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Tumor-infiltrating lymphocytes (TILs) are a powerful prognostic marker in patients with triple negative breast cancer enrolled in the IBCSG phase III randomized clinical trial 22-00

Giancarlo Pruneri, **Kathryn P. Gray**, **Andrea Vingiani**, **Giuseppe Viale**, **Giuseppe Curigliano**, **Carmen Criscitiello**, **István Láng**, **Thomas Ruhstaller**, **Lorenzo Gianni**, **Aron Goldhirsch**, **Roswitha Kammler**, **Karen N. Price**, **Giuseppe Cancello**, **Elisabetta Munzone**, **Richard D. Gelber**, **Meredith M. Regan**, and **Marco Colleoni**

[1]Giancarlo Pruneri. Department of Pathology and Laboratory Medicine, IBCSG Central Pathology Laboratory, European Institute of Oncology, and University of Milan, Milan, Italy. Via Ripamonti 435, Milan, 20141, Italy. giancarlo.pruneri@ieo.it

[2]Kathryn P. Gray. International Breast Cancer Study Group (IBCSG) Statistical Center, Department of Biostatistics and Computational Biology, Dana-Farber Cancer Institute, Harvard T. H. Chan School of Public Health, Boston, MA. 450 Brookline Avenue, Boston, MA 02215, USA. pkruan@jimmy.harvard.edu

[3]Andrea Vingiani. Department of Pathology and Laboratory Medicine, IBCSG Central Pathology Laboratory, European Institute of Oncology, and University of Milan, Milan, Italy. Via Ripamonti 435, Milan, 20141, Italy. andrea.vingiani@unimi.it

[4]Giuseppe Viale. Department of Pathology and Laboratory Medicine, IBCSG Central Pathology Laboratory, European Institute of Oncology, and University of Milan, Milan, Italy. Via Ripamonti 435, Milan, 20141, Italy. giuseppe.viale@ieo.it

[5]Giuseppe Curigliano. Division of Experimental Therapeutics, European Institute of Oncology, Milan, Italy. Via Ripamonti 435, Milan, 20141, Italy. giuseppe.curigliano@ieo.it

[6]Carmen Criscitiello. Division of Experimental Therapeutics, European Institute of Oncology, Milan, Italy. Via Ripamonti 435, Milan, 20141, Italy. carmen.criscitiello@ieo.it

[7]István Láng. National Institute of Oncology, Budapest, Hungary. Ráth György u. 7/9, H-1122 Budapest, Hungary. lang@oncol.hu

Correspondence and reprint requests: Dr. Giancarlo Pruneri. Department of Pathology and Laboratory Medicine, European Institute of Oncology, Via Ripamonti 435, Milan, 20141, Italy, phone: +390257489414, giancarlo.pruneri@ieo.it. **Prepublication Correspondence:** Ms. Karen Price, Department of Biostatistics and Computational Biology, Dana Farber Cancer Institute, 450 Brookline Avenue, Boston, MA 02215, 617 632 3603 (phone) 617 632 2444 (fax), karen.price@jimmy.harvard.edu.

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Informed consent: Informed consent was obtained from all individual participants included in the study.

[8]Thomas Ruhstaller. Breast Center St. Gallen, Switzerland, Swiss Group for Clinical Cancer Research (SAKK), Bern, Switzerland. Kantonsspital St. Gallen 9007, St. Gallen, Switzerland. thomas.ruhstaller@kssg.ch

[9]Lorenzo Gianni. Divisione di Oncologia, Ospedale degli Infermi, Rimini, Italy. Viale Settembrini 2 47923, Rimini, Italy. lorenzo.gianni@auslromagna.it

[10]Aron Goldhirsch. Program for Breast Health, European Institute of Oncology, Milan, Italy. Via Ripamonti 435, Milan, 20141, Italy. aron.goldhirsch@ibcsg.org

[11]Roswitha Kammler. International Breast Cancer Study Group, Translational Research Coordination and Central Pathology Office, IBCSG Coordinating Center, Bern, Switzerland. Effingerstrasse 40 CH-3008, Bern, Switzerland. Rosita.Kammler@ibcsg.org

[12]Karen N. Price, IBCSG Statistical Center and Frontier Science and Technology Research Foundation, Boston, MA. 450 Brookline Avenue, Boston, MA 02215, USA. price@jimmy.harvard.edu

[13]Giuseppe Cancello. Division of Medical Senology, European Institute of Oncology, Milan, Italy. Via Ripamonti 435, Milan, 20141, Italy. giuseppe.cancello@ieo.it

[14]Elisabetta Munzone. Division of Medical Senology, European Institute of Oncology, Milan, Italy. Via Ripamonti 435, Milan, 20141, Italy. elisabetta.munzone@ieo.it

[15]Richard D. Gelber. IBCSG Statistical Center, Department of Biostatistics and Computational Biology, Dana-Farber Cancer Institute, Harvard T.H. Chan School of Public Health, Harvard Medical School, Frontier Science and Technology Research Foundation, Boston, MA. 450 Brookline Avenue, Boston, MA 02215, USA. gelber@jimmy.harvard.edu

[16]Meredith M. Regan. IBCSG Statistical Center, Department of Biostatistics and Computational Biology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA. 450 Brookline Avenue, Boston, MA 02215, USA. mregan@jimmy.harvard.edu

[17]Marco Colleoni. Division of Medical Senology, European Institute of Oncology, Milan, Italy. Via Ripamonti 435, Milan, 20141, Italy. marco.colleoni@ieo.it

Abstract

Purpose—To assess the prognostic and predictive value of tumor-infiltrating lymphocytes (TILs) in the triple negative breast cancer (TNBC) cohort of the phase III IBCSG trial 22-00, comparing low-dose oral ‘metronomic’ cyclophosphamide-methotrexate maintenance chemotherapy (CM-maintenance) to no CM-maintenance (No-CM) in early breast cancer.

