Tumour-infiltrating lymphocytes and prognosis in different subtypes of breast cancer: a pooled analysis of 3771 patients treated with neoadjuvant therapy

Carsten Denkert, Gunter von Minckwitz, Silvia Darb-Esfahani, Bianca Lederer, Barbara I Heppner, Karsten E Weber, Jan Budczies, Jens Huober, Frederick Klauschen, Jenny Furlanetto, Wolfgang D Schmitt, Jens-Uwe Blohmer, Thomas Karn, Berit M Pfitzner, Sherko Kümmel, Knut Engels, Andreas Schneeweiss, Arndt Hartmann, Aurelia Noske, Peter A Fasching, Christian Jackisch, Marion van Mackelenbergh, Peter Sinn, Christian Schem, Claus Hanusch, Michael Untch, Sibylle Loibl

Summary

Background Tumour-infiltrating lymphocytes (TILs) are predictive for response to neoadjuvant chemotherapy in triple-negative breast cancer (TNBC) and HER2-positive breast cancer, but their role in luminal breast cancer and the effect of TILs on prognosis in all subtypes is less clear. Here, we assessed the relevance of TILs for chemotherapy response and prognosis in patients with TNBC, HER2-positive breast cancer, and luminal–HER2-negative breast cancer.

Methods Patients with primary breast cancer who were treated with neoadjuvant combination chemotherapy were included from six randomised trials done by the German Breast Cancer Group. Pretherapeutic core biopsies from 3771 patients included in these studies were assessed for the number of stromal TILs by standardised methods according to the guidelines of the International TIL working group. TILs were analysed both as a continuous parameter and in three predefined groups of low (0–10% immune cells in stromal tissue within the tumour), intermediate (11–59%), and high TILs (≥60%). We used these data in univariable and multivariable statistical models to assess the association between TIL concentration and pathological complete response in all patients, and between the amount of TILs and disease-free survival and overall survival in 2560 patients from five of the six clinical trial cohorts.

Findings In the luminal–HER2-negative breast cancer subtype, a pathological complete response (pCR) was achieved in 45 (6%) of 759 patients with low TILs, 48 (11%) of 435 with intermediate TILs, and 49 (28%) of 172 with high TILs. In the HER2-positive subtype, pCR was observed in 194 (32%) of 605 patients with low TILs, 198 (39%) of 512 with intermediate TILs, and 127 (48%) of 262 with high TILs. Finally, in the TNBC subtype, pCR was achieved in 80 (31%) of 260 patients with low TILs, 117 (31%) of 373 with intermediate TILs, and 136 (50%) of 273 with high TILs ($p<0·0001$ for each subtype, χ^2 test for trend). In the univariable analysis, a 10% increase in TILs was associated with longer disease-free survival in TNBC (hazard ratio [HR] 0·93 [95% CI 0·87–0·98], p=0·011) and HER2-positive breast cancer (0·94 [0·89–0·99], p=0·017), but not in luminal–HER2-negative tumours (1·02 [0·96–1·09], p=0·46). The increase in TILs was also associated with longer overall survival in TNBC (0·92 [0·86–0·99], p=0·032), but had no association in HER2-positive breast cancer (0·94 [0·86–1·02], p=0·11), and was associated with shorter overall survival in luminal–HER2-negative tumours (1·10 [1·02–1·19], p=0·011).

Interpretation Increased TIL concentration predicted response to neoadjuvant chemotherapy in all molecular subtypes assessed, and was also associated with a survival benefit in HER2-positive breast cancer and TNBC. By contrast, increased TILs were an adverse prognostic factor for survival in luminal–HER2-negative breast cancer, suggesting a different biology of the immunological infiltrate in this subtype. Our data support the hypothesis that breast cancer is immunogenic and might be targetable by immune-modulating therapies. In light of the results in luminal breast cancer, further research investigating the interaction of the immune system with different types of endocrine therapy is warranted.

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Institute of Pathology, Charité, Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Berlin, Germany (Prof C Denkert MD, S Darb-Esfahani MD, B I Heppner MD, J Budczies PhD, Prof F Klauschen MD, W D Schmitt MD, B M Pfitzner, MD); **German Cancer Consortium (DKTK), Partner Site Berlin, Berlin, Germany** (Prof C Denkert, J Budczies); **GBG German Breast Group, Neu-Isenburg, Germany** (Prof G von Minckwitz MD, B Lederer PhD, K E Weber PhD, J Furlanetto MD, M van Mackelenbergh MD, Prof S Loibl MD); **Department of Gynecology, University of Ulm, Ulm, Germany** (Prof J Huober MD); **Breast Cancer Center, Charité Universitätsmedizin Berlin, Germany** (Prof J-U Blohmer MD); **Department of Gynecology and Obstetrics, University of Frankfurt, Frankfurt, Germany** (T Karn PhD); **Breast Unit, Kliniken Essen-Mitte, Essen, Germany** (S Kümmel MD); **Zentrum für Pathologie, Zytologie und Molekularpathologie, Neuss, Germany** (K Engels MD); **Universität Heidelberg, Nationales Centrum für Tumorerkrankungen, Heidelberg, Germany** (Prof A Schneeweiss MD); **Institute of Pathology, University of Erlangen-Nürnberg, Erlangen, Germany**

Introduction

Immunological parameters, including tumour-infiltrating lymphocytes (TILs), have been identified as predictors of response to neoadjuvant chemotherapy in breast cancer.[1,2] These data suggest that the immune system might have a major effect on the efficacy of chemotherapy, which opens the possibility for new treatment options through modulation of immune responses. Recent phase 1 trials suggest that a subgroup of breast carcinomas has an enhanced response to immune checkpoint inhibitor therapy in combination with conventional chemotherapy.[3,4] Currently, multiple randomised trials are ongoing to validate this finding in larger cohorts (eg, NCT02819518, NCT03036488, and NCT02425891).

