of Interest

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

References

1. Galon J, Costes A, Sanchez-Cabo F et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science 2006; 313: 1960-1964.
2. Zhang L, Conejo-Garcia JR, Katsaros D et al. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. N Engl J Med 2003; 348: 203-213.
3. Topalian SL, Hodi FS, Brahmer JR et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. N Engl J Med 2012; 366: 2443-2454.
4. Park S, Jiang Z, Mortenson ED et al. The therapeutic effect of anti-HER2/neu antibody depends on both innate and adaptive immunity. Cancer Cell 2010; 18: 160-170.
5. Liu S, Lachapelle J, Leung S et al. CD8+ lymphocyte infiltration is an independent favorable prognostic indicator in basal-like breast cancer. Breast Cancer Res 2012; 14: R48.
6. Mahmoud SM, Paish EC, Powe DG et al. Tumor-infiltrating CD8+ lymphocytes predict clinical outcome in breast cancer. J Clin Oncol 2011; 29: 1949-1955.
7. Baker K, Lachapelle J, Zlobec I et al. Prognostic significance of CD8+ T lymphocytes in breast cancer depends upon both oestrogen receptor status and histological grade. Histopathology 2011; 58: 1107-1116.
8. Liu F, Lang R, Zhao J et al. CD8 cytotoxic T cell and FOXP3 regulatory T cell infiltration in relation to breast cancer survival and molecular subtypes. Breast Cancer Res Treat 2011; 130: 645-655.
9. Shimizu J, Yamazaki S, Sakaguchi S. Induction of tumor immunity by removing CD25+CD4+ T cells: a common basis between tumor immunity and autoimmunity. J Immunol 1999; 163: 5211-5218.
10. Mahmoud SM, Paish EC, Powe DG et al. An evaluation of the clinical significance of FOXP3+ infiltrating cells in human breast cancer. Breast Cancer Res Treat 2011; 127: 99-108.
11. West NR, Kost SE, Martin SD et al. Tumour-infiltrating FOXP3(+) lymphocytes are associated with cytotoxic immune responses and good clinical outcome in oestrogen receptor-negative breast cancer. Br J Cancer 2013; 108: 155-162.
12. Denkert C, Loibl S, Noske A et al. Tumor-associated lymphocytes as an independent predictor of response to neoadjuvant chemotherapy in breast cancer. J Clin Oncol 2010; 28: 105-113.
13. Loi S, Sirtaine N, Piette F et al. Prognostic and predictive value of tumor-infiltrating lymphocytes in a phase III randomized adjuvant breast cancer trial in node-positive breast cancer comparing the addition of docetaxel to doxorubicin with doxorubicin-based chemotherapy: BIG 02-98. Journal of clinical oncology : official journal of the American Society of Clinical Oncology 2013; 31: 860-867.
14. Ali HR, Dawson SJ, Blows FM et al. Cancer stem cell markers in breast cancer: pathological, clinical and prognostic significance. Breast Cancer Res 2011; 13: R118.
15. Earl HM, Hiller L, Dunn JA et al. Adjuvant epirubicin followed by cyclophosphamide, methotrexate and fluorouracil (CMF) vs CMF in early breast cancer: results with over 7 years median follow-up from the randomised phase III NEAT/BR9601 trials. Br J Cancer 2012; 107: 1257-1267.
16. Blows F, Driver K, Schmidt M et al. Subtyping of breast cancer by immunohistochemistry to investigate a relationship between subtype and short and long term survival: a collaborative analysis of data for 10,159 cases from 12 studies. PLoS Med 2010; 7: e1000279.
17. McShane LM, Altman DG, Sauerbrei W et al. Reporting recommendations for tumor marker prognostic studies (REMARK). J Natl Cancer Inst 2005; 97: 1180-1184.
18. Nagalla S, Chou JW, Willingham MC et al. Interactions between immunity, proliferation and molecular subtype in breast cancer prognosis. Genome biology 2013; 14: R34.
19. Gruver AL, Hudson LL, Sempowski GD. Immunosenescence of ageing. J Pathol 2007; 211: 144-156.

20. Hoppin JA, Tolbert PE, Taylor JA et al. Potential for selection bias with tumor tissue retrieval in molecular epidemiology studies. Ann Epidemiol 2002; 12: 1-6.
21. Janssen KJ, Donders AR, Harrell FE, Jr. et al. Missing covariate data in medical research: to impute is better than to ignore. Journal of clinical epidemiology 2010; 63: 721-727.
22. Ali AM, Dawson SJ, Blows FM et al. Comparison of methods for handling missing data on immunohistochemical markers in survival analysis of breast cancer. Br J Cancer 2011; 104: 693-699.
23. Berke G. The binding and lysis of target cells by cytotoxic lymphocytes: molecular and cellular aspects. Annu Rev Immunol 1994; 12: 735-773.
24. Zitvogel L, Kepp O, Kroemer G. Immune parameters affecting the efficacy of chemotherapeutic regimens. Nat Rev Clin Oncol 2011; 8: 151-160.
25. Segal NH, Parsons DW, Peggs KS et al. Epitope landscape in breast and colorectal cancer. Cancer Res 2008; 68: 889-892.
26. Bates GJ, Fox SB, Han C et al. Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse. J Clin Oncol 2006; 24: 5373-5380.
27. Ignatiadis M, Singhal SK, Desmedt C et al. Gene modules and response to neoadjuvant chemotherapy in breast cancer subtypes: a pooled analysis. Journal of clinical oncology : official journal of the American Society of Clinical Oncology 2012; 30: 1996-2004.
28. Loi S, Michiels S, Salgado R et al. Tumor infiltrating lymphocytes are prognostic in triple negative breast cancer and predictive for trastuzumab benefit in early breast cancer: results from the FinHER trial. Ann Oncol 2014.

Tables

Table 1

Multivariate Cox-regression analysis for breast cancer-specific survival based on multiple imputation for ER-positive and ER-negative breast cancer

Table 2

Multivariate Cox-regression analysis for breast cancer-specific survival based on multiple imputation for ER-positive HER2-positive breast cancer

Figure legends

Figure 1

Kaplan-Meier survival plot of patient groups defined by the presence of iT-CD8$^+$ and S-CD8$^+$ cells in ER-negative disease. Unadjusted survival estimates at five and ten 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.

Figure 2

Subgroup analyses by patient and tumour characteristics of the prognostic effect of iT-CD8$^+$ cells in ER-positive (left) and ER-negative (right) breast cancer. Definitions of molecular subtypes within ER-positive breast cancer (Lunminal 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 4.

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 (Lunminal 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 4.

Supporting Information

Supplementary Tables

Supplementary Table 1

Summary of participating studies.

Supplementary Table 2

Patient and tumour characteristics of cases included in the analysis.

Supplementary Table 3

Protocols and reagents for immunohistochemistry by study.

Supplementary Table 4

Scheme used molecular subtyping of tumours.

Supplementary Table 5

Univariate Cox-regression analyses for BCSS based on multiple imputation.

Supplementary Table 6

Univariate Cox-regression analyses for BCSS based on the complete case analysis.

Supplementary Table 7

Multivariate Cox-regression analysis for BCSS based on the complete case analysis in ER-positive and ER-negative breast cancer including $CD8^+$ T-cells.

Supplementary Table 8

Multivariate Cox-regression analysis for BCSS based on multiple imputation and including hormone therapy as a covariate.

Supplementary Table 9

Multivariate Cox-regression analysis for BCSS based on the complete case analysis and including hormone therapy as a covariate.

Supplementary Table 10

Multivariate Cox-regression analysis for BCSS based on multiple imputation including iT-FOXP3$^+$ in ER-positive breast cancer.

Supplementary Table 11

Multivariate Cox-regression analysis for BCSS based on the complete case analysis including iT-FOXP3$^+$ in ER-positive breast cancer.

Supplementary Table 12

Cox-regression analyses for BCSS based on multiple imputation including an interaction term for all combinations of intratumoral or stromal cytotoxic and regulatory T-cells in ER-positive and ER-negative breast cancer.

Supplementary Table 13

Multivariate Cox-regression analysis based on the complete case analysis of iT-CD8$^+$ cells in ER-positive HER2-positive breast cancer.

Supplementary Figures

Supplementary Figure 1

Histograms illustrating the relative distributions of observed values and imputed values by variables included in the imputation model.

Supplementary Figure 2

Kaplan-Meier survival plots of subgroups of patients defined by whether data was missing for CD8, FOXP3, ER and HER2.

Supplementary Figure 3

Spine plots illustrating the percentage distributions of clinical and molecular characteristics by study.

Supplementary Figure 4

Distribution of T-cell counts by study, histological type and molecular subtype illustrated as box-plots. Note T-cell counts for the BCCA study were capped at an upper limit of 100.

Supplementary Figure 5

Forest plots illustrating the univariate HR and 95% CI for association between iT-CD8$^+$ status and survival in different subgroups. Estimates are based on the complete case analysis (CCA).

Supplementary Figure 6

Forest plots illustrating the univariate HR and 95% CI for association between S-CD8$^+$ status and survival in different subgroups. Estimates are based on imputed data.

Supplementary Figure 7

Forest plots illustrating the univariate HR and 95% CI for association between S-CD8$^+$ status and survival in different subgroups. Estimates are based on the complete case analysis (CCA).

Supplementary Figure 8

Forest plots illustrating the univariate HR and 95% CI for association between iT-FOXP3$^+$ status and survival in different subgroups. Estimates are based on imputed data.

Supplementary Figure 9

Forest plots illustrating the univariate HR and 95% CI for association between iT-FOXP3$^+$ cell status and survival in different subgroups. Estimates are based on the complete case analysis.

Supplementary Figure 10

Forest plots illustrating the univariate HR and 95% CI for association between S-FOXP3$^+$ status and survival in different subgroups. Estimates are based on imputed data.

Supplementary Figure 11

Forest plots illustrating the univariate HR and 95% CI for association between S-FOXP3$^+$ cell status and survival in different subgroups. Estimates are based on the complete case analysis.

Supplementary Figure 12

Forest plot illustrating the prognostic effect of CD8$^+$ and FOXP3$^+$ lymphocytes in ER-negative breast cancer according to whether adjuvant chemotherapy was administered. HRs and 95%CIs are based on imputed analyses and are adjusted for tumour size, grade and number of positive lymph nodes.

Supplementary Figure 13

Forest plot illustrating the prognostic effect of CD8$^+$ and FOXP3$^+$ lymphocytes in ER-negative breast cancer according to whether adjuvant chemotherapy was administered. HRs and 95%CIs are based on the complete case analysis and are adjusted for tumour size, grade and number of positive lymph nodes.

Supplementary Figure 14

Forest plot illustrating the prognostic effect of iT-CD8$^+$ lymphocytes in ER-negative and ER-positive breast cancer according to whether adjuvant chemotherapy was administered separately for the three

observational studies (SEARCH, NBCS, BCCA). HRs and 95%CIs are adjusted for tumour size, grade and number of positive lymph nodes (MI = multiple imputation, CCA = complete case analysis).

Supplementary Figure 15

Forest plot illustrating the treatment effect of E-CMF versus CMF by subgroups defined by the presence of $CD8^+$ and $FOXP3^+$ lymphocytes in ER-positive and ER-negative disease. HRs and 95%CIs are based on imputed data and are adjusted for tumour size, grade and number of positive lymph nodes.

Supplementary Figure 16

Forest plot illustrating the treatment effect of E-CMF versus CMF by subgroups defined by the presence of $CD8^+$ and $FOXP3^+$ lymphocytes in ER-positive and ER-negative disease. HRs and 95%CIs are based on the complete case analysis (CCA) and are adjusted for tumour size, grade and number of positive lymph nodes.

Supplementary Figure 17

Trends in hazard ratios and 95% confidence intervals for T-cell status according to tumour proliferation and diagnosis age. Estimates are adjusted for tumour size, histological grade and positive axillary lymph nodes. Analyses are based on imputed data. Estimates and confidence intervals associated with iT-$CD8^+$ cells are represented as dark blue, those with S-$CD8^+$ as light blue, those with iT-$FOXP3^+$ as dark red and those with S-$FOXP3^+$ as light red. Box sizes are proportional to the number of subjects in each subgroup.

Table 1: Multivariate Cox-regression analysis for breast cancer-specific survival based on multiple imputation for ER-positive and ER-negative breast cancer

Variable	Categories	Subjects[†]	HR	95% CI		*P*	T[‡]	95% CI		*P*
ER-positive										
Age	≤55 years, >55 years		1.89	1.42	2.50	0.00001	0.77	0.64	0.91	0.0031
Positive lymph nodes	0, 1, 2-4, 5-9, ≥10		1.56	1.41	1.74	<0.00001	0.94	0.88	1.0	0.06
Tumour size	<10mm, 10-19mm, 20-29mm, 30-49mm, ≥50mm		1.32	1.24	1.40	<0.00001				
Grade	1, 2, 3	8775	2.53	1.93	3.31	<0.00001	0.76	0.64	0.90	0.001
Chemotherapy	No, Yes		1.62	1.41	1.86	<0.00001				
PR status	Negative, Positive		0.32	0.24	0.44	<0.00001	1.57	1.30	1.91	<0.00001
HER2 status	Negative, Positive		1.50	1.29	1.74	<0.00001				
iT-CD8	Negative, Positive		0.95	0.85	1.07	0.43				
ER-negative										
Positive lymph nodes	0, 1, 2-4, 5-9, ≥10		1.43	1.36	1.51	<0.00001				
Tumour size	<10mm, 10-19mm, 20-29mm, 30-49mm, ≥50mm		1.23	1.15	1.32	<0.00001				
Grade	1, 2, 3		2.05	1.48	2.83	0.00002	0.72	0.57	0.91	0.007
Chemotherapy	No, Yes	3591	1.40	1.19	1.64	0.00003				
PR status	Negative, Positive		0.31	0.18	0.51	0.00001	1.93	1.33	2.81	0.0006
HER2 status	Negative, Positive		1.29	1.13	1.49	0.0002				
iT-CD8	Negative, Positive		0.72	0.62	0.84	0.00003				
S-CD8	Negative, Positive		0.79	0.67	0.93	0.004				

[†]Sample size varied between imputed datasets. Reported sizes are the smallest of fifty imputations.

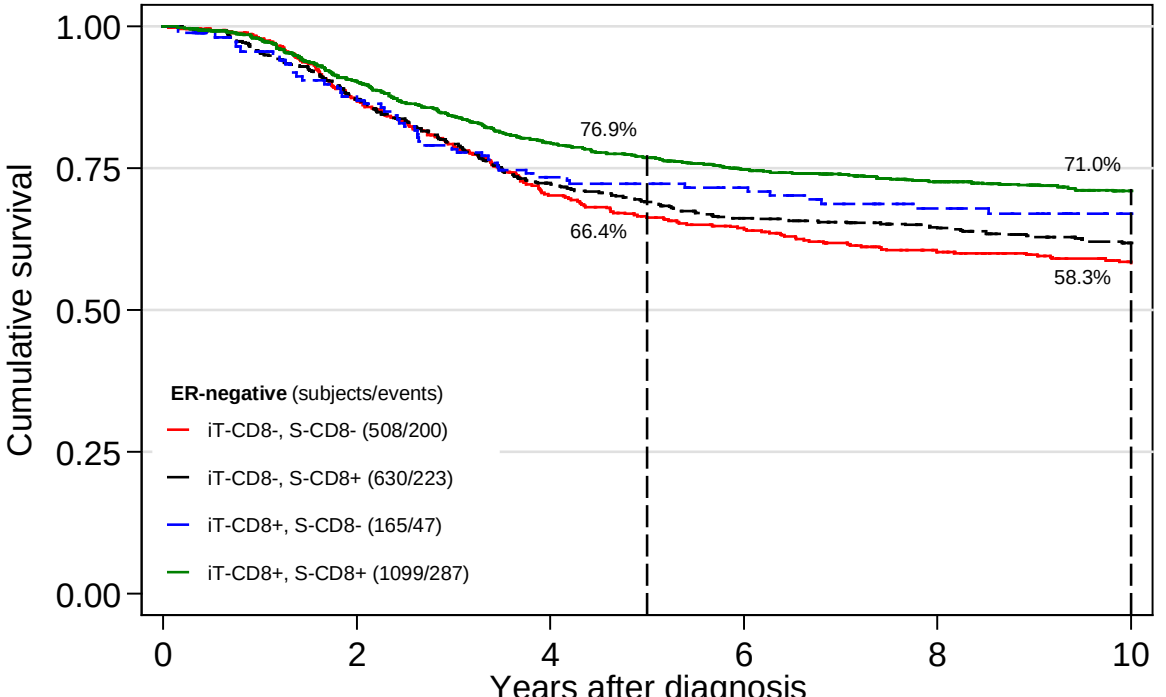
[‡] Variables which violated the proportional hazards assumption were accounted for by an extended Cox-model where the beta-coefficient varies linearly with the natural logarithm of time. 'T' represents the exponent of the extended coefficient where a value >1 implies increasing hazard over time whereas a value of <1 implies decreasing hazard over time.

