Influence of mutagenic and non-mutagenic pre-operative chemotherapy on the immune infiltration of breast cancer

Anna-Mária Tőkés[1*], Orsolya Rusz[2*], Gábor Cserni[3,4], Erika Tóth[5], Gábor Rubovszky[5], Tímea Tőkés[6], Laura Vízkeleti[1,7], Lilla Reiniger[7,8], Renáta Kószó[2], Zsuzsanna Kahán[2], Janina Kulka[1], Marco Donia[9,10], András Vörös[3], Zoltán Szállási[7,11,12#]

[1]Semmelweis University, 2nd Department of Pathology, Budapest, Hungary

[2]University of Szeged, Department of Oncotherapy, Szeged, Hungary

[3]University of Szeged, Department of Pathology, Szeged, Hungary

[4]Department of Pathology, Bács-Kiskun County Teaching Hospital, Kecskemét, Hungary

[5]National Institute of Oncology, Budapest, Hungary

[6]Semmelweis University, Oncology Center, Budapest, Hungary

[7]MTA-SE-NAP B Brain Metastasis Research Group, 2nd Department of Pathology, Semmelweis University, Budapest, Hungary.

[8]1st Department of Pathology and Experimental Cancer Research, Semmelweis University, 1085, Üllői út 26., Budapest, Hungary

[9]Center for Cancer Immune Therapy, Department of Hematology, Herlev Hospital, University of Copenhagen, Copenhagen, Denmark.

[10]Department of Oncology, Herlev Hospital, University of Copenhagen, Copenhagen, Denmark

[11]Department of Bio and Health Informatics, Technical University of Denmark, Kemitorvet 208, 2800 Lyngby, Denmark

[12]Computational Health Informatics Program, Boston Children's Hospital, USA, Harvard Medical School, Boston, USA

*These authors contributed equally

[#]to whom correspondence should be addressed: Zoltan.szallasi@childrens.harvard.edu

Abstract

Background: Induction of neo-epitopes is one of the mechanisms by which neoadjuvant chemotherapy of breast cancer is thought to increase the number of stromal tumor-infiltrating lymphocytes (StrTILs). It is not known, however, whether treatment with agents with significantly different mutagenic ability and thus presumably inducing a different number of neo-epitopes is also inducing a significantly different number of TILs in the clinical setting. Investigating whether such a correlation exists was the aim of the current study.

Patients and Methods: Patients with residual breast carcinoma receiving platinum-based, cyclophosphamide-based or anthracycline-based pre-operative chemotherapy followed by breast surgery, were retrospectively selected. The percentage of StrTIL was evaluated on hematoxylin-eosin stained slides of core biopsy (pre-StrTIL) and surgical tumor samples (post-StrTIL) according to the most recent recommendation of International TILs Working Group. In survival analyses, TIL changes (ΔStrTIL) were calculated from the difference between post-StrTIL and pre-StrTIL.

Results: Of the 112 cases, 58.0% (n=65) were hormone receptor (HR) positive and 42.0% (n=47) were HR negative. The platinum-based, cyclophosphamide-based and anthracycline-based therapy groups consisted of 28, 42 and 42 patients, respectively. . Following the pre-operative chemotherapies, the median post-StrTIL increased significantly to 6.25% (interquartile range: 3.00-25.00; $p<0.001$). Significantly more positive StrTIL changes were observed in cases with HR negative ($p<0.001$) and HR positive/HER2negative/grade 3 ($p=0.007$) carcinomas, but not in HR positive/HER2 negative/grade 1-2 ($p=0.075$). We found only a weak association between StrTIL changes and used treatment regimens.

Both post-StrTIL and ΔStrTIL had independent prognostic role in HR negative cases. Each 1% increase in post-StrTIL reduced the hazard of distant metastases development by 2.6% (hazard ratio: 0.974; CI: 0.948-1.000; P=0.05) and for each 1% ΔStrTIL increment, the risk of distant metastases was reduced by 4.3% (hazard ratio: 0.957; CI: 0932-0.983; P=0.001). The prognostic role of TIL in HR positive cases could not be proven.

Conclusions: Pre-operative treatment with the highly mutagenic agent platinum and moderately mutagenic cyclophosphamide induced somewhat more significant increase in TILs relative to the anthracycline based therapy with no apparent capacity to induce neo-epitopes. This difference was, however, moderate and does not seem to justify preference of one treatment option over another one from an onco-immunological point of view.

Keywords: Tumor infiltrating lymphocytes, mutagenic capacity, neoadjuvant treatment, breast cancer

Introduction

Appropriate stimulation of a patient's immune response can result in durable control of widely metastatic solid tumors. However, such clear clinical benefit is observed only in a relatively small proportion of patients [1,2].

An increasing body of experimental evidence underlines the importance of somatic coding genetic alterations, i.e. mutations, for recognition of cancer by the human immune system. The various forms of coding mutations are often translated into altered proteins including novel peptide sequences, which can become "neo-epitopes" on the surface of tumor cells ready to be scanned by the patient's immune repertoire of T cells. These neo-epitopes are believed to be particularly immunogenic because they are not encoded in the normal genome of the individual patient, thus reactive T cells are not subjected to central tolerance. Recognition of neo-epitopes by cytotoxic T cells can lead to immune-mediated tumor regression. However, given the highly variable and individual nature of somatic tumor mutations, specific interventions to target neo-epitopes are technically very challenging.

The burden of neo-epitopes (which is strongly correlated with the total mutational load) is a strong predictor of response to current cancer immunotherapies [3,4]. This association is thought to reflect the level of how "foreign" is a given tumor, which would then be associated with stronger immune responses.