Methods—TILs were evaluated in full-face hematoxylin and eosin–stained sections of tumor samples confirmed centrally as TNBC (<1% of ER and PgR immunoreactivity and absence of HER2 overexpression or amplification). Mononuclear cells were evaluated in the stromal area within the borders of the invasive tumor. The primary endpoint was breast cancer-free interval (BCFI). Cox proportional hazards regression model assessed the association of BCFI and secondary endpoints with TILs score.

Results—In the 647 tumor samples, the median percentage of TILs was 18% (IQR=8%–40%), with 18% having TILs ≥50% (lymphocyte predominant breast cancer, LPBC). At a median follow-

up of 6.9 years, TILs were associated with better prognosis. For every 10% increase of TILs, BCFI risk reduction was 13% (HR=0.87,95% CI:0.79–0.95,*P*=0.003). DFS, DRFI and OS risk reductions were 11% (*P*=0.005), 16% (*P*=0.003), and 17% (*P*<0.001), respectively. Multivariable analysis confirmed the independent prognostic value of TILs. No significant TILs-by-treatment interaction was observed (*P*=0.39) for associations of TILs with BCFI, although patients with LPBC receiving CM-maintenance had a greater breast cancer risk reduction (HR=0.64,95% CI: 0.23–1.78), than those with non-LPBC (TILs <50%) (HR 0.96,95% CI:0.67–1.40).

Conclusions—TILs score is a potent prognostic factor in patients with TNBC. Low-dose chemotherapy confers a greater (not statistically significant) clinical benefit in patients with LPBC.

Keywords

tumor infiltrating lymphocytes; triple negative; low-dose maintenance chemotherapy; breast cancer; prognosis

INTRODUCTION

Tumor infiltrating lymphocytes (TILs) are associated with a favorable prognosis in breast cancer patents, especially among women with triple negative breast cancer (TNBC) [1–5]. Since the seminal work by Loi et al [1], several groups, including our own [5], demonstrated a positive linear correlation between TILs and breast cancer-specific survival. Furthermore, we recently confirmed the validity of the TILs assessment guidelines in the clinical setting [5], and the International TILs Working Group (TILs WG) [6] has carried out ring studies for improving decentralized TILs scoring reproducibility [Denkert et al., JAMA Oncol, submitted]. These data unequiivocally indicate that TILs may soon enter the clinical arena as a novel prognosticator for patients with TNBC. Nevertheless, there is no convincing evidence that TILs predict sensitivity to different chemotherapy regimens. Most available reports are in patients treated with anthracycline-containing chemotherapy regimens [1–3], although we recently reported that each 10% increase of TILs conferred a significant risk reduction also in patients treated with cyclophosphamide, methotrexate, and fluorouracil (CMF) [5].

Preclinical and clinical studies support the notion that non-toxic, low-dose continuous chemotherapy with cyclophosphamide and methotrexate (CM-maintenance) is of clinical value in TNBC [7–10]. In the present study, we evaluated TILs in patients with TNBC enrolled in the randomized, phase III International Breast Cancer Study Group (IBCSG) Trial 22-00, testing the efficacy of low-dose oral CM-maintenance following standard adjuvant chemotherapy treatment for hormone receptor-negative breast cancer patients.

Trial 22-00 reported a non-significant reduction of the relative risk of recurrence in patients with TNBC treated with CM-maintenance, suggesting that this schedule may be a reasonable therapeutic option, also considering its low cost and tolerability [10]. In this study we evaluated the prognostic value of TILs in the largest phase III randomized TNBC cohort reported to date, and the interaction of TILs with low-dose maintenance chemotherapy.

PATIENTS AND METHODS

Patients and samples

IBCSG Trial 22-00 is a multi-center, randomized, adjuvant phase III trial comparing low-dose CM-maintenance (cyclophosphamide 50 mg/day orally continuously for 1 year and methotrexate 2.5 mg/twice a day orally, days 1 and 2 of every week for 1 year) to no maintenance chemotherapy (No-CM), following breast cancer surgery and standard induction chemotherapy [10]. The trial recruited 1081 patients with estrogen (ER) and progesterone (PgR) receptor-negative tumors, and any HER2 and nodal status. For the present study, we limited our analysis to patients with tumors confirmed centrally as TNBC (<1% of ER and PgR immunoreactivity and absence of HER2 overexpression or amplification) [11]. ER and PgR assessment used the ER/PgR PharmDX kits, Dako, and HER2 status was assessed using the immunohistochemistry with the HercepTest, Dako, and FISH with PathVysion HER2 DNA probe kit, Vysis-Abbott, when necessary [12]. In addition, the IBCSG Central Pathology Office re-evaluated tumor type and grade, and Ki-67 labeling index (MIB-1 monoclonal antibody, 1:200) [12–14]. The eligibility criteria for Trial 22-00 excluded patients with history of previous malignancies (except for primary skin cancer) and prior neoadjuvant therapy for breast cancer. Patients in the analytic cohort consented to use of their tumor tissue for research purposes, and the project was approved by the IBCSG Biological Protocols Working Group.

