Predictive value of improvement in the immune tumour microenvironment in patients with breast cancer treated with neoadjuvant chemotherapy

Wataru Goto,1 Shinichiro Kashiwagi,1 Yuka Asano,1 Koji Takada,1 Katsuyuki Takahashi,2 Takaharu Hatano,3 Tsutomu Takashima,1 Shuhei Tomita,2 Hisashi Motomura,3 Masahiko Ohsawa,4 Kosei Hirakawa,1 Masaichi Ohira1

ABSTRACT
Background Tumour-infiltrating lymphocytes (TILs) can be used to monitor the immune tumour microenvironment (iTME) and predict treatment response and outcome in breast cancer. We evaluated the prognostic significance of the levels of CD8+ TILs and forkhead box protein (FOXP3)-positive TILs before and after neoadjuvant chemotherapy (NAC).

Patients and methods We examined 136 patients with breast cancer treated with NAC. The number of CD8+ TILs and FOXP3+ TILs in biopsy specimens and residual tumours was evaluated by immunohistochemistry.

Results Patients with a high rate of change in the CD8/FOXP3 ratio (CFR) had significantly better recurrence-free survival (RFS) (p<0.001, log-rank). In multivariate analysis, the rates of change in the CD8+ TIL levels and the CFR were independent predictors for RFS (HR=2.304, p=0.036 and HR=4.663, p=0.001). In patients with triple-negative and hormone receptor-positive breast cancer, the rate of change in the CFR had significantly better recurrence-free survival (RFS) (HR=13.021, p=0.002 and HR=4.377, p=0.003).

Conclusion Improvement in the iTME following NAC is correlated with good outcome. The rate of change in the CFR may be a useful biomarker to predict prognosis of patients treated with NAC.

INTRODUCTION
Neoadjuvant chemotherapy (NAC) is the gold standard of care for breast cancer and increases the options for breast-conserving surgery.1-3 Pathological complete response (pCR) after NAC is currently acknowledged as an indicator of good outcome, especially in triple-negative breast cancer (TNBC) and human epidermal growth factor receptor 2 (HER2)-enriched breast cancer (HER2BC).4 One previous study indicated that residual cancer cells after NAC may be more aggressive or have enhanced metastatic potential.5 However, some patients who fail to achieve pCR after NAC have a relatively good outcome. Therefore, novel prognostic markers in residual tumours are needed to identify high-risk patients.

What is already known about this subject?
► Recently, the importance of regulating and improving the immune tumour microenvironment (iTME) has been reported to play an important role in predicting outcomes.
► Tumour-infiltrating lymphocytes (TILs) can be used to monitor the iTME and predict treatment response and outcome in breast cancer.
► CD8/FOXP3 ratio (CFR) in biopsy specimens before neoadjuvant chemotherapy (NAC) is a useful biomarker to predict treatment response to chemotherapy.

What does this study add?
► The predictive value of changes in lymphocytic sub-populations after NAC in all breast cancer subtypes has not been discussed sufficiently.
► The present study investigated the clinical significance and value of changes in the levels of CD8+ TILs and FOXP3+ TILs and the CFR before and after NAC in all breast cancer subtypes.
► To our knowledge, this is the first study to demonstrate the prognostic role of changes in CD8+ TIL levels, FOXP3+ TIL levels and the CFR in patients failing to achieve pathological complete response following NAC in all breast cancer subtypes.

How might this impact on clinical practice?
► These results suggest that by further evaluating the changes in other TILs, such as programmed death-1-positive TILs, along with those in CD8+ TILs and FOXP3+ TILs in patients treated with NAC, more accurate identification of patient-specific immune mechanisms and prediction of prognosis may be possible.
► Improvement in the iTME following NAC is correlated with good outcome.
► The rate of change in the CFR may be a useful biomarker to predict prognosis of patients treated with NAC.