We have previously experimentally classified current chemotherapy regimens as highly (platinum-based), moderately (cyclophosphamide) and marginally/non (paclitaxel, doxorubicine and gemcitabine) mutagenic [5]. Building on these data, we hypothesized that further induction of mutations and neo-epitopes with mutagenic chemotherapy might result in stronger immune reactions in the tumor microenvironment, and this could be reflected by a larger increase in lymphocytic infiltration. This prompted us to investigate whether highly mutagenic chemotherapy induces a larger increase in lymphocytic infiltration compared to low/non-mutagenic chemotherapy when administered as neoadjuvant treatment for breast cancer.

Methods

Patients

112 patients with breast carcinoma treated with pre-operative chemotherapy in four Hungarian institutions (National Institute of Oncology, Onco-Radiology Center of Bács-Kiskun County Teaching Hospital, Semmelweis University and University of Szeged) between 2005 and 2017, were selected and their samples studied retrospectively. The inclusion criteria were as follows: availability of both core biopsy and surgical tumor sample, known clinical and treatment data, at least 2 cycles of chemotherapy administered before surgery, residual tumor after pre-operative chemotherapy. All patients underwent breast surgery. Of the 112 cases, 103 received chemotherapy plus surgery with curative intent, while 9 cases had bone metastases at the beginning of pre-operative chemotherapy. The tumor histological type was defined according to the most recent World Health Organization's classification [6].

Hormone receptor (HR) status was scored according to the respective current Hungarian Guidelines [7] and the American Society of Clinical Oncology/College of American Pathologists' recommendations [8]. A case was considered HR negative if the expression of estrogen receptor and progesterone receptor was <1%. HER2 positivity was evaluated conforming to the United Kingdom recommendations [7, 9]. Based on the HR and HER2 statuses, cases were grouped into 4 different subtypes (Table 1.)

According to the type of pre-operative chemotherapy, the patients were grouped into platinum-based, cyclophosphamide-based and anthracycline-based groups. The treatment regimens are presented in Table 1. All treatment regimens were of conventional doses and schedules, and selected based on valid international guidelines.

Pathology

Formalin fixed, paraffin embedded blocks of core biopsies and surgical specimens were retrieved from the four pathology departments. The study was approved by the Hungarian Medical Research Council (ETT-TUKEB 14383/2017) and it was conducted in accordance with the Declaration of Helsinki.

4μm sections of representative tumor blocks were stained with hematoxylin and eosin (H&E). The percentage of stromal tumor-infiltrating lymphocytes (StrTIL) was evaluated according to the recommendation of International TILs Working Group 2014 [10]. Histopathologic evaluation of StrTILs was performed by GCs, AMT, AV, ET and JK. Controversial cases were reevaluated and discussed.

Statistical analyses

SPSS 20.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The normality of the data was controlled by the Shapiro-Wilk test. The association between changes in StrTIL and clinico-pathological variables (pre-operative chemotherapy received, grade, immunohistochemical subtype and age) was calculated by the Wilcoxon-signed rank test.

The distant metastasis free survival (DMFS) was assessed and defined as the time interval between the first cycle of pre-operative chemotherapy and the date of distant relapse or death. Nine cases with bone metastases at baseline were censored from the DMFS analyses. Database was locked in December 2017. The prognostic value of StrTIL changes (ΔStrTIL: the difference between post-operative stromal tumor-infiltrating lymphocytes in surgical specimen (post-StrTIL) and pre-operative stromal tumor-infiltrating lymphocytes in core biopsy (pre-StrTIL) was tested as continuous variable. Hazard ratios and 95% confidence intervals (95% CI) were calculated with the Cox proportional hazard regression model. Multivariate Cox regression analysis included the following prognostic factors: age, grade, HR status, type of treatment, residual tumor size and post-treatment pathological lymph node status. The Kaplan–Meier method (the log-rank test) was used to analyze the role of ΔStrTIL in DMFS in HR negative and HR positive tumor groups separately.

All applied statistical tests were two-sided.

Results

Baseline disease characteristics

Samples from 112 individuals were available for analysis. Most patients (86.6%, n=97) had invasive carcinoma of no special type (NST), 58.0% (n=65) were HR positive and 42.0% (n=47) were HR negative. The clinico-pathological characteristics are reported in Table 1. At initiation of pre-operative chemotherapy, the patients had a mean age of 55 years (range: 29-80 years). The patients received platinum-based (n=28; 25%), cyclophosphamide-based (n=42; 37.5%) or anthracycline-based (n=42; 37.5%) therapy.

Of the 28 patients undergoing platinum-based therapy, 64.3% (n=18) were HR negative (mainly triple negative, 46.4% (n=13)) and 35.7% (n=10) were HR positive. According to the chemotherapy regimen used, 21 patients were treated with carboplatin + docetaxel or paclitaxel and 7 patients received cisplatin + docetaxel or paclitaxel.

Of the 42 patients undergoing cyclophosphamide-based therapy, 23.8% (n=10) were HR negative and 76.2% (n=32) were HR positive. 57.1% (n=24) received anthracycline (epirubicin (E) or doxorubicin) + cyclophosphamide (C) in combination with or without 5-fluorouracil (F) followed by docetaxel. The other commonly used treatment regimen in this group was FEC without taxane in 35.7% (n=15) of the cases (Table 1).

Of the 42 patients undergoing anthracycline-based therapy, 45.2% (n=19) had HR negative and 54.8% (n=23) had HR positive carcinomas. All cases were treated with anthracycline + taxane combination (of those 32/42 received epirubicin + docetaxel or paclitaxel).