A better understanding of tumour subtypes as well as molecular mechanisms is essential for development of innovative therapeutic strategies for modulation of immune response. The analysis of neoadjuvant

(Prof A Hartmann MD); **Institute of Pathology, Technical University of Munich, Munich, Germany** (A Noske MD); **Department of Gynecology and Obstetrics, University Hospital Erlangen, Friedrich-Alexander University Erlangen-Nürnberg, Comprehensive Cancer Center Erlangen-EMN, Erlangen, Germany** (Prof P A Fasching MD); **Sana Klinikum Offenbach, Offenbach, Germany** (Prof C Jackisch MD); **Institute of Pathology, University of Heidelberg, Heidelberg, Germany** (Prof P Sinn MD); **Department of Gynecology and Obstetrics, University Medical Center Schleswig-Holstein, Campus Kiel, Kiel, Germany** (C Schem MD); **Rotkreuzklinikum, Frauenklinik, München, Germany** (C Hanusch MD); **and Breast Cancer Center, Department of Gynecology and Obstetrics, Helios-Klinikum Berlin Buch, Germany** (Prof M Untch MD)

Correspondence to: Dr Carsten Denkert, Institut für Pathologie, Charité Universitätsmedizin Berlin, Berlin 10117, Germany
carsten.denkert@charite.de

Research in context

Evidence before this study

We did a systematic search of PubMed using the search terms "TILs", "breast cancer", and "neoadjuvant" in one search and "TILs", "breast cancer", and "adjuvant" in another search, each for articles published between Jan 1, 2010, and Dec 31, 2016. Several studies were identified, but most of them focused on moderately sized cohorts of patients with triple-negative breast cancer. To our knowledge, a comprehensive analysis of a large clinical trial cohort of patients with different molecular subtypes of breast cancer treated with neoadjuvant chemotherapy has not been published previously. For luminal breast cancer, some publications have described that tumour-infiltrating lymphocytes (TILs) and immune-related genes were associated with a poor response to aromatase inhibitor treatment. The association of TILs with response to chemotherapy, disease-free survival, and overall survival has not yet been assessed in large clinical trial cohorts.

Added value of this study

Our study shows that increased TIL concentrations are associated with increased response to neoadjuvant chemotherapy in all subtypes assessed and longer survival in triple-negative breast cancer and HER2-positive breast cancer. Our study also shows that the effect of TIL concentration on survival is different in HER2-positive and triple-negative breast cancer compared with that in luminal–HER2-negative tumours. We show a different composition of the immune-cell infiltrate in different breast cancer subtypes and a differential impact of immune-cell subtypes on prognosis in triple-negative breast cancer and luminal–HER2-negative tumours, that might partly explain the different effect of TILs in these subtypes.

Implications of all available evidence

Our results strongly support the hypothesis that breast cancer is immunogenic. This finding suggests that subsets of breast cancer might be targetable by immune-modulating therapeutic approaches. The assessment of TILs—as an indicator of pre-existing immunogenicity—might be useful for further stratification of breast cancer in clinical trials involving chemotherapy, anti-HER2 therapies, and future combinations with immune therapies. An integration of TILs with genomic classifiers might be warranted. Moreover, our findings suggest further research investigating the interaction of the immune system with different types of endocrine therapy is warranted.

treatment of breast carcinomas allows assessment of direct therapy response as well as the associated effect on disease-free survival and overall survival. In particular, patients with HER2-positive breast cancer or triple-negative breast cancer (TNBC) who achieve a pathological complete response after neoadjuvant chemotherapy have an improved prognosis.[5]

The primary aim of this study was the assessment of the association of TILs with response and survival in a large clinical cohort of breast carcinomas treated with neoadjuvant therapy; an additional secondary aim was assessment of immune-cell subpopulations to identify differences between luminal–HER2-negative breast cancer and TNBC.

See Online for appendix

Methods

Study design and clinical cohorts

We included individual patient data from six randomised, multicentre clinical trials done by the German Breast Group of neoadjuvant chemotherapy in patients with newly diagnosed, previously untreated, primary breast cancer: GeparDuo,[6] GeparTrio,[7] GeparQuattro,[8] GeparQuinto,[9,10] GeparSixto,[11] and GeparSepto.[12] All patients had received a neoadjuvant combination therapy as part of the clinical trials; patients with HER2-positive tumours also received an anti-HER2 therapy in all but two trials.[6,7]

In two of the studies[11,12] TILs were assessed prospectively as part of the mandatory central pathology assessment at the Charité Institute of Pathology, Berlin, Germany, and reported to the main study office at the German Breast Group in an individual central pathology report for each patient before randomisation. In the remaining four studies,[6–10] TIL analysis was done retrospectively—after the end of the clinical trial—using hematoxylin and eosin (H&E) stained slides that were prepared as part of the translational research programme from archived paraffin blocks obtained in the biobank of the German Breast Group at the Charité Institute of Pathology. Not all samples from these trials could be assessed for this study because of the lack of availability of tissue or H&E slides. The comparison of cohorts with TIL data (and included in our study) and without TIL data is shown in the appendix (p 1). TNBC and HER2-positive breast cancer samples are enriched in the cohort, because of the research focus of some of the studies.[8–10] For some cohorts, TIL assessments have been published elsewhere.[1,13–15] Data on disease-free survival and overall survival was available for all clinical cohorts except for GeparSepto.[12]

All patients gave informed consent for the use of tissue for research purposes and biomarker assessment, which was approved by the ethical committees for all clinical trials. This biomarker study is reported according to the REMARK guidelines.[16]

Procedures

Stromal TILs were quantified on H&E sections of core biopsies obtained before the start of neoadjuvant chemotherapy. TILs were assessed by use of the guidelines of the international TIL working group.[17] Stromal TILs were measured as percentage of immune

cells in stromal tissue within the tumour that showed a mononuclear immunological infiltrate. The number of TILs was analysed as a continuous measurement; additionally, three predefined categories were used: low TILs (0–10%), intermediate TILs (11–59%), or high TILs (60–100%). The number of intratumoural TILs that directly infiltrated into the tumour cell nests were correlated with the number of stromal TILs, but had generally lower concentrations (data not shown). Therefore, this analysis is focused on stromal TILs. Classification of breast cancer subtypes (TNBC, HER2-positive, and luminal–HER2-negative) were based on immunohistochemistry and in-situ hybridisation (for HER2 2+); tumours with missing subtype information were excluded from the subtype-specific analysis. We did not divide the hormone-receptor-positive (luminal) cohort into luminal A and luminal B-like tumours, but we used Ki67 as an additional parameter for stratification of luminal tumours. For oestrogen receptor, progesterone receptor, HER2, and Ki67 status, the central biomarker result was used if available, otherwise the local assessment from the clinical trial database was used.

TILs contain different subsets of cells, and the distribution of these subsets might be different in different tumour types. Therefore, we analysed the distribution of immune cells in a large cohort of tumours from the Metabric database,[18] by use of specific molecular markers for each immune cell subset.[19] Genome-wide mRNA expression data and clinical and pathological data from the Metabric cohort (n=1980) were downloaded from cBioPortal. Luminal–HER2-negative tumours and TNBCs were included in the analysis. We excluded HER2-positive tumours, because these tumours contain both hormone-receptor-positive and hormone-receptor-negative subsets and are therefore less suitable for identifying differences between datasets.

For **cBioPortal** see http://www.cbioportal.org

Statistical analysis

We hypothesised that patients with breast carcinomas with a high lymphocyte infiltrate would have an increased pathological complete response compared with those with a low lymphocyte infiltrate, which would lead to longer survival in HER2-positive breast cancer, TNBC, and some patients with high-risk luminal–HER2-negative breast cancer.

