



HAL
open science

An evaluation of the clinical significance of FOXP3 infiltrating cells in human breast cancer

Sahar M. A. Mahmoud, Emma C. Paish, Desmond G. Powe, R. Douglas
Macmillan, Andrew H. S. Lee, Ian O. Ellis, Andrew R. Green

► **To cite this version:**

Sahar M. A. Mahmoud, Emma C. Paish, Desmond G. Powe, R. Douglas Macmillan, Andrew H. S. Lee, et al.. An evaluation of the clinical significance of FOXP3 infiltrating cells in human breast cancer. Breast Cancer Research and Treatment, 2010, 127 (1), pp.99-108. 10.1007/s10549-010-0987-8 . hal-00594478

HAL Id: hal-00594478

<https://hal.science/hal-00594478>

Submitted on 20 May 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

An evaluation of the Clinical Significance of FOXP3⁺ Infiltrating Cells in Human Breast Cancer

Mahmoud SMA^{1,2}, Paish EC³, Powe DG³, Macmillan RD⁴, Lee AHS³, Ellis IO^{1,3},
Green AR¹

¹Division of Pathology, School of Molecular Medical Sciences, University of
Nottingham, Nottingham, UK

²Clinical Pathology Department, Faculty of Medicine, Mansoura University,
Mansoura, Egypt

³Department of Histopathology, ⁴The Breast Institute, Nottingham University
Hospitals NHS Trust, Nottingham, UK

Address correspondence to: Andrew R Green, Division of Pathology, School of
Molecular Medical Sciences, University of Nottingham, Queen's Medical Centre,
Nottingham, UK NG7 2UH. e-mail: andrew.green@nottingham.ac.uk Tel: +44
1158230786 Fax: +44 1159709479

Running head: FOXP3 Expression in Breast Cancer

Keywords: FOXP3; Tissue microarray; Immunohistochemistry; breast cancer; T
regulatory cells

Abstract:

Introduction Studies in mice have that shown thymic-derived CD4⁺ CD25⁺ regulatory T cells (T reg; FOXP3⁺ lymphocytes) inhibit an antitumour immune response. Additional studies have also reported that the T reg population increases in peripheral blood and tumour tissues from patients with cancer. However, the relationship between the T reg population and patient prognosis remains controversial. Our aim was to determine the prognostic value of T reg cell density in breast cancer using immunohistochemical assessment of FOXP3, which has been shown to be the optimal marker for T regs.

Patients and methods Tissue microarrays were used, and the density of FOXP3⁺ cells was determined in a series of 1445 cases of well-characterised primary invasive breast carcinoma cases with long-term follow up.

Results FOXP3⁺ cell numbers were counted in tumour nests, in tumour-adjacent stroma, and in distant stroma. The total number of FOXP3⁺ cells significantly correlated with higher tumour grade ($r_s = 0.37$, $p < 0.001$) and ER negativity (Mann-Whitney U test, $p < 0.001$). In addition, FOXP3 infiltration positively correlated with HER2 expression and basal phenotype subclass. On univariate analysis, FOXP3⁺ cells were associated with a worse prognosis ($P = 0.012$, log rank = 6.36). This association was found for intratumoural FOXP3⁺ and for tumour-adjacent stromal FOXP3⁺ cells (tumour-cell associated FOXP3, $p = 0.001$ and log rank 10.35). However, the number of FOXP3⁺ cells was not found to be an independent prognostic factor in multivariate analysis.

Conclusion We therefore conclude that FOXP3⁺ infiltrating cells do not have a dominant role in breast cancer prognosis and suggest that other inflammatory cell subsets may be more critical variables.

Introduction

Breast cancer is a heterogeneous disease with different known biological sub classes some of which are associated with immune cell infiltration [1]. The existing literature is not consistent on the importance of inflammatory cell infiltration in breast cancer development [2]. The presence of inflammatory cells may represent an immune response to the tumour, but could also potentially exhibit pro-tumour effects including stimulation of tumour invasion or angiogenesis. Some studies have reported that a dense lymphohistiocytic infiltrate is associated with a favourable prognosis [3, 4], however, other reports have shown an association between prominent inflammation and a poor patient outcome [5]. A detailed understanding of the functional peculiarities and phenotype of individual T lymphocyte subtypes may explain this paradox [6].

An important mechanism of defence against autoimmunity is the central tolerance which involves deletion of self-reactive T lymphocytes in the thymus at an early stage of development [7]. However, autoreactive T cells that migrate into the tumour periphery are controlled by a number of peripheral mechanisms including the induction of regulatory/suppressor T cells [8]. CD4⁺ T regulatory (T reg) cells include at least four populations that differ in phenotype, cytokine secretion profile, and suppressive mechanisms [9, 10]. One prevailing phenotype is the naturally occurring T reg cells that originate in the thymus and are involved in protection from autoimmune responses. These natural T reg cells have the ability to suppress the activation of conventional T cells in a cell-contact-dependent, IL-10-independent and TGF- β -independent manner [9, 11].

Naturally occurring CD4⁺ T reg cells are characterised by the expression of the IL-2 receptor α -chain (CD25) and constitute 5-10% of peripheral CD4⁺ in healthy individuals [12]. These cells naturally arise in the thymus and after differentiation, the naïve CD4⁺CD25⁺CD45RA⁺ T cells are exported to the periphery where they suppress the activation of T cells potentially reactive to the self [10]. In addition to CD4 and CD25 markers, T reg also constitutively express CD45RO and CD152 (CTLA-4) [13]. *Ex vivo* studies on these cells revealed a poorly proliferative cell population that secretes inhibitory cytokines such as TGF- β and IL-10 [12]. They are

implicated in many autoimmune diseases such as multiple sclerosis, diabetes, thyroiditis, and inflammatory bowel diseases. Besides their role in autoimmunity, Treg cells participate in transplantation tolerance and tumour immunity [14].

Although the expression of CD25 on T cells has been described as a marker of T reg cells, its expression is not restricted to T reg cell function as it is also expressed by activated non-regulatory effector lymphocytes [15]. Previous studies revealed that the most specific marker to identify T reg cells is FOXP3, a member of the forkhead or winged helix family of transcription factors encoded on the X chromosome [16, 17]. The nuclear expression of FOXP3 protein is highly restricted to discrete T reg cell populations and it is therefore considered to be a specific marker to detect T reg cells in vivo [17].

Studies in mice showed that T reg inhibit the antitumour response [18, 19] and that their depletion can result in effective antitumour immune response [20, 21], implicating these cells in immune evasion by cancer cells. Additional studies have reported that the T reg population increases in peripheral blood and tumour tissues in patients with several types of human cancer including breast cancer [13, 22-24]. A high density of tumour-infiltrating FOXP3⁺ cells has been associated with poor outcome in various solid tumours including ovarian [23], pancreatic [25], lung [26], and hepatocellular carcinoma [27]. In contrast, improved patient survival has been shown to be associated with a high density of tumour infiltrating FOXP3⁺ T regs in oesophageal squamous cell carcinoma [28] and colorectal cancer [29].