Table 2: Multivariate Cox-regression analysis for breast cancer-specific survival based on multiple imputation for ER-positive HER2-positive breast cancer

ER-POSITIVE HER2-POSITIVE										
Variable	**Categories**	**Subjects**[†]	**HR**	**95% CI**		***P***	**T**[‡]	**95% CI**		***P***
Positive lymph nodes	0, 1, 2-4, 5-9, ≥10		1.40	1.27	1.54	<0.00001				
Tumour size	<10mm, 10-19mm, 20-29mm, 30-49mm, ≥50mm		1.33	1.16	1.52	0.00004				
		772								
Grade	1, 2, 3		2.73	1.26	5.92	0.01	0.56	0.33	0.95	0.03
PR status	Negative, Positive		0.35	0.17	0.69	0.002	1.73	1.04	2.90	0.04
iT-CD8	Negative, Positive		0.73	0.56	0.96	0.02				

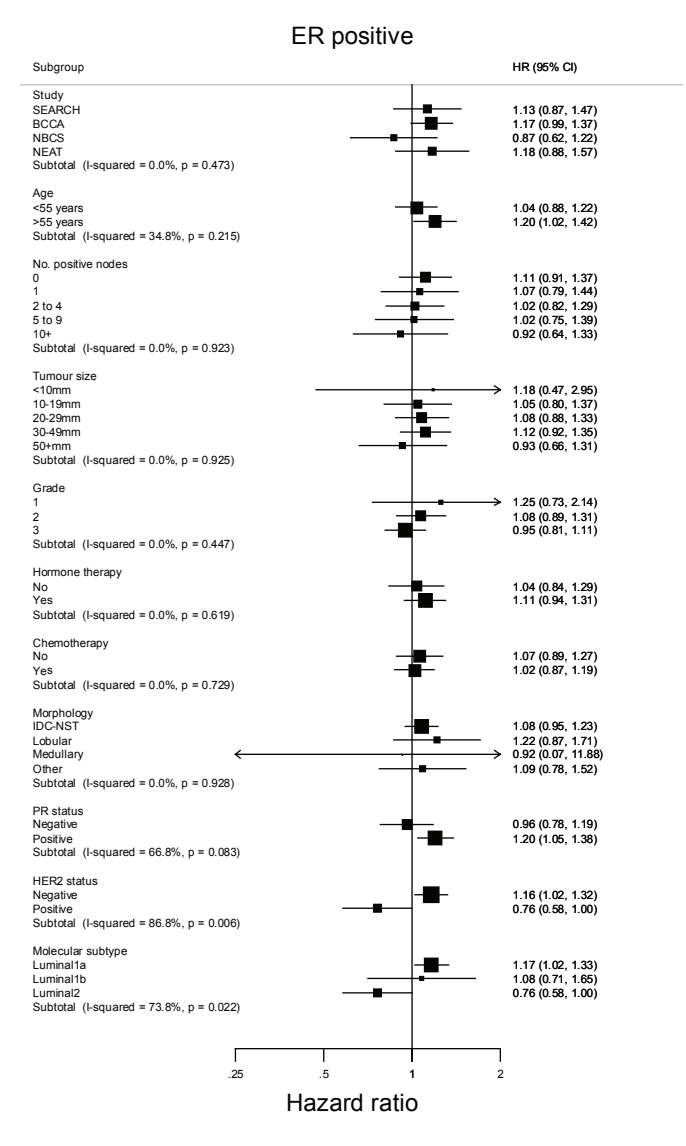
[†]Sample size varied between imputed datasets. Reported sizes are the smallest of fifty imputations.

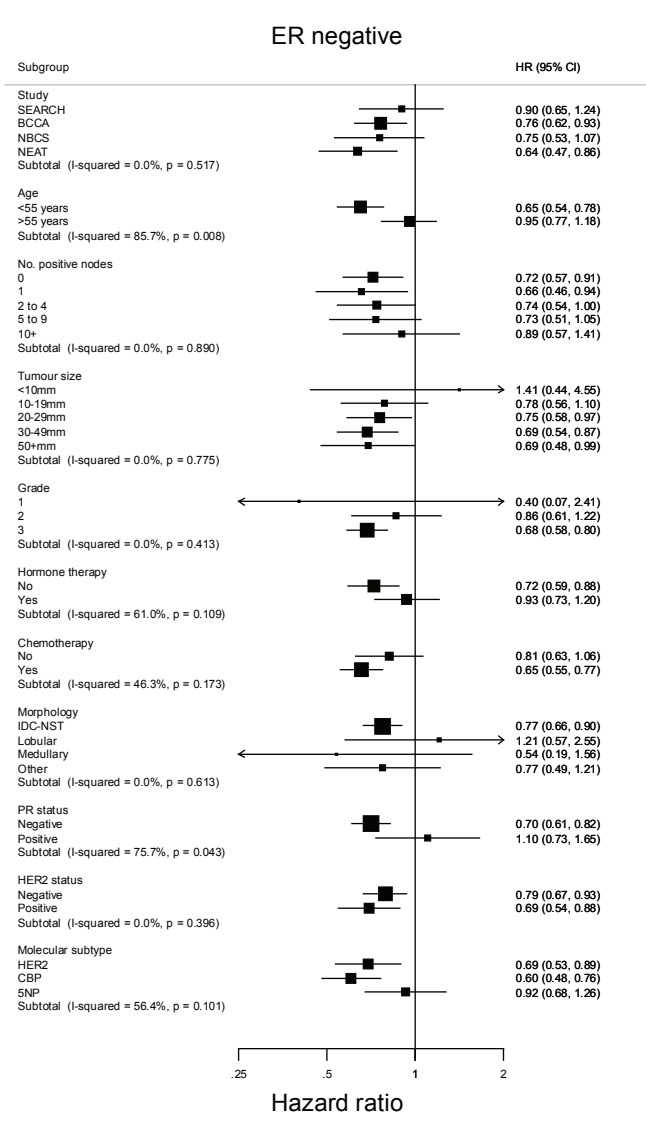
[‡] Variables which violated the proportional hazards assumption were accounted for by an extended Cox-model where the beta-coefficient varies linearly with the natural logarithm of time. 'T' represents the exponent of the extended coefficient where a value >1 implies increasing hazard over time whereas a value of <1 implies decreasing hazard over time.

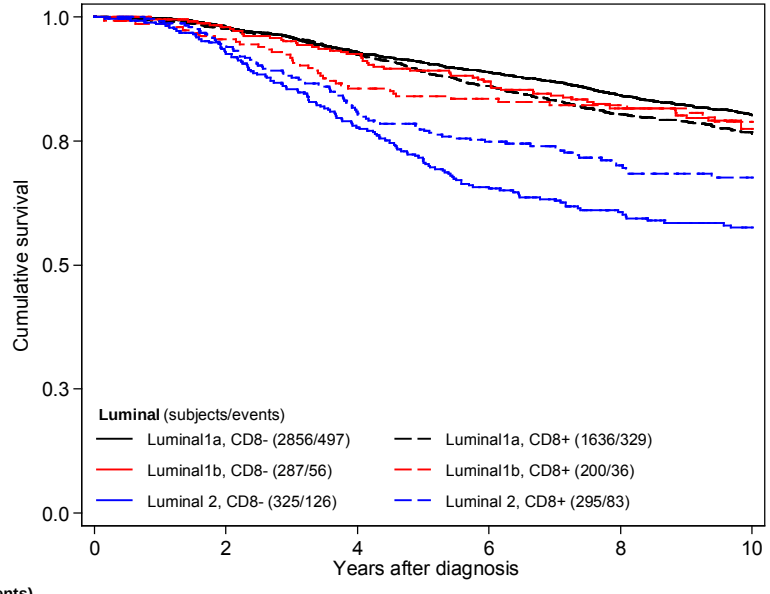


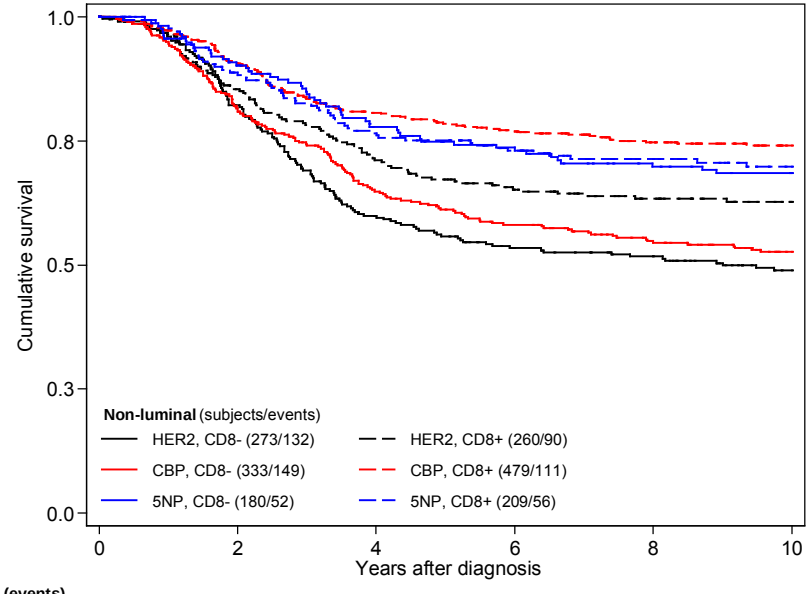
No. at risk (events)
iT-CD8, S-CD8

iT-CD8-, S-CD8-	460	(62)	428	(83)	341	(28)	304	(19)	258	(8)	218
iT-CD8-, S-CD8+	490	(73)	523	(90)	439	(37)	391	(9)	348	(14)	291
iT-CD8+, S-CD8-	94	(16)	129	(22)	122	(3)	106	(5)	79	(1)	52
iT-CD8+, S-CD8+	838	(92)	925	(115)	845	(49)	749	(20)	584	(11)	442


ER positive
Subgroup
HR (95% CI)
Study
SEARCH 1.13 (0.87, 1.47)
BCCA 1.17 (0.99, 1.37)
NBCS 0.87 (0.62, 1.22)
NEAT 1.18 (0.88, 1.57)
Subtotal (I-squared = 0.0%, p = 0.473)
Age
<55 years 1.04 (0.88, 1.22)
>55 years 1.20 (1.02, 1.42)
Subtotal (I-squared = 34.8%, p = 0.215)
No. positive nodes
0 1.11 (0.91, 1.37)
1 1.07 (0.79, 1.44)
2 to 4 1.02 (0.82, 1.29)
5 to 9 1.02 (0.75, 1.39)
10+ 0.92 (0.64, 1.33)
Subtotal (I-squared = 0.0%, p = 0.923)
Tumour size
<10mm 1.18 (0.47, 2.95)
10-19mm 1.05 (0.80, 1.37)
20-29mm 1.08 (0.88, 1.33)
30-49mm 1.12 (0.92, 1.35)
50+mm 0.93 (0.66, 1.31)
Subtotal (I-squared = 0.0%, p = 0.925)
Grade
1 1.25 (0.73, 2.14)
2 1.08 (0.89, 1.31)
3 0.95 (0.81, 1.11)
Subtotal (I-squared = 0.0%, p = 0.447)
Hormone therapy
No 1.04 (0.84, 1.29)
Yes 1.11 (0.94, 1.31)
Subtotal (I-squared = 0.0%, p = 0.619)
Chemotherapy
No 1.07 (0.89, 1.27)
Yes 1.02 (0.87, 1.19)
Subtotal (I-squared = 0.0%, p = 0.729)
Morphology
IDC-NST 1.08 (0.95, 1.23)
Lobular 1.22 (0.87, 1.71)
Medullary 0.92 (0.07, 11.88)
Other 1.09 (0.78, 1.52)
Subtotal (I-squared = 0.0%, p = 0.928)
PR status
Negative 0.96 (0.78, 1.19)
Positive 1.20 (1.05, 1.38)
Subtotal (I-squared = 66.8%, p = 0.083)
HER2 status
Negative 1.16 (1.02, 1.32)
Positive 0.76 (0.58, 1.00)
Subtotal (I-squared = 86.8%, p = 0.006)
Molecular subtype
Luminal1a 1.17 (1.02, 1.33)
Luminal1b 1.08 (0.71, 1.65)
Luminal2 0.76 (0.58, 1.00)
Subtotal (I-squared = 73.8%, p = 0.022)
.25
.5
1
2
Hazard ratio


ER negative
Subgroup
HR (95% CI)
Study
SEARCH 0.90 (0.65, 1.24)
BCCA 0.76 (0.62, 0.93)
NBCS 0.75 (0.53, 1.07)
NEAT 0.64 (0.47, 0.86)
Subtotal (I-squared = 0.0%, p = 0.517)
Age
<55 years 0.65 (0.54, 0.78)
>55 years 0.95 (0.77, 1.18)
Subtotal (I-squared = 85.7%, p = 0.008)
No. positive nodes
0 0.72 (0.57, 0.91)
1 0.66 (0.46, 0.94)
2 to 4 0.74 (0.54, 1.00)
5 to 9 0.73 (0.51, 1.05)
10+ 0.89 (0.57, 1.41)
Subtotal (I-squared = 0.0%, p = 0.890)
Tumour size
<10mm 1.41 (0.44, 4.55)
10-19mm 0.78 (0.56, 1.10)
20-29mm 0.75 (0.58, 0.97)
30-49mm 0.69 (0.54, 0.87)
50+mm 0.69 (0.48, 0.99)
Subtotal (I-squared = 0.0%, p = 0.775)
Grade
1 0.40 (0.07, 2.41)
2 0.86 (0.61, 1.22)
3 0.68 (0.58, 0.80)
Subtotal (I-squared = 0.0%, p = 0.413)
Hormone therapy
No 0.72 (0.59, 0.88)
Yes 0.93 (0.73, 1.20)
Subtotal (I-squared = 61.0%, p = 0.109)
Chemotherapy
No 0.81 (0.63, 1.06)
Yes 0.65 (0.55, 0.77)
Subtotal (I-squared = 46.3%, p = 0.173)
Morphology
IDC-NST 0.77 (0.66, 0.90)
Lobular 1.21 (0.57, 2.55)
Medullary 0.54 (0.19, 1.56)
Other 0.77 (0.49, 1.21)
Subtotal (I-squared = 0.0%, p = 0.613)
PR status
Negative 0.70 (0.61, 0.82)
Positive 1.10 (0.73, 1.65)
Subtotal (I-squared = 75.7%, p = 0.043)
HER2 status
Negative 0.79 (0.67, 0.93)
Positive 0.69 (0.54, 0.88)
Subtotal (I-squared = 0.0%, p = 0.396)
Molecular subtype
HER2 0.69 (0.53, 0.89)
CBP 0.60 (0.48, 0.76)
5NP 0.92 (0.68, 1.26)
Subtotal (I-squared = 56.4%, p = 0.101)
.25
.5
1
2
Hazard ratio





No. at risk (events)
iTCD8 by molecular subtype

Luminal1a, CD8-	2166	(50)	2579	(135)	2511	(109)	2336	(118)	1977	(85)	1568
Luminal1a, CD8+	1123	(33)	1435	(79)	1423	(97)	1276	(78)	981	(42)	722
Luminal1b, CD8-	206	(5)	260	(15)	256	(15)	221	(13)	186	(8)	137
Luminal1b, CD8+	127	(7)	174	(19)	168	(4)	144	(2)	116	(4)	78
Luminal 2, CD8-	268	(21)	285	(47)	240	(37)	196	(14)	145	(7)	117
Luminal 2, CD8+	213	(15)	255	(38)	225	(16)	200	(10)	129	(4)	92

No. at risk (events)
iTCD8 by molecular subtype

HER2, CD8-	243	(46)	217	(59)	157	(17)	133	(4)	120	(6)	96
HER2, CD8+	209	(34)	211	(36)	181	(15)	155	(4)	122	(1)	84
CBP, CD8-	284	(57)	260	(54)	211	(22)	189	(10)	159	(6)	129
CBP, CD8+	358	(37)	397	(46)	375	(17)	338	(9)	270	(2)	213
5NP, CD8-	152	(16)	152	(21)	129	(7)	120	(6)	105	(2)	98
5NP, CD8+	146	(20)	170	(24)	155	(7)	138	(3)	107	(2)	84

Supporting Information

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

H. R. Ali [1,2,4], E. Provenzano[1,4], S-J. Dawson[1,3,4], F. M. Blows[3,], B. Liu[1,3,4], M Shah[3,4,5], H. M. Earl[3,4], C. J. Poole[6], L. Hiller[6], J. A. Dunn[6], S. J. Bowden[7], C. Twelves[8], J. M. S. Bartlett[9], S. M. A. Mahmoud[10, 11], E. Rakha[10], I. O. Ellis[10], S. Liu[12], D. Gao[12], T. O. Nielsen[12], P. D. P. Pharoah[3,4,5], C. Caldas[1,3,4]

Affiliations

1. Cancer Research UK Cambridge Institute, University of Cambridge Cambridge, UK
2. Department of Pathology, University of Cambridge, Cambridge, UK
3. Department of Oncology, University of Cambridge, Cambridge, UK
4. Cambridge Experimental Cancer Medicine Centre and NIHR Cambridge Biomedical Research Centre, Cambridge, UK
5. Strangeways Research Laboratory, Cambridge, UK
6. Warwick Clinical Trials Unit, University of Warwick, Coventry, UK
7. Cancer Research UK Clinical Trials Unit, Institute for Cancer Studies, The University of Birmingham, Birmingham, UK
8. University of Leeds and Leeds Cancer Research UK Centre, St James' Institute of Oncology, Leeds, UK
9. Ontario Institute for Cancer Research, Toronto, Canada and Biomarker and Companion Diagnostics, Edinburgh Cancer Research Centre, Edinburgh, UK.

10. Department of Histopathology and School of Molecular Medical Sciences, The University of Nottingham and Nottingham University Hospitals NHS Trust, Nottingham, UK
11. Current affiliation: Department of Pathology and Laboratory Medicine, University of British Columbia; Integrative Oncology Department, British Columbia Cancer Agency, Vancouver, Canada
12. Genetic Pathology Evaluation Centre, Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver, Canada

Corresponding author:
Professor Carlos Caldas
Cancer Research UK Cambridge Institute,
University of Cambridge,
Li Ka Shing Centre,
Robinson Way,
Cambridge,
CB2 0RE,
UK
Tel: +44 1223 769650
carlos.caldas@cruk.cam.ac.uk

Supplementary Methods

Study populations

Patients recruited to the Study of Epidemiology and Risk Factors in Cancer Heredity (SEARCH) study provided written informed consent; the study is approved by the Cambridgeshire 4 Research ethics committee (02/5/42). SEARCH is a prospective population-based study of women diagnosed with breast cancer in East Anglia, England. Cases are ascertained through the East Anglia Cancer Registry and comprise prevalent cases (women diagnosed under the age of 55 years during 1991-1996 and who were still alive at study commencement in 1996) and incident cases (women under the age of 70 years and diagnosed after 1996). Patients were treated according to regional protocols.

The British Columbia Cancer Agency (BCCA) study was approved by the Clinical Research Ethics Committee Board of the BCCA and the University of British Columbia; since this study utilised anonymised archival specimens, the need for informed consent was waived in accordance with the Canadian Tri-Council Policy Statement for ethical research involving human subjects. This study comprised women diagnosed with breast cancer between 1986 and 1992 in British Columbia and referred to BCCA for consideration of adjuvant therapy. Approximately 75% of women diagnosed with breast cancer in British Columbia were referred to BCCA during the study period; the remaining 25% were those for whom adjuvant therapy was not indicated.

