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Original Articles

BRCA gene mutations do not shape the extent and organization of tumor infiltrating lymphocytes in triple negative breast cancer

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ABSTRACT

This study investigated the prevalence of TIL subpopulations, TLS, PD-1 and PD-L1 in tumors from TNBC patients harboring wild-type or mutated *BRCA1* or *BRCA2* germline genes.

This TNBC cohort included 85% TIL-positive (≥10%) tumors with 21% classified as TIL^{hi} (≥50%). Interestingly, the *BRCA*^{mut} group had a significantly higher incidence of TIL^{pos} tumors compared to the *BRCA*^{wt} group ($P = 0.037$). T cells were dominant in the infiltrate but no statistically significant differences were detected between *BRCA*^{wt} and *BRCA*^{mut} for CD3$^+$, CD4$^+$ and CD8$^+$ T cells or CD20$^+$ B cells. TLS were detected in 74% of tumors but again no significant differences between the *BRCA* groups. PD-1 expression was observed in 33% and PD-L1 in 53% (any cell, cut-off ≥1%) tumors for the entire TNBC cohort. PD-1 expression correlated with PD-L1 and both with TIL and TLS but was not associated with *BRCA* mutational status.

Our analyses reveal that *BRCA*^{wt} and *BRCA*^{mut} TNBC are similar except for a significant increase of TIL^{pos} tumors in the *BRCA*^{mut} group. While *BRCA* gene mutations may not directly drive immune infiltration, the greater number of TIL^{pos} tumors could signal greater immunogenicity in this group.

1. Introduction

Triple negative breast cancer (TNBC), diagnosed in 10–20% of patients, is characterized by a poor prognosis with early onset and relapse predominantly in the visceral organs [1]. Classification as TNBC is based on the absence of estrogen (ER), progesterone (PR) and HER2 receptor expression [2]. Patients with a personal or familial history of breast cancer (BC) or ovarian cancer (OC) often carry pathogenic germline gene mutations, with the *BRCA1* or *BRCA2* genes (*BRCA*^{mut}) most frequently mutated [3]. Among TNBC patients, ~10% carry a *BRCA* gene mutation with gene expression profiling classifying the majority in the basal-like subtype [1,2,4].

Functional BRCA1/2 (BRCA^{wt}) proteins play critical roles in repairing double-stranded DNA breaks via homologous recombination and thereby help to maintain genomic stability. Pathogenic *BRCA* gene defects are associated with higher chromosomal instability, the loss of genetic information, gene rearrangements and cell death. *BRCA* mutations have been linked to an increased cancer risk particularly for BC and OC [5]. Individuals with *BRCA* mutations or alterations have a 57% (*BRCA1*) or 49% (*BRCA2*) lifetime risk of developing BC or OC,

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Abbreviations

BC	breast cancer
BRCA	breast cancer associated antigen
$BRCA^{mut}$	*BRCA* mutated
$BRCA^{wt}$	*BRCA* wild-type
ER	estrogen receptor
FFPE	formalin-fixed and paraffin-embedded
FISH	fluorescence *in situ* hybridization
IC	immune cells
ICC	intraclass correlation coefficient
I-DFS	invasive disease free survival
IHC	immunohistochemistry
IQR	interquartile range
MSI^{hi}	microsatellite instability-high
OC	ovarian cancer
OS	overall survival
PD-1	programmed cell death - 1
PD-L1	programmed death - ligand 1
PR	progesteron receptor
SC	stromal cells
TC	tumor cell
TIL	tumor infiltrating lymphocytes
TIL^{hi}	TIL high
TIL^{int}	TIL intermediate
TIL^{neg}	TIL negative
TIL^{pos}	TIL positive
TLS	tertiary lymphoid structures
TNBC	triple negative breast cancer
yrs	years

respectively [6,7]. Estimates from the growing gene sequencing database indicate that ~50% of hereditary BC or OC patients carry pathogenic mutations in genes other than *BRCA1* or *BRCA2* [7]. Nonetheless, the *BRCA* genes are the most common inherited germline mutation currently associated with BC or OC.