The analysis of TILs was retrospectively performed in full-face hematoxilyn and eosin sections, strictly adhering to the criteria proposed by the TILs WG [6]. Briefly, all mononuclear cells (i.e. lymphocytes and plasma cells) in the stromal compartment within the borders of the invasive tumor were evaluated and reported as a percentage (TILs score). TILs outside of the tumor border, around DCIS and normal breast tissue, as well as in areas of necrosis, if any, were not included in the scoring. Tumors with TILs score of ≥50% were considered lymphocyte predominant breast cancer (LPBC). TILs were assessed as a continuous measure (score) and as a dichotomous measure (LPBC versus non-LPBC).

Statistical analysis

The primary endpoint was breast cancer-free interval (BCFI), defined as the time from randomization to the recurrence of invasive breast cancer (local, regional or distant) or invasive contralateral breast cancer. Secondary endpoints included disease-free survival (DFS): time from randomization to the first appearance of invasive recurrence of breast cancer (local, regional or distant), invasive contralateral breast cancer, second (non-breast) invasive cancer or death without prior cancer event; distant recurrence-free interval (DRFI): time from randomization to the recurrence of breast cancer at a distant site; and overall survival (OS): time from randomization to death from any cause. In the absence of BCFI, DFS, DRFI or OS events, endpoints were censored at date of last follow-up or known alive.

Cox proportional hazards regression models [15] were stratified by menopausal status and type of induction chemotherapy, and assessed the associations of BCFI, DFS, DRFI and OS with TILs and estimated the hazards ratio (HR) and 95% confidence interval (CI).

Multivariable models included age at randomization: <40, 40–49, 50–59, ≥60 years; lymph node status: N0, N+ 1–3, N+ ≥4; tumor size: ≤2, >2 cm; and tumor grade: 1–2, 3.

The distribution of study endpoints according to TILs (LPBC versus non-LPBC) was estimated using the Kaplan-Meier method [16]. The predictive role of TILs was investigated by including the TILs (LPBC)-by-treatment interaction term in the Cox model. Subpopulation Treatment Effect Pattern Plot (STEPP) [17] analysis was also used to explore the relationship between TILs score along the continuum and 5-year BCFI percentages for the two treatment groups.

With an analytic cohort of 647 patients (Fig. 1), assuming 18% of patients with LPBC, the cohort would have 81% power (two-sided type I error of 0.05) to detect a HR of 0.52 comparing LPBC versus non-LPBC. Treatment-by-TILs (LPBC) interaction was tested using Cox models with 1-df Wald test. With our LPBC and breast cancer event distributions, there was 70% power to detect an interaction hazard ratio of 0.44 (treatment HR (0.47) for LPBC relative to treatment HR (0.95) for non-LPBC) using large sample approximation. Therefore, the statistical power for treatment-by-TILs (LPBC) interaction is likely limited, so inference regarding differential treatment effects should be interpreted with caution.

Associations between the TILs as a continuous variable and patient and disease characteristics were evaluated using the Wilcoxon test (two groups comparison) or Kruskal-Wallis test (>2 groups comparison).

All analyses were pre-specified and followed the reporting recommendations for tumor marker prognostic studies criteria (REMARK) [18]. Two-sided p values were reported for all analyses. To assess the analytic validity of the TILs score, a subset of 70 (~10% of the analysis cohort) samples was independently read by two pathologists, and the inter-observer correlation was measured as the proportion with scores within ±10 points, with exact binominal CI.

RESULTS

Patients

After routine central assessment of ER, PgR and HER2 in Trial 22-00, 724 samples were identified as TNBC. TILs were evaluated from 672 tumors with adequate submitted tissue and consent, and 25 specimens could not be assessed, resulting in the analysis cohort of 647 patients, 340 assigned to CM-maintenance and 307 to No-CM (Fig. 1). Characteristics of the analytic cohort are shown in Table 1. The median follow-up was 6.9 years (95% CI: 6.6–7.2), and median age was 52 years (interquartile range (IQR) 44–59), with 45% of the patients being premenopausal at study entry. Breast-conserving surgery was performed in 79%, and the axillary lymph node status was N0 in 57% if the patients. Induction chemotherapy regimens included anthracyclines for 83% of patients.

Distributions of TILs and associations with clinico-pathological characteristics

The median TILs score was 18% (IQR 8%–40%) (Table 1). TILs score distributions according to treatment, age, nodal status, tumor grade, and tumor size are shown in Figure 2.