The Nottingham Tenovus Primary Breast Cancer Series (NBCS) comprises patients diagnosed and treated at Nottingham City Hospital between 1987 and 1998 was approved by Nottingham Research Ethics Committee 2. Data on survival time and locoregional recurrence were prospectively maintained. Patients were treated uniformly according to institutional protocol.

Details of the National Epirubicin Adjuvant Trial and BR9601 trial (here collectively referred to as NEAT) have been published previously[1]. 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); all participants provided written informed consent and the

study was approved by local and central ethics review committees. Patients were recruited to the NEAT trial by 111 clinicians at 65 centres in England between 1996 and 2001; 26 clinicians at 10 centres in Scotland recruited patients to the BR9/601 trial. Patients were eligible for these trials if they had completely excised early breast cancer, required adjuvant chemotherapy and could begin treatment within ten weeks of surgery. Results of this trial were first published in 2006[2]. Additional details are described in Supplementary Table 1. Patient characteristics are detailed in Supplementary Table 2.

Some patients were recruited to both the SEARCH and NEAT studies of which it was possible to identify approximately 60% (44 cases). Although the remaining duplicate instances remain they are likely to be exceptionally rare (approximately 30 cases (0.2%)) in this large study, hence will not influence the validity of analyses.

Immunohistochemistry and scoring

Details of reagents and protocols for IHC by study are provided in Supplementary Table 3. All tumours were represented by one 0.6 mm tissue core on tissue microarrays (TMAs) constructed at the respective host institutions. ER status was assessed based on IHC applied to TMAs and scored using the Allred scoring system for the SEARCH and NEAT studies and the percentage of positive tumour cells for the NBCS and BCCA studies. IHC and scoring were conducted at the host institutions. The same reagents and protocols for IHC were used for staining of the SEARCH and NEAT studies; one pathologist (HRA) scored the samples from these studies for infiltrating lymphocytes. Lymphocytes were manually quantified by counting the absolute number of positive lymphocytes according to their micro-anatomic location (intratumoral or stromal). Tumour infiltrating lymphocytes were classified as ‘intratumoral’ (iT) if they were seen in direct contact with cancer cells and ‘stromal’ (S) if they were not in direct contact with cancer cells. For the NBCS study the stromal category was generated by the addition of the ‘peri-tumoral’ (close to but not touching tumour cells) and ‘distant stroma’ categories previously reported[3]. In the BCCA study, the absolute numbers of $CD8^+$ lymphocytes were counted up to a maximum of 100. To make $CD8^+$ lymphocyte counts comparable between all four studies, scores exceeding 100 were changed to 100. 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-cells was available for the SEARCH, NBCS and NEAT studies only.

Statistical analyses

The primary endpoint was breast cancer specific survival (BCSS). Follow-up time was truncated at 10 years from the date of diagnosis. Following removal of cases with missing follow-up time (NEAT study: 6 patients, NBCS: 61 patients) 12,439 cases remained. Women with ER-positive and ER-negative tumours were analysed separately because they have different patterns of short- and long-term survival. Cox proportional-hazards regression models were used to assess the association between T-cells and BCSS. All regression models were stratified by study. SEARCH is an on-going prospective study to which patients are recruited at different times after their initial diagnosis. Late entry refers to the enrolment of patients into the ongoing SEARCH study at some point after the date of diagnosis. This can be a potential source of bias in observational studies because to be enrolled into the study patients must survive the time between date of diagnosis and enrolment. Since those who do not survive this period are never enrolled, late entry can lead to biased estimates for that time frame. Survival time was left-truncated to adjust for this bias. Left-truncation means that patients are only included in survival time analysis during their time under observation that is, following study enrolment. For time-to-event analyses the date-of-diagnosis was used to define the start of follow-up time. However, cases from the SEARCH study had their survival times left-truncated to study entry (time-under-observation). This method produces unbiased estimates providing the proportional-hazards assumption is not violated and study entry time is unrelated to outcome[4, 5]. The proportional hazards assumption was tested iteratively for all variables by using an extended regression model to include a coefficient which varies linearly as a function of the natural logarithm of time. Variables were modelled as time-varying according to the significance of the p-value of the extended coefficient. Variables which were best modelled as time-varying in univariate analyses were initially modelled as time-varying in multivariate analyses. Variables significantly associated

with outcome on univariate analysis were further assessed in multivariate analyses. In addition to T-cell counts, initial multivariate models included: age (≤55 years, >55 years), number of positive axillary lymph nodes (0, 1, 2-4, 5-9, ≥10) tumour size (<10mm, 10-19mm, 20-29mm, 30-49mm, ≥50mm), histological grade, PR status, HER2 status and whether chemotherapy was administered. Additional models including hormone therapy as a covariate but excluding the NEAT trial for which this data was not available, were also constructed. Tumour size, lymph nodes and grade were modelled as continuous variables. Multivariate models, including estimates for an interaction with time, were modified by backward elimination.

Univariate Cox-regression analyses were also used to test for differences in the effect of T-cells on survival between patient subgroups. Heterogeneity for the prognostic effect of T-cell status by study and patient subgroups was tested using Cochran's Q-test. The I^2 statistic is also reported; this provides an estimate of the percentage of variation across subgroups that is due to heterogeneity rather than chance[6]. Using data from the NEAT trial, assessment of the ability of T-cell counts to predict differential benefit from the addition of Epirubicin to adjuvant chemotherapy was made by comparing estimates of treatment effect (E-CMF vs CMF) in subgroups defined by T-cell status using Cochran's Q-test. Interaction between $CD8^+$ and $FOXP3^+$ T-cells was investigated by including an interaction term in a Cox-regression model. Absolute survival estimates adjusted for node status, tumour size and histological grade were made using the Kaplan-Meier method.

Data were missing in varying proportions for different characteristics and studies (Supplementary Table 2). Missingness can be correlated across variables particularly where some data points are dependent on biological samples so analyses based on the complete cases alone can be biased (Supplementary Figure 2). To adjust for this bias we conducted multiple imputation by chained equations using the *ice* command in Stata[7]. This method produces multiple replicate datasets where missing values have been resolved under a model informed by the rest of the data. All variables which were to be evaluated in multivariate analyses, histological type, Ki67, EGFR and CK56 status were included in the imputation model and fifty replicate datasets produced. The logarithm of tumour size showed a near normal distribution hence was used in the imputation model and back-transformed

subsequently. Continuous T-cell counts were transformed into ordinal scores where a score of zero remained a separate category and successive categories were divided by every tenth count i.e. 0, 1-11, 12-21, 22-31 etc. All other variables were included as dichotomous, categorical or ordinal in imputation models. Ordinal variables (grade, positive lymph nodes, T-cell counts, Ki67 expression) were imputed using ordered logistic regression. The censoring indicator and the Nelson-Aalen cumulative hazard function were also included in the analysis as their inclusion has been shown to produce the least biased estimates[8, 9]. Estimates derived from regression analyses across fifty datasets were combined using Rubin's rules which accounts for within-dataset and between-dataset variation. Relative survival estimates derived from imputed data are presented in the main report and estimates from the complete case analysis detailed in the supplementary material. Reported absolute survival estimates were made using complete data only. The distributions of imputed values compared to observed values for all variables are illustrated as histograms in Supplementary Figure 1. All statistical analyses were compliant with REMARK guidelines[10] and were conducted using Intercooled Stata version 11.2 (Stata Corp, College Station, TX, USA).

References

1. Earl HM, Hiller L, Dunn JA et al. Adjuvant epirubicin followed by cyclophosphamide, methotrexate and fluorouracil (CMF) vs CMF in early breast cancer: results with over 7 years median follow-up from the randomised phase III NEAT/BR9601 trials. Br J Cancer 2012; 107: 1257-1267.

2. Poole CJ, Earl HM, Hiller L et al. Epirubicin and cyclophosphamide, methotrexate, and fluorouracil as adjuvant therapy for early breast cancer. N Engl J Med 2006; 355: 1851-1862.

3. Mahmoud SM, Paish EC, Powe DG et al. Tumor-infiltrating CD8+ lymphocytes predict clinical outcome in breast cancer. J Clin Oncol 2011; 29: 1949-1955.

4. Azzato E, Greenberg D, Shah M et al. Prevalent cases in observational studies of cancer survival: do they bias hazard ratio estimates? Br J Cancer 2009; 100: 1806-1811.

5. Bull K, Spiegelhalter DJ. Survival analysis in observational studies. Stat Med 1997; 16: 1041-1074.

6. Higgins JP, Thompson SG, Deeks JJ, Altman DG. Measuring inconsistency in meta-analyses. BMJ 2003; 327: 557-560.

7. Royston P. Multiple imputation of missing values. Stata Journal 2004; 4: 227-241.

8. Moons KG, Donders RA, Stijnen T, Harrell FE. Using the outcome for imputation of missing predictor values was preferred. J Clin Epidemiol 2006; 59: 1092-1101.

9. White IR, Royston P. Imputing missing covariate values for the Cox model. Stat Med 2009; 28: 1982-1998.

10. McShane LM, Altman DG, Sauerbrei W et al. Reporting recommendations for tumor marker prognostic studies (REMARK). J Natl Cancer Inst 2005; 97: 1180-1184.

Supplementary Tables

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

H. R. Ali [1,2,4], E. Provenzano[1,4], S-J. Dawson[1,3,4], F. M. Blows[3,], B. Liu[1,3,4], M Shah[3,4,5], H. M. Earl[3,4], C. J. Poole[6], L. Hiller[6], J. A. Dunn[6], S. J. Bowden[7], C. Twelves[8], J. M. S. Bartlett[9], S. M. A. Mahmoud[10, 11], E. Rakha[10], I. O. Ellis[10], S. Liu[12], D. Gao[12], T. O. Nielsen[12], P. D. P. Pharoah[3,4,5], C. Caldas[1,3,4]

Affiliations

1. Cancer Research UK Cambridge Institute, University of Cambridge, Li Ka Shing Centre, Robinson Way, Cambridge, CB2 0RE, UK
2. Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QP
3. Department of Oncology, University of Cambridge, Addenbrooke's Hospital, Box 193-R4, Hills Road, Cambridge, CB2 0QQ, UK
4. Cambridge Experimental Cancer Medicine Centre and NIHR Cambridge Biomedical Research Centre, Box 277, Hills Road, Cambridge, CB2 0QQ, UK
5. Strangeways Research Laboratory, Worts Causeway, Cambridge, CB1 8RN, UK
6. Warwick Clinical Trials Unit, University of Warwick, Gibbet Hill Road, Coventry CV4 7AL, UK
7. Cancer Research UK Clinical Trials Unit, Institute for Cancer Studies, The University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

8. University of Leeds and Leeds Cancer Research UK Centre, St James' Institute of Oncology, Leeds, LS9 7TF
9. Ontario Institute for Cancer Research, MaRS Centre, South Tower, 661 University Avenue, Suite 510, Toronto, Ontario, M5G 0A3, Canada and Biomarker and Companion Diagnostics, Edinburgh Cancer Research Centre, Edinburgh, UK.
10. Department of Histopathology and School of Molecular Medical Sciences, The University of Nottingham and Nottingham University Hospitals NHS Trust, Nottingham City Hospital, Nottingham, UK
11. Current affiliation: Department of Pathology and Laboratory Medicine, University of British Columbia; Integrative Oncology Department, British Columbia Cancer Agency, Vancouver, BC, Canada
12. Genetic Pathology Evaluation Centre, Department of Pathology and Laboratory Medicine, University of British Columbia, 2660 Oak Street, Vancouver, BC, V6H 3Z6, Canada

Corresponding author:
Professor Carlos Caldas
Cancer Research UK Cambridge Institute,
University of Cambridge,
Li Ka Shing Centre,
Robinson Way,
Cambridge,
CB2 0RE,
UK
Tel: 01223 769650
carlos.caldas@cruk.cam.ac.uk

Supplementary Table 1: Summary of participating studies

Study (*N*)	Type	Deaths from breast cancer	Median time at-risk (years)	Study design	Chemotherapy
SEARCH (4,079)	Population-based	508 (12%)	7.9	Cases ascertained through the East Anglia Cancer Registry. There are (a) prevalent cases diagnosed under the age of 55 years during 1991-1996 and still alive in 1996 and (b) incident cases under the age of 70 years and diagnosed after the age of 70 years.	Variable between hospitals and over time
BCCA[†] (4,520)	Hospital-based	1252 (28%)	12.31	Women diagnosed with invasive breast cancer during 1986-1992 and referred to the BCCA for consideration of adjuvant therapy.	Variable regimens, including (i) Doxorubicin and cyclophosphamide, (ii) cyclophosphamide, methotrexate and fluorouracil, (iii) fluorouracil, doxorubicin, cyclophosphamide, (iv) cyclophosphamide, epirubicin, fluorouracil
NBCS[†] (1,842)	Hospital-based	429 (23%)	10.6	Consecutive series of patients diagnosed between 1987 and 1998 and enrolled in the Nottingham Tenovus Primary Breast Cancer series study.	Cyclophosphamide, methotrexate, fluorouracil (CMF)
NEAT[‡] (1,998)	Randomised controlled trial	485 (24%)	6.9	A UK-based phase III trial	Patients were randomised on a 1:1 basis to receive CMF alone or Epirubicin plus CMF (E-CMF).

[†]For these studies analyses based on T-cell data have been published previously.

[‡]Also includes patients enrolled in the BR9601 trial (NEAT, N=1,684 and BR9601, N=341).

[§]Summary statistics reflect survival analyses where follow-up time was truncated at ten years.

Supplementary Table 2: Patient and tumour characteristics of cases included in the analysis

Variable	Study SEARCH		BCCA		NBCS		NEAT		Total	
	No.	%	No.	%	No.	%	No.	%	No.	%
Age										
≤55 years	2529	62	1755	39	963	52	1504	75	6751	54
>55 years	1550	38	2765	61	879	48	494	25	5688	46
Positive lymph nodes										
0	2292	56	2539	56	1037	56	575	29	6443	52
1	569	14	614	14	288	16	341	17	1812	15
2 to 4	508	12	733	16	231	13	514	26	1986	16
5 to 9	184	5	343	8	58	3	211	11	796	6
10+	120	3	182	4	14	1	80	4	396	3
Missing	406	10	109	2	214	12	277	14	1006	8
Tumour size										
<10mm	444	11	0	0	147	8	82	4	673	5
10-19mm	1741	43	833	18	822	45	564	28	3960	32
20-29mm	1003	25	1632	36	642	35	687	34	3964	32
30-49mm	497	12	1486	33	213	12	464	23	2660	21
50+mm	145	4	472	10	17	1	159	8	793	6
Missing	249	6	97	2	1	0	42	2	389	3
Grade										
1	799	20	231	5	344	19	117	6	1491	12
2	1701	42	1731	38	611	33	670	34	4713	38
3	1102	27	2303	51	887	48	1182	59	5474	44
Missing	477	12	255	6	0	0	29	1	761	6
Morphology										
Ductal	3057	75	3555	79	1034	56	1617	81	9263	74
Lobular	594	15	308	7	131	7	182	9	1215	10
Medullary	45	1	70	2	45	2	25	1	185	1
other	375	9	587	13	597	32	142	7	1701	14
Missing	8	0	0	0	35	2	32	2	75	1
Endocrine therapy										
No	837	21	2712	60	1103	60	0[†]	0	4652	37
Yes	3231	79	1808	40	655	36	0	0	5694	46
Missing	11	0	0	0	84	5	1998	100	2093	17
Chemotherapy										
No	2472	61	2993	66	1460	79	2[‡]	0	6927	56
Yes	1242	30	1527	34	303	16	1996	100	5068	41
Missing	365	9	0	0	79	4	0	0	444	4
ER status										
Negative	757	19	1335	30	451	24	644	32	3187	26
Positive	2267	56	3064	68	1269	69	992	50	7592	61
Missing	1055	26	121	3	122	7	362	18	1660	13
PR status										
Negative	874	21	1959	43	694	38	800	40	4327	35
Positive	2173	53	2011	44	1004	55	1002	50	6190	50
Missing	1032	25	550	12	144	8	196	10	1922	15
HER2 status										
Negative	2533	62	3720	82	1550	84	1515	76	9318	75
Positive	345	8	558	12	236	13	391	20	1530	12
Missing	1201	29	242	5	56	3	92	5	1591	13
Molecular subtype										
Luminal 1a	1618	40	2268	50	825	45	632	32	5343	43
Luminal 1b	214	5	138	3	183	10	83	4	618	5
Luminal 2	200	5	251	6	113	6	189	9	753	6
HER2	118	3	276	6	111	6	144	7	649	5
CBP	210	5	375	8	189	10	170	9	944	8
5NP	127	3	178	4	89	5	85	4	479	4
Missing	1592	39	1034	23	332	18	695	35	3653	29
iT-CD8 status										
Negative	1138	28	2587	57	920	50	591	30	5236	42
Positive	1115	27	1216	27	367	20	1044	52	3742	30
Missing	1826	45	717	16	555	30	363	18	3461	28
S-CD8 status										
Negative	468	11	1533	34	229	12	448	22	2678	22
Positive	1785	44	2270	50	1058	57	1187	59	6300	51
Missing	1826	45	717	16	555	30	363	18	3461	28
iT-FOXP3 status										
Negative	1728	42	0	0	1038	56	837	42	3603	29
Positive	458	11	0	0	357	19	821	41	1636	13
Missing	1893	46	4520	100	447	24	340	17	7200	58
S-FOXP3 status										
Negative	1117	27	0	0	364	20	581	29	2062	17
Positive	1069	26	0	0	1031	56	1077	54	3177	26
Missing	1893	46	4520	100	447	24	340	17	7200	58

[†]Data on endocrine therapy was not available for the NEAT trial.
[‡]A small proportion of patients randomised did not ultimately receive chemotherapy.