The majority of patients received more than four cycles of chemotherapy, and the average cycle number was the same in each group (Table 1).

Of the 22 HER2 positive cases, 68.2% (n=15) received pre-operative trastuzumab therapy. This was administered in combination with platinum-based (n=8), cyclophosphamide-based (n=5) or anthracycline-based (n=2) therapy (Table 1).

StrTIL changes before and after chemotherapy

In the pre-operative core biopsy samples, the median pre-StrTIL was 3.00% (interquartile range (IQR): 1.00-7.50) and more than 50% StrTIL (lymphocyte predominant) was detected in only one case. The post-StrTIL reached 50% or above in 10 cases (the pre-operative therapy was platinum-based (n=4), FEC (n=1) or docetaxel + epirubicin (n=5)), (Fig. 1). The median post-StrTIL rose significantly to 6.25% (IQR: 3.00-25.00; $p<0.001$) after treatment. Pre-StrTIL <1% was observed in 14 cases, while StrTIL <1% in the residual tumor occurred in only two cases.

The increase in post-StrTIL was significant both in HR positive (ΔStrTIL positive: n=32 (49.2%); zero: n=21 (32.3%); negative: 12 (18.5%)) and HR negative (ΔStrTIL positive: n=29 (61.7%); zero: n=5 (10.6%); negative: n=13 (27.7%)) cases ($p<0.001$ in both groups; Table 2). In the subgroup of HR positive/HER2 negative cases, the changes in StrTIL was significant in grade 3 cases (ΔStrTIL positive: n=14 (66.7%); zero: n=3 (14.3%); negative: n=4 (19.0%); $p=0.007$) but not in grade 1-2 cases (ΔStrTIL positive: n=11 (36.6%); zero: n=14 (46.7%); negative: n=5 (16.7%); $p=0.075$; Table 2).

We did not detect any association between changes in StrTIL and other features (shown in Table 2). When analyzing the pre-StrTIL and post-StrTIL among the three treatment groups, we experienced significant StrTIL increase independently from the treatment applied (Table 2; Fig. 1a, 1d, 1g; Supplementary Table 1). Interestingly, in the subgroup analysis, only the administration of cyclophosphamide resulted in a significant increase in StrTIL in HR positive cases (ΔStrTIL positive: n=18 (56.3%); zero: n=10 (31.2%); negative: n=4 (12.5%); $p<0.001$; Table 2; Fig. 1c, 1f, 1i; Supplementary Table 1).

Survival analyses

Data on DMFS was available for 103 cases. The median DMFS was 28.2 months (range: 2.6-118.3 months). Distant metastases occurred in 31/103 (30.1%) cases. In 21/31 (67.7%) cases, the primary breast carcinoma was HR negative, and in 19/31 (61.3%) cases the post-StrTIL was lower than 10.0% or showed a decrease in comparison with the pre-StrTIL. As reported in Table 3, in univariate analyses, the HR status and the post-treatment pathological lymph node status were the only significant factors influencing DMFS. In the multivariate model, changes of StrTIL showed a strong prognostic value (Table 3). The Cox analysis in HR negative cases confirmed both post-StrTIL and ΔStrTIL as playing independent prognostic role in DMFS. Each 1% increase in post-StrTIL reduced the hazard of distant metastases development by 2.6% (hazard ratio: 0.974; CI: 0.948-1.000; $p=0.05$) and for each 1% ΔStrTIL increment, the risk of distant metastases was reduced by 4.3% (hazard ratio: 0.957; CI: 0932-0.983; $p=0.001$), but according to our results, the pre-StrTIL did not influence the DMFS. The prognostic role of TIL in HR positive cases could not be proven (Supplementary Table 2). The Kaplan-Meier analysis was carried out in HR negative and HR positive cases separately. Among HR negative cases, increased or unchanged post-StrTIL was associated with better survival (Fig. 2c).

Discussion

It has been established in several studies that tumor infiltrating lymphocytes in baseline, pretreatment biopsies of breast cancer are powerful prognostic markers of outcome in triple negative and HER2 positive breast cancers [11,12]. The correlation between posttreatment levels of TIL and outcome is less clear, perhaps due to the fact that cases with pathological complete response are often eliminated from further analysis. High TIL levels in posttreatment biopsies were associated with better outcome in some studies [13,14] but others did not find similar correlations [15,16]. Most recently, the prognostic role of change in TIL levels between the pre- and post-treatment biopsy was investigated and an increase in TIL levels was associated with better survival [15]. Here we are confirming this observation, suggesting that the increase of TIL ratio in tumor biopsies as a surrogate measure of anti-tumor immune activation may in fact reflect significant therapeutic benefit.

Cytotoxic chemotherapy has been shown to increase T-cell response in breast cancer [13,15]. The various agents may improve immune response in vivo by a wide array of biological mechanisms. Previous studies have shown that anthracyclines, such as doxorubicin may induce T cell activation in breast cancer by a toll-like receptor driven mechanism [17], while cyclophosphamide may enhance anti-tumorigenic immune response by suppressing T regulatory cell function [18]. Importantly, it was shown in preclinical studies that artificial inactivation of MutL homologue 1 (MLH1) increased the mutational burden and led to dynamic mutational profiles, which resulted in the persistent renewal of neoantigens in vitro and in vivo, improving immune surveillance [19]. Therefore, we hypothesized that induction of somatic tumor mutations via mutagenic chemotherapy would increase the immunogenicity of tumors and improve immune responses in patients with breast cancer. Furthermore, if a cytotoxic chemotherapy agent induces significantly more mutations/neo-epitopes it is also expected to induce a more intense immune reaction, as reflected in the number of TILs. Such a correlation would justify the combination of highly mutagenic agents with checkpoint inhibitor therapy [20]. This was the rationale behind comparing the TIL induction by three classes of cytotoxic agents, each with a distinct level, high, medium and low, mutagenic capacity. Previous studies did not attempt to perform such a comparison and in particular no data were reported on the induction of TILs by platinum based neoadjuvant chemotherapy in breast cancer.