The endpoints of interest were pathological complete response, disease-free survival, and overall survival. We defined pathological complete response as ypT0/ypN0 on the basis of the local histopathological analysis of the resection specimen after neoadjuvant chemotherapy. We defined disease-free survival as the time from randomisation until any breast cancer relapse or death from any cause; and overall survival as the time from randomisation to death irrespective of cause.

The analysis was based on a predefined statistical analysis plan (appendix pp 8–14) and we did these analyses using SPSS version 23 (IBM Corporation, Somers, NY, USA). We assessed correlations between TIL categories

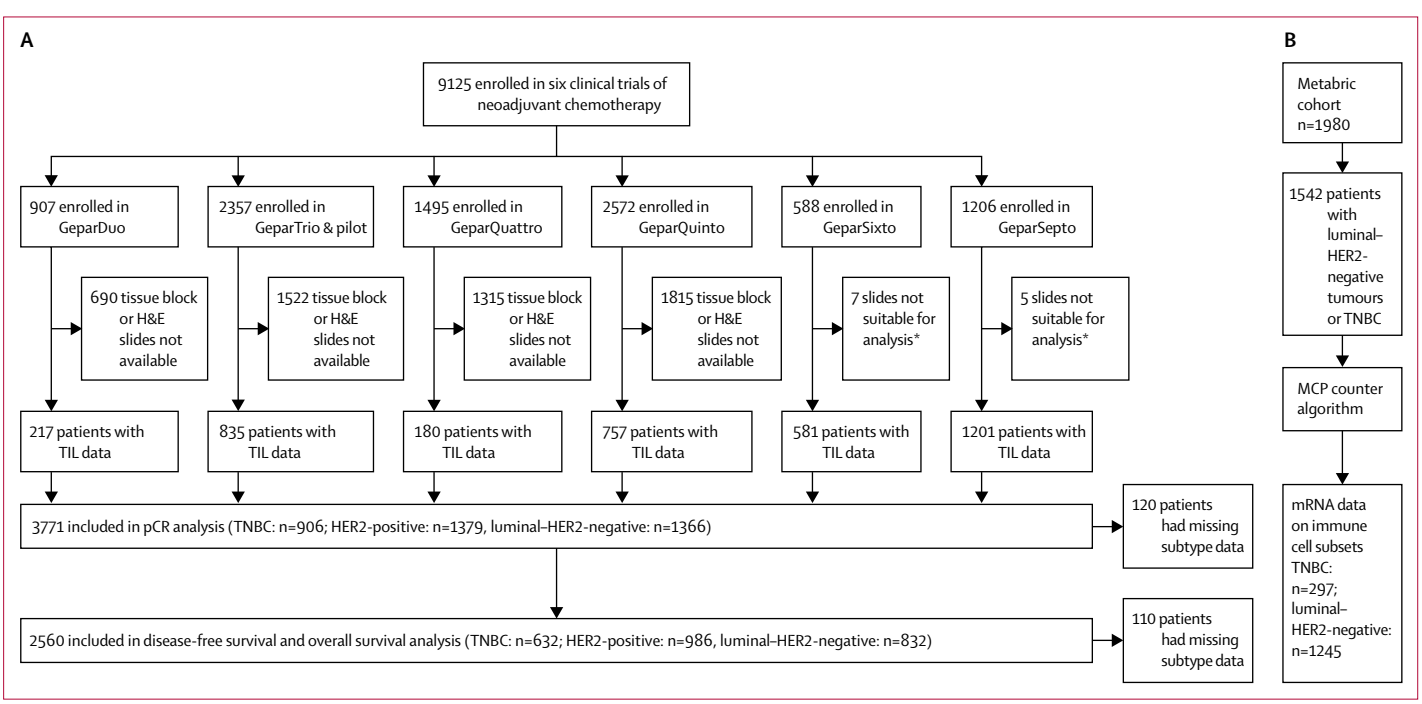

Figure 1: **Study cohorts.**
3771 tumours with available TIL data were included in this pooled analysis (A). A cohort of TNBC and luminal–HER2-negative tumours from the METABRIC were investigated for mRNA based immune-cell subtyping (B). TIL=tumour-infiltrating lymphocytes. H&E=haematoxylin and eosin. pCR=pathological complete response. TNBC=triple-negative breast cancer. MCP=microenvironment cell populations. *Eg, core biopsy with lymph node tissue.

	Number of patients	Stromal TILs low (0–10%)	Stromal TILs intermediate (11–59%)	Stromal TILs high (≥60%)	p value
Age (n=3771)					
≤40 years	729 (19%)	279 (38%)	294 (40%)	156 (21%)	0·0006*
41–50 years	1368 (36%)	619 (45%)	477 (35%)	272 (20%)	..
>50 years	1674 (44%)	779 (47%)	598 (36%)	297 (18%)	..
Tumour stage (n=3752)					
cT1	703 (19%)	266 (38%)	292 (42%)	145 (21%)	<0·0001*
cT2	2227 (60%)	991 (45%)	784 (35%)	452 (20%)	..
cT3	479 (13%)	236 (49%)	163 (34%)	80 (17%)	..
cT4a–c	160 (4%)	86 (54%)	57 (36%)	17 (11%)	..
cT4d	183 (5%)	91 (50%)	64 (35%)	28 (15%)	..
Nodal status (n=3705)					
cN0	2031 (55%)	968 (48%)	717 (35%)	346 (17%)	<0·0001*
cN1	1501 (41%)	619 (41%)	559 (37%)	323 (22%)	..
cN2	134 (4%)	51 (38%)	48 (36%)	35 (26%)	..
cN3	39 (1%)	8 (21%)	22 (56%)	9 (23%)	..
Tumour grading (n=3712)					
G1	114 (3%)	79 (69%)	28 (25%)	7 (6%)	<0·0001*
G2	1783 (48%)	958 (54%)	616 (35%)	209 (12%)	..
G3	1815 (49%)	618 (34%)	703 (39%)	494 (27%)	..
Tumour type (n=3767)					
Ductal	3223 (86%)	1382 (43%)	1201 (37%)	640 (20%)	<0·0001†
Lobular	272 (7%)	185 (68%)	74 (27%)	13 (5%)	..
Other‡	272 (7%)	109 (40%)	92 (34%)	71 (26%)	..
Molecular subtypes (n=3651)					
Luminal–HER2-negative breast cancer	1366 (37%)	759 (56%)	435 (32%)	172 (13%)	<0·0001†
HER2-positive	1379 (38%)	605 (44%)	512 (37%)	262 (19%)	..
TNBC	906 (25%)	260 (29%)	373 (41%)	273 (30%)	..
Clinical trial (n=3771)					
GeparDuo[9]	217 (6%)	96 (44%)	96 (44%)	25 (12%)	<0·0001†
GeparTrio[10]	835 (22%)	408 (49%)	250 (30%)	177 (21%)	..
GeparQuattro[11]	180 (5%)	71 (39%)	61 (34%)	48 (27%)	..
GeparQuinto[12]	757 (20%)	349 (46%)	259 (34%)	149 (20%)	..
GeparSixto[14]	581 (15%)	183 (32%)	257 (44%)	141 (24%)	..
GeparSepto[15]	1201 (32%)	570 (48%)	446 (37%)	185 (15%)	..
Type of taxane (n=3771)					
Docetaxel	1911 (51%)	887 (46%)	638 (33%)	386 (20%)	<0·0001†
Paclitaxel	1258 (33%)	507 (40%)	508 (40%)	243 (19%)	..
Nab-paclitaxel	602 (16%)	283 (47%)	223 (37%)	96 (16%)	..
Neoadjuvant anti-HER2 (n=3771)					
None	2518 (67)	1117 (44%)	901 (36%)	500 (20%)	0·092
Trastuzumab	381 (10%)	178 (47%)	127 (33%)	76 (20%)	..
Lapatinib	212 (6%)	97 (46%)	79 (37%)	36 (17%)	..
Trastuzumab plus lapatinib	267 (7%)	99 (37%)	115 (43%)	53 (20%)	..
Trastuzumab plus pertuzumab	393 (10%)	186 (47%)	147 (37%)	60 (15%)	..
pCR (ypT0ypN0) (n=3771)					
No	2757 (73%)	1349 (49%)	1000 (36%)	408 (15%)	<0·0001†
Yes	1014 (27%)	328 (32%)	369 (36%)	317 (31%)	..