Supplementary Table 3: Protocols and reagents for immunohistochemistry by study

Study	Marker	Clone	Clonality	Source	Dilution	Antigen retrieval	Scoring system	Cut-off
SEARCH & NEAT	ER	6F11/2	Mouse monoclonal	Novocastra	1 in 70	Citrate buffer pH6, 30 minutes	Allred	>2
	PR	PgR636	Mouse monoclonal	Dako	1 in 50	Citrate buffer pH6, 30 minutes	Allred	>2
	HER2	c-erbB-2	Rabbit monoclonal	Dako	1 in 250	Citrate buffer pH6, 40 minutes	HercepTest	≥ 2
	CK5/6	D5/16 B4	Mouse monoclonal	Dako	1 in 50	Tris-EDTA buffer pH9, 30 minutes	Binary estimate	>10%
	EGFR	31G7	Mouse monoclonal	Zymed	1 in 25	Proteinase K digestion, 10 minutes	Allred	≥ 2
	Ki67	MIB-1	Mouse monoclonal	Dako	1 in 200	Tris-EDTA buffer pH9, 30 minutes	Allred proportion (SEARCH) and percentage (NEAT)	NA
	CD8	SP16	Rabbit monoclonal	Thermo Fisher	1 in 100	Tris-EDTA buffer pH9, 20 minutes	Absolute count	>0
	FOXP3	236A/E7	Mouse monoclonal	AbCam	1 in 50	Citrate buffer pH6, 20 minutes	Absolute count	>0
NBCS	ER	1D5	Mouse monoclonal	Dako	1 in 80	Microwave	H-score	>10%
	PR	PgR636	Mouse monoclonal	Dako	1 in 50	Microwave	H-score	>10%
	HER2	c-erbB-2	Rabbit monoclonal	Dako	1 in 250	None	H-score	>10%
	CK5/6	D5/16134	Mouse monoclonal	Boehringer Biochemica	1 in 100	Microwave	H-score	>10%
	EGFR	EGFR.113	Mouse monoclonal	Novocastra	1 in 10	Microwave	H-score	>10%
	Ki67	MIB-1	Mouse monoclonal	Dako	1 in 100	Citrate buffer pH6, 20 minutes	Percentage	NA
	CD8	1A5	Mouse monoclonal	Vector laboratories	1 in 50	Citrate buffer pH6, 20 minutes	Absolute count	>0
	FOXP3	236A/E7	Mouse monoclonal	Abcam	1 in 100	Tris-EDTA buffer pH8, 20 minutes	Absolute count	>0
BCCA	ER	SP1	Rabbit monoclonal	Lab Vision	1 in 250	Citrate buffer pH6, 8 minutes	Percentage	>0%
	PR	1E2	Rabbit monoclonal	Ventana	Ready-to-use	Ventana CC1 protocol	Percentage	>0%
	HER2	SP3	Rabbit monoclonal	Lab Vision	1 in 100	Tris-EDTA buffer pH9, 30 minutes	HercepTest	≥ 2[†]
	CK5/6	D5/16B4	Mouse monoclonal	Zymed	1 in 100	Ventana CC1 protocol	Binary estimate	Any cytoplasmic and/or membranous staining
	EGFR	2-18C9	Mouse monoclonal	Dako (PharmDx kit)	Ready-to-use	Proteinase K digestion, 5 minutes	Binary estimate	
	Ki67	SP6	Rabbit monoclonal	Lab Vision	1 in 200	Ventana CC1 protocol	Percentage	NA
	CD8	C8/144B	Mouse monoclonal	Dako	1 in 100	Dako target retrieval solution High pH 9, 20 minutes	Absolute count up to a maximum of 100	>0

Allred Scoring System: Staining intensity score: 1 = weak, 2 = moderate, 3 = strong; Proportion score: 1 = <1%, 2 = 1-10%, 3 = 11-33%, 4 = 34-66%, 5 = >66%; Total score = intensity score + proportion score = 0-8

HercepTest™ : 0 = No staining or weak staining in ≤ 10% of cells; 1 = Weak incomplete membranous staining in >10% of cells; 2 = Moderate circumferential membranous staining in > 10% of cells; 3 = Strong circumferential membranous staining in >10% of cells

[†]Cases with a score of '2' were subjected to fluorescence in situ hybridisation (FISH) analysis for assessment of the number of copies of the HER2 gene relative to the centromere of chromosome 17 using the PathVysion DNA probe kit (Abbott Molecular, Inc., Chicago, IL, USA).

Supplementary Table 4: Scheme for molecular subtyping of tumours

Molecular subtype	ER and/or PR	HER2	CK56 and/or EGFR
Luminal 1a	Positive	Negative	Negative
Luminal 1b	Positive	Negative	Positive
Luminal 2	Positive	Positive	NA
HER2	Negative	Positive	NA
CBP	Negative	Negative	Positive
5NP	Negative	Negative	Negative

Abbreviations: CBP – core basal phenotype, CK56 – cytokeratin 56, ER – estrogen receptor, EGFR – epidermal growth factor receptor, HER2 – human epidermal growth factor receptor 2, PR – progesterone receptor, 5NP – five marker negative phenotype.

Supplementary Table 5: Univariate Cox-regression analyses for BCSS based on multiple imputation

ER-Positive										
Variable	**Categories**	**Subjects**[†]	**HR**	**95% CI**		***P***	**T**[‡]	**95% CI**		***P***
Age	≤55 years, >55 years	8775	1.73	1.30	2.30	0.0006	0.70	0.59	0.84	0.0001
Positive lymph nodes	0, 1, 2-4, 5-9, ≥10	8775	1.93	1.74	2.14	<0.00001	0.91	0.85	0.98	0.008
Tumour size	<10mm, 10-19mm, 20-29mm, 30-49mm, ≥50mm	8775	1.98	1.71	2.29	<0.00001	0.90	0.82	0.98	0.02
Grade	1, 2, 3	8775	3.73	2.87	4.85	<0.00001	0.70	0.59	0.82	0.00001
Hormone therapy	No, Yes	7576	2.65	1.91	3.69	<0.00001	0.73	0.60	0.90	0.003
Chemotherapy	No, Yes	8775	2.75	2.46	3.08	<0.00001				
PR status	Negative, Positive	8775	0.22	0.17	0.30	<0.00001	1.83	1.51	2.21	<0.00001
HER2 status	Negative, Positive	8775	3.94	2.85	5.45	<0.00001	0.66	0.52	0.82	0.0002
iT-CD8	Negative, Positive	8775	1.12	0.99	1.25	0.07				
S-CD8	Negative, Positive	8775	0.99	0.87	1.11	0.82				
iT-FOXP3	Negative, Positive	5635	1.30	1.09	1.56	0.004				
S-FOXP3	Negative, Positive	5635	1.07	0.91	1.26	0.39				
ER-Negative										
Variable		**Subjects**	**HR**	**95% CI**		***P***	**T**	**95% CI**		***P***
Age	≤55 years, >55 years	3591	1.11	0.98	1.26	0.11				
Positive lymph nodes	0, 1, 2-4, 5-9, ≥10	3591	1.57	1.50	1.65	<0.00001				
Tumour size	<10mm, 10-19mm, 20-29mm, 30-49mm, ≥50mm	3591	1.70	1.51	1.91	<0.00001	0.87	0.79	0.96	0.006
Grade	1, 2, 3	3591	2.61	1.90	3.58	<0.00001	0.65	0.52	0.82	0.0003
Hormone therapy	No, Yes	2800	1.28	1.11	1.49	0.00101				
Chemotherapy	No, Yes	3591	2.25	1.95	2.60	<0.00001				
PR status	Negative, Positive	3591	0.26	0.15	0.42	<0.00001	2.14	1.49	3.08	0.00005
HER2 status	Negative, Positive	3591	1.60	1.40	1.82	<0.00001				
iT-CD8	Negative, Positive	3591	0.75	0.66	0.87	0.00009				
S-CD8	Negative, Positive	3591	0.80	0.70	0.93	0.003				
iT-FOXP3	Negative, Positive	2213	0.86	0.72	1.04	0.12				
S-FOXP3	Negative, Positive	2213	0.94	0.77	1.14	0.53				

[†]Sample size varied between imputed datasets. Reported sizes are the smallest of fifty imputations.

‡ 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 exponentiated extended coefficient where a value >1 implies increasing hazard over time whereas a value of <1 implies decreasing hazard over time.

Supplementary Table 6: Univariate Cox-regression analyses for BCSS based on the complete case analysis

ER-positive											
Variable	**Categories**	**Subjects**	**Events**	**HR**	**95% CI**		***P***	**T**[†]	**95% CI**		***P***
Age	≤55 years, >55 years	7591	1412	1.76	1.30	2.36	0.0002	0.71	0.59	0.86	0.0004
Positive lymph nodes	0, 1, 2-4, 5-9, ≥10	7030	1265	1.96	1.76	2.19	<0.00001	0.91	0.84	0.97	0.006
Tumour size	<10mm, 10-19mm, 20-29mm, 30-49mm, ≥50mm	7431	1384	1.95	1.68	2.27	<0.00001	0.91	0.82	1.00	0.04
Grade	1, 2, 3	7146	1331	3.62	2.75	4.76	<0.00001	0.72	0.60	0.85	0.00010
Hormone therapy	No, Yes	6549	1194	2.86	2.04	3.99	<0.00001	0.71	0.57	0.87	0.0001
Chemotherapy	No, Yes	7348	1392	2.63	2.34	2.96	<0.00001				
PR status	Negative, Positive	7045	1319	0.22	0.16	0.30	<0.00001	1.84	1.51	2.23	<0.00001
HER2 status	Negative, Positive	7165	1356	3.81	2.73	5.30	<0.00001	0.67	0.53	0.84	0.0005
iT-CD8	Negative, Positive	5956	1184	1.17	1.03	1.32	0.01				
S-CD8	Negative, Positive	5956	1184	1.03	0.91	1.17	0.62				
iT-FOXP3	Negative, Positive	3263	541	1.33	1.10	1.62	0.004				
S-FOXP3	Negative, Positive	3263	541	1.07	0.90	1.28	0.45				
ER-negative											
Variable	**Categories**	**Subjects**	**Events**	**HR**	**95% CI**		***P***	**T**	**95% CI**		***P***
Age	≤55 years, >55 years	3187	967	1.13	0.99	1.29	0.06				
Positive lymph nodes	0, 1, 2-4, 5-9, ≥10	2967	873	1.71	1.56	1.86	<0.00001	0.93	0.86	1.00	0.05
Tumour size	<10mm, 10-19mm, 20-29mm, 30-49mm, ≥50mm	3087	941	1.72	1.52	1.94	<0.00001	0.87	0.78	0.96	0.004
Grade	1, 2, 3	3034	934	2.61	1.88	3.61	<0.00001	0.65	0.51	0.83	0.0006
Hormone therapy	No, Yes	2505	772	1.27	1.09	1.48	0.002				
Chemotherapy	No, Yes	3080	942	2.27	1.95	2.63	<0.00001				
PR status	Negative, Positive	2877	888	0.26	0.16	0.44	<0.00001	2.16	1.50	3.12	0.00004
HER2 status	Negative, Positive	2948	911	1.54	1.34	1.76	<0.00001				
iT-CD8	Negative, Positive	2402	757	0.75	0.65	0.87	0.0001				
S-CD8	Negative, Positive	2402	757	0.82	0.70	0.95	0.01				
iT-FOXP3	Negative, Positive	1391	357	0.83	0.67	1.03	0.09				
S-FOXP3	Negative, Positive	1391	357	1.02	0.80	1.28	0.90				

[†] 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 exponentiated extended coefficient where a value >1 implies increasing hazard over time whereas a value of <1 implies decreasing hazard over time.

Supplementary Table 7: Multivariate Cox-regression analysis for BCSS based on the complete case analysis in ER-positive and ER-negative breast cancer including CD8$^+$ T-cells

Variable	Categories	Subjects	Events	HR	95% CI		*P*	T†	95% CI		*P*
ER-positive											
Age (>55 years)	≤55 years, >55 years			2.19	1.51	3.18	0.00004	0.74	0.59	0.94	0.01
Positive lymph nodes	0, 1, 2-4, 5-9, ≥10			1.53	1.34	1.74	<0.00001	0.92	0.85	1.00	0.06
Tumour size	<10mm, 10-19mm, 20-29mm, 30-49mm, ≥50mm	4801	927	1.32	1.22	1.42	<0.00001				
Grade	1, 2, 3			2.62	1.84	3.73	<0.00001	0.73	0.59	0.91	0.005
Chemotherapy	No, Yes			1.89	1.59	2.26	<0.00001				
PR status	Negative, Positive			0.30	0.20	0.43	<0.00001	1.70	1.33	2.16	0.00002
HER2 status	Negative, Positive			1.54	1.29	1.84	<0.00001				
iT-CD8	Negative, Positive			1.07	0.93	1.23	0.35				
ER-negative											
Age (>55 years)	≤55 years, >55 years			1.31	1.09	1.58	0.004				
Positive lymph nodes	0, 1, 2-4, 5-9, ≥10			1.52	1.37	1.70	<0.00001	0.90	0.83	0.99	0.03
Tumour size	<10mm, 10-19mm, 20-29mm, 30-49mm, ≥50mm	1953	614	1.26	1.15	1.38	<0.00001				
Grade	1, 2, 3			1.56	1.27	1.93	0.00004				
Chemotherapy	No, Yes			1.82	1.45	2.28	<0.00001				
PR status	Negative, Positive			0.20	0.10	0.39	<0.00001	3.06	1.92	4.90	<0.00001
HER2 status	Negative, Positive			1.17	0.98	1.39	0.09				
iT-CD8	Negative, Positive			0.70	0.59	0.84	0.0001				
S-CD8	Negative, Positive			0.75	0.63	0.91	0.003				

† 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 exponentiated extended coefficient where a value >1 implies increasing hazard over time whereas a value of <1 implies decreasing hazard over time.

Supplementary Table 8: Multivariate Cox-regression analysis for BCSS based on multiple imputation and including hormone therapy as a covariate

Variable	Categories	Subjects	HR	95% CI		*P*	T	95% CI		*P*
ER-positive										
Age (>55 years)	≤55 years, >55 years	7576	2.13	1.54	2.94	0.00001	0.75	0.61	0.91	0.003
Positive lymph nodes	0, 1, 2-4, 5-9, ≥10		1.54	1.37	1.73	<0.00001	0.93	0.87	1.00	0.04
Tumour size	<10mm, 10-19mm, 20-29mm, 30-49mm, ≥50mm		1.36	1.27	1.45	<0.00001				
Grade	1, 2, 3		2.65	1.95	3.59	<0.00001	0.75	0.62	0.90	0.002
Hormone therapy	No, Yes		1.08	0.95	1.24	0.23				
Chemotherapy	No, Yes		1.74	1.50	2.01	<0.00001				
PR status	Negative, Positive		0.36	0.26	0.50	<0.00001	1.42	1.16	1.75	0.0007
HER2 status	Negative, Positive		1.50	1.27	1.78	<0.00001				
iT-CD8	Negative, Positive		0.93	0.82	1.07	0.30				
ER-negative										
Positive lymph nodes	0, 1, 2-4, 5-9, ≥10	2800	1.40	1.32	1.49	<0.00001				
Tumour size	<10mm, 10-19mm, 20-29mm, 30-49mm, ≥50mm		1.20	1.11	1.30	<0.00001				
Grade	1, 2, 3		2.25	1.55	3.27	0.00002	0.72	0.55	0.94	0.02
Hormone therapy	No, Yes		1.24	1.06	1.45	0.008				
Chemotherapy	No, Yes		1.50	1.27	1.77	<0.00001				
PR status	Negative, Positive		0.28	0.15	0.51	0.00005	1.88	1.21	2.91	0.005
HER2 status	Negative, Positive		1.28	1.09	1.49	0.002				
iT-CD8	Negative, Positive		0.72	0.60	0.85	0.0002				
S-CD8	Negative, Positive		0.80	0.67	0.96	0.01				

†Sample size varied between imputed datasets. Reported sizes are the smallest of fifty imputations.

‡ 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 exponentiated extended coefficient where a value >1 implies increasing hazard over time whereas a value of <1 implies decreasing hazard over time.

Supplementary Table 9: Multivariate Cox-regression analysis for BCSS based on the complete case analysis and including hormone therapy as a covariate

Variable	Categories	Subjects	Events	HR	95% CI		*P*	T	95% CI		*P*
ER-positive											
Age	≤55 years, >55 years	4170	809	2.28	1.50	3.46	0.0001	0.76	0.59	0.98	0.03
Positive lymph nodes	0, 1, 2-4, 5-9, ≥10			1.55	1.34	1.79	<0.00001	0.90	0.83	0.99	0.03
Tumour size	<10mm, 10-19mm, 20-29mm, 30-49mm, ≥50mm			1.36	1.25	1.48	<0.00001				
Grade	1, 2, 3			2.71	1.83	4.02	<0.00001	0.72	0.57	0.91	0.006
Hormone Therapy	No, Yes			1.00	0.84	1.18	0.96				
Chemotherapy	No, Yes			1.99	1.66	2.39	<0.00001				
PR status	Negative, Positive			0.32	0.21	0.48	<0.00001	1.56	1.21	2.02	0.0007
HER2 status	Negative, Positive			1.55	1.28	1.88	0.00001				
iT-CD8	Negative, Positive			1.03	0.88	1.19	0.74				
ER-negative											
Age	≤55 years, >55 years	1510	509	1.37	1.11	1.68	0.003				
Positive lymph nodes	0, 1, 2-4, 5-9, ≥10			1.52	1.35	1.71	<0.00001	0.88	0.80	0.98	0.02
Tumour size	<10mm, 10-19mm, 20-29mm, 30-49mm, ≥50mm			1.19	1.08	1.32	0.0008				
Grade	1, 2, 3			1.75	1.39	2.22	<0.00001				
Hormone Therapy	No, Yes			1.23	1.00	1.51	0.05				
Chemotherapy	No, Yes			2.01	1.58	2.54	<0.00001				
PR status	Negative, Positive			0.12	0.05	0.28	<0.00001	3.89	2.17	7.00	0.00001
HER2 status	Negative, Positive			1.21	1.00	1.46	0.06				
iT-CD8	Negative, Positive			0.67	0.55	0.81	0.00006				
S-CD8	Negative, Positive			0.78	0.64	0.96	0.02				

† 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 exponentiated extended coefficient where a value >1 implies increasing hazard over time whereas a value of <1 implies decreasing hazard over time.