Based on our previous findings [5], we expected significantly more TIL induction by the highly mutagenic platinum agent relative to treatments without platinum or cyclophosphamide. However, while platinum and cyclophosphamide based treatment seemed to have induced a

somewhat more significant increase in TIL, neoadjuvant chemotherapy containing none of these agents also increased post-treatment TIL. This does not support the notion that chemotherapeutic agents showing higher mutagenic potential in experimental systems would be better candidates to be combined with immune checkpoint inhibitors.

There are several possible explanations for this lack of difference. First, the mutagenicity of the therapeutic agents was tested in cell lines, and currently there are no reliable measures of mutagenic capacity of these agents in human tumor samples. Therefore, we do not know whether the difference seen in cell lines also holds in human tumors in vivo. It is also possible that the higher mutagenicity of a given agent, e.g. platinum, is compensated by another mechanism, such as the downregulation of the MHC complex [21]. While this is possible, it should be noted that platinum treatment was reported to induce HLA expression in breast cancer [22]. Finally, it was suggested that in breast cancer, the main increase in therapeutic TIL response is driven by gamma delta lymphocytes and not alpha beta lymphocytes, and the former response is typically not induced by neo-epitopes [23].

In summary, we did not find a significantly higher level of TIL induction by more mutagenic chemotherapeutic agents. Therefore, combining those with checkpoint inhibitors may not lead to enhanced therapeutic benefit.

Competing interests: The authors declare that they have no potential conflicts of interest.

Acknowledgement:

New National Excellence Program (ÚNKP-17-4-II-SE-65) (L.V)

New National Excellence Program (ÚNKP-17-4-III-SE-71) (AMT)

NVKP_16-1-2016-0004 (AMT)

STIA 19/2017, 6800313113, 68003F0043 (AMT)

The Research and Technology Innovation Fund (KTIA_NAP_13-2014-0021 to Z.S.)

Breast Cancer Research Foundation (Z.S.)

Table 1 Clinico-pathological characteristics

	All, n=112 (%)	Platinum based group, n=28 (%)	Cyclophosphamide based group, n=42 (%)	Anthracycline based group, n=42 (%)
mean age (years; range)	55 (29-80)	53 (29-80)	55 (35-79)	57 (32-78)
histological type (core biopsy)				
invasive carcinoma NST	97 (86.6)	23 (82.1)	41 (97.6)	33 (78.6)
ILC	12 (10.7)	4 (14.3)	1 (2.4)	7 (16.6)
other	3 (2.7)	1 (3.6)	0 (0.0)	2 (4.8)
immunohistochemical type (core biopsy)				
HR positive	65 (58.0)	10 (35.7)	32 (76.2)	23 (54.8)
HR positive/HER2 negative	53 (47.3)	4 (14.3)	27 (64.3)	22 (52.4)
HR positive/HER2 positive	12 (10.7)	6 (21.4)	5 (11.9)	1 (2.4)
HR negative	47 (42.0)	18 (64.3)	10 (23.8)	19 (45.2)
HR negative/HER2 positive	10 (8.9)	5 (17.9)	1 (2.4)	4 (9.5)
triple negative	37 (33.1)	13 (46.4)	9 (21.4)	15 (35.7)
histological grade (core biopsy)				
grade 1	3 (2.7)	0 (0.0)	1 (2.4)	2 (4.8)
grade 2	40 (35.7)	8 (28.6)	16 (38.1)	16 (38.1)
grade 3	66 (58.9)	19 (67.8)	23 (54.8)	24 (57.1)
unknown	3 (2.7)	1 (3.6)	2 (4.8)	0 (0.0)
HR positive				
grade 1	3 (2.7)	0 (0.0)	1 (2.4)	2 (4.8)
grade 2	31 (27,7)	3 (10.7)	15 (35.7)	13 (31.0)
grade 3	29 (25.9)	7 (25.0)	14 (33.3)	8 (19.0)
unknown	2 (1.8)	0 (0.0)	2 (4.8)	0 (0.0)
HR negative				
grade 1	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
grade 2	9 (8.0)	5 (17.9)	1 (2.4)	3 (7.1)
grade 3	37 (33.0)	12 (42.8)	9 (21.4)	16 (38.1)