Data are n (%). TILs=tumour infiltrating lymphocytes. pCR=pathological complete response. *χ^2 test for trend. †χ^2 test. ‡Includes all tumours that were neither ductal nor lobular; 255 (96%) of the 265 tumours in this category with available information on grading were grade 2 or grade 3.

Table: **Baseline parameters and distribution of TILs in clinicopathological subgroups**

and clinical–pathological parameters as well as pathological complete response by χ^2 test, χ^2 test for trend, or Fisher's exact tests, and by logistic regression; correlations with disease-free survival and overall survival were assessed by Cox proportional hazard regression. All p values were two-sided; p values less than 0·05 were deemed significant. We calculated 95% CIs for logistic regression and Cox regression using the function provided in the SPSS software. Continuous TIL variable unit odds ratios (for pathological complete response) and unit hazard ratios (for disease-free survival and overall survival) are reported for units of 10% TILs. We did regression analyses as univariable and multivariable models; the covariates for multivariable models were age (≤40 years *vs* 40–50 years *vs* >50 years, T stage (T1 *vs* T2 *vs* T3 *vs* T4a–c *vs* T4d), N stage (N0 *vs* N1 *vs* N2 *vs* N3), histopathological type (ductal invasive *vs* lobular invasive *vs* other), tumour grading (G1 *vs* G2 *vs* G3), study, and molecular subtype (hormone-receptor-negative and HER2-negative *vs* hormone-receptor-negative and HER2-positive *vs* hormone-receptor-positive and HER2-negative *vs* hormone-receptor-positive and HER2-positive). Some of these parameters were missing in some subgroups and thus were omitted in the analysis of these subgroups. The number of samples available for the multivariable models is slightly smaller than the one for the corresponding univariable analyses because of missing values (appendix p 1). For significance testing in Kaplan-Meier survival analysis, we used the log-rank test. In a post-hoc analysis of survival, we assessed a large set of clinicopathological parameters in the different breast cancer subtypes by logistic regression and Cox regression.

Abundance of immune cell populations in the Metabric cohort, focusing on differences between luminal–HER2-negative tumours and TNBC, was estimated from $\log_2$ scale mRNA expression data. We used the micro-environment-cell-populations (MCP)-counter method on the basis of specific molecular markers for each immune cell subset.[19] For analysis of overall survival with Cox proportional hazard models, we used the median level of each immune cell population as the cutoff. Differences between immune cell populations in each breast cancer subtype were assessed using Welch's *t* test.

Role of the funding source

The funding source had no role in study design, data collection, data analysis, interpretation of data, or writing of the report. CD, KEW, JB, and BL had access to the raw data. The corresponding author had full access to all of the data and the final responsibility to submit for publication.

Results

Of 9125 patients enrolled in the six included clinical trials, 3771 pre-therapeutic core biopsies from patients with primary breast cancer were eligible to be assessed for TILs (figure 1). Of these, 1366 (37%) were luminal–HER2-negative tumours, 906 (25%) were TNBC, and 1379 (38%) were HER2-positive breast cancer (table). The table shows the association of TILs with clinical–pathological parameters. By use of the predefined cutpoints, 1677 (44%) tumours had low TILs, 1369 (36%) had intermediate TILs, and 725 (19%) had high TILs. The percentage of tumours with high TILs was higher in TNBC (273 [30%] of 906) and HER2-postive breast cancer (262 [19%] of 1379) than in luminal–HER2-negative tumours (172 [13%] of 1366; p<0·0001, χ^2 test for trend; table). We used predefined cutoff points to distinguish three TIL subgroups; nevertheless, the actual distribution suggests that the proportion of TILs in a tumour is a continuous variable (figure 2).

Increased concentration of TILs was a predictor of response to neoadjuvant chemotherapy: a pathological complete response was achieved in 328 (20%) of 1677 tumours with low TILs, 369 (27%) of 1369 tumours with intermediate TILs, and 317 (44%) of 725 tumours with high TILs (p<0·0001, χ^2 test for trend, figure 3A). This positive correlation was also observed using TILs as a continuous measurement (figure 3B, C). Increased TIL concentrations were linked to increased pathological complete response in all subtypes when TILs were assessed either as a categorical or a continuous variable (figure 3). In patients with the luminal–HER2-negative subtype, a pathological complete response was achieved in 45 (6%) of 759 patients with low TILs, 48 (11%) of 435 with intermediate TILs, and 49 (28%) of 172 with high TILs. In patients with the HER2-positive subtype, a pathological complete response was achieved in 194 (32%) of 605 patients with low TILs, 198 (39%) of 512 with intermediate TILs, and 127 (48%) of 262 with high TILs. In patients with TNBC, a pathological complete response was achieved in 80 (31%) of 260 patients with low TILs, 117 (31%) of 373 with intermediate TILs, and 136 (50%) of 273 with high TILs (p<0·0001 for each subtype, χ^2 test for trend).