TNBC is an aggressive disease that disproportionally afflicts young women and because this is a heterogeneous group with no targeted drugs available, uniformly treating patients is difficult. The strikingly durable responses some cancer patients have shown to immune checkpoint inhibitors, particularly the newer combination therapies, stimulated interest in these drugs, particularly for those with TNBC [8,9]. Better response rates to immunomodulatory drugs are generally associated with high levels of tumor infiltrating lymphocytes (TIL) because the extent of the infiltrate reflects higher expression of the target molecules, including programmed cell death - 1 (PD-1) and programmed death - ligand 1 (PD-L1) [8–10]. The extent of TIL in BC varies widely but higher TIL are more frequently observed in the aggressive subtypes, TNBC and HER2-positive (HER2+), compared to the luminal A and B subtypes [9–12]. Close examination of high TIL BC found immune cells are principally located in the peri-tumoral stroma with 40–60% of tumors (most frequently HER2+ and TNBC) organizing their TIL in one or more tertiary lymphoid structures (TLS) [8,10,13]. An important link between TIL and better clinical outcomes was demonstrated in a number of BC clinical trials [14–21]. Furthermore, high TIL TNBC is associated with better survival after adjuvant treatment [15,16,18,22] and a higher incidence of pathologic complete response following neoadjuvant therapy [19,20]. A TLS presence has also been shown to be a good prognostic factor in some studies of BC and other solid tumors (reviewed in Ref. [21]).

Patients with advanced melanoma, non-small cell lung cancer, renal cancer, urothelial carcinoma, Hodgkin lymphoma, head and neck squamous cell carcinoma, Merkel cell and mismatch repair deficient tumors now receive immune checkpoint inhibitors as standard of care. Unfortunately, only a minority of patients actually derive clinical benefit making the identification of reliable predictive biomarkers an important clinical need [23]. PD-L1 and/or PD-1 expression [24,25] and tumor mutation burden [26] in lung cancer, circulating lactate dehydrogenase levels in melanoma [27] and microsatellite instability-high (MSI^{hi}) in colorectal carcinoma [28] are currently being investigated as biomarkers for patient selection. BC clinical trials using anti-PD-1 or PD-L1 antibodies alone or with other immunotherapy, chemotherapy, radiotherapy or targeted agents are ongoing with initial results showing promise, but again only in some patients [9]. In metastatic TNBC, PD-L1 expression, stromal TIL and $CD8^+$ TIL have been associated with a benefit from immunotherapy [29–31].

The lack of robust biomarkers that identify potential responder patients together with an abundance of single and combination treatments targeting the immune system under study highlight the need for clinically relevant biomarkers of response. In BC, scoring TIL has underlined the relationship between higher TIL and the response to treatment, including immunotherapy [30,31]. High $CD3^+$ and $CD8^+$ TIL together with increased PD-1 and PD-L1 expression in $BRCA^{mut}$ high-grade serous OC have been linked with clinical response, leading to their proposal as predictive biomarkers for immunotherapy [32,33]. The present study investigated whether germline *BRCA1/2* gene mutations are associated with enhanced immune infiltration, TLS formation and/or PD-1 and PD-L1 expression in the TNBC tumor microenvironment.

2. Material and methods

2.1. Patients

Formalin-fixed and paraffin-embedded (FFPE) tumor blocks selected from BC patients whose untreated primary tumors were surgically removed between 1985 and 2015 and who underwent genetic counseling and testing for germline *BRCA1/2* gene mutations for their personal or familial history of BC or OC were used in this study. Patient characteristics and cohort selection are detailed in Table 1 and Fig. 1. This study was approved by the Institut Jules Bordet's ethics committee (EC2038). Informed consent was obtained.

2.2. Immunohistochemistry

Immunohistochemical (IHC) staining of FFPE tumor tissues sections (4 µm) was performed using a Ventana Benchmark XT IHC/ISH automated staining instrument (Ventana Medical Systems). The dual CD3/CD20, CD4/CD8 and PD-1/PD-L1 IHC stains were performed as previously detailed [10] using the primary antibodies listed in Table S1.

2.3. Pathological assessment

IHC-stained full-face tumor sections were independently scored for $CD3^+$ and $CD20^+$ TIL, TLS, $CD4^+$ and $CD8^+$ TIL subpopulations, PD-1 and PD-L1 by two experienced pathologists (GvdE, AdW) blinded to the clinical data. TIL infiltration, as a continuous variable, was scored as a percentage of the defined tumor area infiltrated with $CD3^+$ T cells (also scored separately for $CD4^+$ and $CD8^+$ T cells) plus $CD20^+$ B cells. TIL in direct contact with tumor cells were counted as intratumoral TIL and those in the peri-tumoral areas as stromal TIL [14]. Dense aggregates of B cells in follicles with an adjacent T cell zone were scored as TLS with the number of TLS normalized to the total tumor area. TIL-positive (TIL^{pos}) tumors have ≥10% stromal TIL while TIL high (TIL^{hi}) are ≥50% stromal or intratumoral TIL and TIL-intermediate (TIL^{int}) lie in between these two thresholds [8,10,14]. TIL-negative (TIL^{neg}) tumors have < 10% stromal TIL. The threshold for PD-1 and PD-L1 positivity

Table 1
Clinicopathological parameters of TNBC patients grouped by germline BRCA gene status.