unknown	1 (0.9)	1 (3.6)	0 (0.0)	0 (0.0)
average number of pre-operative chemotherapy cycles (range)	5.3 (2-8)	5.1 (2-8)	5.1 (2-6)	5.5 (3-8)
number of pre-operative chemotherapy cycles				
≤4	34 (30.4)	9 (32.1)	15 (35.7)	10 (23.8)
>4	78 (69.6)	19 (67.9)	27 (64.3)	32 (76.2)
chemotherapy regimens				
carboplatin + docetaxel or paclitaxel	21 (18.8)	21 (75.0)	-	-
cisplatin + docetaxel or paclitaxel	7 (6.3)	7 (25.0)	-	-
AC	2 (1.8)	-	2 (4.8)	-
FEC	15 (13.4)	-	15 (35.7)	-
CMF	1 (0.9)	-	1 (2.4)	-
AC + docetaxel/FEC+docetaxel	24 (21.4)	-	24 (57.1)	-
epirubicin + docetaxel or paclitaxel	32 (28.5)	-	-	32 (76.2)
doxorubicin + docetaxel or paclitaxel	10 (8.9)	-	-	10 (23.8)
pre-operative trastuzumab	15 (13.4)	8 (28.6)	5 (11.9)	2 (4.8)
ypT				
<2cm	42 (37.5)	11 (39.3)	17 (40.5)	14 (33.3)
≥2cm	69 (61.6)	16 (57.1)	25 (59.5)	28 (66.7)
unknown	1 (0.9)	1 (3.6)	0 (0.0)	0 (0.0)
ypN				
negative	31 (27.7)	9 (32.1)	11 (26.2)	11 (26.2)
positive	75 (67.0)	18 (64.3)	29 (69.0)	28 (66.7)
unknown	6 (5.3)	1 (3.6)	2 (4.8)	3 (7.1)
ΔStrTIL				
zero or positive	87 (77.7)	22 (78.6)	35 (83.3)	30 (71.4)
negative	25 (22.3)	6 (21.4)	7 (16.7)	12 (28.6)

AC: doxorubicin plus cyclophosphamide; CMF: cyclophosphamide plus metotrexat plus 5-fluorouracyl, ILC: invasive lobular carcinoma, FEC: 5-fluorouracyl plus epirubicin plus

cyclophosphamide; HR: hormone receptor, NST: no special type, StrTIL: stromal tumor-infiltrating lymphocytes

Table 2 Changes in stromal tumor-infiltrating lymphocytes: median StrTIL levels before and after pre-operative chemotherapy

	pre-StrTIL; median [%]; (IQR)	post-StrTIL; median [%]; (IQR)	P value (Wilcoxon Signed-test)
all population n=112	3.00 (1.00-7.50)	6.25 (3.00-25.00)	<0.001
age <50 n=35	3.00 (1.00-7.50)	5.00 (1.00-25.00)	0.001
age ≥50 n=77	3.00 (1.00-8.75)	7.50 (3.00-26.25)	<0.001
grade 1-2 n=43	1.00 (1.00-3.00)	3.00 (1.00-7.50)	0.011
grade 3 n=66	5.00 (1.00-11.25)	15.00 (3.00-35.00)	<0.001
HR positive n=65	1.00 (1.00-3.00)	3.00 (1.00-8.75)	<0.001
HR positive/HER2 negative n=53	1.00 (1.00-3.00)	3.00 (1.00-7.50)	0.002
HR positive/HER2 positive n=12	2.00 (1.00-3.00)	4.00 (1.50-16.88)	0.020
HR negative n=47	10.00 (3.00-20.00)	20.00 (5.00-35.00)	<0.001
HR negative/HER2 positive n=10	3.00 (0.88-13.1)	27.5 (8.75-32.50)	0.012
triple negative n=37	10.00 (5.00-20.00)	20.00 (5.00-35.00)	0.008
HR positive/HER2 negative/Grade 1-2 n=30	1.0 (1.00-3.00)	3.0 (1.00-5.62)	0.075
HR positive/HER2 negative/Grade 3 n=21	1.00 (1.00-6.25)	5.0 (1.00-16.25)	0.007
platinum-based group n=28	4.00 (1.00-13.75)	10.00 (3.00-33.75)	0.007
HR positive n=10	2.00 (1.00-3.50)	3.00 (1.00-12.50)	0.094
HR negative n=18	10.00 (2.50-20.00)	18.75 (7.50-35.00)	0.026
cyclophosphamide-based group	1.00	5.00	<0.001

n=42	(1.00-5.00)	(1.00-17.50)	
HR positive n=32	1.00 (1.00-3.00)	4.00 (1.00-14.38)	<0.001
HR negative n=10	6.25 (2.50-20.00)	17.50 (1.00-36.25)	0.049
anthracycline-based group n=42	4.00 (1.00-10.00)	5.00 (2.50-30.00)	0.047
HR positive n=23	3.00 (1.00-7.50)	3.00 (1.00-7.50)	0.502
HR negative n=19	10.00 (3.00-25.00)	30.00 (5.00-40.00)	0.063
cycle number ≤4 n=34	2.00 (1.00-5.63)	4.00 (1.00-18.13)	<0.001
cycle number >4 n=78	3.00 (1.00-10.00)	7.50 (3.00-28.13)	<0.001
HER2 positive with trastuzumab n=15	1.00 (1.00-3.00)	7.50 (3.00-25.00)	0.006
HER2 positive without trastuzumab n=7	5.00 (1.00-7.50)	30.00 (3.00-35.00)	0.031

HR: hormone receptor, IQR: interquartile range, pre-StrTIL: pre-operative stromal tumor-infiltrating lymphocytes in core biopsy, post-StrTIL: post-operative stromal tumor-infiltrating lymphocytes in surgical specimen

Table 3 Factors associated with distant metastasis free survival

		Univariate analysis			Multivariate analysis		
		Hazard ratio	95% CI	P value	Hazard ratio	95% CI	P value
ΔStrTIL		0.976	0.950-1.004	0.091	0.973	0.948-0.999	0.044
age	<50 years	1.000					
	≥50 years	1.545	0.690-3.455	0.290	0.892	0.344-2.318	0.815
grade	1-2	1.000					
	3	2.178	0.973-5.062	0.071	2.236	0.841-5.943	0.107
HR status	negative	1.000					
	positive	0.237	0.111-0.505	<0.001	0.169	0.072-0.398	<0.001
ypT	<2cm	1.000					
	≥2cm	2.107	0.964-4.602	0.062	3.854	1.520-9.775	0.004
ypN	negative	1.000					
	positive	1.644	1.562-17.147	0.007	6.984	2.011-24.261	0.002
pre-operative chemotherapy	anthracycline-based group	1.000					
	platinum-based group	0.737	0.297-1.830	0.511	0.741	0.269-2.044	0.563
	cyclophosphamide-based group	0.642	0.284-1.451	0.287	0.961	0.388-2.379	0.931