A few studies have recently examined the role of FOXP3⁺ cells in breast cancer and have utilised relatively small patient cohorts [22, 30]. In the largest study, Bates et al [22] studied 222 invasive breast carcinomas and focused on the prognostic value of FOXP3⁺ cells in ER-positive tumours, while Ladoire et al [30] concentrated on the pathologic response to neoadjuvant chemotherapy. There is still limited information delineating the mechanisms behind T reg accumulation within the breast cancer microenvironment and their expansion locoregionally. Thus, it is important to evaluate the localisation of infiltrating T regs in relation to clinical outcome. The aim of this study was therefore to determine the prognostic value of FOXP3⁺ cell density and localisation in a large well-characterised series of invasive breast cancer with long

follow-up. Furthermore, we have correlated the number of FOXP3⁺ cells infiltrating breast carcinomas with other recognised prognostic factors.

Patients and methods

Study population

This retrospective study is based on a consecutive unselected series of 1902 patients from the Nottingham Tenovus Primary Breast Carcinoma series who presented during the period 1987 to 1998. This is a well-characterised series of primary operable invasive breast carcinomas patients less than 71 years of age, treated in a single institution, which has been previously investigated for a wide range of prognostic factors [31-34]. Data on a range of patient, pathology and biomarkers were available. These include histologic grade [35], histologic tumour type [36], vascular invasion (VI) [37], primary tumour size, and lymph node (LN) stage, and Nottingham prognostic index (NPI) [38] which were routinely assessed and recorded in the database as well as patients' information such as age, family history, and menopausal status.

All patients received standard surgical treatment of either mastectomy or wide local excision with radiotherapy. Prior to 1988, patients did not routinely receive systemic adjuvant treatment. From 1988, patients were offered systemic adjuvant treatment based on NPI score and hormone receptor status [39]. Patients with an NPI score \leq 3.4 received no adjuvant therapy, those with a NPI score $>$ 3.4 received Tamoxifen if oestrogen receptor (ER) positive (\pm Zoladex if pre-menopausal) or classical cyclophosphamide, methotrexate and 5-fluorouracil if ER negative and fit enough to tolerate chemotherapy. Information on local, regional and distant recurrence and survival was maintained on a prospective basis. Patients were followed up at 3-month intervals initially, then every 6 months, then annually for a median follow-up period of 128 months (range 4-243). Breast cancer specific survival (BCSS) was defined as the time (in months) from the date of the primary surgical treatment to the time of death from breast cancer. Patients who died from other causes or were still alive were censored at the time of last follow-up. Disease-free interval (DFI) was calculated from the date of first operation until the first recurrence (local, regional, or distant). The number of deaths due to breast cancer was 398 while the number of relapses was 581.

The clinicopathological characteristics of the series are presented in Table 1. Reporting Recommendations for Tumour Marker Prognostic Studies (REMARK) criteria were followed throughout, as recommended by McShane et al [40].

This study was approved by Nottingham Research Ethics Committee 2 under the title of “Development of a molecular genetic classification of breast cancer”.

Tissue microarrays and immunohistochemistry

Breast cancer tissue microarrays (TMA) and whole breast cancer tissue sections were prepared and immunohistochemistry performed employing the standard streptavidin-biotin complex method as previously described [41]. In short, tissue sections were deparaffinised with xylene then rehydrated through 3 changes of alcohol. Endogenous peroxidase activity was blocked by 0.3% hydrogen peroxide for 10 minutes. Antigen retrieval was performed by microwave treatment in 0.01M TRIS-EDTA buffer (pH 8) for 20 min. Slides were incubated for 1 hour with monoclonal mouse anti-human FOXP3 (clone 236A/E7, Abcam, Cambridge, UK, 1:100 dilution). After washing with Tris-buffered saline (TBS, pH 7.6), sections were incubated with biotinylated secondary antibody (1:100 dilution) for 30 minutes, followed by streptavidin coupled to biotinylated horse radish peroxidase (1:100 dilution) for a further 55 minutes using streptABC kit (streptABCComplex/HRP Duet, biotinylated goat antimouse/rabbit immunoglobulin kit, Dako Corporation, Denmark, K0492). Antigen-antibody reaction was visualised with 3, 3' Diaminobenzidine tetrahydrochloride (Dako, K3468). After counterstaining with Mayer's haematoxylin, sections were dehydrated through ascending alcohols to xylene and mounted. Positive staining controls were performed in parallel with paraffin sections of normal human tonsil. Negative control was performed by omitting the primary antibody.

To assess the topographical distribution and heterogeneity of FOXP3⁺ cell distribution, immunoreactivity was also determined in 30 cases (included in the TMA) using full-face tissue sections.

Quantification of FOXP3⁺ cells

The number of FOXP3⁺ cells was counted in each tumour core (0.6 mm diameter) using an eyepiece graticule. The scorer was blinded to the patients' clinical

information (S.M.). Scores were audited by a second assessor (I.O.E) and a very good correlation was found between the two investigators' scores (κ value = 0.9). FOXP3⁺ cells were counted in three compartments in each tumour; intratumoural compartment (within the tumour cell nests); within distant stroma not touching tumour cells (defined as the distance between positive FOXP3 cell and tumour nest is more than the size of one tumour cell); and within adjacent stroma and touching tumour cells (the distance between positive FOXP3 cell and tumour nest is equal or less than the size of one tumour cell) (Fig. 1 C and D). The total number of FOXP3⁺ cells was determined by adding the counts for the three compartments.

Statistical analyses

Analysis of the correlation between the number of FOXP3⁺ cells and clinicopathological characteristics was conducted using Spearman rank order correlation or Mann-Whitney U test where appropriate. Concordance between scores from both observers was obtained using Kappa (κ) measure of agreement test. All tests were 2-tailed.

X-tile software program (version 3.4.7, Robert L Camp, 2005) was utilised in order to define the single optimal cut-off point for FOXP3⁺ cell count with corrected *p*-value and relative risk against BCSS in each tumour compartment [42]. X-tile software provides a method to determine the optimal divisions of a continuous population and then does statistical testing using training set/validation set method for *p*-value estimation when no foreknowledge about how a marker parses a population into subsets. In addition, the software can perform standard Monte Carlo cross-validation to produce corrected *p*-values to assess statistical significance of data assessed by multiple cut-points [42, 43].

Survival rates were determined using Kaplan-Meier method and compared by the log-rank test. Multivariate analysis using Cox proportional hazards regression model determined the influence of T regs, when adjusted to other variables, on BCSS. All analyses were conducted using Statistical Package for the Social Sciences (SPSS Inc, version 15, Chicago, IL, USA) software for windows. *P*-value < 0.05 was identified as statistically significant.

Cut-off values for ER α , PgR, and HER2 included in this study were chosen before statistical analyses as previously described for this patients' series [32, 44]. Basal phenotype positivity was defined as detection of expression in 10% or more of invasive malignant cells for CK5/6 and/or CK14 [33].