	BRCA^{wt}[a]	*BRCA*^{mut}[b]	Test
	N = 41[c] (48%)[d]	*N* = 44[c] (52%)[d]	***P*- value**[e]
Age (years)			**U- Mann Whitney**[f]
median (years)	43	38	**0.008**[e]
			Chi-square[f]
< 50	28[c] (68%)[d]	42[c] (95%)[d]	**0.0013**[e]
≥50	13[c] (32%)[d]	2[c] (5%)[d]	
Surgery			**Chi-square**[f]
Lumpectomy or Quadrantectomy	27[c] (66%)[d]	32[c] (73%)[d]	0.64[e]
Mastectomy (not specified)	14[c] (34%)[d]	12[c] (27%)[d]	
Axillary Surgery			**Chi-square**[f]
Sentinel Node Biopsy	17[c] (41%)[d]	14[c] (32%)[d]	0.38[e]
Dissection	24[c] (58%)[d]	30[c] (68%)[d]	
Histology			**Chi-square**[f]
Ductal	36[c] (88%)[d]	37[c] (84%)[d]	0.78[e]
Lobular	1[c] (2.5%)[d]	0[c] (0%)[d]	
Medullary-like	3[c] (7%)[d]	4[c] (9%)[d]	
Other	1[c] (2.5%)[d]	3[c] (7%)[d]	
Size			**Chi-square**[f]
< 20 mm	28[c] (68%)[d]	29[c] (66%)[d]	0.39[e]
20 mm < 50 mm	9[c] (22%)[d]	12[c] (27%)[d]	
≥50 mm	2[c] (5%)[d]	1[c] (2%)[d]	
Unknown	2[c] (5%)[d]	2[c] (5%)[d]	
Nodes			**Chi-square**[f]
0	27[c] (66%)[d]	32[c] (73%)[d]	0.72[e]
1-3	12[c] (29%)[d]	10[c] (23%)[d]	
4-9	1[c] (2.5%)[d]	2[c] (5%)[d]	
> 10	1[c] (2.5%)[d]	0[c] (0%)[d]	
Histological grade			**Chi-square**[f]
1	0[c] (0%)[d]	1[c] (2%)[d]	0.64[e]
2	4[c] (10%)[d]	3[c] (7%)[d]	
3	33[c] (80%)[d]	38[c] (86%)[d]	
Unknown	4[c] (10%)[d]	2[c] (5%)[d]	
%Ki67			**Chi-square**[f]
< 20	4[c] (10%)[d]	4[c] (9%)[d]	0.99[e]
≥20	34[c] (83%)[d]	37[c] (84%)[d]	
Unknown	3[c] (7%)[d]	3[c] (7%)[d]	
Stage			**Chi-square**[f]
1	21[c] (51%)[d]	21[c] (48%)[d]	0.71[e]
2	15[c] (37%)[d]	20[c] (45%)[d]	
3	4[c] (10%)[d]	3[c] (7%)[d]	
Unknown	1[c] (2%)[d]	0[c] (0%)[d]	
Menopausal status			**Chi-square**[f]
Yes	12[c] (29%)[d]	3[c] (7%)[d]	**0.02**[e]
No	26[c] (63%)[d]	38[c] (86%)[d]	
NA	3[c] (7%)[d]	3[c] (7%)[d]	
Lymphovascular emboli			**Chi-square**[f]
Yes	12[c] (29%)[d]	13[c] (30%)[d]	0.96[e]
No	19[c] (46%)[d]	19[c] (43%)[d]	
Unknown	10[c] (24%)[d]	12[c] (27%)[d]	
Adjuvant chemotherapy			**Chi-square**[f]
Anthracyclines/Taxanes	27[c] (66%)[d]	23[c] (52%)[d]	0.45[e]
Anthracyclines/CMF	1[c] (2%)[d]	3[c] (7%)[d]	
Anthracyclines	6[c] (15%)[d]	9[c] (20%)[d]	
Others	6[c] (15%)[d]	7[c] (16%)[d]	
None	1[c] (2%)[d]	2[c] (5%)[d]	
Family history of breast or ovarian cancer			**Chi-square**[f]
Yes	30[c] (73%)[d]	35[c] (80%)[d]	0.70[e]
No	10[c] (24%)[d]	9[c] (20%)[d]	
Unknown	1[c] (2.5%)[d]	0[c] (0%)[d]	
Prophylactic surgery			**Chi-square**[f]
Contralateral breast mastectomy	1[c] (2%)[d]	12[c] (27%)[d]	**0.002**[e]
Oophorectomy	4[c] (10%)[d]	35[c] (80%)[d]	**< 0.0001**[e]
Median survival (years)			**Log-rank**[f]
I-DFS (years)	3.47	5.81	0.84[e]
OS (years)	5.03	9.3	0.23[e]