In the assessment of stromal lymphocytic infiltration the two pathologists had an agreement rate of 96% (95% CI 88%–99%), and no evidence of bias was found in the Bland-Altman plot (data not shown). Patients with grade 3 had a higher median TIL score (20%, IQR 8%–40%) than those with grade 1 or 2 (8%, IQR 5%–16%); younger patients (age 23-<40) had a highest median TIL score (26%, IQR 10%–52%) compared with patients aged 40-<50 (20%, IQR 10%–45%), 50-<60 years (15%, IQR 6%–30%) and 60–80 years (15%, IQR 7%–32%) (Supplementary Appendix Fig. 1).

Associations of TILs with BCFI

Continuous TILs was associated with BCFI in univariable analysis, with an estimated HR for a 10-point change in TILs of 0.87 (95% CI 0.79–0.95, P=0.003). Multivariable analysis stratified by menopausal status and induction therapy type confirmed TILs as a prognostic marker for BCFI with an estimated HR of 0.87 (95% CI: 0.79–0.96, P=0.006) after adjusting for age, nodal status, tumor size, and tumor grade. Consistent results were observed when TILs score was assessed as a binary variable (LPBC versus non-LPBC), with an estimated HR of 0.56 (95% CI: 0.33 to 0.94, P=0.03) (HR=0.61, 95% CI: 0.36 to 1.03, P=0.06, in MVA) (Fig. 2 and Table 2).

Associations of TILs with DFS, DRFI and OS

In multivariable analyses, continuous TILs were associated with DFS, DRFI and OS, with an estimated HR for a 10-point change in TILs of 0.90 (95% CI: 0.82–0.97, P=0.01), 0.83 (95% CI: 0.74–0.94, P=0.004), and 0.83 (95% CI: 0.74–0.93, P<0.001), respectively. When assessed as a binary variable (LPBC versus non-LPBC), the estimated HRs for DFS, DRFI and OS were 0.70 (95% CI: 0.44–1.10, P=0.13); 0.42 (95% CI: 0.2–0.89, P=0.02) and 0.48 (95% CI: 0.25–0.90, P=0.02), respectively (Fig. 2 and Table 2).

Predictive Value of TILs

No TILs (LPBC)-by-treatment interaction was observed (interaction p-values range 0.32–0.77), although patients with LPBC receiving CM-maintenance had greater reduction in risk of breast cancer (HR=0.64, 95% CI: 0.23–1.78) *versus* No-CM than was observed among non-LPBC patients (HR=0.96, 95% CI: 0.67–1.40) (Table 3).

A STEPP analysis to explore the TILs-by-treatment interaction according to TILs score as a continuous measure, also indicated a possible greater beneficial effect of CM-maintenance for patients with higher TILs scores, with the differential treatment effect emerging when TILs score was about 30% (Fig. 3). Similar results were observed for DFS, DRFI and OS (not shown).

DISCUSSION

A growing body of evidence suggests that tumor immune response is associated with the clinical behavior of TNBC patients. Preclinical data suggest that both high-dose and low-dose chemotherapy regimens play an active role in modulating anti-tumor immunity. Anthracyclines are capable of sustaining an anticancer immune response via different mechanisms, as recruitment and differentiation of anti-tumoral antigen presenting cells or

impairing of pro-tumoral myeloid-derived suppressor cells [19–21]. Besides exerting its antitumoral effects through tumor angiogenesis inhibition [22,23], low-dose chemotherapy has also been shown to selectively deplete the immune-inhibitory T-reg subpopulation, eventually enhancing effector T-cell antitumoral activity [24]. Along this line, low-dose cyclophosphamide plus gemcitabine administered metronomically has been reported to induce anti-tumor T cell immunity in CT26 colon carcinoma-bearing mice [25].

In the present study, we confirm that TILs—a surrogate of adaptive anti-tumor immunity—are a powerful prognostic indicator in the adjuvant setting for patients with TNBC, independent of age, nodal status and tumor size. The TILs effect size in the cohort as a whole was remarkably similar to previous findings, with an adjusted overall survival HR=0.84 for each 10% increment in TILs, as compared to 0.81–0.83 reported by Loi et al. [1,2] and 0.76 by our group in a previous retrospective study [5]. We also provide further evidence that TILs WG guidelines have clinical validity and are highly reproducible: median TILs scores (21%) and LPBC prevalence (18%) were consistent with previous observations, and the interobserver agreement between two different readers was excellent (0.96 intra-class correlation coefficient).

The aforementioned data corroborate the notion that TILs are likely a prognostic marker in TNBC. However, their putative predictive value has not yet been elucidated, as most of the studies included patients treated with anthracycline-containing chemotherapy regimens, and we recently reported that TILs was also a prognostic factor in patients treated with CMF alone [5]. Likewise, the interaction of TILs with trastuzumab, originally postulated by Loi et al. in the FinHER trial [2], has recently been questioned. Perez et al. investigated the association of TILs with recurrence-free survival in 945 women with HER2 positive breast cancer randomized to chemotherapy or chemotherapy plus trastuzumab within the N9831 trial, reporting that TILs were associated with survival in patients treated with chemotherapy alone, but not with chemotherapy plus trastuzumab [26]. Along this line, TILs have been recently reported to be a prognostic marker in patients with HER2-positive, early-stage breast cancer randomized in the neoadjuvant NeoALTTO trial to three treatment arms: trastuzumab, lapatinib, or the combination for six weeks followed by the addition of weekly paclitaxel for 12 weeks before surgery, followed by 3 cycles of fluorouracil, epirubicin, and cyclophosphamide after surgery. Although these data suggest a possible role of TILs in predicting benefit of anti-HER2 therapy, the authors did not exclude the likelihood that the prognostic effect of TILs could predominantly result from chemotherapy [27].