Recently, the importance of regulating and improving the immune tumour microenvironment has been reported to play an
important role in predicting outcomes. Tumour-infiltrating lymphocytes (TILs) can be used to monitor the tumour microenvironment and are important in predicting treatment efficacy and clinical outcomes in many types of cancer, including breast cancer. Various cells of the immune system can play varying roles in tumour progression; for instance, cytotoxic T cells (CD8+ T cells), natural killer cells, dendritic cells and macrophages are associated with improved clinical outcomes, whereas regulatory T (Treg) cells and myeloid-derived suppressor cells suppress antitumour immunity. Specific TIL subsets, such as CD3+, CD8+ and forhead box protein 3 (FOXP3)-positive TILs, have been reported to be clinically significant and reliable in predicting treatment response. In addition, since Treg cells suppress the induction of cytotoxic T cells in response to cancer cells, the CD8+/FOXP3 ratio (CFR) has been reported to be associated with high pCR rates. We have also suggested that the CFR in biopsy specimens before NAC is a useful biomarker to predict treatment response to chemotherapy in TNBC and HER2BC.

Chemotherapy enhances the immune activity or the reversal of immunosuppression. Some studies revealed that changes in the levels of CD8+ or FOXP3+ TILs induced by chemotherapy can be used as a prognostic marker in aggressive breast cancer subtypes, such as TNBC. However, the predictive value of changes in lymphocytic subpopulations after NAC in all breast cancer subtypes has not been discussed sufficiently.

The present study investigated the clinical significance and value of changes in the levels of CD8+ TILs and FOXP3+ TILs and the CFR before and after NAC in all breast cancer subtypes. To our knowledge, this is the first study to demonstrate the prognostic role of changes in CD8+ TIL levels, FOXP3+ TIL levels and the CFR in patients failing to achieve pCR following NAC in all breast cancer subtypes.

**PATIENTS AND METHODS**

**Ethics**

This study was conducted at the Osaka City University Graduate School of Medicine, Osaka, Japan according to the Reporting Recommendations for Tumour Marker Prognostic Studies guidelines and following a retrospectively written research proposal, pathological evaluation and statistical plan. This study conformed to the provisions of the Declaration of Helsinki. All patients were informed of the investigational nature of this study and provided their written informed consent.

**Patient background**

A total of 214 patients with resectable, early-stage, primary infiltrating ductal breast cancer who were treated with NAC between 2007 and 2015 were included. Tumour stage and T and N factors were stratified based on the TNM Classification of Malignant Tumours, The Union for International Cancer Control Seventh Edition.
the stroma surrounding the stained cancer cells in each FOV was measured microscopically at 400× magnification (figure 1). The mean number of CD8+ or FOXP3+ lymphocytes in each FOV was counted. The CFR was defined as the number of CD8+ TILs divided by the number of FOXP3+ TILs.

Statistical analyses
Statistical analysis was performed using the JMP13 software programme (SAS Institute). The associations between levels of CD8+ TILs and FOXP3+ TILs and clinicopathological variables were analysed using $X^2$ tests or Fisher’s exact tests, as appropriate. OS and RFS were estimated using the Kaplan-Meier method and compared using the log-rank test. Univariate and multivariate HRs were computed for the study parameters with 95% CIs using a Cox proportional hazards model and used in a backward stepwise method for variable selection in multivariate analyses. A p value <0.05 was considered significant.

Results
CD8+ TILs and FOXP3+ TILs before NAC and outcome
Among 214 patients, 78 (36.4%) patients achieved pCR. Therefore, 136 (63.6%) patients with residual tumour after NAC were included in the study. Except for one patient who had insufficient tissue for immunohistochemical staining, the baseline levels of CD8+ TILs and FOXP3+ TILs and the CFR before NAC are presented in online supplementary table 1. Unstained TILs (%) ranged from 0 to 90 (mean, 16; median, 18; SD, 5). CD8+ TILs ranged from 0 to 138 (mean, 38; median, 36; SD, 22). FOXP3+ TILs ranged from 0 to 55 (mean, 14; median, 17; SD, 11). In each breast cancer subtype, the proportion of unstained TILs tended to be higher in patients with TNBC than in other breast cancer subtypes (p=0.055), but there was no relationship between CD8+ TILs and FOXP3+ TILs and breast cancer subtypes (p=0.838 and p=0.570, respectively). The cut-off levels for high or low infiltration were based on the mean number of infiltrating cells per field as follows: CD8+ TILs, 38; FOXP3+ TILs, 14 and CFR, 3.1. Ki-67 was significantly higher in patients with low levels of FOXP3+ TILs (p=0.016). The proportion of unstained TILs was significantly higher in patients with high levels of CD8+ TILs (p=0.015), low levels of FOXP3+ TILs (p=0.003) and high levels of CFR (p<0.001). There were significant positive correlations among the levels of CD8+ TILs and FOXP3+ TILs and the CFR (CD8+ vs FOXP3+: p<0.001, CD8+ vs CFR: p<0.001, FOXP3+ vs CFR: p<0.001). No correlations between any other tested clinicopathological parameter and the levels of CD8+ TILs and FOXP3+ TILs and the CFR were found. RFS was significantly longer in the high CFR group than in the low CFR group (p=0.013),
but OS was not significantly different (p=0.054, log-rank) (online supplementary figure 1).