Supplementary Table 10: Multivariate Cox-regression analysis for BCSS based on multiple imputation including iT-FOXP3^{+} in ER-positive breast cancer

ER-POSITIVE										
Variable	**Categories**	**Subjects**[†]	**HR**	**95% CI**		***P***	**T**[‡]	**95% CI**		***P***
Age	≤55 years, >55 years	5635	1.85	1.22	2.81	0.004	0.66	0.51	0.86	0.002
Positive lymph nodes	0, 1, 2-4, 5-9, ≥10		1.61	1.37	1.90	<0.00001	0.99	0.89	1.10	0.79
Tumour size	<10mm, 10-19mm, 20-29mm, 30-49mm, ≥50mm		1.27	1.17	1.37	<0.00001				
Grade	1, 2, 3		2.62	1.80	3.82	<0.00001	0.79	0.63	0.99	0.04
Chemotherapy	No, Yes		1.02	0.83	1.27	0.82				
PR status	Negative, Positive		0.34	0.22	0.53	<0.00001	1.56	1.15	2.11	0.004
HER2 status	Negative, Positive		2.82	1.73	4.60	0.00003	0.64	0.45	0.90	0.001
iT-CD8	Negative, Positive		0.91	0.76	1.08	0.27				
iT-FOXP3	Negative, Positive		1.05	0.87	1.26	0.64				

[†]Sample size varied between imputed datasets. Reported sizes are the smallest of fifty imputations.

[‡] 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 exponentiated extended coefficient where a value >1 implies increasing hazard over time whereas a value of <1 implies decreasing hazard over time.

Supplementary Table 11: Multivariate Cox-regression analysis for BCSS based on the complete case analysis including iT-FOXP3^{+} in ER-positive breast cancer

ER-POSITIVE											
Variable	**Categroies**	**Subjects**	**Events**	**HR**	**95% CI**		***P***	**T**	**95% CI**		***P***
Age (>55 years)	≤55 years, >55 years	2295	371	1.99	1.10	3.59	0.02	0.67	0.46	0.98	0.04
Positive lymph nodes	0, 1, 2-4, 5-9, ≥10			1.47	1.18	1.85	0.0008	1.06	0.91	1.22	0.4
Tumour size	<10mm, 10-19mm, 20-29mm, 30-49mm, ≥50mm			1.24	1.11	1.39	0.0002				
Grade	1, 2, 3			2.32	1.38	3.91	0.001	0.86	0.62	1.18	0.3
Chemotherapy	No, Yes			0.98	0.72	1.34	0.90				
PR status	Negative, Positive			0.24	0.13	0.46	0.00001	2.03	1.32	3.12	0.001
HER2 status	Negative, Positive			1.63	1.26	2.12	0.0003				
iT-CD8	Negative, Positive			1.06	0.85	1.33	0.58				
iT-FOXP3	Negative, Positive			1.09	0.86	1.38	0.47				

† 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 exponentiated extended coefficient where a value >1 implies increasing hazard over time whereas a value of <1 implies decreasing hazard over time.

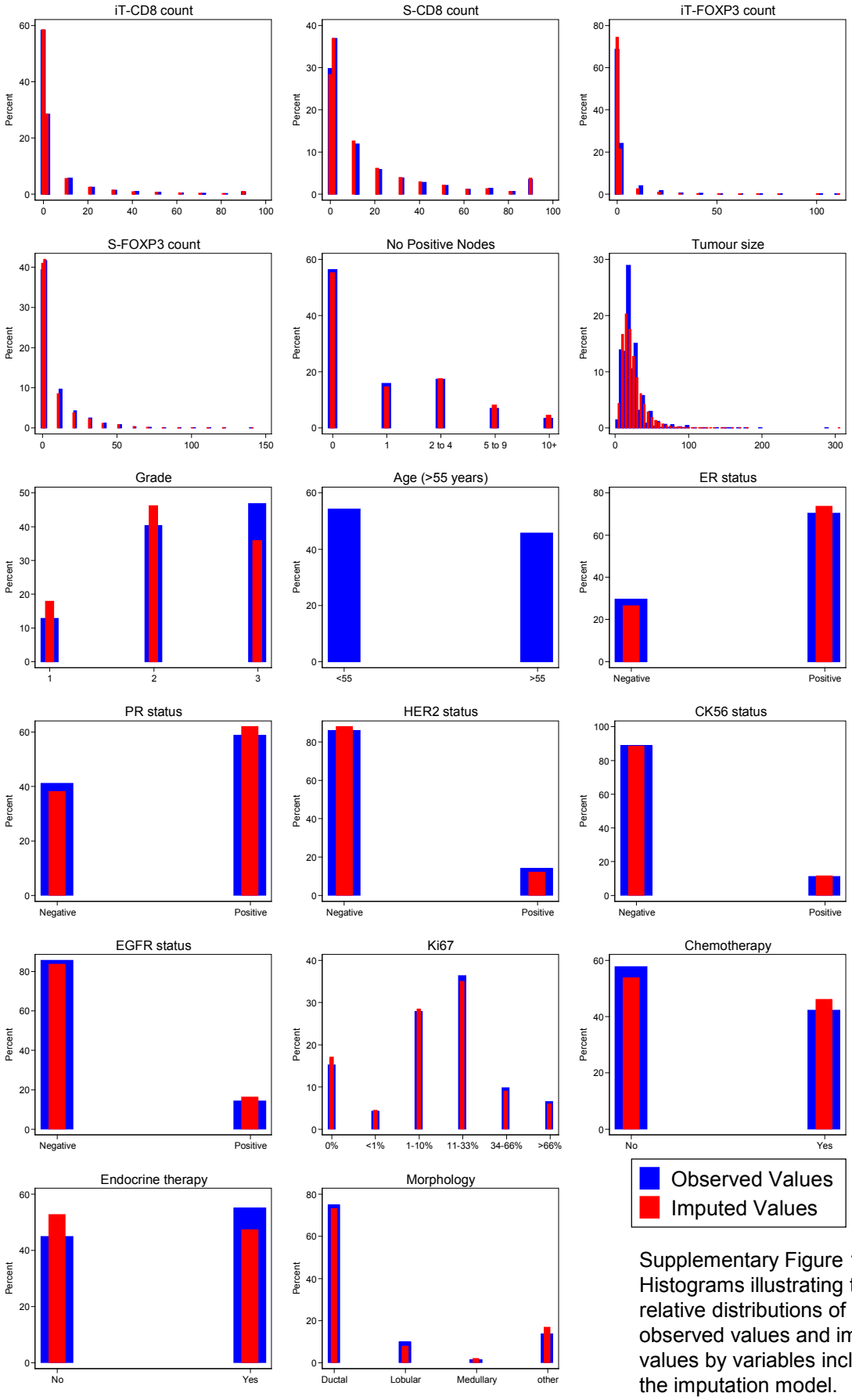
Supplementary Table 12: Cox-regression analyses for BCSS based on multiple imputation including an interaction term for all combinations of intratumoral or stromal cytotoxic and regulatory T-cells

Variable	HR	95% CI		*P*
ER-POSITIVE				
iT-CD8+	1.01	0.82	1.25	0.91
iT-FOXP3+	1.56	1.16	2.10	0.004
Interaction (iT-CD8*iT-FOXP3)	0.94	0.63	1.39	0.75
iT-CD8+	1.12	0.84	1.50	0.44
S-FOXP3+	1.24	0.99	1.55	0.06
Interaction (iT-CD8*S-FOXP3)	0.92	0.64	1.33	0.67
S-CD8+	0.87	0.69	1.09	0.23
iT-FOXP3+	1.46	0.98	2.16	0.06
Interaction (S-CD8*iT-FOXP3)	1.05	0.66	1.65	0.84
S-CD8+	0.77	0.59	1.00	0.05
S-FOXP3+	1.10	0.78	1.56	0.59
Interaction (S-CD8*S-FOXP3)	1.21	0.81	1.81	0.35
ER-NEGATIVE				
iT-CD8+	0.83	0.62	1.12	0.24
iT-FOXP3+	1.11	0.81	1.54	0.51
Interaction (iT-CD8*iT-FOXP3)	0.82	0.53	1.27	0.38
iT-CD8+	0.90	0.61	1.31	0.57
S-FOXP3+	1.27	0.93	1.73	0.13
Interaction (iT-CD8*S-FOXP3)	0.78	0.50	1.22	0.27
S-CD8+	0.66	0.49	0.89	0.006
iT-FOXP3+	0.78	0.53	1.17	0.23
Interaction (S-CD8*iT-FOXP3)	1.26	0.79	1.99	0.33
S-CD8+	0.70	0.47	1.04	0.08
S-FOXP3+	1.22	0.82	1.82	0.32
Interaction (S-CD8*S-FOXP3)	0.97	0.57	1.64	0.90

Supplementary Table 13: Multivariate Cox-regression analysis based on the complete case analysis of iT-CD8^{+} cells in ER-positive HER2-positive breast cancer

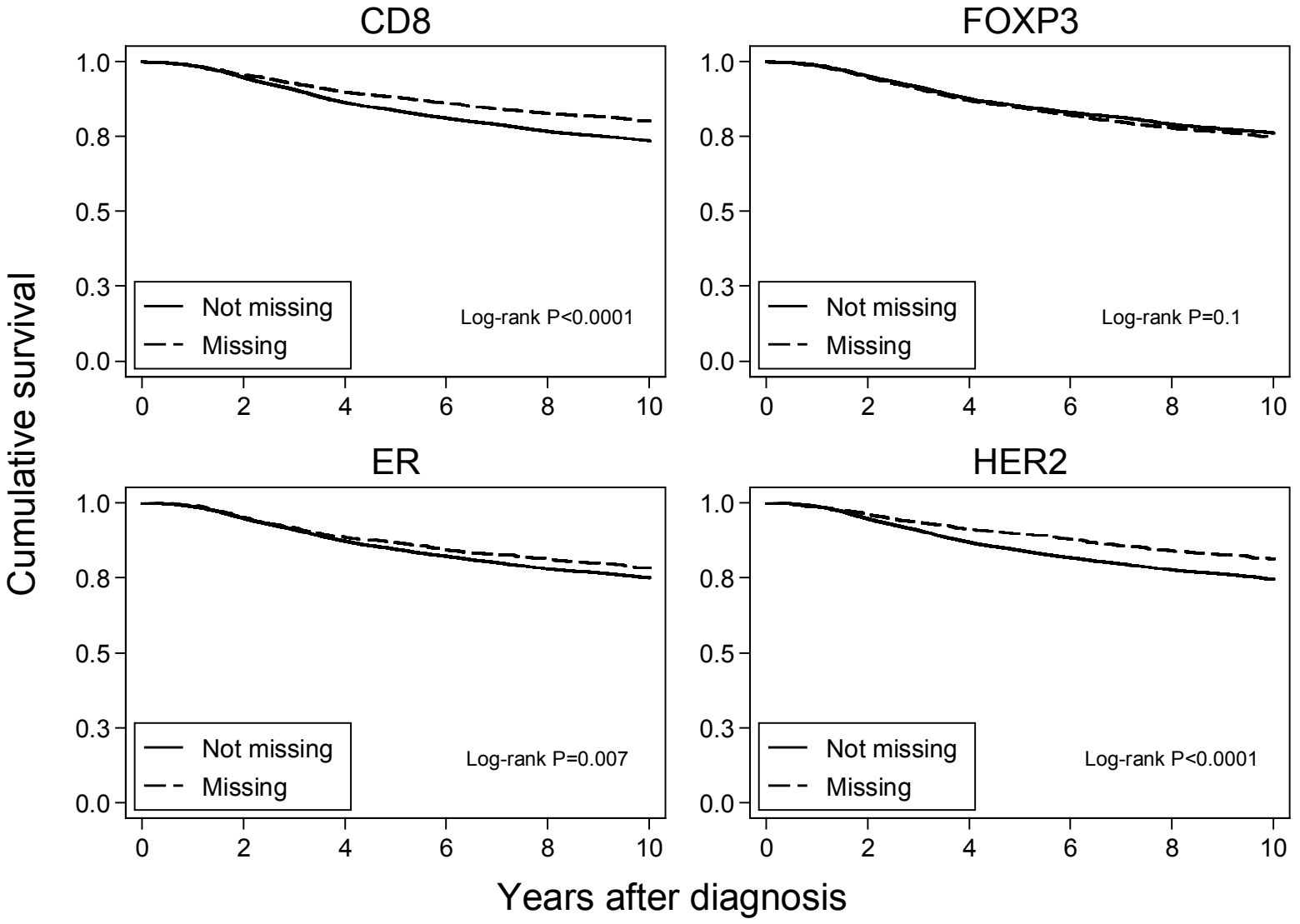
ER-POSITIVE, HER2-POSITIVE											
Variable	**Categories**	**Subjects**	**Events**	**HR**	**95% CI**		***P***	**T**	**95% CI**		***P***
Positive lymph nodes	0, 1, 2-4, 5-9, ≥10	483	162	1.31	1.17	1.47	<0.00001				
Tumour size	<10mm, 10-19mm, 20-29mm, 30-49mm, ≥50mm			1.33	1.12	1.58	0.001				
Grade	1, 2, 3			2.37	0.93	6.07	0.07	0.62	0.33	1.18	0.15
PR status	Negative, Positive			0.46	0.21	1.03	0.06	1.63	0.90	2.94	0.10
iT-CD8	Negative, Positive			0.69	0.50	0.94	0.02				

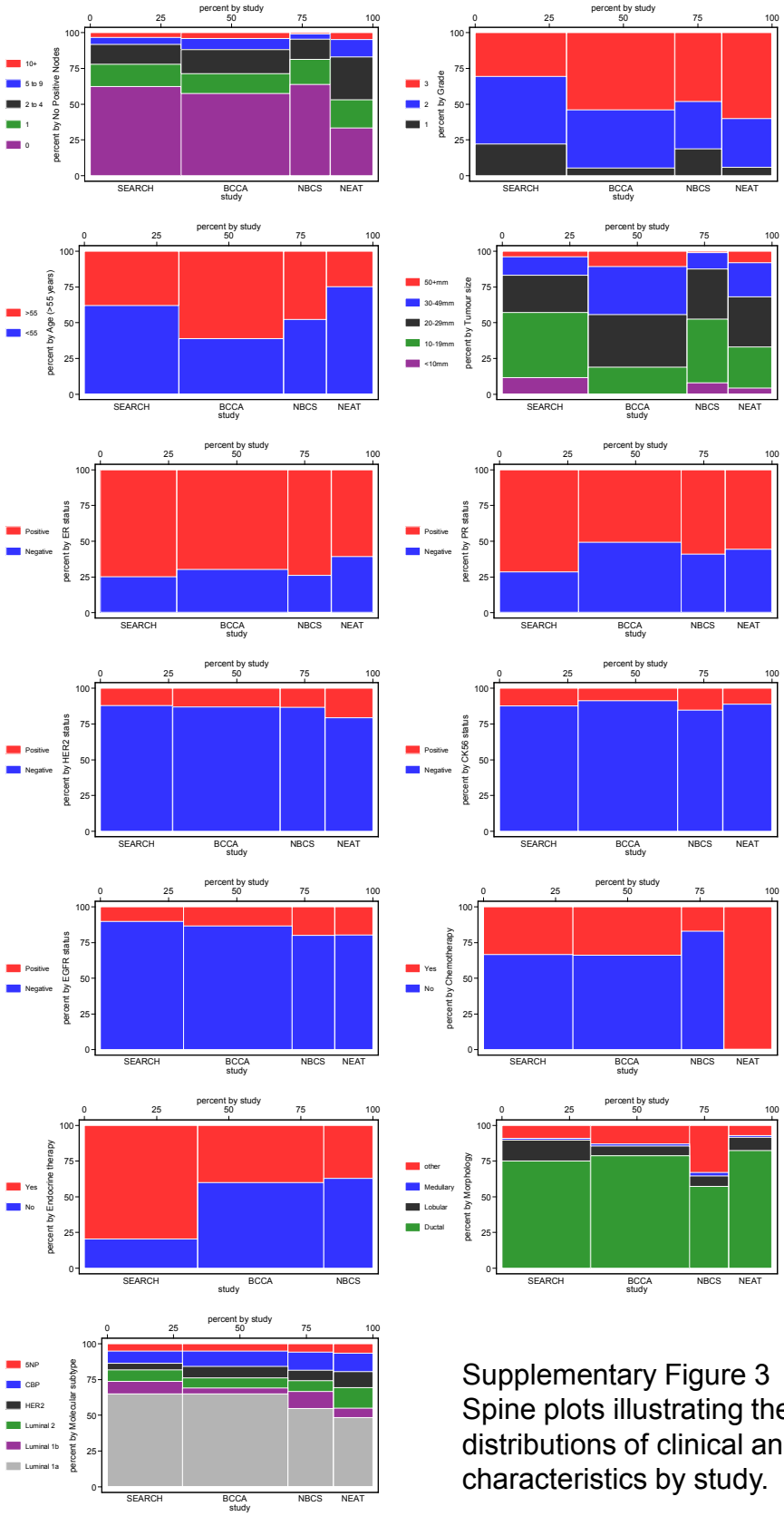
† Variables which violated the proportional hazards assumption were accounted for by an extended Cox-model where the beta-coefficient varies linearly with the natural logarithm of time. 'T' represents the exponentiated extended coefficient where a value >1 implies increasing hazard over time whereas a value of <1 implies decreasing hazard over time



Supplementary Figure 1 Histograms illustrating the relative distributions of observed values and imputed values by variables included in the imputation model.

Supplementary Figure 2
Kaplan-Meier survival plots of patient groups defined by whether data was missing or not missing for investigated markers.