In the present study, we explored the predictive role of TILs in patients randomized to receive low-dose CM-maintenance or No-CM after standard induction chemotherapy. The published results of the trial showed a trend toward a benefit for the triple-negative node-positive disease subgroup, prompting the authors to conclude that CM-maintenance should be further evaluated in high risk patients with TNBC [10]. In the present study, the TILs-by-treatment interaction test did not show a statistically significant association between TILs and CM-maintenance, but we found that LPBC was associated with a greater observed magnitude of CM-maintenance treatment benefit than non-LBPC.. Also, the exploratory STEPP analysis showed a possible greater effect of CM-maintenance for patients with higher TILs score, emerging as TILs score reached about 30%. Collectively, these data

suggest that further studies addressing whether TILs may identify a patient population possibly benefiting from low-dose maintenance chemotherapy strategies would be valuable. Our results confirm that TILs are a potent prognostic indicator in patients with TNBC, and that CM-maintenance may confer a greater clinical benefit for patients with LPBC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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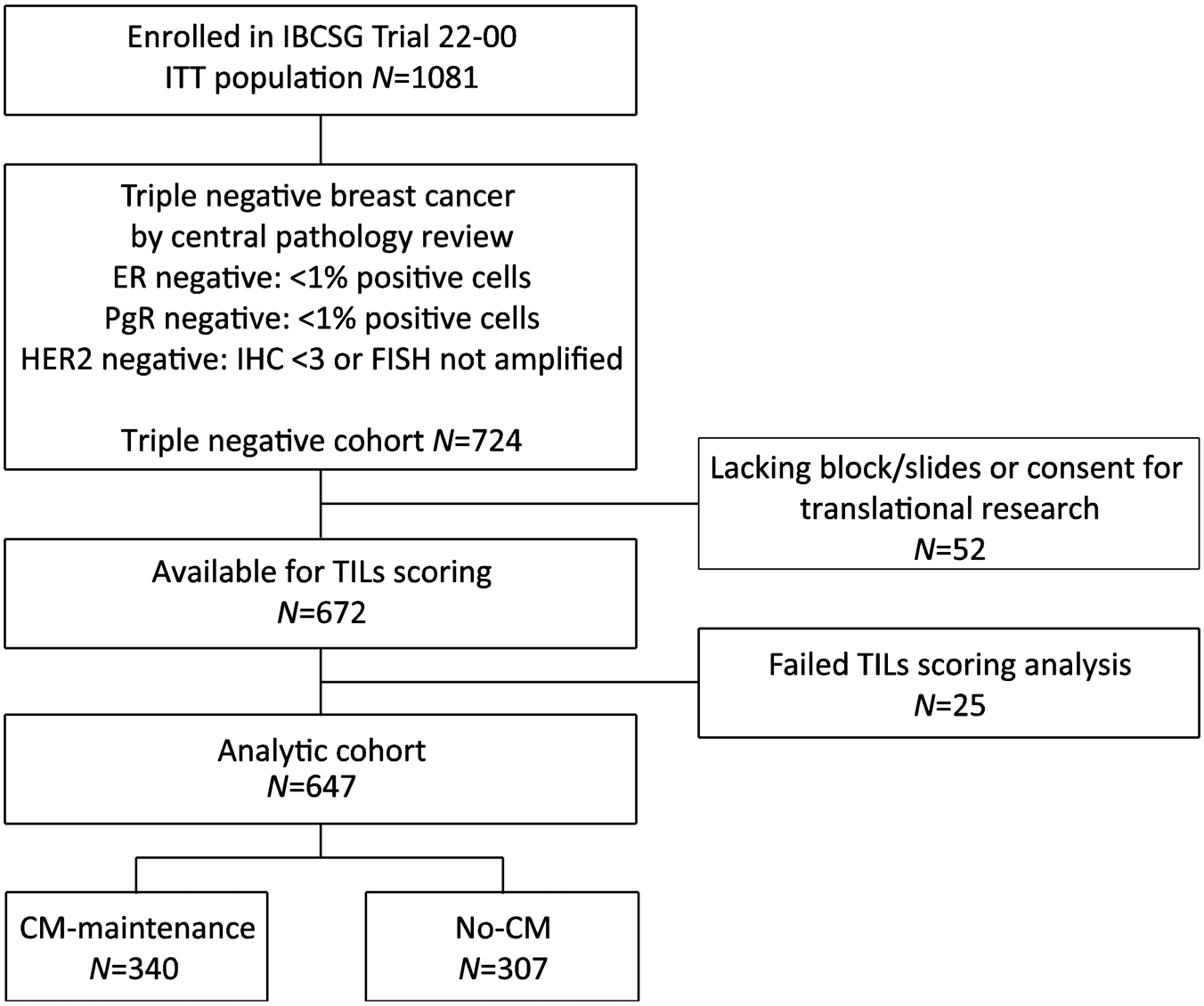

Figure 1.