**CD8+ TILs and FOXP3+ TILs after NAC and outcome**

Except for six patients who had either insufficient tissue or no available tissue for immunohistochemical staining, the levels of CD8+ TILs and FOXP3+ TILs and the CFR after NAC are presented in online supplementary table 2. Unstained TILs (%) ranged from 0 to 94 (mean, 23; median, 29; SD, 7). CD8+ TILs ranged from 0 to 141 (mean, 42; median, 53; SD, 23). FOXP3+ TILs ranged from 0 to 67 (mean, 7; median, 6; SD, 6). In each breast cancer subtype, there was no relationship between unstained TILs, CD8+ TILs, FOXP3+ TILs and breast cancer subtypes (p=0.168, p=0.772 and p=0.579, respectively). The cut-off levels for high or low infiltration of the residual tumour after NAC were as follows: CD8+ TILs, FOXP3+ TILs, and CFR, 7.0. Younger patients (≤56 years) had significantly higher levels of CD8+ (p=0.035) and FOXP3+ (p=0.024) TILs than older patients (>56 years). The partial response (PR) rate was significantly higher in the high CFR group than in the low CFR group (p=0.012). The proportion of unstained TILs was significantly higher in patients with high levels of CD8+ TILs (p=0.001) and high levels of FOXP3+ TILs (p<0.001), but there was no significant correlation between unstained TILs and the CFR (p=0.364). The high CD8+ TILs group had significantly better RFS and OS than the low CD8+ TILs group (p=0.001, p=0.017, log-rank, respectively). The low FOXP3+ TILs group had significantly better RFS and OS than the high FOXP3+ TILs group (p=0.006, p=0.005, log-rank, respectively). A high CFR was also significantly correlated with better RFS and OS (p<0.001, both end points) (online supplementary figure 2).

**Changes in CD8+ TILs and FOXP3+ TILs before and after NAC and their association with prognosis**

The mean rates of change in CD8+ TIL levels, FOXP3+ TIL levels and the CFR before and after NAC were as follows: CD8+ TILs, 1.2; FOXP3+ TILs, 0.5 and CFR, 2.3. Of 129 patients, 56 (43.4%) had a high rate of change in CD8+ TILs, 82 (63.6%) had a low rate of change in FOXP3+ TILs and 68 (52.7%) had a high rate of change in the CFR. In addition, 62 (48.1%) patients had a high rate of change in unstained TILs. Younger patients (≤56 years) were significantly more likely to have a high rate of change in CD8+ TIL levels than older patients (>56 years) (p=0.013). Patients with TNBC had a significantly higher rate of change in FOXP3+ TIL levels than patients with other subtypes (p=0.014) (table 1). Patients with a high rate of change in unstained TILs were significantly higher in patients with a high rate of change in CD8+ TIL levels (p<0.001) and a high rate of change in FOXP3+ TILs (p=0.003), but there was no significant correlation between the rate of change in unstained TILs and the CFR (p=0.479). Patients with a high rate of change in CD8+ TIL levels had significantly better RFS and OS than those with a low rate of change (p=0.005, p=0.032, log-rank, respectively). Patients with a low rate of change in FOXP3+ TIL levels had significantly better RFS and OS than those with a high rate of change (p=0.044, p=0.025, log-rank, respectively). Patients with a high rate of change in the CFR also had significantly better RFS and OS than those with a low rate of change (p<0.001, log-rank, both end points) (figure 2).