TNBC: triple negative breast cancer.
BRCA: germline *BRCA* gene.
wt: wild-type.
mut: mutated.
CMF: cyclophosphamide, methotrexate, 5-fluorouracil.
I-DFS: invasive disease-free survival.
OS: overall survival.
[a] *BRCA*^{wt}: wild-type germline *BRCA1* or *BRCA2* genes.
[b] *BRCA*^{mut}: mutated germline *BRCA1* or *BRCA2* genes.
[c] Number of patients.
[d] Percentage of patients.
[e] *P* - value; significance < 0.05.
[f] Statistical test.

was set at ≥1% surface positive cells. Regions of *in situ* carcinoma, normal glandular epithelium and necrosis were excluded from evaluation [14,33]. The pathology lab defined ER and PR positive status using a cut-off of > 1% ER + or PR + cells on IHC stained tissue sections. HER2 negative status was defined as 0 or 1 + on IHC stained tissue sections. IHC 2 + and IHC3+ scores were subsequently analyzed by fluorescence *in situ* hybridization (FISH) to confirm HER2 positivity.

2.4. Statistical analysis

All statistical analyses were performed using the R programming language and its available packages for specific statistical approaches (all of the references for statistical tests are listed in Table S2). The intraclass correlation coefficient (ICC) was used to measure the inter-observer variance between the two pathologists who scored the slides. ICC was calculated using the mixed model [ICC(3,k) as defined by Shrout and Fleiss]. The data shown are mean scores derived from pathologists independent readings when their concordance was good (> 0.75). Discrepancies between the two pathologists were discussed and a consensus score was based on discussion and examination of each case together. We carefully studied pathologists' concordance in a previous publication [34]. The U-Mann-Whitney non-parametric test was used to compare the distribution for continuous variables. Chi-square or Fisher exact tests were used to assess the contingency tables based on categorical data. Test power equaled 0.6323 and 0.9818 for medium and large effect sizes (df = 2). Spearman tests were used for analyzing correlations between variables. All reported *P*-values are two-tailed and a test comparison was considered as statistically significant if the associated *P*-value was < 0.05. Survival analyses were performed with the use of Kaplan-Meier estimator and the Cox proportional hazard model. Invasive disease free survival (I-DFS) and overall survival (OS) were defined as the time from diagnosis to the first invasive relapse (local, contralateral or distant) and as the time from diagnosis to death, respectively.

3. Results

3.1. TNBC study population

We first selected BC patients whose untreated primary tumors were surgically removed and who, due to their familial history of BC or OC, were tested for germline *BRCA* gene mutations after genetic counseling (*N* = 1155). We retained TNBC patients only using the inclusion criteria detailed in Fig. 1. The final cohort included 85 TNBC patients divided in two groups: *BRCA*^{wt} (*N* = 41) and *BRCA*^{mut} (*N* = 44; 38 *BRCA1*^{mut} and 6 *BRCA2*^{mut}). A list of types of germline *BRCA* mutations is provided in Table S3. The clinicopathological characteristics of these patients are presented in Table 1.

Predictably, *BRCA*^{mut} patients were younger than *BRCA*^{wt} patients (mean age: 39 *versus* 45 yrs; *P* = 0.008), and thus had a higher prevalence of pre-menopausal patients. Both groups were well balanced for all other clinicopathological parameters (Table 1). A majority of patients had a familial history of BC or OC: 73% in the *BRCA*^{wt} and 80% in the *BRCA*^{mut} group. Invasive relapses occurred in 31% of patients with