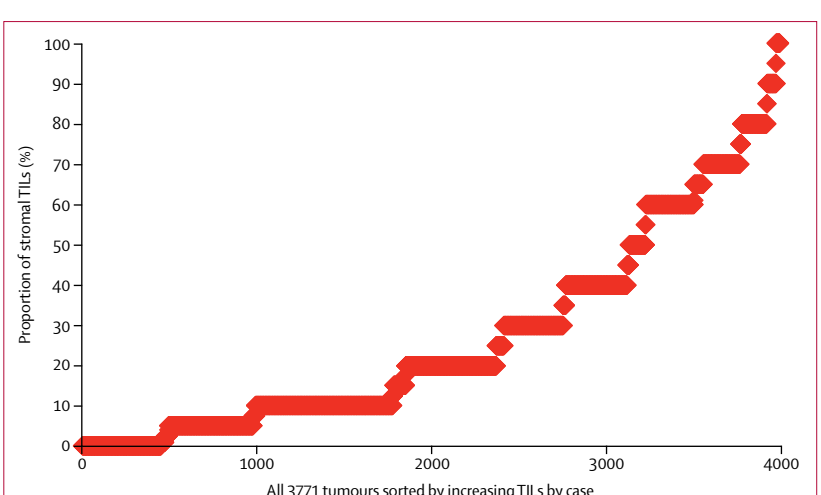

Figure 2: **TILs as a continuous measurement**
3771 tumours sorted by increasing stromal TIL levels. TIL=tumour-infiltrating lymphocyte. TNBC=triple-negative breast cancer.

In a post-hoc univariable logistic regression analysis, TIL concentrations predicted pathological complete response in most clinicopathological subgroups of TNBC, HER2-positive breast cancer, and luminal–HER2-negative breast cancer, suggesting that the association between immunological infiltrates and chemotherapy response is similar across subtypes and clinicopathological subgroups (appendix p 3).

We assessed TILs as a prognostic marker for disease-free survival and overall survival in 2570 patients from five clinical trial cohorts.[6–9,11] The median follow-up for overall survival was 62·8 months (IQR 37·6–73·2) and the median follow-up for disease-free survival was 63·3 months (IQR 38·0–73·5). When TIL concentration was assessed as a continuous variable, patients with increased TIL concentrations and TNBC had significantly longer disease-free survival and overall survival than did patients with TNBC and lower TIL concentrations; increased TIL concentrations in patients with HER2-positive breast cancer had a significantly longer disease-free survival than did patients with HER2-positive breast cancer with lower TIL concentrations but there was no association for overall survival (figure 4A, D). By contrast, in the luminal–HER2-negative patients, TIL concentration was not significantly associated with disease-free survival, and a low TIL concentration was significantly associated with longer overall survival than was a high TIL concentration (figure 4A, D). The association between TIL concentration and survival was similar between univariable analysis and multivariable analysis including baseline parameters (figure 4B, E) for all subtypes. However, when pathological complete response was included as a parameter in the multivariable analysis, TILs were no longer significantly associated with disease-free survival in patients with TNBC or with overall survival in patients with TNBC or HER2-positive breast cancer (figure 4C, F).

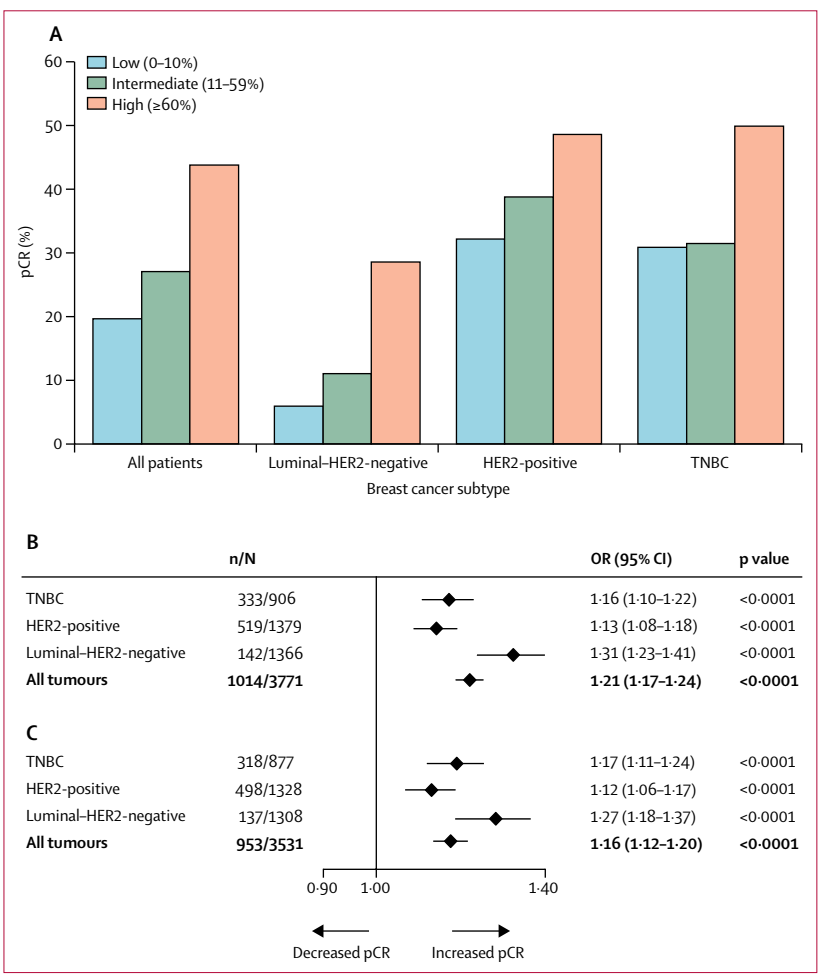

Figure 3: **TIL concentration and response to neoadjuvant therapy**
(A) pCR in three predefined TIL groups in all breast cancer subtypes. p values are from the χ^2 test for trend. (B) Univariable analysis of TILs as a continuous marker for prediction of pCR. p values are from the log-rank test. (C) Multivariable analysis of TILs as a continuous marker for prediction of pCR. p values are from the log-rank test. TIL=tumour infiltrating lymphocyte. pCR=pathological complete response. TNBC=triple negative breast cancer. OR=odds ratio.

A total of 632 patients with TNBC, 986 patients with HER2-positive breast cancer, and 832 patients with luminal–HER2-negative breast cancer were included in the Kaplan-Meier survival analysis. By use of the three predefined TIL subgroups, the analysis showed that high TILs were a positive prognostic factor for disease-free survival in TNBC (figure 5A) and HER2-positive breast cancer (figure 5C). By contrast, in the luminal–HER2-negative group, low TILs were a positive prognostic factor for disease-free survival (figure 5E). Results for overall survival were similar to those for disease-free survival in all subtypes (figure 5B, D, F). When the overall survival analysis was stratified for pathological complete response within the subtypes of TNBC and luminal–HER2-negative tumours, patients with a pathological complete response had a good prognosis regardless of TILs (appendix p 4). Patients with TNBC without a pathological complete response had a similar prognosis across all three TIL groups; by contrast, in patients with luminal–HER2-negative tumours without a pathological complete response, the prognosis was better in the low TILs subgroup than in the high or intermediate TILs subgroups (appendix p 4).