Results

The frequency and localisation pattern of FOXP3⁺ cells in breast cancer

The evaluation of full size tissue sections demonstrated random distribution of the FOXP3-positive cells indicating suitability for use of TMAs. In addition, both full sections and arrays showed similarity in intensity of staining and area of positive staining. FOXP3-positive cells exhibited distinct nuclear staining, consistent with the expected transcription function role of FOXP3. In the tonsil positive control section, FOXP3⁺ cells were distributed mainly in interfollicular (T-cell) areas, although occasionally low numbers of FOXP3⁺ cells were found within follicles (Fig. 1 A and B). [None of the carcinoma cells or mesenchymal cells exhibited staining.](#)

A total of 1445 tumours were examined after exclusion of the uninformative cores. FOXP3⁺ cells were distributed among the three defined tumour compartments with higher numbers of positive cells in the stroma. Figure 1 shows representative photomicrographs of immunohistochemical staining of Treg using FOXP3 antibody. There were more FOXP3⁺ cells in the distant stroma compared to FOXP3⁺ cells within the tumour and adjacent stroma (Table 2).

Correlation with other prognostic factors

There was a moderate positive correlation between the total number of FOXP3⁺ cells and tumour grade (Table 1). Furthermore, there was a significant correlation between total number of FOXP3⁺ cells and both ER and PgR negativity (Mann-Whitney U test). On the other hand, total number of FOXP3⁺ cells was positively correlated with HER2 expression and basal phenotype subclasses. FOXP3 expression was weakly inversely correlated with patient age (Table 1).

Survival analysis

Using X-tile software, the optimal cutpoints identified for positivity and negativity for the total number of FOXP3⁺ cells and distant stromal FOXP3⁺ cells was ≥ 3 positive cells and for intratumoural and adjacent stromal FOXP3⁺ cells was ≥ 1 positive cells.

In univariate analysis, patients with positive FOXP3 expression, irrespective of locality, had a significantly shorter BCSS than those tumours negative for FOXP3⁺ cells ($P= 0.012$, log rank= 6.36, Figure 2A), with hazard ratio of 1.32 (95% CI, 1.06 to 1.63). This effect on survival was most evident during the first 180 months of follow-up. Similarly, an association between intratumoural FOXP3 cell number positivity and shorter BCSS was observed ($P= 0.037$, log rank= 4.34, Figure 2B), with hazard ratio of 1.26 (95% CI, 1.01 to 1.56). Again, the effect on survival was most apparent before 180 months of follow-up. In addition, adjacent stromal FOXP3 cell number positivity was associated with shorter BCSS ($P= 0.001$, log rank= 10.60, Figure 2D), with hazard ratio of 1.39 (95% CI, 1.14 to 1.70). However, there was no significant difference in survival between patients with positive distant stromal FOXP3 cell numbers and those with negative distant stromal FOXP3 cell numbers ($P= 0.235$, log rank= 1.41, Figure 2C).

In multivariate Cox regression model including well-recognised parameters related to patient outcome (histological grade, tumour size, LN stage, and vascular invasion), total FOXP3 cell number did not retain significance (Table 3). Likewise, neither intratumoural FOXP3 nor adjacent stromal FOXP3 cell numbers kept significance in the same multivariate model (HR = 0.97, 95% CI = 0.78-1.22, $p= 0.81$; HR = 1.10., 95% CI = 0.90-1.36, $p= 0.35$, respectively).

With regard to DFI, total FOXP3⁺ cell number positivity was associated with decline in DFI however that did not reach a significant level ($P= 0.068$, log rank= 3.33). Neither intratumoural nor distant stromal FOXP3⁺ cell numbers had a significant prognostic effect ($P= 0.06$, log rank= 3.61; $P= 0.58$, log rank= 0.31 respectively). Conversely, patients who were positive for adjacent stromal FOXP3⁺ cell numbers had a significantly reduced DFI ($P= 0.013$, log rank= 6.23).

Relationship between tumour-cell associated FOXP3 and patient outcome

To further assess the role of FOXP3⁺ cell localisation in this patient group, we combined intratumoural FOXP3⁺ cells and adjacent stromal FOXP3⁺ cells as an inclusive tumour cell associated FOXP3⁺ populations. We defined, using X-tile software, the cut-off value for the new variable as ≥ 1 and categorised patients based on this cut-off point into tumour-cell associated FOXP3⁺ cell number positive and negative patients. Tumour-cell associated FOXP3⁺ cell number positivity was accompanied with significantly shorter BCSS ($P= 0.001$, log rank= 10.35, Figure 3), with hazard ratio of 1.38 (95% CI, 1.13 to 1.68). In multivariate analysis, after adjustment for histological grade, tumour size, LN stage, and vascular invasion, tumour-cell associated FOXP3⁺ cell numbers lost significance and was not therefore an independent prognostic factor for patient survival (HR = 1.063, 95% CI = 0.865-1.306, $p= 0.562$). Of note, after exclusion of tumour grade from the multivariate model, tumour-cell associated FOXP3 expression was an independent predictor of patient survival (HR = 1.27, 95% CI = 1.04-1.55, $p= 0.019$). In the same way, patients with tumours positive for tumour-cell associated FOXP3⁺ cells had a significantly shorter DFI ($P= 0.003$, log rank= 9.01). Nevertheless, that was not significant in multivariate analysis (HR = 1.13, 95% CI = 0.95-1.34, $p= 0.17$).

Survival in estrogen receptor-positive patients

Bates et al [22] found that FOXP3⁺ cells were an independent prognostic factor in patients with ER-positive tumours, but not in patients with ER-negative tumours. We therefore investigated the prognostic significance of T regs in both ER-positive and –negative tumours. Within the ER-positive group total FOXP3⁺ cell number positivity was significantly associated with shorter BCSS on univariate analysis ($P= 0.012$, log rank = 6.32, Figure 4A), with hazard ratio of 1.39 (95% CI, 1.07 to 1.81), but was not independent of other prognostic factors in multivariate analysis. Also, tumour-cell associated FOXP3⁺ cell number positivity was significantly associated with worse outcome in ER-positive patients ($P= 0.004$, log rank = 8.48, Figure 4B), with hazard ratio of 1.45 (95% CI, 1.13 to 1.86), however, was no longer significant in multivariate analysis (data not shown). In contrast, FOXP3⁺ cell numbers had no significant influence on BCSS within the ER-negative group on univariate analysis

($P= 0.79$, log rank = 0.07, for total FOXP3 and $P= 0.88$, log rank = 0.02, for tumour-cell associated FOXP3, [Figure 4 C and D](#), respectively).

Prognostic value of FOXP3⁺ cells in different adjuvant therapy groups

The patients were categorised according to the systemic adjuvant treatment received. The numbers of FOXP3⁺ cells were not of prognostic significance in either the group that received chemotherapy (n=223) or the group that received endocrine therapy (n=441) on univariate analysis. FOXP3⁺ cell number positivity was associated with a worse prognosis in univariate analysis of the group not receiving systemic adjuvant treatment (n=491), but not in multivariate analysis. There were too few patients receiving both treatments for analysis. No patients received trastuzumab.