In univariate analysis, pathological response (HR=6.33, 95% CI 2.893 to 13.13, p<0.001), the rate of change in CD8+ TIL levels (HR=3.114, 95% CI 1.430 to 7.773, p=0.003) and the CFR (HR=5.612, 95% CI 2.581 to 14.001, p<0.001) were found to be favourable prognostic factors. The rate of change in unstained TILs was not a significant prognostic factor (HR=1.276, 95% CI 0.656 to 2.536, p=0.473). Multivariate analysis showed that PR (HR=5.260, 95% CI 2.373 to 11.145, p<0.001), a high rate of change in CD8+ TIL levels (HR=2.304, 95% CI 1.052 to 5.776, p=0.056) and the CFR (HR=4.663, 95% CI 2.133 to 11.682, p<0.001) were independent good prognostic factors (table 1). With respect to OS, in univariate analysis, intrinsic subtype (HR=2.933, 95% CI 1.186 to 7.586, p=0.020), pathological response (HR=13.771, 95% CI 5.397 to 35.763, p<0.001), the rate of change in CD8+ TIL levels (HR=3.103, 95% CI 1.147 to 10.797, p=0.024), FOXP3+ TILs (HR=2.586, 95% CI 1.086 to 6.230, p=0.032) and the CFR (HR=8.279, 95% CI 2.800 to 35.365, p<0.001) were good prognostic factors. The rate of change in unstained TILs was not a significant prognostic factor (HR=1.316, 95% CI 0.568 to 3.191, p=0.524). Multivariate analysis revealed that TNBC subtype (HR=4.024, 95% CI 1.395 to 12.522, p=0.010), PR (HR=15.564, 95% CI 5.368 to 48.819, p<0.001) and a high rate of change in the CFR (HR=7.1877, 95% CI 1.921 to 34.687, p=0.003) had strong prognostic significance (table 2).

**Prognostic value of changes in CD8+ TILs and FOXP3+ TILs before and after NAC in breast cancer subtypes**

Additionally, we investigated the prognostic value of changes in the levels of CD8+ TILs and FOXP3+ TILs in each breast cancer subtype. Of the 39 patients with TNBC, pathological response (HR=25.642, 95% CI 6.873 to 123.724, p<0.001) and the rate of change in the CFR (HR=11.420, 95% CI 2.215 to 208.742 p=0.002) were significantly correlated with RFS in univariate analysis. Multivariate analysis showed that PR (HR=34.290, 95% CI 7.314 to 265.738, p<0.001) and the rate of change in the CFR (HR=13.021, 95% CI 2.241 to 258.136, p=0.002) were independent prognostic factors for survival. Moreover, pathological response (HR=11.812, 95% CI 0.043 to 23.141, p<0.001) and the rate of change in the CFR (HR=9.847, 95% CI 1.883 to 180.764, p=0.004) were significantly correlated with OS in univariate analysis. Multivariate analysis showed that PR (HR=11.243, 95% CI 20.791 to 20.892, p<0.001) and a high rate of change in the CFR (HR=8.346, 95% CI 1.538 to 155.128, p=0.010) were independent prognostic factors for survival. Of the 77 patients with hormone receptor-positive breast cancer (HRBC), the rate of change in CD8+ TILs...
levels (HR=3.167, 95% CI 1.134 to 11.196, p=0.027) and the CFR (HR=4.740, 95% CI 1.779 to 14.833, p=0.002) was significantly correlated with RFS in univariate analysis. Multivariate analysis revealed only a high rate of change in the CFR (HR=4.377, 95% CI 1.641 to 13.712, p=0.003) as an independent prognostic factor for recurrence. Additionally, lymph node status before NAC (HR=7.640, 95% CI 1.184 to 16.852, p=0.035) and the rate of change in the CFR (HR=11.081, 95% CI 1.969 to 207.323, p=0.004) were significantly correlated with OS in univariate analysis. Multivariate analysis showed that lymph node metastasis before NAC (HR=6.548, 95% CI 1.037 to 1.083, p=0.047) and a high rate of change in the CFR (HR=10.333, 95% CI 1.832 to 193.336, p=0.006) were independent prognostic factors for survival (tables 2 and 3). Since the number of patients with HER2BC was small (n=20), it could not be analysed.

**DISCUSSION**

In the present study, the proportion of unstained TILs, the number of CD8+ TILs and the CFR increased, and
the number of FOXP3+ TILs decreased in breast tumours after NAC. Those results indicated that a regimen of FEC followed by paclitaxel±trastuzumab enhances antitumour immunity and reversal of immunescape in cancer cells. This improvement in the immune microenvironment following NAC was significantly correlated with prognosis.