CI: confidence interval, HR: hormone receptor, StrTIL: stromal tumor-infiltrating lymphocytes

Supplementary Table 1 Changes of StrTIL in the three treatment groups

ΔStrTIL	Platinum-based			Cyclophosphamide-based			Anthracycline-based		
	all n=28 (n;%)	HR negative n=18 (n;%)	HR positive n=10 (n;%)	all n=42 (n;%)	HR negative n=10 (n;%)	HR positive n=32 (n;%)	all n=42 (n;%)	HR negative n=19 (n;%)	HR positive n=23 (n;%)
positive	16 (57.2)	11 (61.1)	5 (50.0)	25 (59.5)	7 (70.0)	18 (56.3)	20 (47.6)	11 (57.9)	9 (39.2)
zero	6 (21.4)	2 (11.1)	4 (40.0)	10 (23.8)	0 (0.0)	10 (31.2)	10 (23.8)	3 (15.8)	7 (30.4)
negative	6 (21.4)	5 (27.8)	1 (10.0)	7 (16.7)	3 (30.0)	4 (12.5)	12 (28.6)	5 (26.3)	7 (30.4)

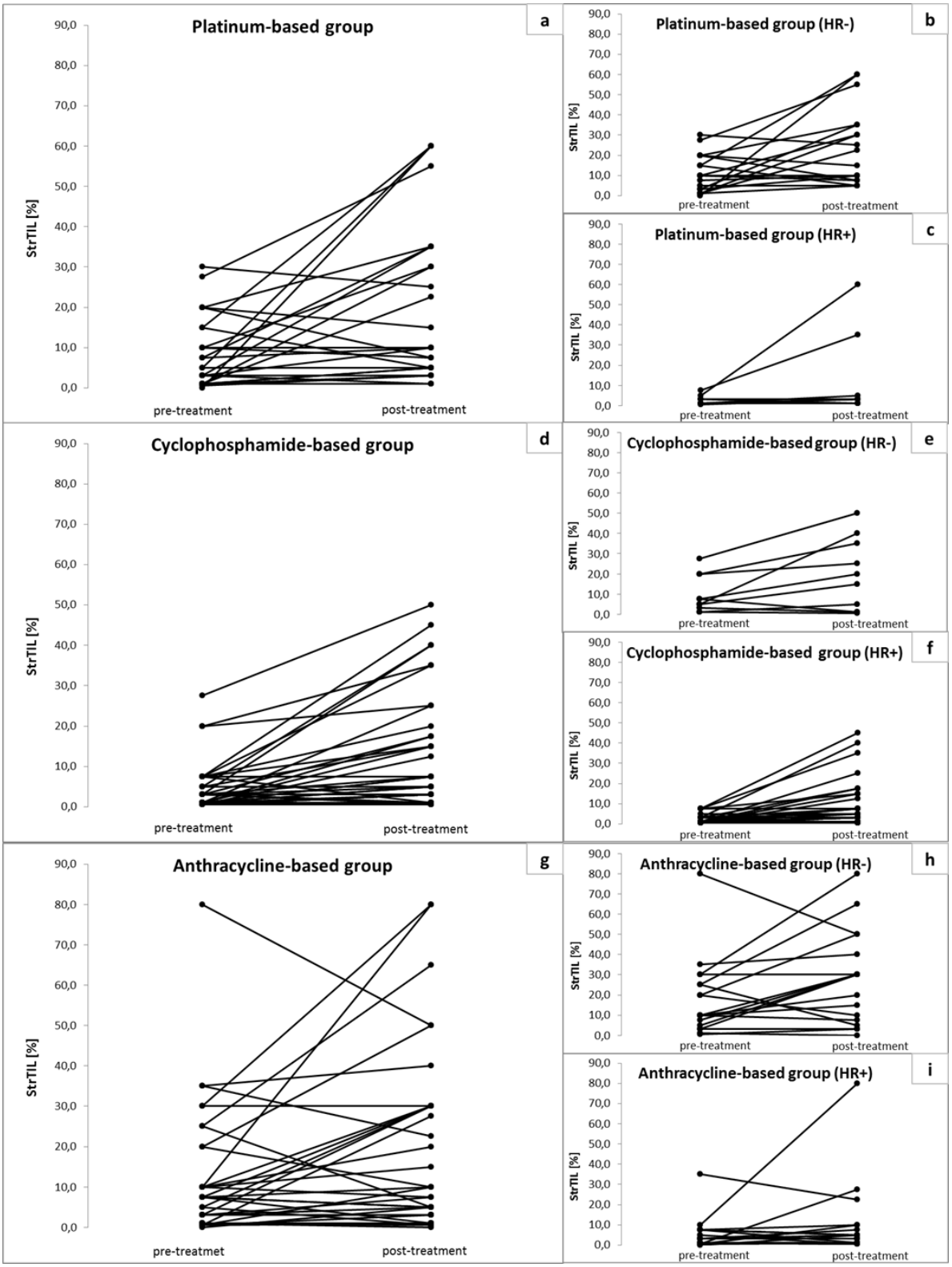
HR: hormone receptor; StrTIL: stromal tumor-infiltrating lymphocytes