Discussion

Naturally occurring T regulatory cells (T regs) have functionally suppressive actions on other effector T cells and have been characterised as a CD4⁺ CD25⁺ population among CD4⁺ T lymphocytes [12, 45]. Consequently, an inhibitory effect of T regs on anti-tumour immunity has been proposed implicating them in impeding immunosurveillance against cancer [46]. In agreement with this hypothesis, higher numbers of tumour-infiltrating T reg were shown to be associated with poor outcome in pancreatic [25] and hepatocellular [27] carcinomas. In follicular lymphoma, head and neck, and ovarian cancer the number of infiltrating FOXP3⁺ immune cells has shown to be an independent prognostic factor [47-49]. However, studies of other tumours have led to conflicting results. No prognostic effect of T reg was observed in a study of anal carcinomas [50] whereas in colorectal and oesophageal carcinomas, tumour-infiltrating T reg were associated with favourable prognosis [28, 29]. Limited data are available about T reg in breast cancer [22, 30, 51, 52]. Furthermore, no data has been previously published on the localisation patterns of FOXP3⁺ cells in breast cancer. The present study is, to the best of our knowledge, the first report describing the density and localisation of FOXP3⁺ infiltrating cells in relation to breast prognosis in a large series of patients.

It has been recently reported that FOXP3 is the most reliable marker of T regs [15]. Moreover, It was confirmed that the 236A/E7 antibody clone specifically recognises

FOXP3 and not other FOXP proteins [17]. In fact, this clone has been shown the best currently available to study FOXP3 expression by immunohistochemistry [22]. More importantly, although the reactivity spectrum of FOXP3 is composed of T reg and may include small number of CD8⁺ cells, it has been recently shown that it is the most accurate single marker of T regs [53]. In this study, we observed FOXP3⁺ cell infiltration in most of the tumours. Recent studies have reported the expression of FOXP3 mRNA and protein in a limited number of pancreatic carcinoma cells, cell lines [54], and other tumour cell lines of several origins, including breast cancer [55]. In contrast to these studies we did not detect any immunoreactive carcinoma cells in our series. Our data showed significant correlations between T reg infiltration and high tumour grade and ER negativity, consistent with previous reports in breast cancer [22, 52]. In accord with previously published data in breast cancer, we have demonstrated an association between elevated numbers of FOXP3⁺ infiltrating cells and HER2 positivity. Furthermore, we found a similar relationship in basal phenotype tumours. The association between HER-2 and FOXP3⁺ cell infiltration can probably be explained by the association of both with histological grade. Eighty-five percent of HER-2 positive tumours were grade 3 and high FOXP3⁺ cell counts were also associated with higher grade. There is no correlation between HER-2 expression and FOXP3⁺ cell counts in grade 3 carcinomas (data not shown). By contrast although 73% of basal carcinomas were grade 3, FOXP3⁺ cell counts in grade 3 carcinomas were higher in basal tumours. This suggests that factors other than grade are important in the correlation between basal types and FOXP3⁺ cell counts.

We found that higher numbers of FOXP3⁺ infiltrating cells were associated with a worse prognosis on univariate analysis. This association was found for intratumoural FOXP3⁺ cells and for adjacent stromal FOXP3⁺ cells. However, the number of FOXP3⁺ cells was not identified as an independent prognostic factor in multivariate analysis. This is probably because of the correlation with histological grade, which is a well recognised and powerful prognostic factor in breast cancer. In the only previous prognostic study, Bates et al [22], in a much smaller series (222 patients), found that FOXP3⁺ cell infiltration was associated with a shorter relapse free survival ($p= 0.04$) independent of other prognostic factors, but this was not seen for overall survival ($p= 0.07$).

Bates et al [22] found that **FOXP3+ infiltrating cells** were an independent prognostic factor in patients with ER-positive tumours, but not in patients with ER-negative tumours. We found no such distinction, FOXP3⁺ cells were not identified as an independent prognostic factor in either group in our series. The number of events (deaths/relapses) in the ER-positive group was small in the Bates study and their results must be interpreted with caution.

In summary, in the present large study we found that **FOXP3+ infiltrating cells** in human breast cancer are associated with histological grade, but are not an independent prognostic factor. There are numerous other infiltrating inflammatory cell types observed in breast cancer and the association between T regs and other cells is not clear. Our data suggest that predicting clinical outcome is not possible using T regs alone. We are currently investigating other cell types.

Acknowledgement

Sahar M. A. Mahmoud would like to thank the Egyptian Ministry of Higher Education for funding her during this work.

References

1. Callagy, G., E. Cattaneo, Y. Daigo, L. Happerfield, L.G. Bobrow, P.D. Pharoah, et al. (2003) Molecular classification of breast carcinomas using tissue microarrays. *Diagn Mol Pathol* 12(1):27-34.
2. Stewart, T.H. and G.H. Heppner (1997) Immunological enhancement of breast cancer. *Parasitology* 115 Suppl(S141-53).
3. Lee, A.H., C.E. Gillett, K. Ryder, I.S. Fentiman, D.W. Miles, and R.R. Millis (2006) Different patterns of inflammation and prognosis in invasive carcinoma of the breast. *Histopathology* 48(6):692-701.
4. Rilke, F., M.I. Colnaghi, N. Cascinelli, S. Andreola, M.T. Baldini, R. Bufalino, et al. (1991) Prognostic significance of HER-2/neu expression in breast cancer and its relationship to other prognostic factors. *Int J Cancer* 49(1):44-9.
5. Carlomagno, C., F. Perrone, R. Lauria, M. de Laurentiis, C. Gallo, A. Morabito, et al. (1995) Prognostic significance of necrosis, elastosis, fibrosis and inflammatory cell reaction in operable breast cancer. *Oncology* 52(4):272-7.
6. Yu, P. and Y.X. Fu (2006) Tumor-infiltrating T lymphocytes: friends or foes? *Lab Invest* 86(3):231-45.
7. Sprent, J. and C.D. Surh (2003) Knowing one's self: central tolerance revisited. *Nat Immunol* 4(4):303-4.
8. Curiel, T.J. (2007) Tregs and rethinking cancer immunotherapy. *J Clin Invest* 117(5):1167-74.
9. Buckner, J.H. and S.F. Ziegler (2004) Regulating the immune system: the induction of regulatory T cells in the periphery. *Arthritis Res Ther* 6(5):215-22.
10. Kosmaczewska, A., L. Ciszak, S. Potoczek, and I. Frydecka (2008) The significance of Treg cells in defective tumor immunity. *Arch Immunol Ther Exp (Warsz)* 56(3):181-91.
11. Ramsdell, F. (2003) Foxp3 and natural regulatory T cells: key to a cell lineage? *Immunity* 19(2):165-8.
12. Dieckmann, D., H. Plottner, S. Berchtold, T. Berger, and G. Schuler (2001) Ex vivo isolation and characterization of CD4(+)CD25(+) T cells with regulatory properties from human blood. *J Exp Med* 193(11):1303-10.
13. Liyanage, U.K., T.T. Moore, H.G. Joo, Y. Tanaka, V. Herrmann, G. Doherty, et al. (2002) Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma. *J Immunol* 169(5):2756-61.
14. Sakaguchi, S. (2000) Regulatory T cells: key controllers of immunologic self-tolerance. *Cell* 101(5):455-8.
15. Fontenot, J.D., J.P. Rasmussen, L.M. Williams, J.L. Dooley, A.G. Farr, and A.Y. Rudensky (2005) Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity* 22(3):329-41.
16. Hori, S., T. Nomura, and S. Sakaguchi (2003) Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299(5609):1057-61.
17. Roncador, G., P.J. Brown, L. Maestre, S. Hue, J.L. Martinez-Torrecuadrada, K.L. Ling, et al. (2005) Analysis of FOXP3 protein expression in human