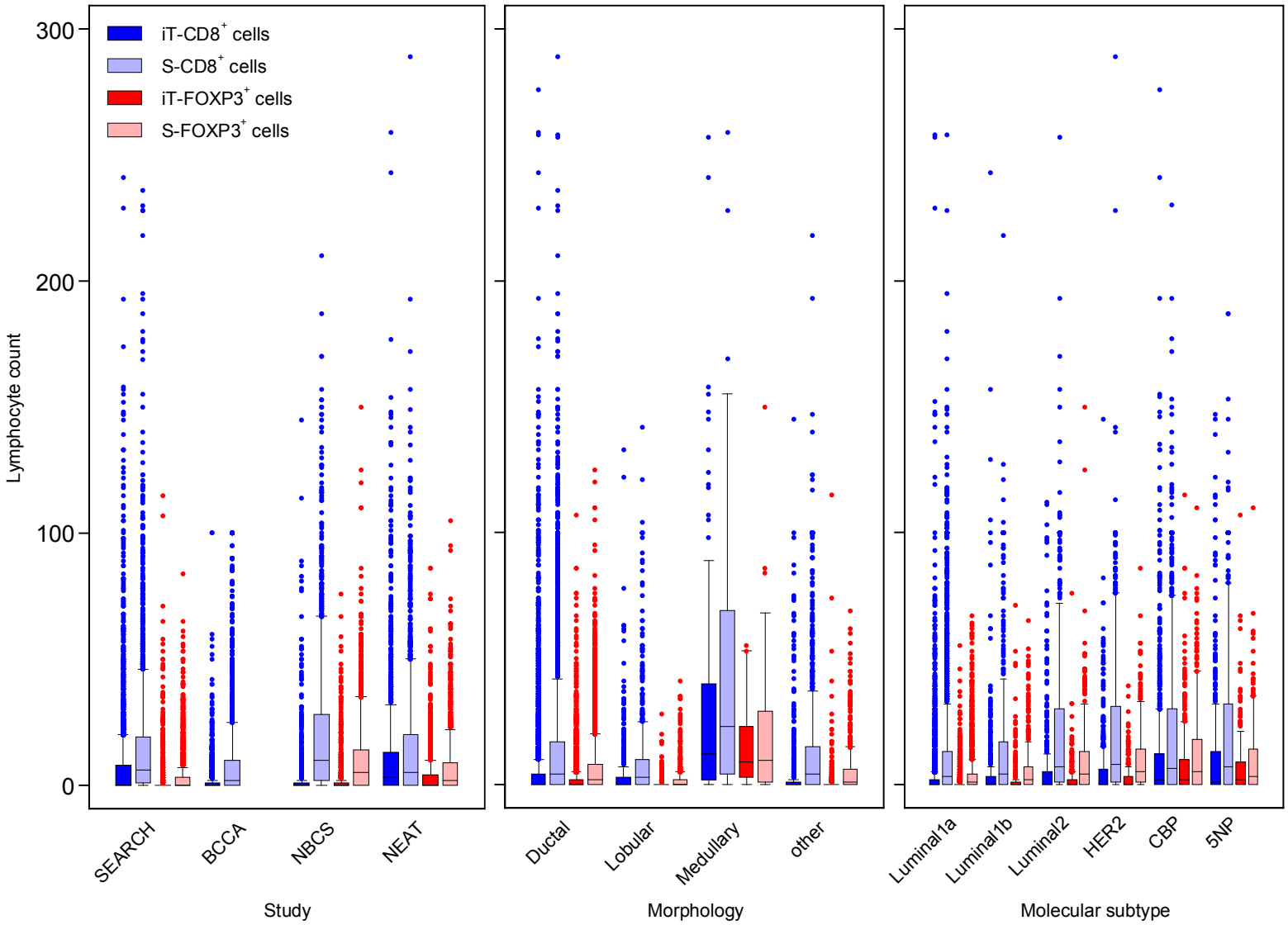




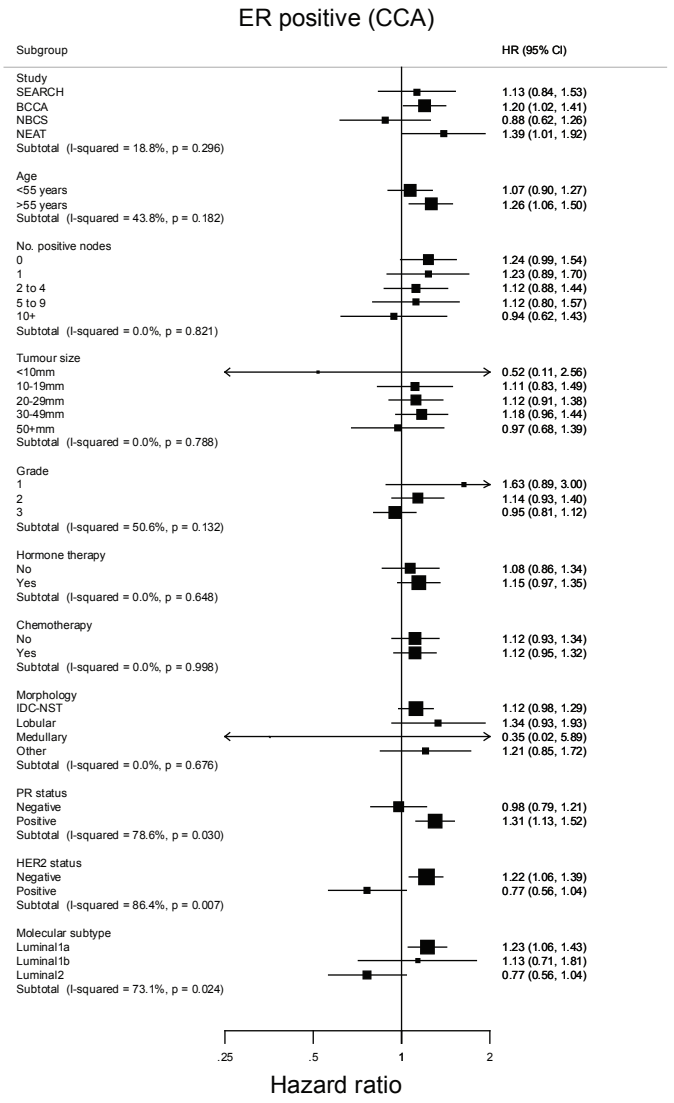
Supplementary Figure 3
Spine plots illustrating the percentage distributions of clinical and molecular characteristics by study.

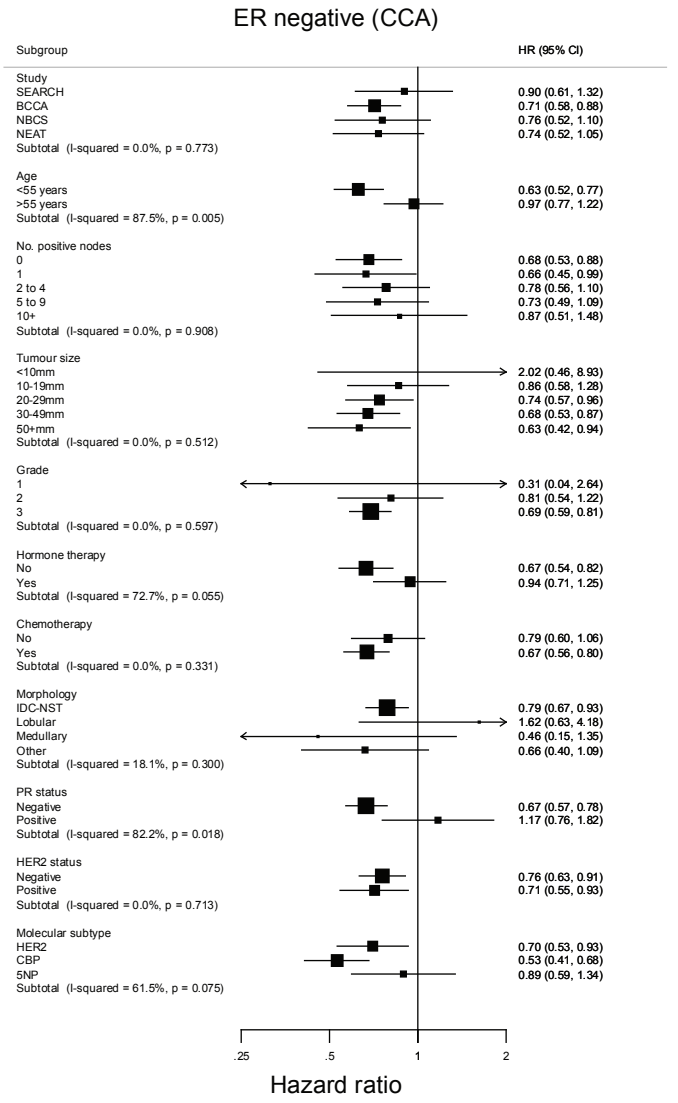
Supplementary Figure 4

Distribution of T-cell counts by study, histological type and molecular subtype illustrated as box-plots. Note T-cell counts for the BCCA study were capped at an upper limit of 100.

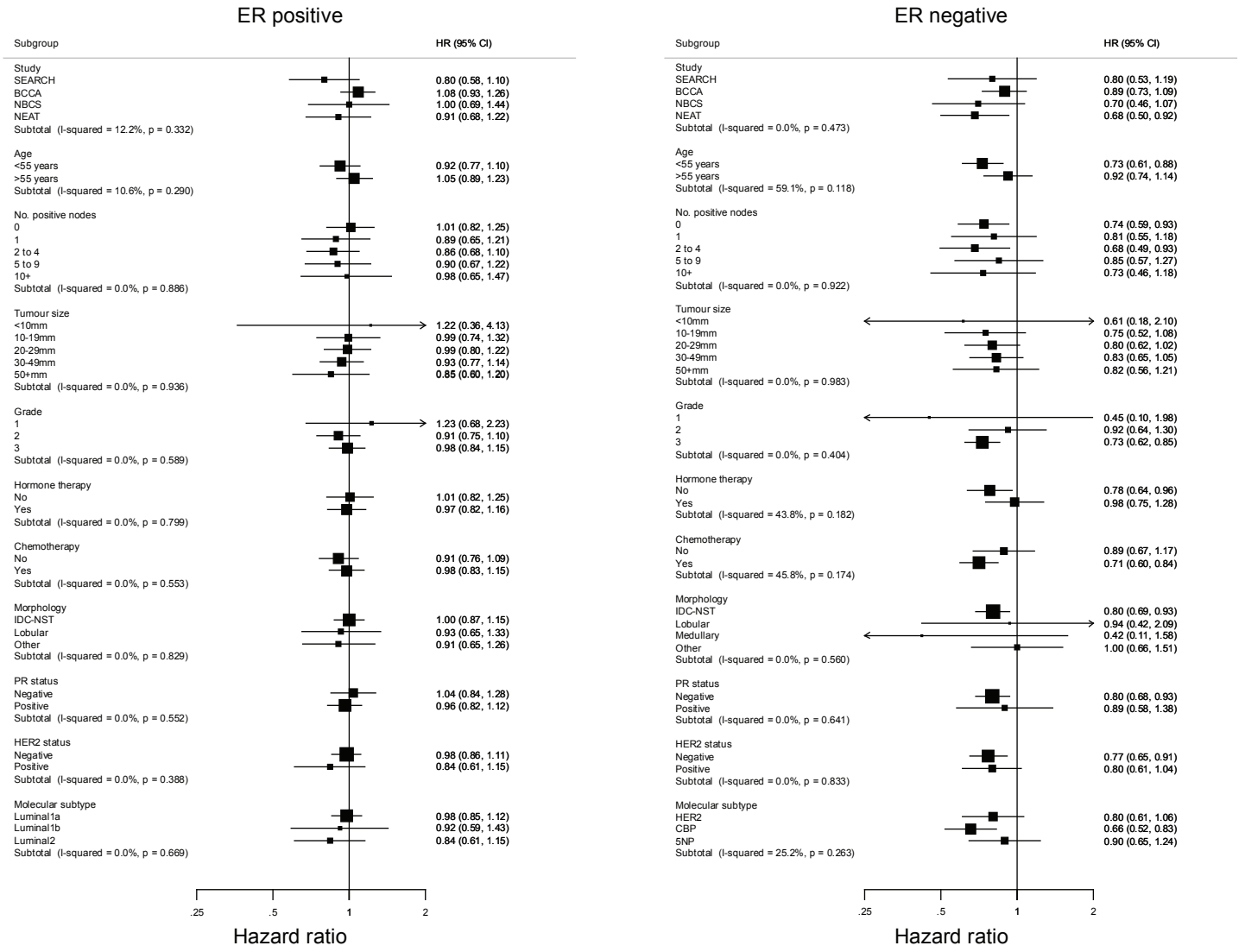


Supplementary Figure 5
Forest plots illustrating the univariate HR and 95% CI for association between iT-CD8+ status and survival in different subgroups. Estimates are based on the complete case analysis (CCA).

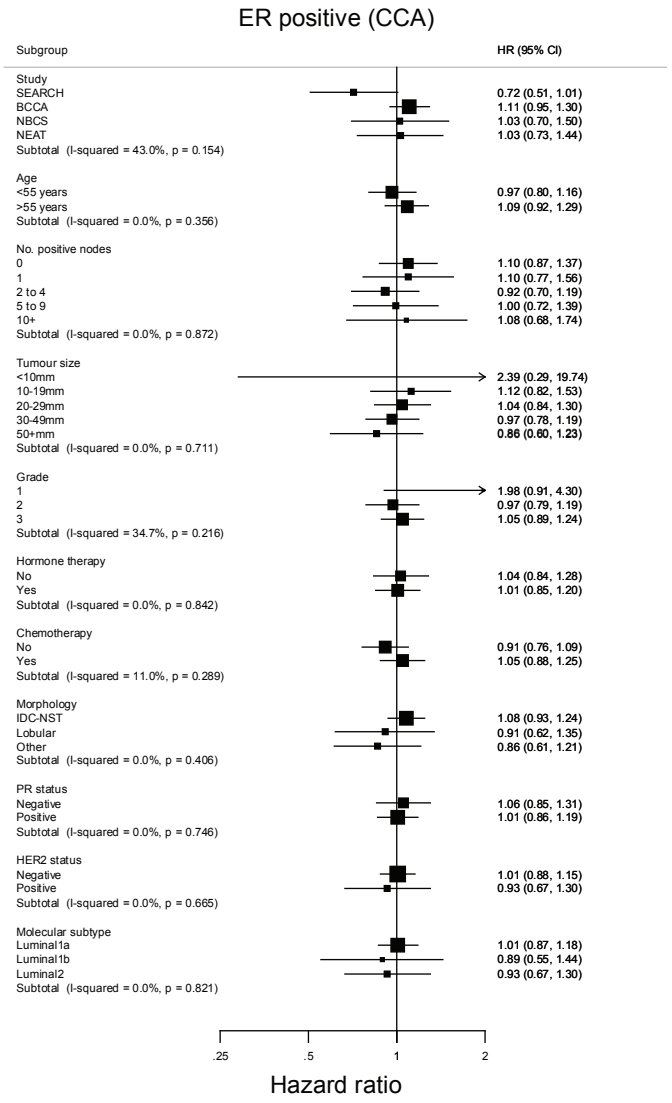


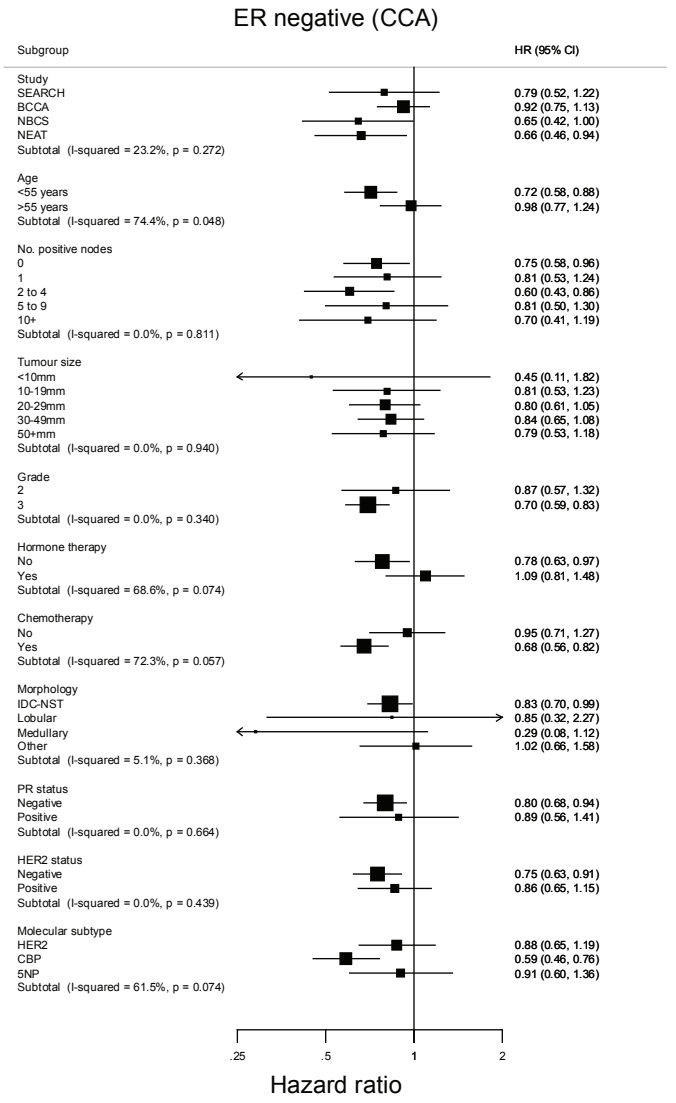


Supplementary Figure 6
Forest plots illustrating the univariate HR and 95% CI for association between S-CD8+ status and survival in different subgroups. Estimates are based on imputed data.