In a post-hoc analysis of survival on the basis of molecular subtypes and clinicopathological parameters, higher TIL concentrations were associated with longer disease-free survival and overall survival than were lower TIL concentrations in most subgroups of TNBC and HER2-positive breast cancer (appendix pp 5–6). By contrast, the effects were mixed for both disease-free survival and overall survival in different clinicopathological subgroups for patients with luminal–HER2-negative breast cancer (appendix pp 5–6).

1245 luminal–HER2-negative tumours and 297 TNBCs were included in the METABRIC analysis. We noted a distinct distribution of immune cell types in the breast cancer subtypes (appendix p 7). The effect of these immune cell types on prognosis was also different

among the breast cancer subtypes. For TNBC, the presence of most immune cell types, including different types of T cells, natural killer cells, B cells, monocytes, and myeloid-derived dendritic cells were significantly associated with better prognosis for overall survival (appendix p 7). By contrast, in luminal–HER2-negative tumours, most T-cell markers were not associated with overall survival (appendix p 7), myeloid-derived dendritic cells and B cells were linked to improved prognosis, though the presence of monocytes or macrophages was associated with poor prognosis.

Discussion

In our pooled assessment of TIL concentrations in patients with breast cancer treated in six randomised trials of neoadjuvant chemotherapy we found that pathological complete response was associated with TIL concentrations in all breast cancer subtypes with a strong positive correlation, but the effect of TIL concentration on disease-free survival and overall survival differed between HER2-positive breast cancer or TNBC (positive survival effect in both subtypes) and luminal–HER2-negative breast cancer (negative survival effect).

The neoadjuvant therapy approach has the advantage over adjuvant therapy in that it allows the separation of biological predictive markers of pathological complete response from prognostic factors. For neoadjuvant therapy response, we observed a consistent positive association of increased TILs with increased pathological complete response. This effect was observed in most subgroups defined by the standard clinicopathological parameters, with only a few exceptions. In logistic regression analysis, for every 10% of increased TILs, there was an increase in the odds ratio of pathological complete response (on the basis of data for the complete cohort).

In this study, we focused on stromal TILs, because this was the predominant location of TILs in breast cancer in our study. The number of intratumoural TILs were correlated with the number of stromal TILs (data not shown), but typically had a much lower density and therefore are less suitable as a biomarker.

Although the association of TILs with response to neoadjuvant chemotherapy was similar across breast cancer subtypes, there were major differences between subtypes for survival endpoints. For TNBC and HER2-positive breast cancer, increased numbers of TILs were linked to longer disease-free survival than that for lower numbers of TILs; for TNBC, increased TILs were linked with improved overall survival. In the multivariable analysis, the prognostic role of TILs in these subtypes remained significant with inclusion of baseline parameters. However, when pathological complete response was included in the multivariable analysis, the association was no longer significant, which can be explained by the significant correlation of TILs with increased pathological complete response. For luminal–HER2-negative tumours, TILs were not prognostic for disease-free survival. Notably, for overall survival in this subtype, there was an improved prognosis associated with low TIL concentrations. Therefore, the effect of TILs on overall survival had the opposite effect for luminal tumours, compared with TNBC and HER2-positive breast cancer.

It is unlikely that the different effect of TILs on survival in TNBC and luminal breast cancer is because of differences in chemotherapy response, as pathological complete response increased with increased TIL concentrations in all subtypes. For luminal grade 3

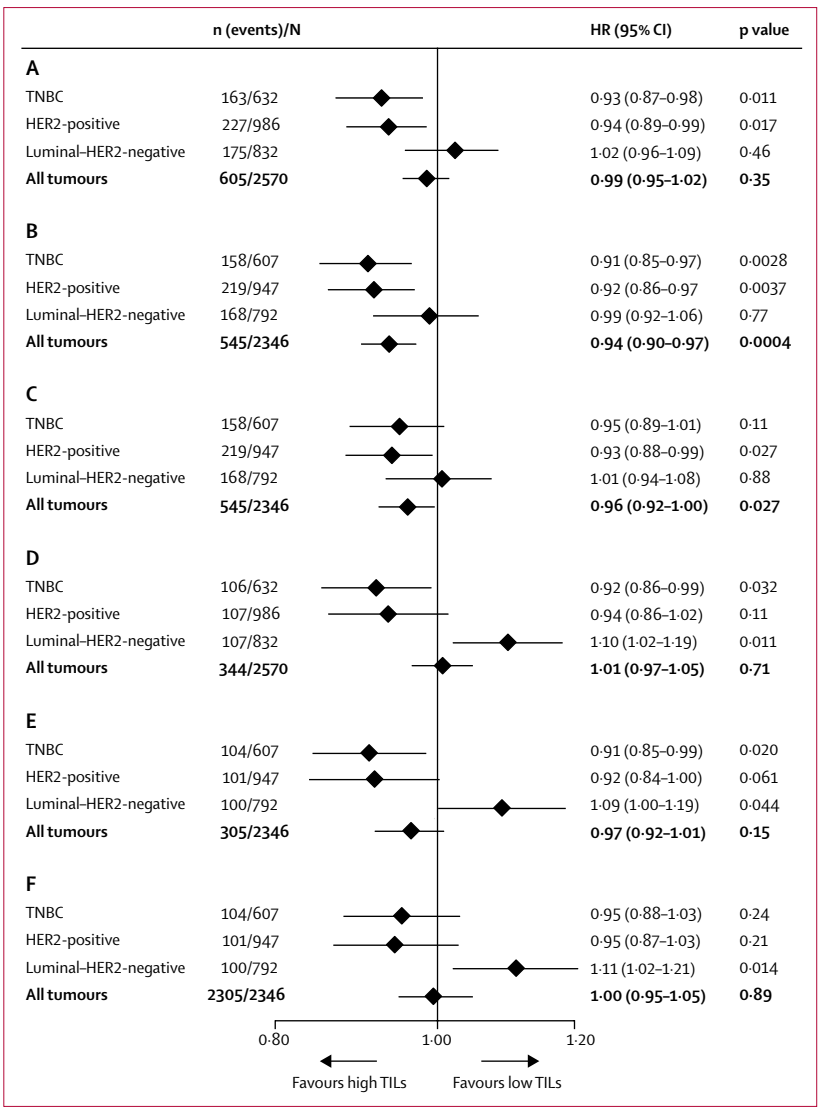

Figure 4: **Continuous TIL concentration as a prognostic marker for disease-free survival and overall survival for all tumour subtypes**

p values have been obtained from a logistic regression analysis. Disease-free survival by univariable analysis (A), multivariable analysis including all baseline parameters (B), and multivariable analysis including all baseline parameters and pCR (C). Overall survival by univariable analysis (D), multivariable analysis including all baseline parameters (E), and multivariable analysis including all baseline parameters and pCR (F). TIL=tumour-infiltrating lymphocyte. TNBC=triple-negative breast cancer. pCR=pathological complete response.