- CD4+CD25+ regulatory T cells at the single-cell level. *Eur J Immunol* 35(6):1681-91.
18. Onizuka, S., I. Tawara, J. Shimizu, S. Sakaguchi, T. Fujita, and E. Nakayama (1999) Tumor rejection by in vivo administration of anti-CD25 (interleukin-2 receptor alpha) monoclonal antibody. *Cancer Res* 59(13):3128-33.
 19. Shimizu, J., S. Yamazaki, and S. Sakaguchi (1999) Induction of tumor immunity by removing CD25+CD4+ T cells: a common basis between tumor immunity and autoimmunity. *J Immunol* 163(10):5211-8.
 20. Sutmuller, R.P., L.M. van Duivenvoorde, A. van Elsas, T.N. Schumacher, M.E. Wildenberg, J.P. Allison, et al. (2001) Synergism of cytotoxic T lymphocyte-associated antigen 4 blockade and depletion of CD25(+) regulatory T cells in antitumor therapy reveals alternative pathways for suppression of autoreactive cytotoxic T lymphocyte responses. *J Exp Med* 194(6):823-32.
 21. Tanaka, H., J. Tanaka, J. Kjaergaard, and S. Shu (2002) Depletion of CD4+ CD25+ regulatory cells augments the generation of specific immune T cells in tumor-draining lymph nodes. *J Immunother* 25(3):207-17.
 22. Bates, G.J., S.B. Fox, C. Han, R.D. Leek, J.F. Garcia, A.L. Harris, et al. (2006) Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse. *J Clin Oncol* 24(34):5373-80.
 23. Curiel, T.J., G. Coukos, L. Zou, X. Alvarez, P. Cheng, P. Mottram, et al. (2004) Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med* 10(9):942-9.
 24. Wolf, A.M., D. Wolf, M. Steurer, G. Gastl, E. Gunsilius, and B. Grubeck-Loebenstien (2003) Increase of regulatory T cells in the peripheral blood of cancer patients. *Clin Cancer Res* 9(2):606-12.
 25. Hiraoka, N., K. Onozato, T. Kosuge, and S. Hirohashi (2006) Prevalence of FOXP3+ regulatory T cells increases during the progression of pancreatic ductal adenocarcinoma and its premalignant lesions. *Clin Cancer Res* 12(18):5423-34.
 26. Petersen, R.P., M.J. Campa, J. Sperlazza, D. Conlon, M.B. Joshi, D.H. Harpole, Jr., et al. (2006) Tumor infiltrating Foxp3+ regulatory T-cells are associated with recurrence in pathologic stage I NSCLC patients. *Cancer* 107(12):2866-72.
 27. Gao, Q., S.J. Qiu, J. Fan, J. Zhou, X.Y. Wang, Y.S. Xiao, et al. (2007) Intratumoral balance of regulatory and cytotoxic T cells is associated with prognosis of hepatocellular carcinoma after resection. *J Clin Oncol* 25(18):2586-93.
 28. Yoshioka, T., M. Miyamoto, Y. Cho, K. Ishikawa, T. Tsuchikawa, M. Kadoya, et al. (2008) Infiltrating regulatory T cell numbers is not a factor to predict patient's survival in oesophageal squamous cell carcinoma. *Br J Cancer* 98(7):1258-63.
 29. Salama, P., M. Phillips, F. Grieu, M. Morris, N. Zeps, D. Joseph, et al. (2009) Tumor-infiltrating FOXP3+ T regulatory cells show strong prognostic significance in colorectal cancer. *J Clin Oncol* 27(2):186-92.
 30. Ladoire, S., L. Arnould, L. Apetoh, B. Coudert, F. Martin, B. Chauffert, et al. (2008) Pathologic complete response to neoadjuvant chemotherapy of breast carcinoma is associated with the disappearance of tumor-infiltrating foxp3+ regulatory T cells. *Clin Cancer Res* 14(8):2413-20.

31. Abd El-Rehim, D.M., G. Ball, S.E. Pinder, E. Rakha, C. Paish, J.F. Robertson, et al. (2005) High-throughput protein expression analysis using tissue microarray technology of a large well-characterised series identifies biologically distinct classes of breast cancer confirming recent cDNA expression analyses. *Int J Cancer* 116(3):340-50.
32. Abd El-Rehim, D.M., S.E. Pinder, C.E. Paish, J. Bell, R.W. Blamey, J.F. Robertson, et al. (2004) Expression of luminal and basal cytokeratins in human breast carcinoma. *J Pathol* 203(2):661-71.
33. Rakha, E.A., D.A. El-Rehim, C. Paish, A.R. Green, A.H. Lee, J.F. Robertson, et al. (2006) Basal phenotype identifies a poor prognostic subgroup of breast cancer of clinical importance. *Eur J Cancer* 42(18):3149-56.
34. Zhang, H., E.A. Rakha, G.R. Ball, I. Spiteri, M. Aleskandarany, E.C. Paish, et al. (2009) The proteins FABP7 and OATP2 are associated with the basal phenotype and patient outcome in human breast cancer. *Breast Cancer Res Treat* *In Press*.
35. Elston, C.W. and I.O. Ellis (1991) Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. *Histopathology* 19(5):403-10.
36. Ellis, I.O., M. Galea, N. Broughton, A. Locker, R.W. Blamey, and C.W. Elston (1992) Pathological prognostic factors in breast cancer. II. Histological type. Relationship with survival in a large study with long-term follow-up. *Histopathology* 20(6):479-89.
37. Pinder, S.E., I.O. Ellis, M. Galea, S. O'Rourke, R.W. Blamey, and C.W. Elston (1994) Pathological prognostic factors in breast cancer. III. Vascular invasion: relationship with recurrence and survival in a large study with long-term follow-up. *Histopathology* 24(1):41-7.
38. Galea, M.H., R.W. Blamey, C.E. Elston, and I.O. Ellis (1992) The Nottingham Prognostic Index in primary breast cancer. *Breast Cancer Res Treat* 22(3):207-19.
39. Green, A.R., C. Burney, C.J. Granger, E.C. Paish, S. El-Sheikh, E.A. Rakha, et al. (2008) The prognostic significance of steroid receptor co-regulators in breast cancer: co-repressor NCOR2/SMRT is an independent indicator of poor outcome. *Breast Cancer Res Treat* 110(3):427-37.
40. McShane, L.M., D.G. Altman, W. Sauerbrei, S.E. Taube, M. Gion, and G.M. Clark (2005) Reporting recommendations for tumor marker prognostic studies. *J Clin Oncol* 23(36):9067-72.
41. Rakha, E.A., S.E. El-Sheikh, M.A. Kandil, M.E. El-Sayed, A.R. Green, and I.O. Ellis (2008) Expression of BRCA1 protein in breast cancer and its prognostic significance. *Hum Pathol* 39(6):857-65.
42. Camp, R.L., M. Dolled-Filhart, and D.L. Rimm (2004) X-tile: a new bioinformatics tool for biomarker assessment and outcome-based cut-point optimization. *Clin Cancer Res* 10(21):7252-9.
43. Dolled-Filhart, M., A. McCabe, J. Giltane, M. Cregger, R.L. Camp, and D.L. Rimm (2006) Quantitative in situ analysis of beta-catenin expression in breast cancer shows decreased expression is associated with poor outcome. *Cancer Res* 66(10):5487-94.
44. Rakha, E.A., M.E. El-Sayed, A.R. Green, A.H. Lee, J.F. Robertson, and I.O. Ellis (2007) Prognostic markers in triple-negative breast cancer. *Cancer* 109(1):25-32.