Supplementary Table 2 Prognostic value of pre-operative StrTIL, postoperative StrTIL and changes in StrTIL

	HR negative cases			HR positive cases		
	Hazard ratio	95% CI	p value	Hazard ratio	95% CI	p value
pre-StrTIL	1.022	0.997-1.049	0.088	1.028	0.872-1.212	0.745
post-StrTIL	0.974	0.948-1.000	0.050	1.009	0.980-1.040	0.545
ΔStrTIL	0.957	0.932-0.983	0.001	1.010	0.978-1.044	0.546

CI: confidence interval, pre-StrTIL: pre-operative stromal tumor-infiltrating lymphocytes, post-StrTIL: post-operative stromal tumor-infiltrating lymphocytes

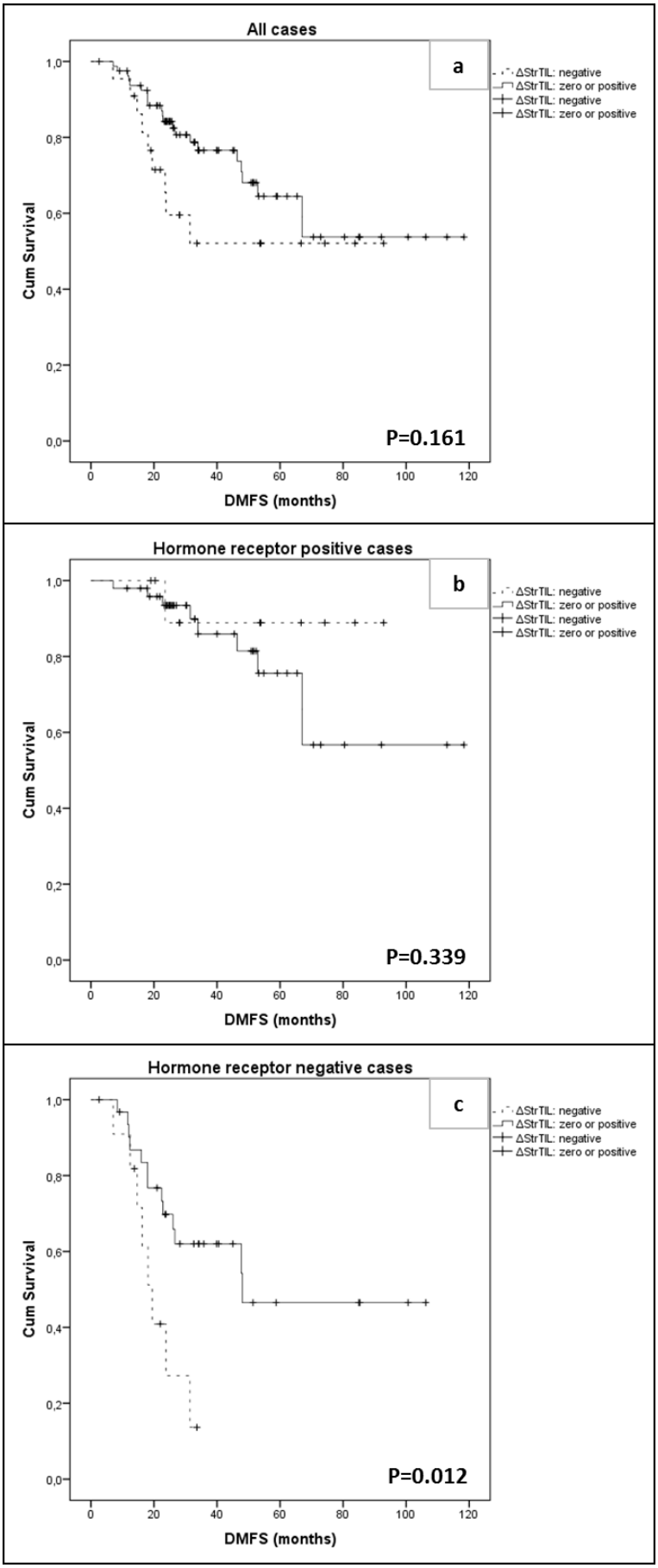
Figure 1 Stromal tumor-infiltrating lymphocytes (StrTIL) before and after pre-operative chemotherapy



Significant ΔStrTIL increase was observed in the three treatment groups (platinum-based: P=0.007; cyclophosphamide-based: P<0.001; anthracycline-based: P=0.047; Fig. 1a, d, g). By analysis separately the HR positive and HR-negative cases we experienced only the

administration of cyclophosphamide resulted a significant increase in HR positive cases ($P<0.001$, Fig. 1c, f, i), whereas in HR negative cases StrTIL changes seemed independently from the treatment applied (platinum-based: $P=0.026$; cyclophosphamide-based: $P=0.049$; anthracycline-based: $P=0.063$).

Figure 2 Kaplan-Meier curves of survival analyses



By analyzing the whole study cohort no significant correlation was detected between ΔStrTIL and distant metastasis free survival (DMFS) (P=0.161, Fig. 2a), the same result was observed in hormone receptor positive cases (P=0.339, Fig. 2b).

In hormone receptor negative cases the estimated median DMFS was significantly higher, if the ΔStrTIL was zero or positive (48.0 months; standard error: 8.7) compare to the cases where ΔStrTIL was negative (19.4 months; standard error: 2.4) (Fig. 2c).

References

1. Rosenberg SA. Decade in review-cancer immunotherapy: entering the mainstream of cancer treatment. Nat Rev Clin Oncol. Nature Publishing Group; 2014 Nov;:630–2.