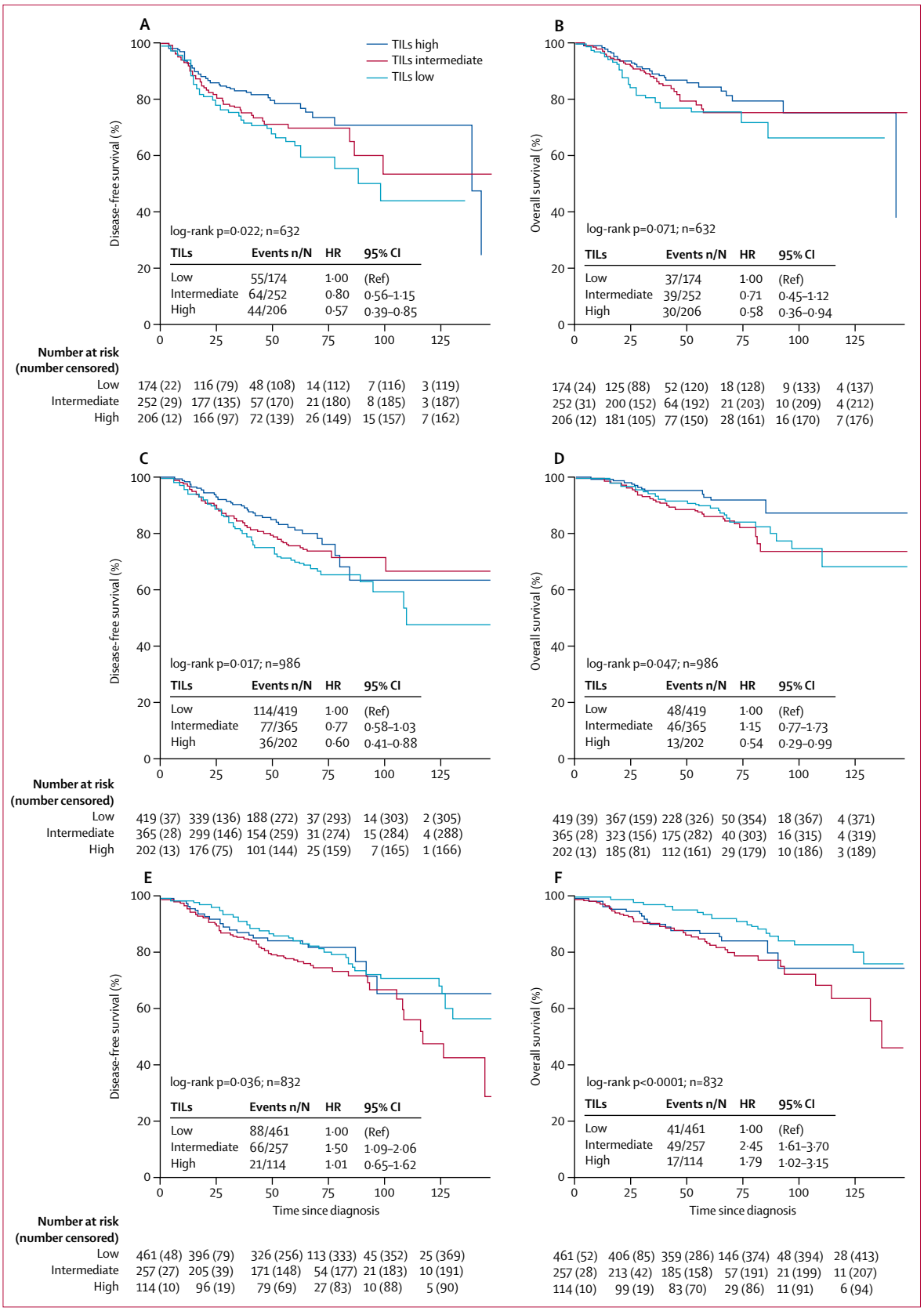

Figure 5: **Kaplan-Meier analysis for prognosis of patients with high, intermediate, and low TILs in different molecular subtypes**
(A) Disease-free survival in TNBC. (B) Overall survival in TNBC. (C) Disease-free survival in HER2-positive breast cancer. (D) Overall survival in HER2-positive breast cancer. (E) Disease-free survival in luminal–HER2-negative breast cancer. (F) Overall survival in luminal–HER2-negative breast cancer. p values were derived from a log-rank test; HR and 95% CI for comparison of the three TIL groups were derived from univariate Cox-regression using the three TIL categories. TIL=tumour-infiltrating lymphocytes. TNBC=triple-negative breast cancer. HR=hazard ratio.

tumours, increased TILs were a positive prognostic factor, similar to TNBC, which might be explained by the biological similarity between the subtypes. By contrast, for luminal tumours with a high T stage, the prognosis was significantly improved with low TIL concentrations. This might be explained by the fact that patients with this tumour subset are unlikely to reach a pathological complete response.[20] Stratification in the luminal subtype by progesterone receptor status or Ki67 did not change the overall effects of TILs. Nevertheless, this finding does not exclude that more advanced ways to characterise luminal tumours, such as gene expression signatures, mutation-based subtyping, or mutational signatures, might result in more refined breast cancer subtypes with different immunobiology.

In general, the negative effect of TILs on survival in patients with luminal–HER2-negative tumours was most pronounced in patients who did not have a pathological complete response and was stronger for overall survival than for disease-free survival. The number of patients with pathological complete response was too small to do statistical assessments; nevertheless, the results suggest that the poor prognosis of luminal tumours with high TILs was mainly driven by the large group of patients without a pathological complete response. This finding suggests that therapies that were given after surgery and first recurrence, particularly after endocrine therapy, might be relevant. Some publications have shown that TILs and immune-related genes were associated with a poor response to aromatase inhibitor treatment.[21,22] Therefore, the adverse prognostic effect of increased TILs in patients with luminal tumours without a pathological complete response might also be explained by a relative resistance to adjuvant and metastatic endocrine treatment. Additional validations in large endocrine-treated clinical study cohorts comparing different types of endocrine treatment would be interesting.

One possible explanation of the differences between luminal tumours and TNBC could be the contribution of different immune cell types. Most types of immune cell were increased in TNBC compared with luminal–HER2-negative breast cancer. In TNBC, the presence of many immune cell subtypes, including B cells, T cells, and macrophages, were linked to improved survival. By contrast, in luminal–HER2-negative breast cancer, the presence of T cells were not prognostic for survival and the only cell types linked to improved prognosis were B cells and myeloid dendritic cells, although macrophages were linked to reduced survival. This suggests differences in the cellular composition and the prognostic effect of immune cells in TNBC versus luminal–HER2-negative breast cancer.