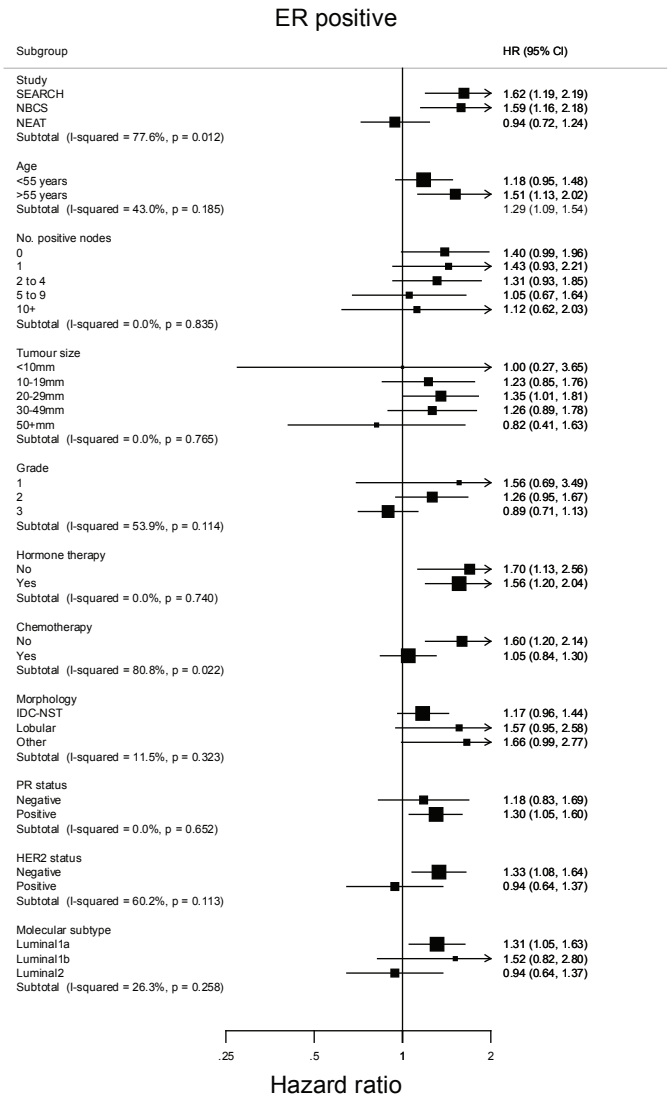


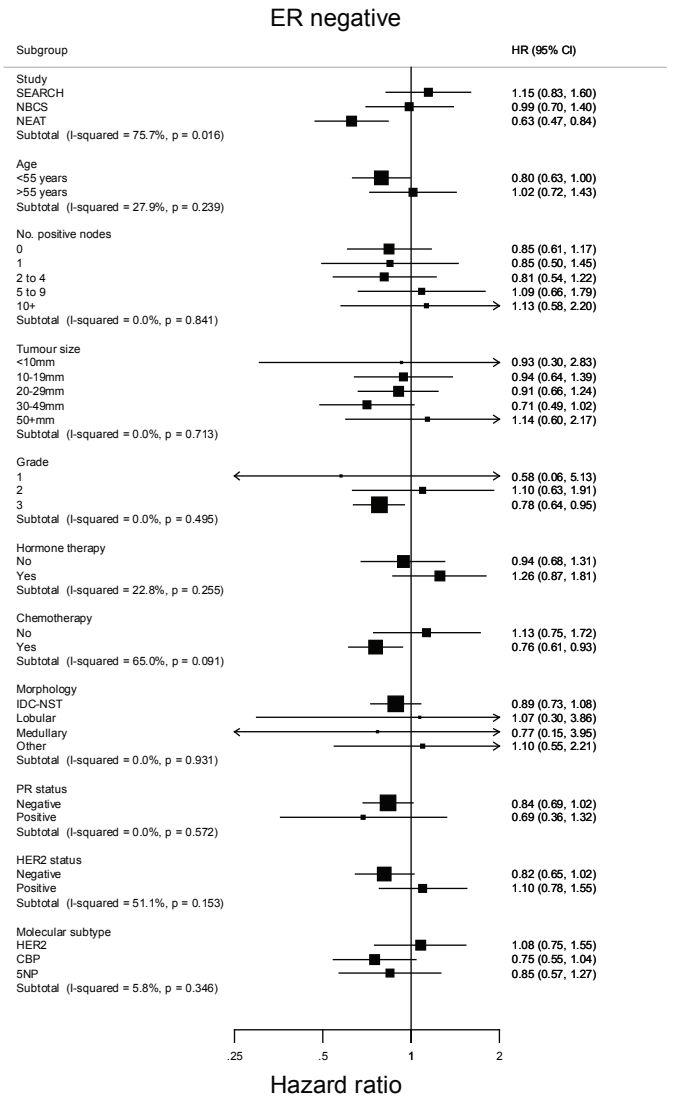
Supplementary Figure 7
Forest plots illustrating the univariate HR and 95% CI for association between S-CD8+ status and survival in different subgroups. Estimates are based on the complete case analysis (CCA).



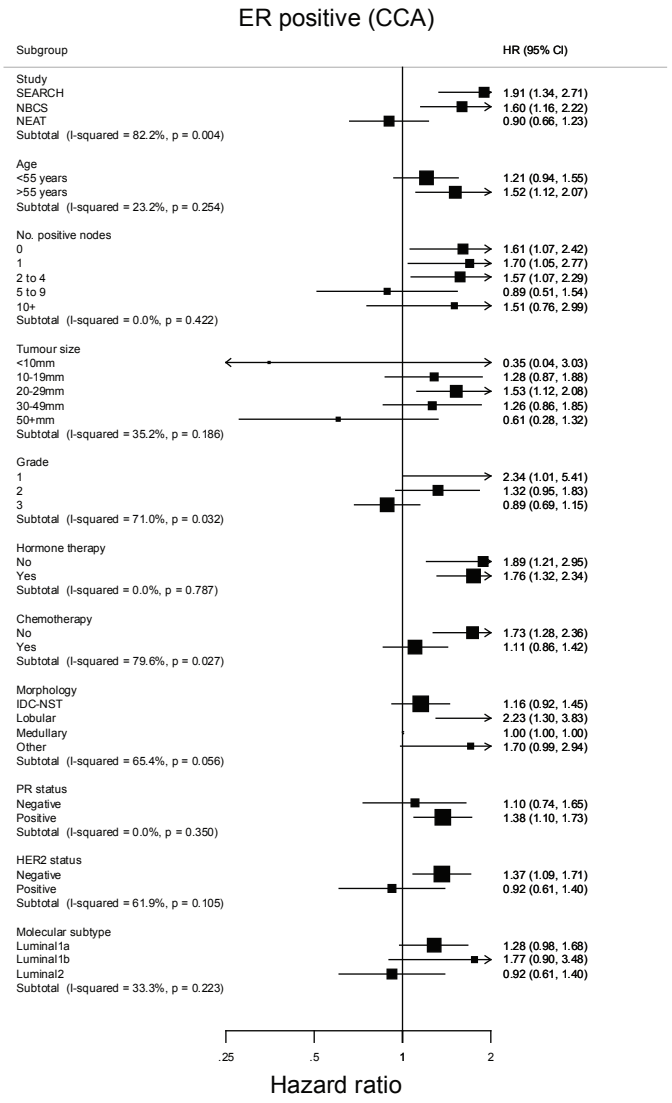


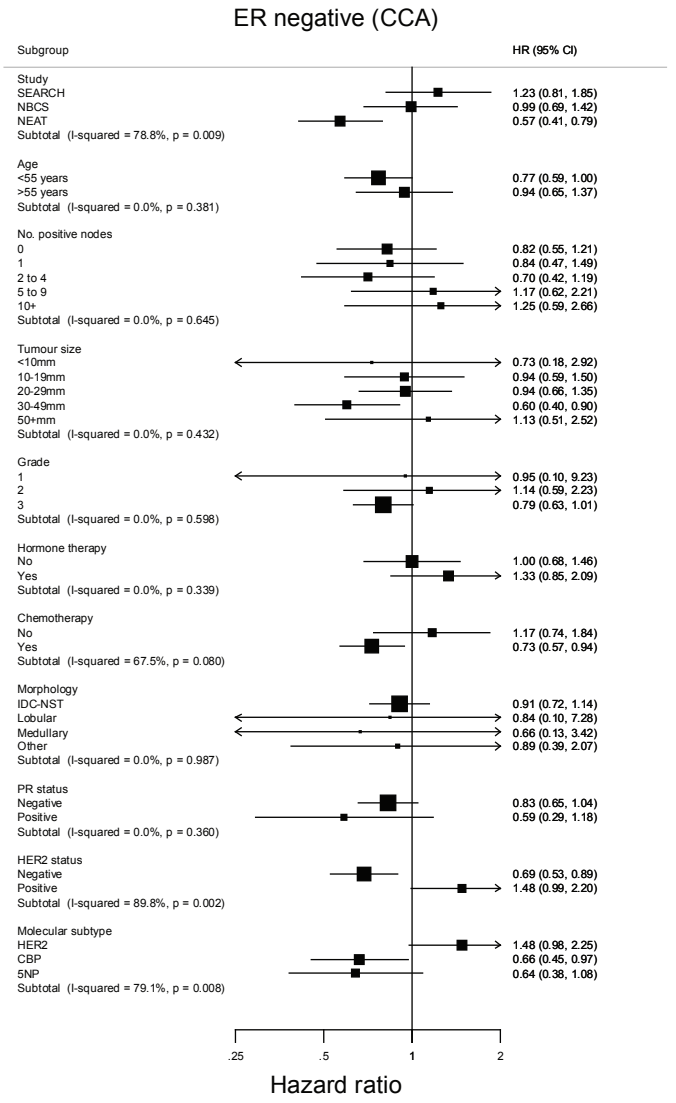
Supplementary Figure 8
Forest plots illustrating the univariate HR and 95% CI for association between iT-FOXP3+ status and survival in different subgroups. Estimates are based on imputed data.



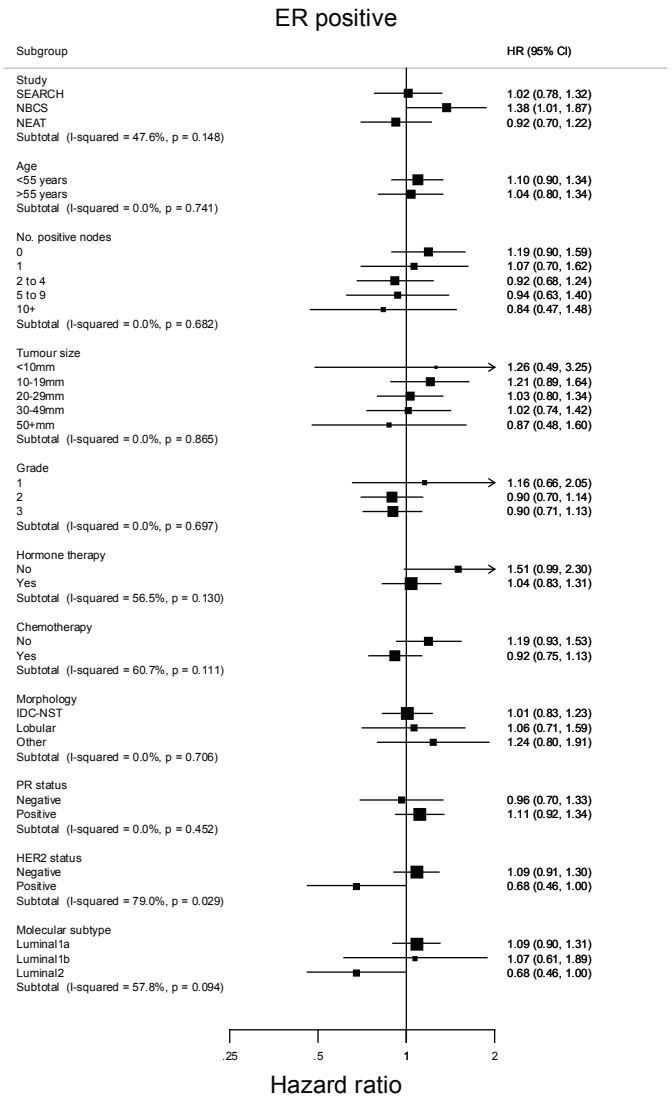


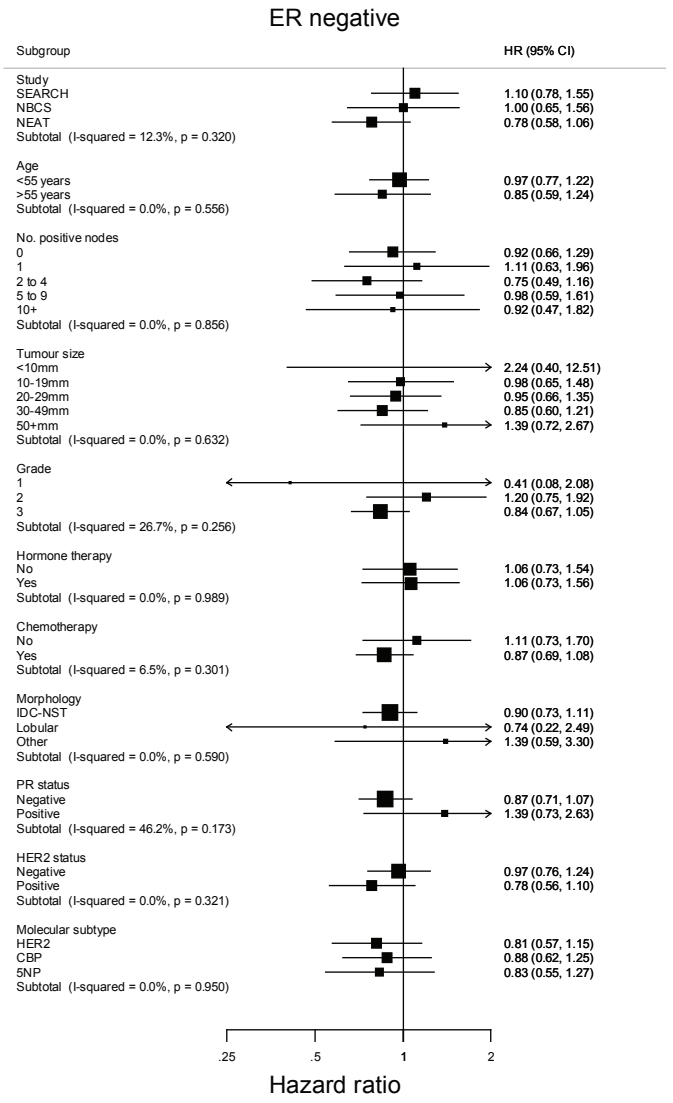
Supplementary Figure 9
Forest plots illustrating the univariate HR and 95% CI for association between iT-FOXP3+ cell status and survival in different subgroups. Estimates are based on the complete case analysis.



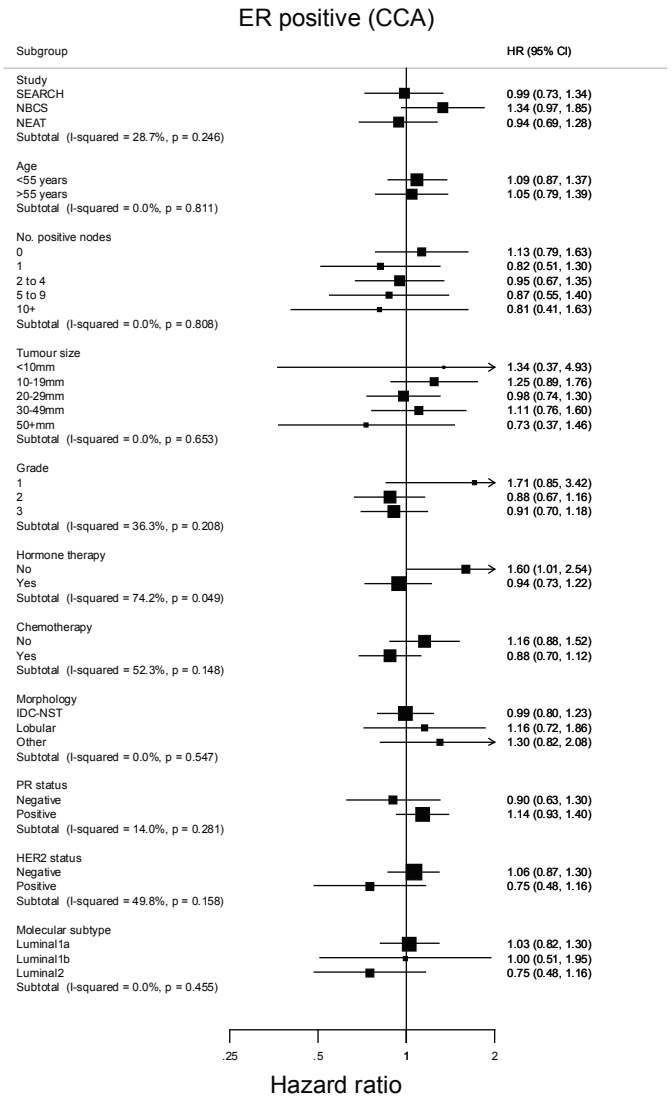


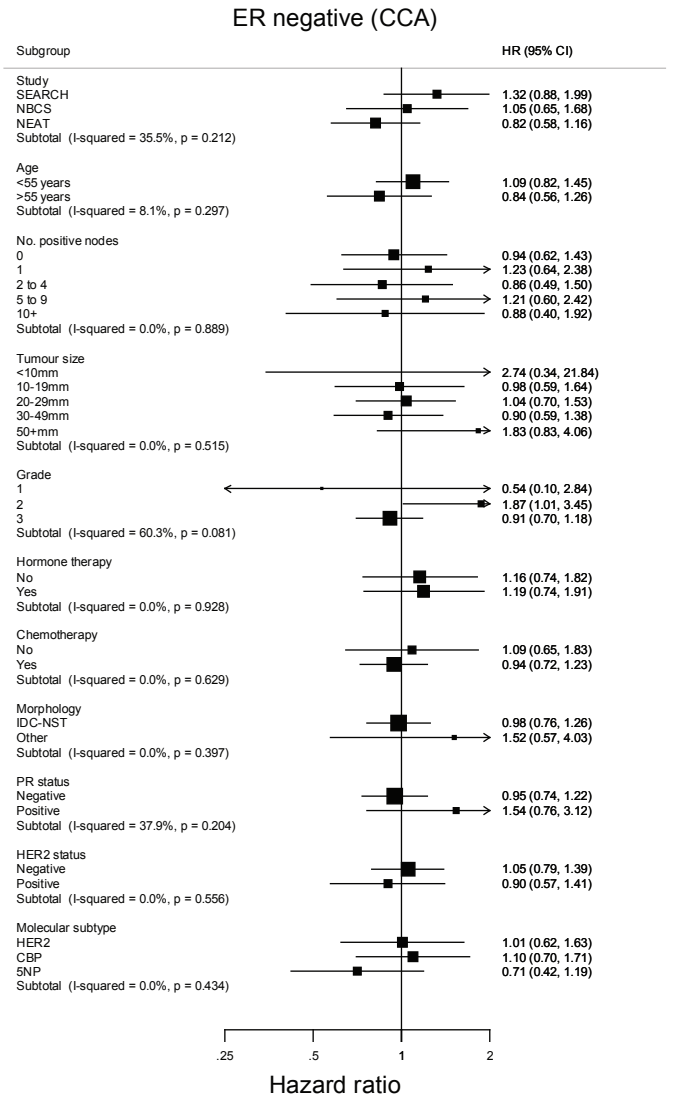
Supplementary Figure 10
Forest plots illustrating the univariate HR and 95% CI for association between S-FOXP3+ cell status and survival in different subgroups. Estimates are based on imputed data.