Flow diagram showing the derivation of the analytic cohort of patients enrolled in IBCSG Trial 22-00 whose tumors were triple negative by central assessment and were successfully assessed for tumor-infiltrating lymphocytes (TILs)

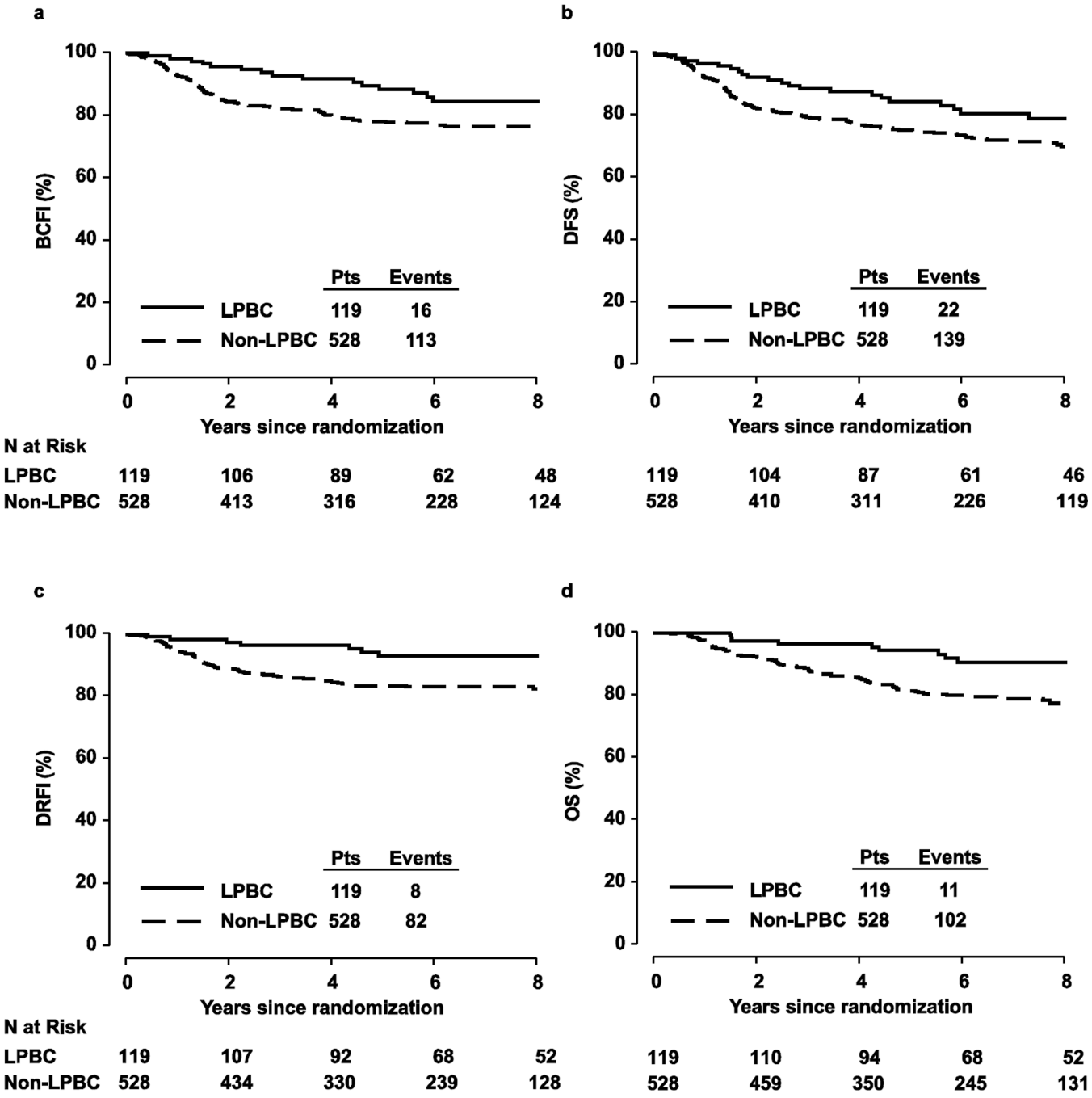

Figure 2.

Kaplan-Meier estimates of (a) breast cancer-free interval (BCFI), (b) disease-free survival (DFS), (c) distant recurrence-free interval (DRFI), and (d) overall survival (OS) according to TILs status dichotomized as lymphocyte predominant breast cancer (LPBC, TILs score ≥50%) versus non-LPBC (TILs score <50%)