45. Sakaguchi, S., N. Sakaguchi, M. Asano, M. Itoh, and M. Toda (1995) Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 155(3):1151-64.
46. Zou, W. (2006) Regulatory T cells, tumour immunity and immunotherapy. *Nat Rev Immunol* 6(4):295-307.
47. Badoual, C., S. Hans, J. Rodriguez, S. Peyrard, C. Klein, H. Agueznay Nel, et al. (2006) Prognostic value of tumor-infiltrating CD4+ T-cell subpopulations in head and neck cancers. *Clin Cancer Res* 12(2):465-72.
48. Carreras, J., A. Lopez-Guillermo, B.C. Fox, L. Colomo, A. Martinez, G. Roncador, et al. (2006) High numbers of tumor-infiltrating FOXP3-positive regulatory T cells are associated with improved overall survival in follicular lymphoma. *Blood* 108(9):2957-64.
49. Wolf, D., A.M. Wolf, H. Rumpold, H. Fiegl, A.G. Zeimet, E. Muller-Holzner, et al. (2005) The expression of the regulatory T cell-specific forkhead box transcription factor FoxP3 is associated with poor prognosis in ovarian cancer. *Clin Cancer Res* 11(23):8326-31.
50. Grabenbauer, G.G., G. Lahmer, L. Distel, and G. Niedobitek (2006) Tumor-infiltrating cytotoxic T cells but not regulatory T cells predict outcome in anal squamous cell carcinoma. *Clin Cancer Res* 12(11 Pt 1):3355-60.
51. Bohling, S.D. and K.H. Allison (2008) Immunosuppressive regulatory T cells are associated with aggressive breast cancer phenotypes: a potential therapeutic target. *Mod Pathol* 21(12):1527-32.
52. Ghebeh, H., E. Barhoush, A. Tulbah, N. Elkum, T. Al-Tweigeri, and S. Dermime (2008) FOXP3+ Tregs and B7-H1+/PD-1+ T lymphocytes co-infiltrate the tumor tissues of high-risk breast cancer patients: Implication for immunotherapy. *BMC Cancer* 8(57).
53. Ahmadzadeh, M., A. Felipe-Silva, B. Heemskerk, D.J. Powell, Jr., J.R. Wunderlich, M.J. Merino, et al. (2008) FOXP3 expression accurately defines the population of intratumoral regulatory T cells that selectively accumulate in metastatic melanoma lesions. *Blood* 112(13):4953-60.
54. Hinz, S., L. Pagerols-Raluy, H.H. Oberg, O. Ammerpohl, S. Grussel, B. Sipos, et al. (2007) Foxp3 expression in pancreatic carcinoma cells as a novel mechanism of immune evasion in cancer. *Cancer Res* 67(17):8344-50.
55. Karanikas, V., M. Speletas, M. Zamanakou, F. Kalala, G. Loules, T. Kerenidi, et al. (2008) Foxp3 expression in human cancer cells. *J Transl Med* 6(19).

Table 1: Correlation between presence of FOXP3⁺ cells and clinicopathological and some immunohistochemical markers in invasive breast cancer.

Pathological/clinical parameter	FOXP3 positive n (%)	FOXP3 negative n (%)	Significance
Age, years			
<40	81 (72)	31 (28)	$r_s = -0.11$ $p < 0.001$
40-50	283 (68)	134 (32)	
51-60	292 (61)	188 (39)	
>60	268 (62)	167 (38)	
Tumour size			
≤2cm	563 (62)	341 (38)	$r_s = 0.08$ $p = 0.002$
>2cm	355 (67)	177 (33)	
Nodal status			
1 (negative)	562 (63)	333 (37)	$r_s = 0.06$ $p = 0.015$
2 (1-3 LN)	268 (64)	151 (36)	
3 (>3 LN)	90 (73)	34 (27)	
Grade			
1	115 (47)	131 (53)	$r_s = 0.37$ $p < 0.001$
2	241 (51)	233 (49)	
3	562 (79)	154 (22)	
Vascular invasion			
Absent	612 (63)	366 (37)	$P = 0.106^*$
Present	300 (67)	148 (33)	
Distant metastases			
Absent	617 (62)	372 (38)	$P = 0.121^*$
Present	298 (67)	145 (33)	
Any recurrence			
Absent	514 (62)	311 (38)	$P = 0.516^*$
Present	387 (67)	195 (33)	
ER alpha status			
Negative	297 (82)	67 (18)	$P < 0.001^*$
Positive	573 (58)	409 (42)	
PgR status			
Negative	401 (74)	138 (26)	$P < 0.001^*$
Positive	461 (58)	333 (42)	
HER 2 status			
Negative	731 (61)	473 (39)	$P < 0.001^*$
Positive	151 (86)	24 (14)	
Basal phenotype			
Negative	648 (61)	412 (39)	$P < 0.001^*$
Positive	221 (78)	63 (22)	

Significant p -values are in bold; * Mann-Whitney U test.
FOXP3 positive is ≥ 3 cells/core.

Table 2: Expression of FOXP3⁺ cells in the three compartments per tumour core (0.28 mm² s.a.) in breast cancer.

<i>Location</i>	<i>Mean</i>	<i>Median</i>	<i>Interquartile range</i>
Total No. of FOXP3+ cells *	12.7	6	1 – 17
Intratumoural FOXP3+ cells *	2.3	0	0 – 1
Distant stromal FOXP3+ cells *	8.8	3	0 – 11
Adjacent stromal FOXP3+ cells *	1.6	0	0 – 1

* Number of patients= 1445.

Table 3: Cox proportional hazard model including tumour size, LN stage, grade, vascular invasion, and total number of FOXP3 in whole series.

Variable	Hazard ratio	95% CI	<i>P</i>-value
Tumour size/ cm	1.303	1.177-1.441	< 0.001
LN stage	1.579	1.359-1.835	< 0.001
Grade	1.801	1.512-2.146	< 0.001
Vascular invasion	1.578	1.271-1.959	< 0.001
Total no. of FOXP3	0.974	0.776-1.221	0.817

Abbreviation: CI, confidence interval.