TILs are mononuclear immune cells in the tumour microenvironment. Infiltration of TILs before NAC is a useful biomarker to predict treatment response in patients with TNBC and HER2BC, two subtypes of highly malignant breast cancer.11 30–33 In these subtypes, high TILs group before NAC is significantly associated with higher pCR rate, good prognostic factor. In our study, investigating patients with non-pCR after NAC, the proportion of unstained TILs alone was not a useful predictor of outcome (online supplementary figure 3). Therefore, more detailed evaluation of TILs becomes necessary. In a previous study, Ladoire et al examined changes in the levels of CD8+ TILs and FOXP3+ TILs after NAC in 56 patients with breast cancer and reported that a high rate of change in the CFR was associated with pCR.20 Miyashita et al also analysed 78 patients with TNBC and reported that high rates of change in the level of CD8+ TILs and the CFR were significantly correlated with good RFS and OS.15 However, there have been few studies stratifying by intrinsic subtype of breast cancer. Our study is the first to indicate that a high rate of change in the CFR is an independent prognostic factor for good outcome in patients with TNBC and HRBC who do not achieve pCR after NAC.

Figure 2  Analysis of the rate of changes in CD8+ TILs, FOXP3+ TILs and the CFR and RFS and OS in patients with all breast cancer subtypes. Patients with a high rate of change in CD8+ TIL levels had significantly better RFS (A) and OS (B) than those with a low rate of change (p=0.005, p=0.032, log-rank, respectively). Patients with a low rate of change in FOXP3+ TIL levels had significantly better RFS (C) and OS (D) than those with a high rate of change (p=0.044, p=0.025, log-rank, respectively). Patients with a high rate of change in the CFR also had significantly better RFS (E) and OS (F) than those with a low rate of change (p<0.001, log-rank, both end points). Goto et al.17 CFR, CD8/FOXP3 ratio; FOXP3, forkhead box protein 3; OS, overall survival; RFS, recurrence-free survival; TIL, tumour-infiltrating lymphocyte.
TILs are mononuclear immune cells in the tumour microenvironment. Infiltration of TILs is a useful biomarker to predict treatment response in patients with TNBC and HER2BC, two subtypes of highly malignant breast cancer.11 30–33 These studies suggest that TNBC and HER2BC have high immunoactivity. However, based on the detailed subclassification of TILs as CD8+ TILs or FOXP3+ TILs, studies evaluating the prognostic significance of CD8+ TILs or FOXP3+ TILs or the CFR in the intrinsic molecular subtypes of breast cancer have shown conflicting results.15 17 34–40 One possible explanation consistent with these discrepant findings is that HRBC is also considered to be associated with some kind of immunity.

The CFR reflects the interplay between CD8+ TILs and Treg cells in a tumour and indicates the activity of the immune microenvironment. A higher CFR has been shown to be significantly associated with better survival in hormone receptor-negative tumours.15–17 In the present study, we focused on the rate of changes in the CFR induced by NAC and demonstrated that an increase in the CFR was significantly associated with improved clinical outcomes in not only TNBC but also HRBC. This result suggests that the change in the CFR after NAC may