Different approaches to analyse immune cell phenotypes in mRNA datasets of malignant tumours have been published.[23–27] The MCP counter[18] method, which we used, measures the absolute abundance of immune cell subtypes and is therefore suitable for tumours with low amounts of immune cells, such as breast carcinomas. The Cibersort method,[28,29] which is also used widely, focuses on relative quantification of immune cell subtypes, and is less suitable for tumours with very low infiltrates. Nevertheless, previous studies using Cibersort have similarly identified different immune cell subpopulations, including different types of macrophages, in luminal breast cancer and TNBC.[30,31] B-cell-related mRNA signatures are prognostic in luminal breast cancer and TNBC,[26] although T-cell-related mRNA signatures are prognostic only in TNBC and HER2-positive breast cancer.[27]

At present, it is not feasible to do an mRNA based subtyping of immune cells in our entire clinical trial cohort. Nevertheless, our study shows that some immunological differences detected in complex molecular profiling studies can also be observed and validated in large clinical study cohorts by use of the relatively simple approach of assessment of TILs in H&E sections. Both approaches complement and validate each other and together strongly indicate that differences in immunological interactions in TNBC and luminal breast cancer can be molecularly defined and are clinically relevant. For further substratification, particularly in luminal tumours, genomic parameters such as mutational signatures or copy number variations[18] could be integrated. This approach might be of interest because it has been shown that apolipoprotein B mRNA editing enzyme catalytic polypeptide (APOBEC)-type substitution signatures are linked to increased TIL concentrations and immune-gene expression in oestrogen-receptor-positive breast cancer.[32]

In our study, we used predefined cutoff points to distinguish three groups with different TIL concentrations, but it should be emphasised that the actual distribution of TILs suggests that these were artificial cutoffs. The number of TILs was a continuous variable, which could reach any proportion between 0 and nearly 100%. This suggests that TILs—similar to the proliferation marker Ki67—are a continuous parameter of tumour-immune cell interaction rather than a marker of a specific immune-activated tumour subtype. This is in line with results on genomic parameters that were published by Lehmann and colleagues,[33] showing that gene expression profiling could not be used to define an immune-enriched subtype of TNBC, but that different levels of immune-gene expression were observed across all breast cancer subtypes and were correlated with TIL concentrations. The continuous analysis of immune markers is more relevant from a statistical point of view and gives a more accurate description of tumour biology. Nevertheless, the categorisation into low, intermediate, and high concentrations of TILs might be relevant for future clinical applications, since stratification in clinical trials can be more easily done on the basis of categorical variables that divide patients in different groups. Therefore, in our study, we have presented both the categorical and the continuous analysis and both

approaches give similar results for prediction of pathological complete response as well as prognosis.

As a limitation, it should be mentioned that a substantial number of tissue samples from the earlier trials[6–10] were not available for TIL analysis and translational projects within those trials were focused on TNBC and HER2-positive breast cancer. Therefore, our cohort is enriched for TNBC and HER2-positive breast cancer as well as for tumours from the remaining trials.[11,12] The enrichment for TNBC and HER2-positive breast cancer has the advantage that the three subtype cohorts (TNBC, HER2-positive breast cancer, and luminal–HER2-negative breast cancer) are of similar sizes for statistical analysis, but it generates some differences compared with the original clinical trial cohorts. Despite the evidence discussed above, at this time, there is no clear biological explanation for the different prognoses of TIL concentration in TNBC versus luminal–HER2-negative tumours. It can therefore not be excluded that these different prognoses might be due to chance.

It would be interesting to further explore the prognostic value of TILs in cohorts treated with endocrine therapy. The GeparSepto trial[12] provides prospective results regarding TILs and pathological complete response from a large clinical cohort of 1201 tumours, but survival data, including for patients with HER2-positive tumours treated with the trastuzumab–pertuzumab combination, is not yet available.

In summary, we show that increased TIL concentrations are associated with increased frequency of response to neoadjuvant chemotherapy in all breast cancer subtypes and that they are also associated with longer survival for patients with TNBC and HER2-positive breast cancer, although they are not associated with longer survival for patients with luminal–HER2-negative tumours. However, it should be emphasised that even in luminal tumours—depending on the clinical setting—TILs can have a positive effect on the frequency of pathological complete response. Therefore, it would also be interesting to further assess whether a modulation of immune cells in luminal breast cancers—particularly in tumours that have at least some basal immune infiltrate—might increase pathological complete response and create a stronger link between pathological complete response and survival. This approach is currently being tested in the ULTIMATE trial (NCT02997995). In the ongoing GeparNuevo trial (NCT02685059), we are assessing TIL concentrations prospectively as a stratification parameter for a combination of neoadjuvant chemotherapy and the Programmed death-ligand 1 inhibitor durvalumab in TNBC. For combined risk assessment, the combination of gene expression-based risk predictors and immunological parameters might offer new options for the stratification of breast cancer allowing different therapeutic strategies.

Contributors

The study was designed by CD, GvM, and SL. CD, SD-E, BIH, FK, WDS, BMP, KE, and AN contributed to data acquisition. Data analysis was done by CD, BL, KEW, and JB. Patient recruitment, sample collection, and data collection was done by GvM, JH, JF, J-UB, SK, KE, AS, AH, PAF, CJ, MvM, PS, CS, CH, MU, and SL. All authors interpreted the data. The first draft of the report was written by CD. The decision to submit the report for publication was made by all the authors. All authors contributed to the review of the manuscript.

Declaration of interests

CD reports grant support from the European Commission (Responsify FP7 project and Transcan UGI1 project) as well as from the German Cancer Aid (Deutsche Krebshilfe, TransLuminal-B project) during the conduct of the study; ownership interest in Sividon Diagnostics outside the submitted work; and honoraria from Pfizer, Merck, Sharp & Dohme, Amgen, Myriad, Teva, Celgene, Roche, and AstraZeneca outside the submitted work. GvM reports grants from Pfizer, Sanofi-Aventis, Amgen, Roche, Novartis, Celgene, Teva, AstraZeneca, AbbVie, and Vifor outside the submitted work. KEW reports grant support from the European Commission (Responsify FP7 project and Transcan UGI1 project) and German Cancer Aid (Deutsche Krebshilfe, TransLuminal-B project) during the conduct of the study and ownership interest in Sividon Diagnostics outside the submitted work. JB reports grant support from the German Cancer Aid (Deutsche Krebshilfe, TransLuminal-B project) during the conduct of the study. AS reports honoraria from Roche, Celgene, AstraZeneca, Novartis, Pfizer, and Amgen outside the submitted work. CH reports personal fees from Roche, Novartis, Pfizer, Celgene, and Amgen outside the submitted work. All other authors declare no competing interests.

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