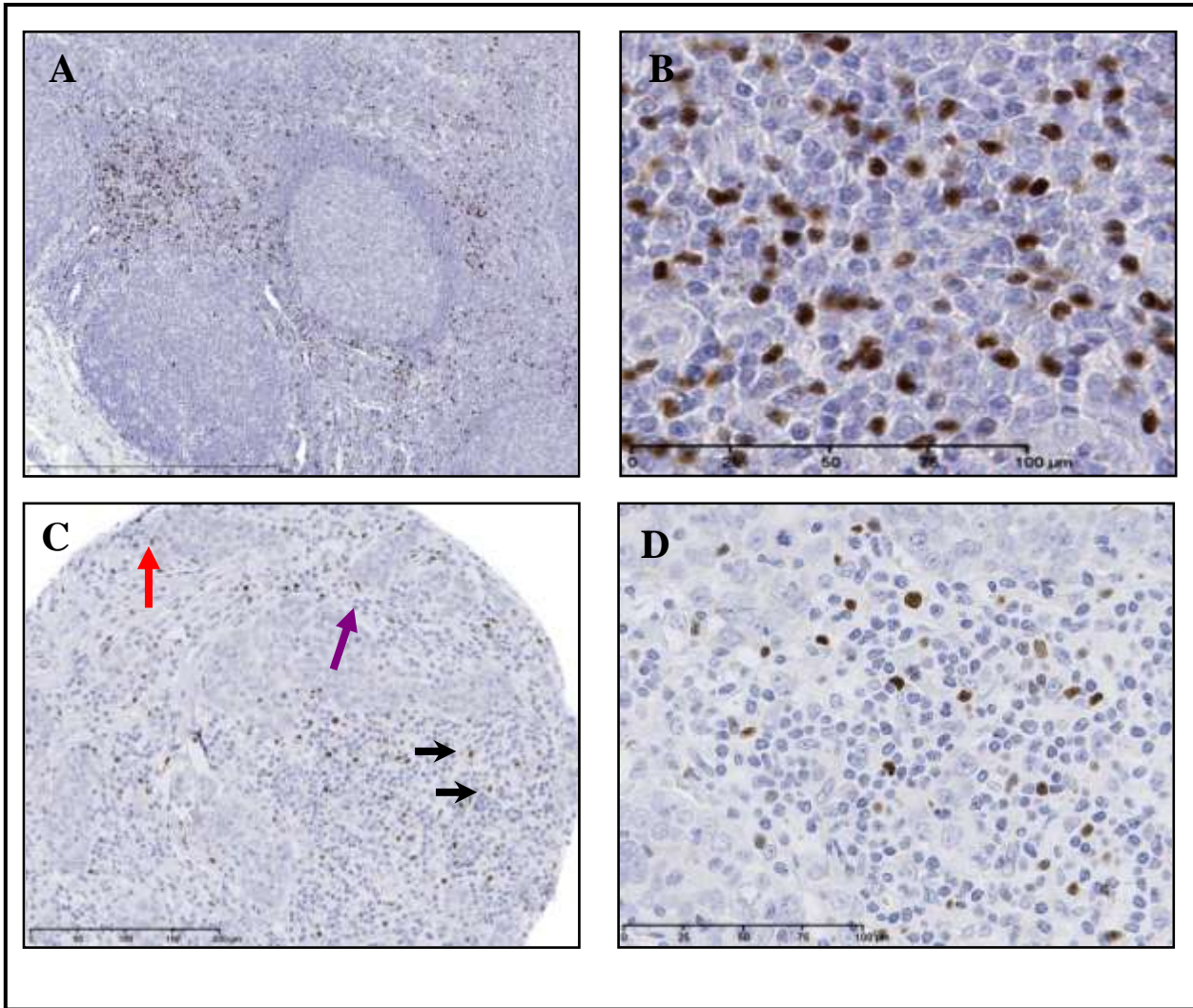


Figure 1

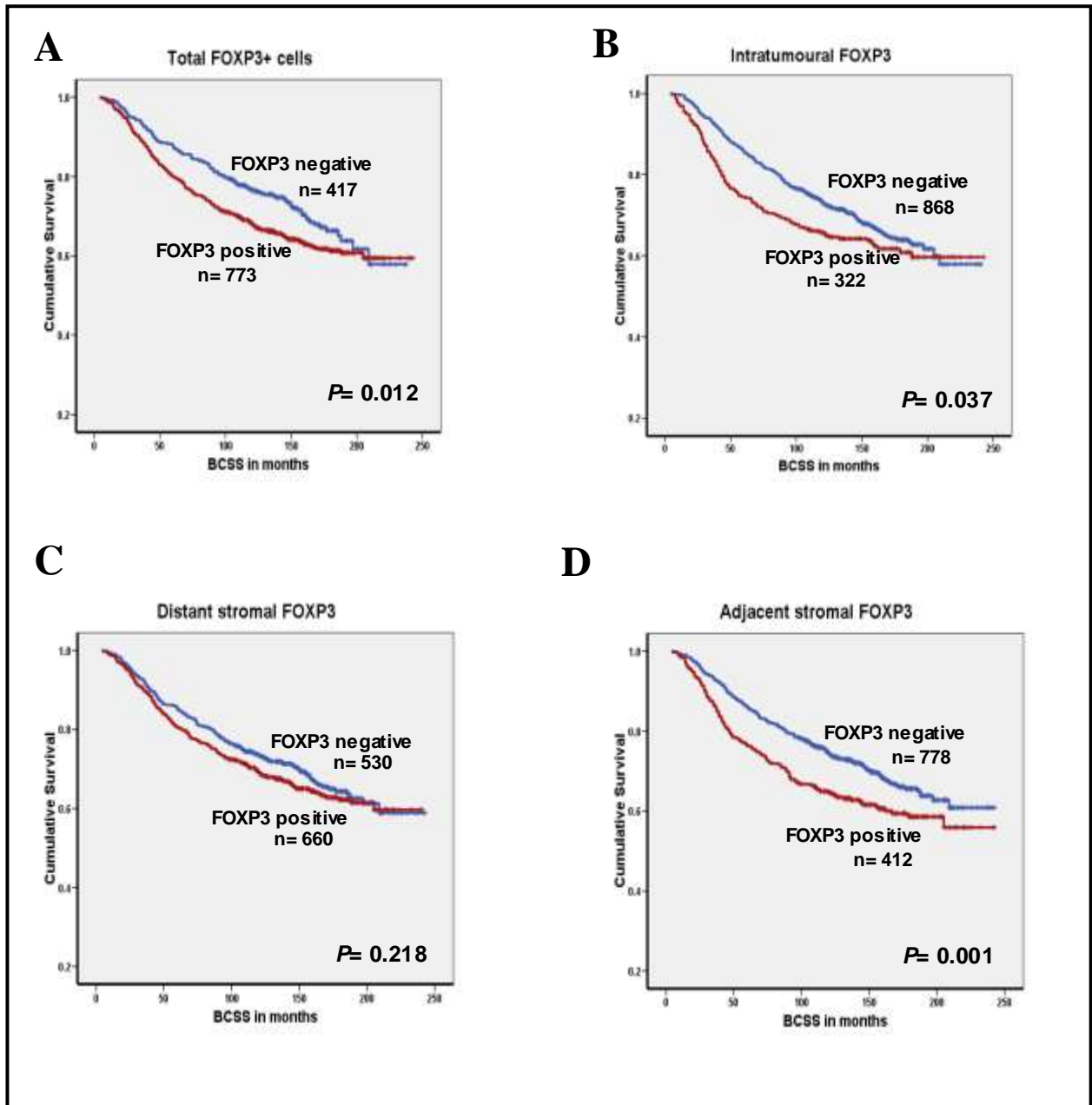


Figure 2

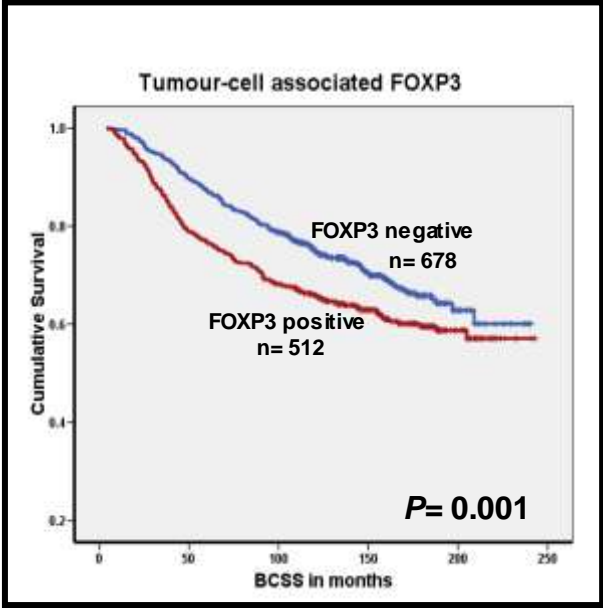


Figure 3

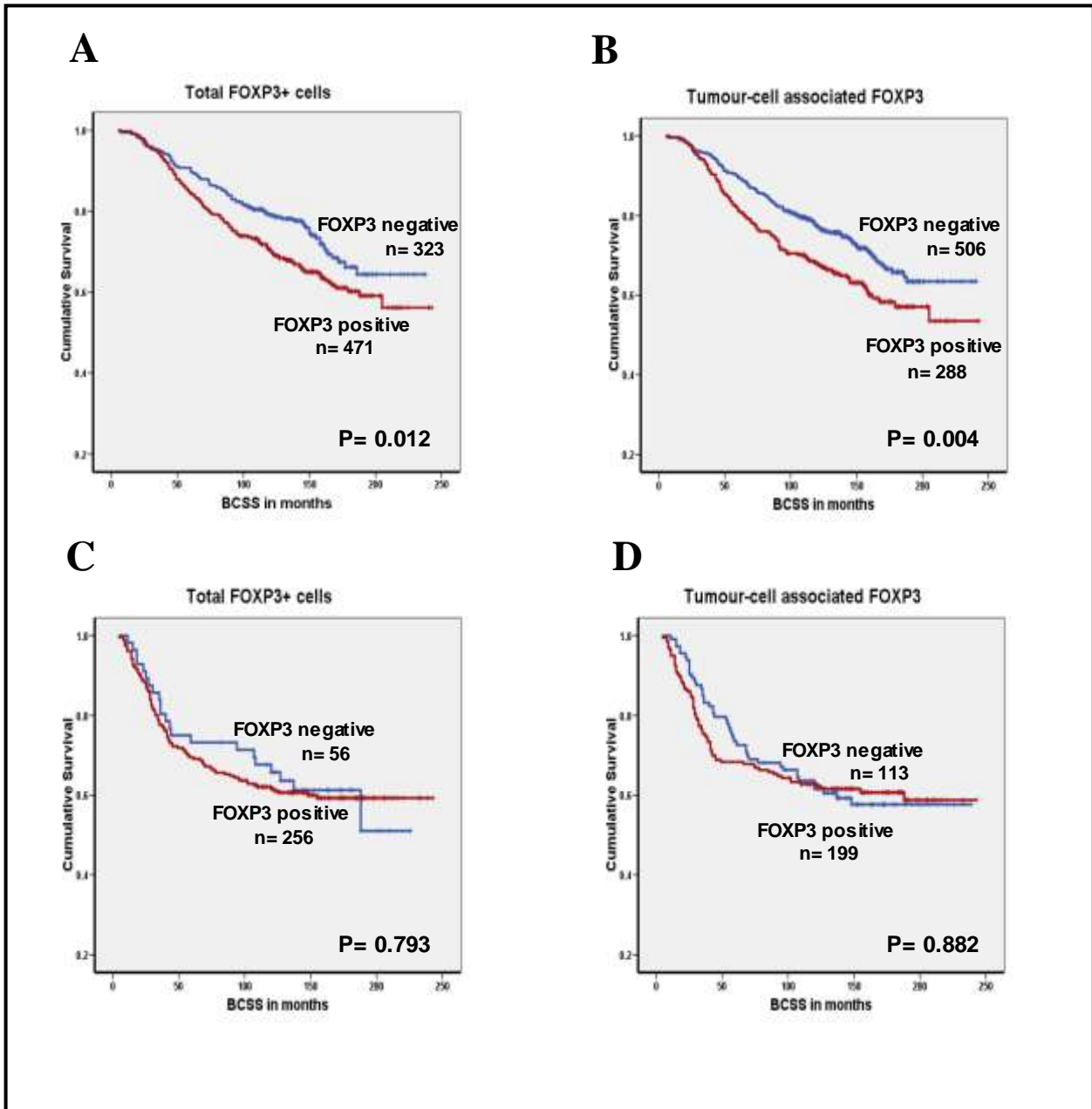


Figure 4:

Figure 1: Immunohistochemical detection of FOXP3⁺ cells. **A** and **B**, normal human tonsil control was immunostained with FOXP3 and counterstained with haematoxylin. FOXP3⁺ cells were distributed mainly in parafollicular area, and sometimes within the follicles, **A** x 200 and **B** x 400. **C** and **D**, representative tumour tissue infiltrated by FOXP3⁺ cells depicting the localisation patterns, red arrow points to the intratumoural FOXP3⁺ cells, black arrows direct distant stromal FOXP3⁺ cells, while the violet arrow indicates the adjacent stroma FOXP3⁺ cells, **C** x 200 and **D** x 400.

Figure 2: Kaplan-Meier curves for breast cancer specific survival (BCSS) stratified by the predefined cut-off points (for the whole series). (A) Survival curve for total FOXP3. (B) Survival curve for intratumoural FOXP3. (C) Survival curve for distant stromal FOXP3. (D) Survival curve for adjacent stromal FOXP3.

Figure 3: Kaplan-Meier curve showing significant association between positive tumour-cell associated FOXP3 expression and shorter breast cancer specific survival (BCSS). Tumour-cell associated FOXP3 consists of the sum of intratumoural FOXP3⁺ cells and adjacent stromal FOXP3⁺ cells.

Figure 4: Kaplan-Meier curves for breast cancer specific survival (BCSS) of FOXP3⁺ cell expression in ER-positive tumours (A and B) and ER-negative tumours (C and D). (A) Kaplan-Meier blot shows the significance of total number of FOXP3⁺ cell expression in ER-positive patient outcome. (B) Kaplan-Meier blot depicts the significance of tumour-cell associated FOXP3⁺ cell expression in ER-positive patient outcome. Neither total number nor tumour-cell associated FOXP3⁺ cell expression had an impact on BCSS in ER-negative group (C and D respectively).