2. Sharma P, Allison JP. Immune checkpoint targeting in cancer therapy: toward combination strategies with curative potential. Cell. 2015;161:205–14.

3. Van Allen EM, Miao D, Schilling B, Shukla SA, Blank C, Zimmer L, et al. Genomic correlates of response to CTLA-4 blockade in metastatic melanoma. Science. American Association for the Advancement of Science; 2015;350:207–11.

4. Le DT, Uram JN, Wang H, Bartlett BR, Kemberling H, Eyring AD, et al. PD-1 Blockade in Tumors with Mismatch-Repair Deficiency. N. Engl. J. Med. Massachusetts Medical Society; 2015;372:2509–20.

5. Szikriszt B, Póti Á, Pipek O, Krzystanek M, Kanu N, Molnár J, et al. A comprehensive survey of the mutagenic impact of common cancer cytotoxics. Genome Biol. BioMed Central; 2016;17:99.

6. Lakhani SR, Ellis IO, Schnitt SJ. WHO/IARC Classification of Tumours of the breast 4th, vol. 4. 2012.

7. Cserni G, Kulka J, Francz M, Járay B, Kálmán E, Kovács I, et al. [Pathological diagnosis, work-up and reporting of breast cancer. Recommendations of the 3rd Hungarian Consensus Conference on Breast Cancer]. Magy Onkol. 2016;60:209–28.

8. Hammond MEH, Hayes DF, Dowsett M, Allred DC, Hagerty KL, Badve S, et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer. Arch. Pathol. Lab. Med. 2010;134:907–22.

9. Rakha EA, Pinder SE, Bartlett JMS, Ibrahim M, Starczynski J, Carder PJ, et al. Updated UK Recommendations for HER2 assessment in breast cancer. J. Clin. Pathol. BMJ Publishing Group; 2015. pp. 93–9.

10. Salgado R, Denkert C, Demaria S, Sirtaine N, Klauschen F, Pruneri G, et al. The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014. Ann. Oncol. 2015. pp. 259–71.

11. Wein L, Savas P, Luen SJ, Virassamy B, Salgado R, Loi S. Clinical Validity and Utility of Tumor-Infiltrating Lymphocytes in Routine Clinical Practice for Breast Cancer Patients: Current and Future Directions. Front Oncol. Frontiers; 2017;7:156.

12. Pruneri G, Gray KP, Vingiani A, Viale G, Curigliano G, Criscitiello C, et al. 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. Breast Cancer Res. Treat. 2016;158:323–31.

13. Dieci MV, Criscitiello C, Goubar A, Viale G, Conte P, Guarneri V, et al. Prognostic value of tumor-infiltrating lymphocytes on residual disease after primary chemotherapy for triple-negative breast cancer: a retrospective multicenter study.

Ann. Oncol. 2014;25:611–8.

14. Bianchini G, Pusztai L, Pienkowski T, Im Y-H, Bianchi GV, Tseng L-M, et al. Immune modulation of pathologic complete response after neoadjuvant HER2-directed therapies in the NeoSphere trial. Ann. Oncol. 2015;26:2429–36.

15. Pelekanou V, Carvajal-Hausdorf DE, Altan M, Wasserman B, Carvajal-Hausdorf C, Wimberly H, et al. Effect of neoadjuvant chemotherapy on tumor-infiltrating lymphocytes and PD-L1 expression in breast cancer and its clinical significance. Breast Cancer Res. BioMed Central; 2017;19:91.

16. Lo CS, Sanii S, Kroeger DR, Milne K, Talhouk A, Chiu DS, et al. Neoadjuvant Chemotherapy of Ovarian Cancer Results in Three Patterns of Tumor-Infiltrating Lymphocyte Response with Distinct Implications for Immunotherapy. Clin. Cancer Res. 2017;23:925–34.

17. Apetoh L, Ghiringhelli F, Tesniere A, Obeid M, Ortiz C, Criollo A, et al. Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. Nat. Med. Nature Publishing Group; 2007;13:1050–9.

18. Lutsiak MEC, Semnani RT, De Pascalis R, Kashmiri SVS, Schlom J, Sabzevari H. Inhibition of CD4(+)25+ T regulatory cell function implicated in enhanced immune response by low-dose cyclophosphamide. Blood. American Society of Hematology; 2005;105:2862–8.

19. Germano G, Lamba S, Rospo G, Barault L, Magrì A, Maione F, et al. Inactivation of DNA repair triggers neoantigen generation and impairs tumour growth. Nature. 2017;552:116–20.

20. Stanton SE, Disis ML. Clinical significance of tumor-infiltrating lymphocytes in breast cancer. J Immunother Cancer. BioMed Central; 2016;4:59.

21. Seliger B. Novel insights into the molecular mechanisms of HLA class I abnormalities. Cancer Immunol. Immunother. Springer-Verlag; 2012;61:249–54.

22. Wan S, Pestka S, Jubin RG, Lyu YL, Tsai Y-C, Liu LF. Chemotherapeutics and radiation stimulate MHC class I expression through elevated interferon-beta signaling in breast cancer cells. Fernandez-Sesma A, editor. PLoS ONE. 2012;7:e32542.

23. Bense RD, Sotiriou C, Piccart-Gebhart MJ, Haanen JBAG, van Vugt MATM, de Vries EGE, et al. Relevance of Tumor-Infiltrating Immune Cell Composition and Functionality for Disease Outcome in Breast Cancer. J. Natl. Cancer Inst. 2017;109:djw192.