| Table 2  | Univariate and multivariate analyses with respect to recurrence-free survival in breast cancer subtypes |
|----------------|-----------------------------------------------------------------------------------|
|                | Univariate analysis |                             | Multivariate analysis |                             |
|                | HR          | 95% CI      | P values | HR          | 95% CI      | P values |
| All breast cancer (n=129) |          |                             |                     |          |                             |                     |
| Age (≤56 years) | 1.406       | 0.729 to 2.755 | 0.309 |          |                             |                     |
| Tumour size (>2 cm) | 1.098       | 0.434 to 3.693 | 0.859 |          |                             |                     |
| Lymph node (+) | 2.004       | 0.895 to 5.342 | 0.095 |          |                             |                     |
| Ki-67 (>14) | 1.012       | 0.523 to 1.974 | 0.971 |          |                             |                     |
| Subtype (TNBC) | 1.494       | 0.553 to 4.706 | 0.441 |          |                             |                     |
| Pathological response (non-PR) | 6.327       | 2.893 to 13.133 | <0.001 | 5.260       | 2.373 to 11.145 | <0.001 |
| TIL (%) change (low) | 1.276       | 0.656 to 2.536 | 0.473 |          |                             |                     |
| CD8 change (low) | 3.114       | 1.430 to 7.773 | 0.003 | 2.304       | 1.052 to 5.776 | 0.036 |
| FOXP3 change (high) | 1.978       | 0.996 to 3.894 | 0.051 |          |                             |                     |
| CFR change (low) | 5.612       | 2.581 to 14.001 | <0.001 | 4.663       | 2.133 to 11.682 | <0.001 |
| TNBC (n=39) |          |                             |                     |          |                             |                     |
| Age (≤56 years) | 1.547       | 0.491 to 5.252 | 0.455 |          |                             |                     |
| Tumour size (>2 cm) | 0.261       | 0.066 to 1.721 | 0.139 |          |                             |                     |
| Lymph node (+) | 0.934       | 0.279 to 4.212 | 0.919 |          |                             |                     |
| Ki-67 (>14) | 1.138       | 0.358 to 4.264 | 0.832 |          |                             |                     |
| Pathological response (non-PR) | 25.642       | 6.873 to 123.724 | <0.001 | 34.290       | 7.314 to 265.738 | <0.001 |
| TIL (%) change (low) | 1.701       | 0.542 to 5.758 | 0.361 |          |                             |                     |
| CD8 change (low) | 2.339       | 0.697 to 10.551 | 0.177 |          |                             |                     |
| FOXP3 change (high) | 2.106       | 0.660 to 7.922 | 0.212 |          |                             |                     |
| CFR change (low) | 11.420       | 2.215 to 208.742 | 0.002 | 13.021       | 2.241 to 258.136 | 0.002 |
| HRBC (n=77) |          |                             |                     |          |                             |                     |
| Age (≤56 years) | 1.182       | 0.475 to 2.982 | 0.717 |          |                             |                     |
| Tumour size (>2 cm) | 3.622       | 0.743 to 65.248 | 0.128 |          |                             |                     |
| Lymph node (+) | 2.803       | 0.796 to 17.749 | 0.118 |          |                             |                     |
| Ki-67 (>14) | 0.781       | 0.305 to 1.969 | 0.597 |          |                             |                     |
| Pathological response (non-PR) | 2.132       | 0.488 to 6.625 | 0.277 |          |                             |                     |
| TIL (%) change (low) | 1.069       | 0.404 to 2.885 | 0.892 |          |                             |                     |
| CD8 change (low) | 3.167       | 1.134 to 11.196 | 0.027 | 2.746       | 0.976 to 9.741 | 0.056 |
| FOXP3 change (high) | 1.985       | 0.682 to 5.237 | 0.196 |          |                             |                     |
| CFR change (low) | 4.740       | 1.779 to 14.833 | 0.002 | 4.377       | 1.641 to 13.712 | 0.003 |

Values in parentheses are 95% CIs.
CFR, CD8/FOXP3 ratio; FOXP3, forkhead box protein; HRBC, hormone receptor-positive breast cancer; PR, partial response; TIL, tumour-infiltrating lymphocyte; TNBC, triple-negative breast cancer.

Goto W, et al. ESMO Open 2018;3:e000305. doi:10.1136/esmoopen-2017-000305
be a more accurate indicator of immune activity induced by chemotherapy.

The recently identified immune checkpoint markers, programmed death-1 (PD-1) and programmed death-ligand 1 (PD-L1), are present in some breast cancers.\textsuperscript{41}

The PD-1/PD-L1 axis, a major immune checkpoint pathway, leads to a reduction in the immune response by inducing T cell tolerance.\textsuperscript{42}

In previous studies, Muenst \textit{et al} reported that PD-1 + TILs are significantly associated with worse OS in the luminal B and basal-like subtypes.\textsuperscript{43}

Ali \textit{et al} also reported that PD-L1 expression is significantly enriched in the basal-like subtype and is correlated to the presence of TILs.\textsuperscript{44}

In addition, one recent study indicated that PD-L1 expression in residual tumours is significantly associated with the levels of CD8\textsuperscript{+} TILs and FOXP3\textsuperscript{+} TILs and may be a useful prognostic marker in patients with breast cancer following NAC.\textsuperscript{45}

These results suggest that by further evaluating the changes in other TILs, such as PD-1\textsuperscript{+} TILs, along with those in CD8\textsuperscript{+} TILs and FOXP3\textsuperscript{+} TILs in patients treated with NAC, more accurate identification of patient-specific immune mechanisms and prediction of prognosis may be possible.