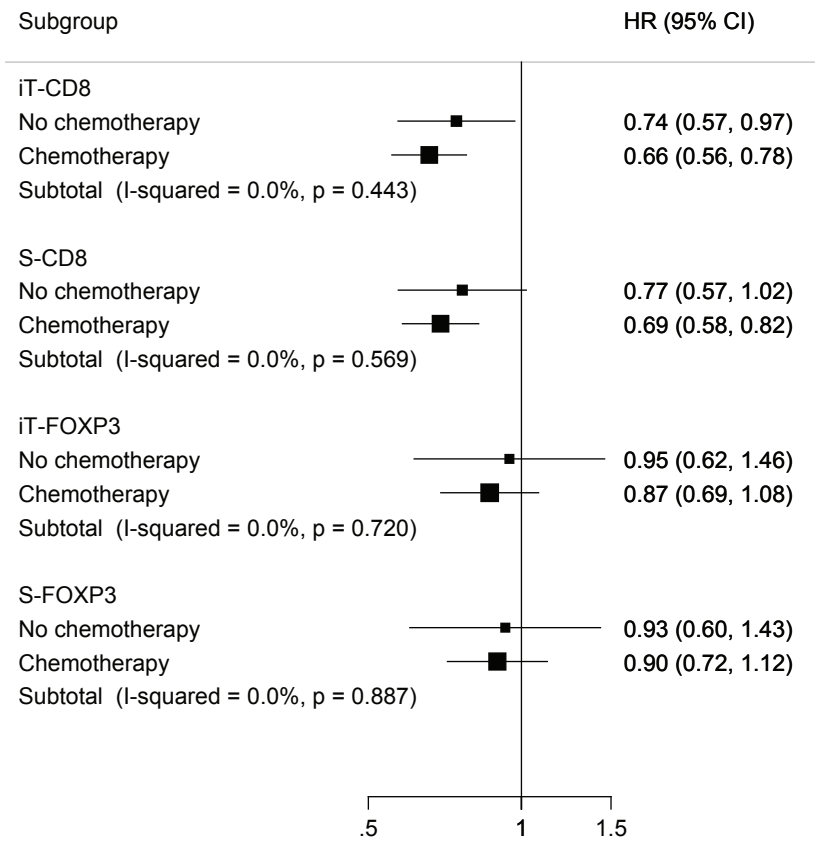


Supplementary Figure 11
Forest plots illustrating the univariate HR and 95% CI for association between S-FOXP3+ cell status and survival in different subgroups. Estimates are based on the complete case analysis.

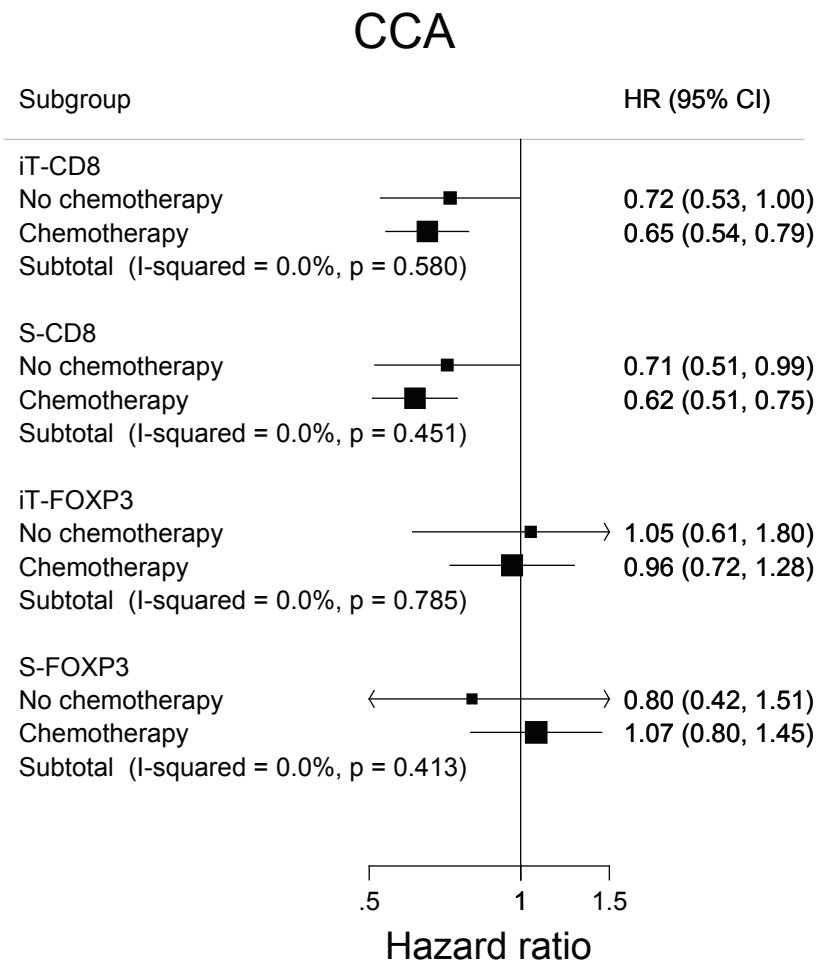




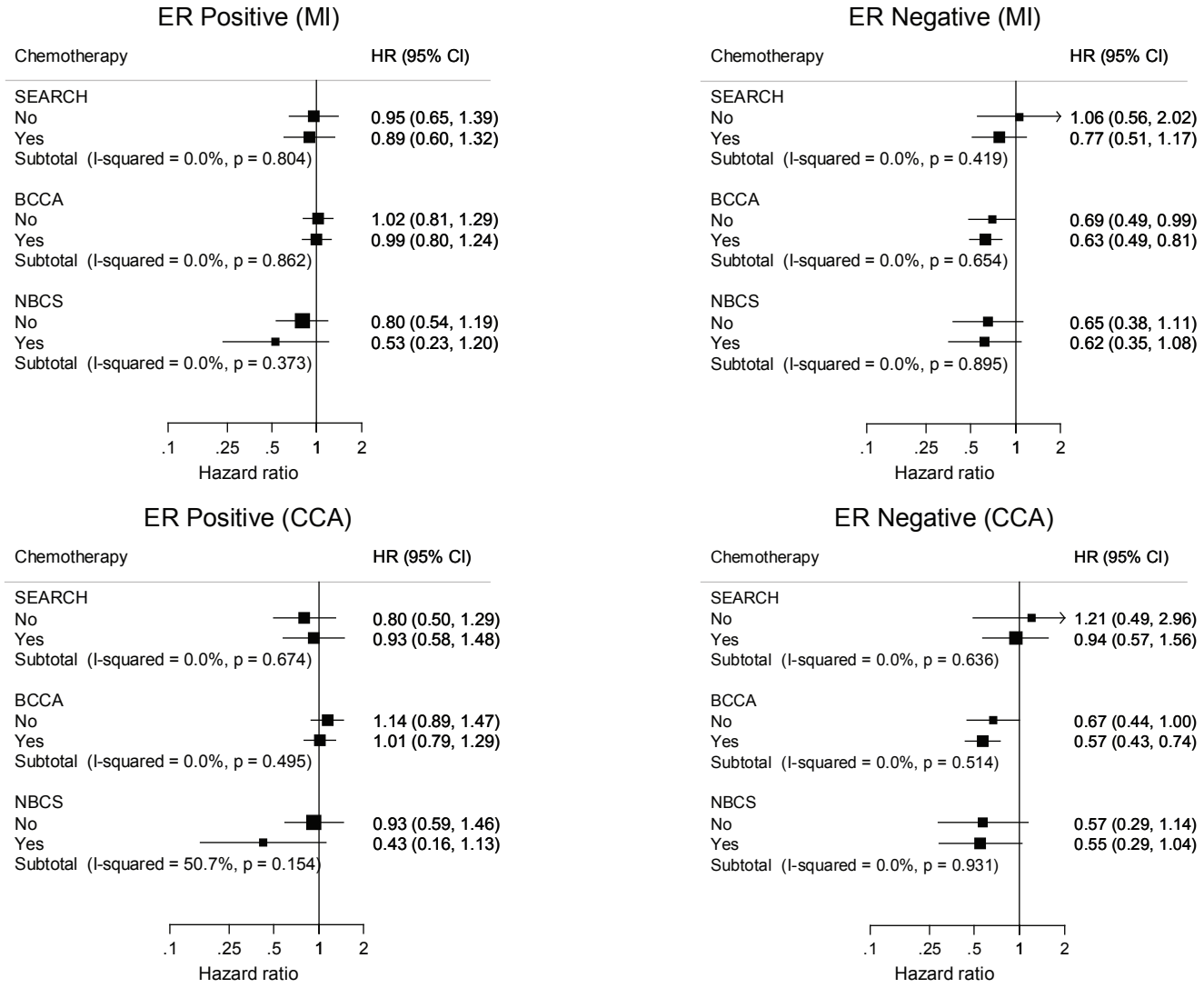
Supplementary Figure 12
Forest plot illustrating the prognostic effect of CD8+ and FOXP3+ lymphocytes in ER-negative breast cancer according to whether adjuvant chemotherapy was administered. HRs and 95%CIs are based on imputed analyses and are adjusted for tumour size, grade and number of positive lymph nodes.



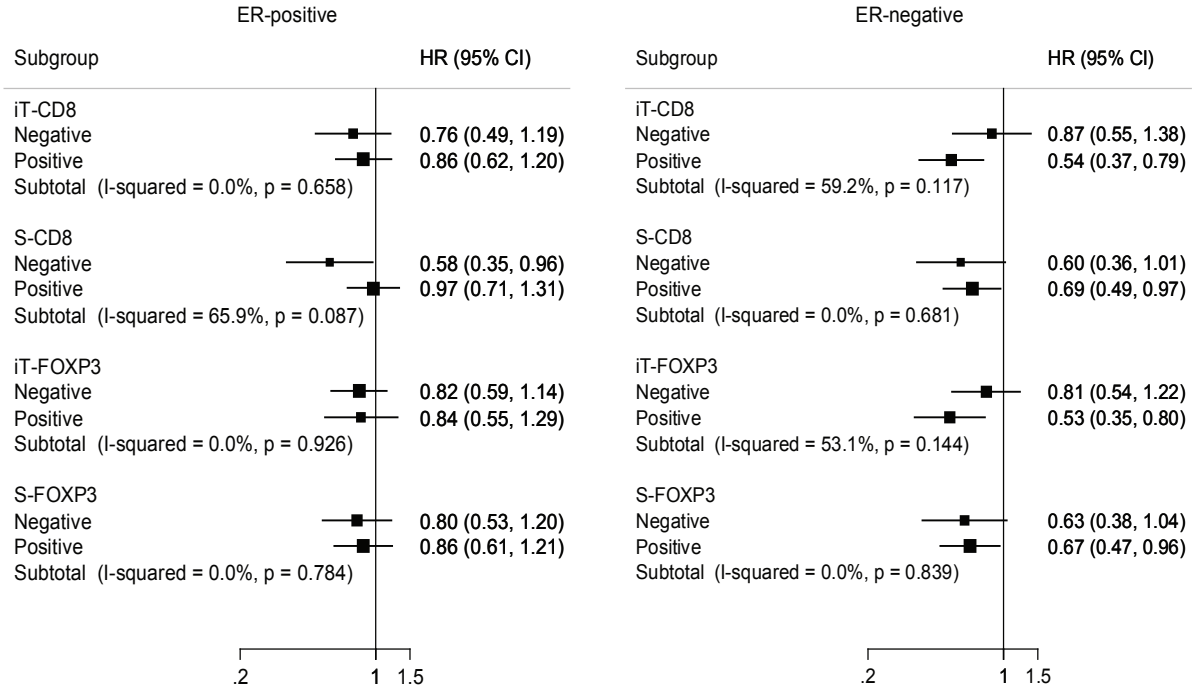
Supplementary Figure 13
Forest plot illustrating the prognostic effect of CD8+ and FOXP3+ lymphocytes in ER-negative breast cancer according to whether adjuvant chemotherapy was administered. HRs and 95%CIs are based on the complete case analysis (CCA) and are adjusted for tumour size, grade and number of positive lymph nodes.



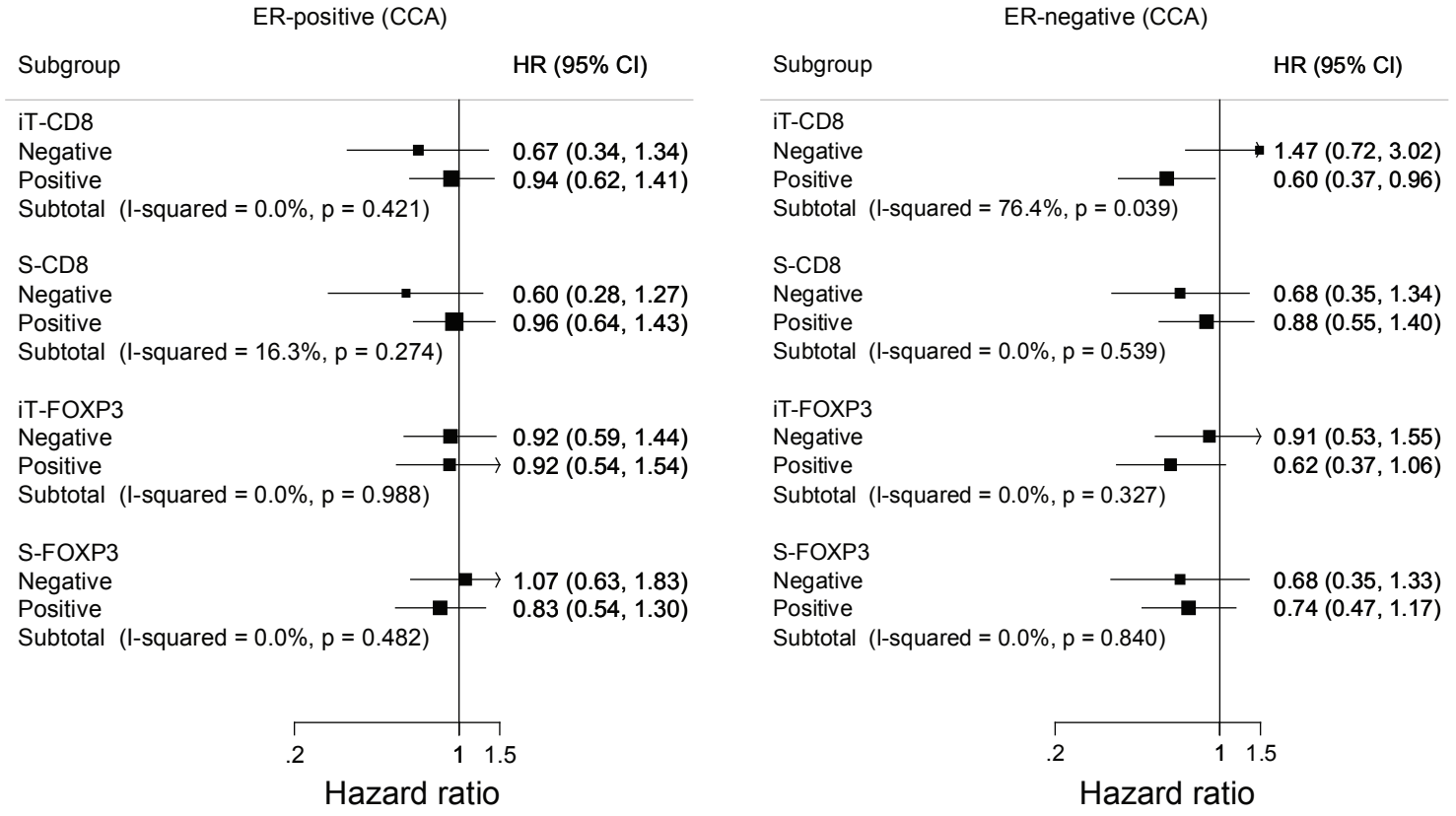
Supplementary Figure 14
Forest plot illustrating the prognostic effect of iT-CD8+ lymphocytes in ER-negative and ER-positive breast cancer according to whether adjuvant chemotherapy was administered separately for the three observational studies (SEARCH, NBCS, BCCA). HRs and 95%CIs are adjusted for tumour size, grade and number of positive lymph nodes (MI = multiple imputation, CCA = complete case analysis).



Supplementary Figure 15
Forest plot illustrating the treatment effect of E-CMF versus CMF by subgroups deÿned by the presence of CD8+ and FOXP3+ lymphocytes in ER-positive and ER-negative disease. HRs and 95%CIs are based on imputed data and are adjusted for tumour size, grade and number of positive lymph nodes.



Supplementary Figure 16
Forest plot illustrating the treatment effect of E-CMF versus CMF by subgroups defined by the presence of CD8+ and FOXP3+ lymphocytes in ER-positive and ER-negative disease. HRs and 95%CIs are based on the complete case analysis (CCA) and are adjusted for tumour size, grade and number of positive lymph nodes.



Supplementary Figure 17
Trends in hazard ratios and 95% confidence intervals for T-cell status according to tumour proliferation and diagnosis age. Estimates are adjusted for tumour size, histological grade and positive axillary lymph nodes. Analyses are based on imputed data. Estimates and confidence intervals associated with iT-CD8+ cells are represented as dark blue, those with S-CD8+ as light blue, those with iT-FOXP3+ as dark red and those with S-FOXP3+ as light red. Box sizes are proportional to the number of subjects in each subgroup.