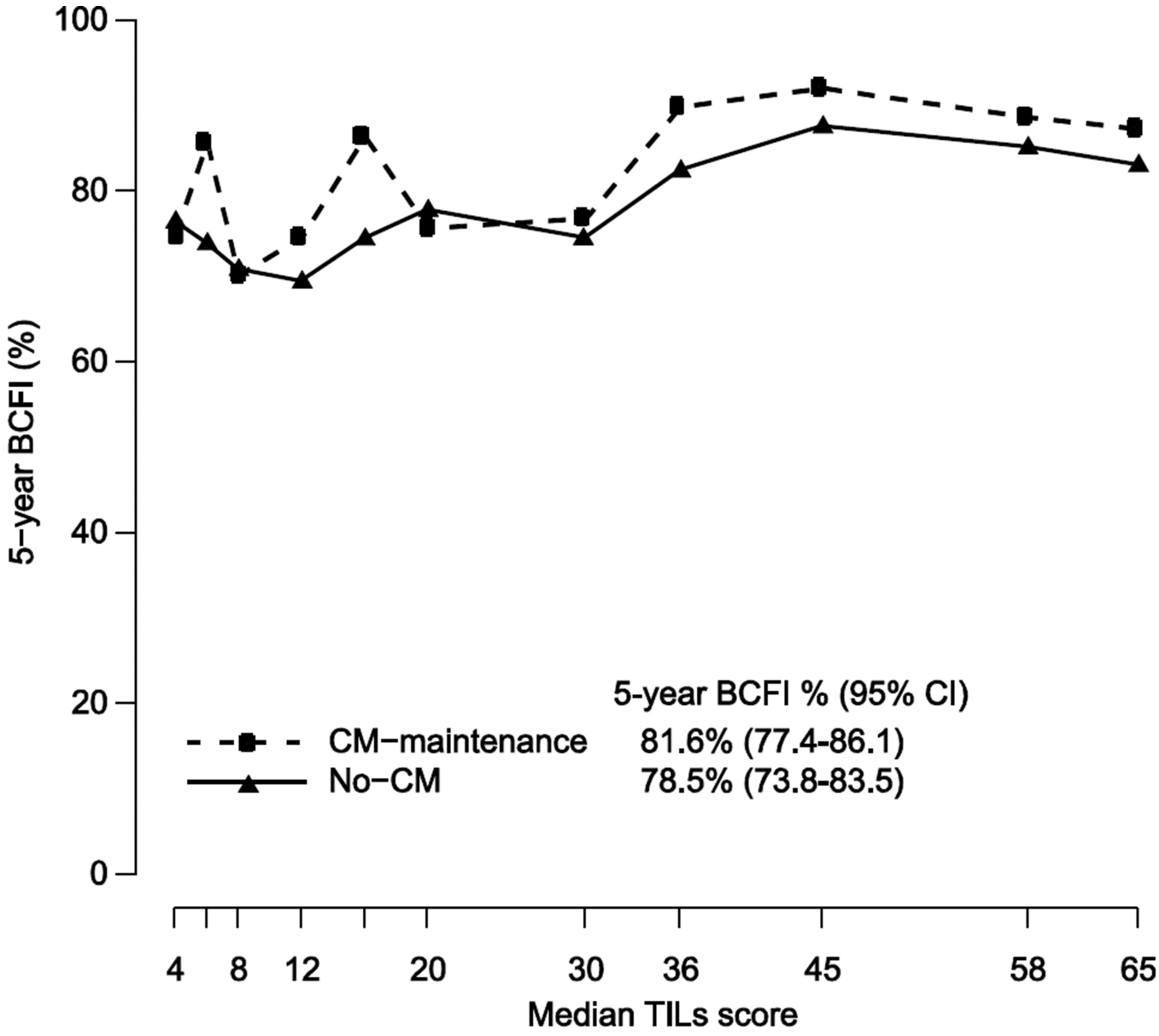

Figure 3.

STEPP analysis showing estimates of 5-year breast cancer-free interval % (BCFI) (y-axis) separately for the IBCSG Trial 22-00 randomized treatment groups (CM-maintenance versus No-CM) for subpopulations of patients defined according to increasing median values of TILs score (from left to right along the x-axis)

Table 1

Characteristics of patients with centrally-confirmed TNBC in IBCSG Trial 22-00 overall and according to TILs (LPBC) status

Characteristics		Total (n=647)	Non-LPBC (n=528)	LPBC (n=119)
Median follow-up, years		6.9 (6.6 to 7.2)	6.8 (6.6 to 7)	8.0 (6.9 to 9.0)
Treatment assignment, *N*(%)	CMM	340 (53)	270 (51)	70 (59)
	OBS	307 (47)	258 (49)	49 (41)
Premenopausal, *N*(%)		289 (45)	223 (42)	66 (55)
Induction chemotherapy, *N*(%)	Anthracycline+Taxanes ±CMF	171 (26)	149 (28)	22 (18)
	Anthracycline±CMF	367 (57)	293 (55)	74 (62)
	CMF	109 (17)	86 (16)	23 (19)
Age at randomization (yrs)	Median (IQR)	52 (44,59)	53 (44,60)	49 (41,56)
	<40	88 (14)	64 (12)	24 (20)
	40-<50	185 (29)	147 (28)	38 (32)
	50-<60	217 (34)	180 (34)	37 (31)
	>=60	157 (24)	137 (26)	20 (17)
White race, *N*(%)		626 (97)	511 (97)	115 (97)
Nodal status, *N*(%)	N0	370 (57)	290 (55)	80 (68)
	N+ 1-3	171 (27)	144 (27)	27 (23)
	N+ >=4	103 (16)	92 (17)	11 (9)
	Unknown	3	2	1
Tumor size, *N*(%)	T1 (0-2 cm)	291 (45)	230 (44)	61 (51)
	T2 (>2-5 cm)	329 (51)	272 (52)	57 (48)
	T3 (>5 cm)	27 (4)	26 (5)	1 (1)
Tumor grade (CPR+local), *N*(%)	1	3 (0)	3 (1)	0 (0)
	2	76 (12)	70 (13)	6 (5)
	3	568 (88)	455 (86)	113 (95)
Histology by CPR, *N*(%)	Ductal	604 (93)	488 (92)	116 (97)
	Lobular	5 (1)	5 (1)	0 (0)
	Other/unknown history	38 (6)	35 (7)	3 (3)
Ki-67 labeling index (CPR)	Median (IQR)	45 (31,65)	45 (30,65)	54 (40,75)
Breast-conserving surgery, *N*(%)		513 (79)	412 (78)	101 (85)
TILs score (% of cells)	Median (IQR)	18 (8,40)	14 (6,25)	60 (55,70)