### Table 3

Univariate and multivariate analyses with respect to overall survival in breast cancer subtypes

|                      | Univariate analysis |                      |                      |
|----------------------|---------------------|----------------------|----------------------|
|                      | HR                  | 95% CI               | P values             |
|                      | Multivariate analysis | HR                  | 95% CI               | P values             |
| **All breast cancer (n=129)** |                     |                      |                      |
| Age (≤56 years)      | 1.700               | 0.733 to 4.124       | 0.217                |
| Tumour size (>2 cm)  | 1.421               | 0.415 to 8.901       | 0.619                |
| Lymph node (+)       | 2.280               | 0.776 to 9.710       | 0.145                |
| Ki-67 (>14)          | 1.272               | 0.547 to 3.092       | 0.578                |
| Subtype (TNBC)       | 2.933               | 1.186 to 7.586       | 0.020                |
| Pathological response (non-PR) | 13.771 | 5.397 to 35.763 | <0.001                | 15.564 | 5.368 to 48.819 | <0.001                |
| TIL (%) change (low) | 1.316               | 0.568 to 3.191       | 0.524                |
| CD8 change (low)     | 3.103               | 1.147 to 10.797      | 0.024                |
| FOXP3 change (high)  | 2.586               | 1.086 to 6.230       | 0.032                |
| CFR change (low)     | 8.279               | 2.800 to 35.365      | <0.001                |
| **TNBC (n=39)**      |                      |                      |                      |
| Age (≤56 years)      | 2.264               | 0.674 to 8.756       | 0.187                |
| Tumour size (>2 cm)  | 0.349               | 0.089 to 2.303       | 0.232                |
| Lymph node (+)       | 0.798               | 0.229 to 3.654       | 0.744                |
| Ki-67 (>14)          | 1.648               | 0.475 to 7.542       | 0.446                |
| Pathological response (non-PR) | 11.812 | 0.043 to 23.141 | <0.001                | 11.243 | 20.791 to 20.892 | <0.001                |
| TIL (%) change (low) | 1.270               | 0.380 to 4.431       | 0.694                |
| CD8 change (low)     | 1.822               | 0.525 to 8.340       | 0.358                |
| FOXP3 change (high)  | 3.324               | 0.949 to 15.311      | 0.061                |
| CFR change (low)     | 9.847               | 1.883 to 180.764     | 0.004                |
| **HRBC (n=77)**      |                      |                      |                      |
| Age (≤56 years)      | 1.876               | 0.460 to 9.155       | 0.381                |
| Tumour size (>2 cm)  | 6.474               | 0.716 to 0.716       | 0.092                |
| Lymph node (+)       | 7.640               | 1.184 to 16.852      | 0.035                |
| Ki-67 (>14)          | 0.455               | 0.225 to 4.054       | 0.948                |
| Pathological response (non-PR) | 3.235 | 0.473 to 14.085 | 0.198                |
| TIL (%) change (low) | 1.369               | 0.336 to 6.678       | 0.664                |
| CD8 change (low)     | 5.283               | 0.939 to 98.783      | 0.060                |
| FOXP3 change (high)  | 2.231               | 0.455 to 9.159       | 0.295                |
| CFR change (low)     | 11.081              | 1.969 to 207.323     | 0.004                |

Values in parentheses are 95% CIs.

CFR, CD8/FOXP3 ratio; FOXP3, forkhead box protein; HRBC, hormone receptor-positive breast cancer; PR, partial response; TIL, tumour-infiltrating lymphocyte; TNBC, triple-negative breast cancer.
between HER2BC and the rate of change in the levels of CD8+ TILs and FOXP3+ TILs or the CFR. In addition, we analysed without dividing patients with HRBC into a HER2-positive and HER2-negative group.

This is the first study to indicate that improvement in the immune microenvironment following NAC has a relationship with good outcome, and that a high rate of change in the CFR could be a potential prognostic marker in patients with TNBC and HRBC who do not achieve pCR after NAC.

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Contributors All authors were involved in the preparation of the manuscript. WG collected the data and wrote the manuscript. SK, YA, KiTakah, KiTakah, TH and TT performed the operation and designed the study. WG, SK and ST summarised the data and revised the manuscript. MOhs performed the pathological diagnosis. HM, KH and MOhi made substantial contribution to the study design, performed the operation and revised the manuscript. All authors read and approved the final manuscript.

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Patient consent Obtained.

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