Abbreviations: TILs: tumor-infiltrating lymphocytes; LPBC: Lymphocyte-predominant breast cancer (TILs score ≥50%); CPR: central pathology review; IQR: inter-quartile range

Table 2

Association of outcome with TILs as a continuous measure (linear) and dichotomized as LPBC, TILs (≥50%) versus non-LPBC (<50%) in multivariable Cox models.

Study endpoints	Patients (events)	Univariable HR (95%CI)	*P*-value	*Multivariable HR (95%CI)	*P*-value
TILs (10% increase)					
BCFI	647 (129)	0.87 (0.79 to 0.95)	0.003	0.87 (0.79 to 0.96)	0.006
DFS	647 (161)	0.89 (0.82 to 0.97)	0.005	0.9 (0.82 to 0.97)	0.01
DRFI	647 (90)	0.84 (0.74 to 0.94)	0.003	0.83 (0.74 to 0.94)	0.004
OS	647 (113)	0.83 (0.74 to 0.92)	<0.001	0.83 (0.74 to 0.93)	<0.001
LPBC vs. no LPBC (reference)					
BCFI	119 (16) vs. 528 (113)	0.56 (0.33 to 0.94)	0.03	0.61 (0.36 to 1.03)	0.06
DFS	119 (22) vs. 528 (139)	0.65 (0.41 to 1.02)	0.06	0.7 (0.44 to 1.11)	0.13
DRFI	119 (8) vs. 528 (82)	0.39 (0.19 to 0.8)	0.01	0.42 (0.2 to 0.89)	0.02
OS	119 (11) vs. 528 (102)	0.42 (0.22 to 0.78)	0.006	0.47 (0.25 to 0.89)	0.02

*adjusted for age, nodal status, tumor size, and tumor grade, stratified by menopausal status and induction chemotherapies

Abbreviations: TILs: tumor-infiltrating lymphocytes; LPBC: Lymphocyte-predominant breast cancer (TILs score ≥50%); BCFI: breast cancer-free interval; DFS: disease-free survival; DRFI: distant recurrence-free interval; OS: overall survival; HR: hazards ratio; CI: confidence interval

Table 3

Association of outcomes with treatment assignment according to TILs score dichotomized into LPBC (TILs ≥50%) and non-LPBC (TILs <50%)

			TILs as predictive marker				
	Overall CM-Maintenance vs. No-CM		LPBC CM-Maintenance vs. No-CM		Non-LPBC CM-Maintenance vs. No-CM		
Endpoint	Patients (events)	HR (95%CI)	Patients (events)	HR (95%CI)	Patients (events)	HR (95%CI)	Int-*P*
BCFI	340 (62) vs. 307 (67)	0.89 (0.63 to 1.26)	70 (7) vs. 49 (9)	0.64 (0.23 to 1.78)	270 (55) vs. 258 (58)	0.96 (0.67 to 1.40)	0.41
DFS	340 (79) vs. 307 (82)	0.92 (0.67 to 1.26)	70 (11) vs. 49 (11)	0.74 (0.3 to 1.79)	270 (68) vs. 258 (71)	0.96 (0.68 to 1.34)	0.77
DRFI	340 (44) vs. 307 (46)	0.92 (0.6 to 1.39)	70 (3) vs. 49 (5)	0.5 (0.11 to 2.25)	270 (41) vs. 258 (41)	1.02 (0.66 to 1.57)	0.32
OS	340 (54) vs. 307(59)	0.89 (0.61 to 1.29)	70 (5) vs. 49 (6)	0.64 (0.18 to 2.26)	270 (49) vs. 258 (53)	0.93 (0.63 to 1.38)	0.70

All estimates were from multivariable analysis adjusted for age, nodal status, tumor size, and tumor grade, stratified by menopausal status and induction chemotherapies.

Abbreviations: Int-*P*=LPBC-by-treatment interaction test *P*-value; CM: low-dose cyclophosphamide and methotrexate; TILs: tumor-infiltrating lymphocytes; LPBC: Lymphocyte-predominant breast cancer (TILs score ≥50%); BCFI: breast cancer-free interval; DFS: disease-free survival; DRFI: distant recurrence-free interval; OS: overall survival; HR: hazards ratio; CI: confidence interval