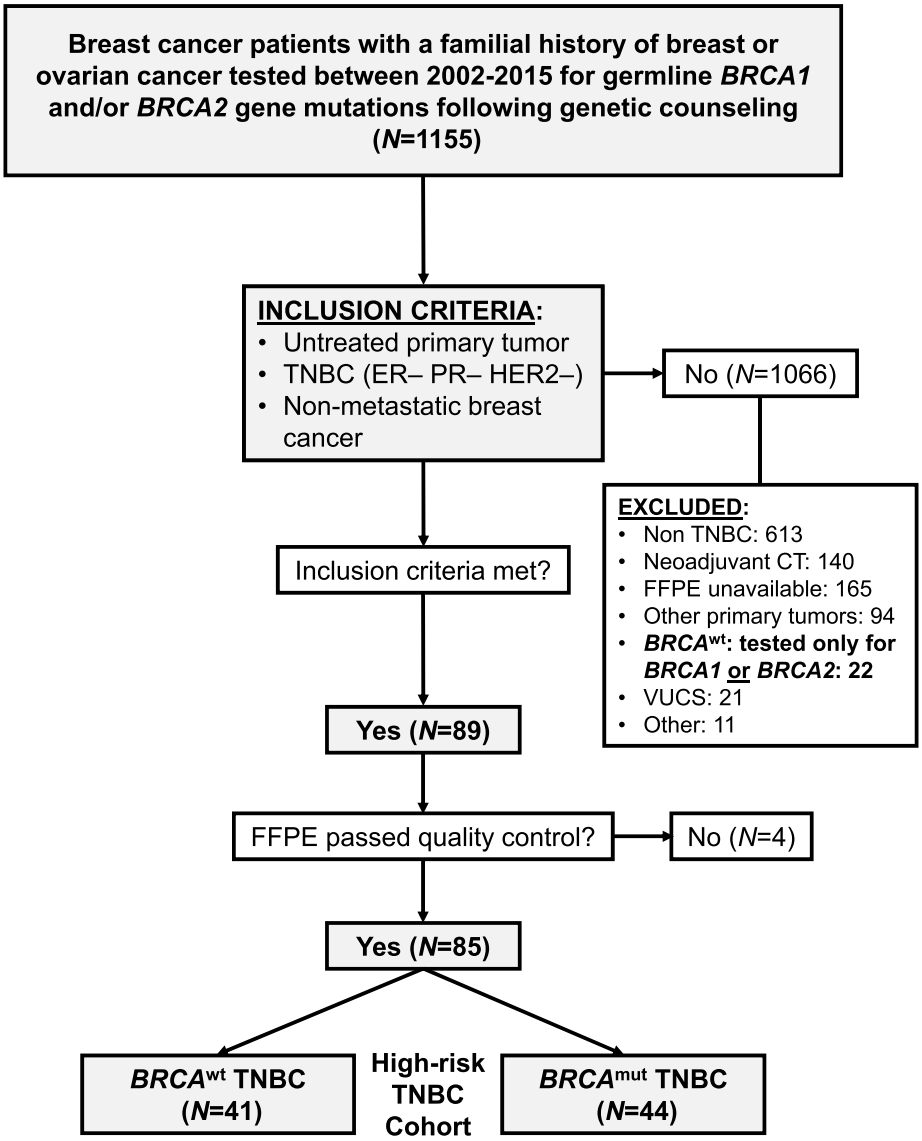

Fig. 1. Breast cancer patient selection and inclusion criteria. Flow chart diagraming the patient selection process and criteria.

eight events in *BRCA*^{wt} (20%) and 16 events in *BRCA*^{mut} (36%). For the entire cohort, median I-DFS was 4.725 yrs and median OS was 6.66 yrs. No significant differences were observed between the *BRCA*^{wt} and *BRCA*^{mut} groups, despite a trend toward longer survival in *BRCA*^{mut} patients (I-DFS: 3.47 *versus* 5.81 yrs, respectively; OS: 5.03 *versus* 9.3 yrs, respectively; log-rank test, p = 0.84 and 0.23, respectively).

3.2. Variation in the evaluation of TIL

The ICC for evaluation of stromal, intratumoral and global TIL [14] was good to excellent. Remarkably, the ICC for tumor cell (TC) PD-L1 expression was 0.82 (0.73–0.89; $P < 0.0001$) and PD-1 expression within TLS was 0.72 (0.57–0.82; $P < 0.0001$) (Table S4). The variance between the two pathologists was higher for the other variables, including PD-L1 expression on stromal cells (SC), PD-L1 expression on immune cells (IC) and PD-1 expression on TIL. Consensus scores were given for final analyses in these latter cases.

3.3. Immune infiltration levels, composition and organization in TNBC patients

We first asked whether *BRCA*^{mut} TNBC was distinguished by elevated TIL similar to MSI^{hi} colorectal carcinomas [35]. Immune infiltrates were scored as a continuous variable on dual CD3/CD20 IHC stained tumor sections (Fig. 2A) to quantify both the level of infiltrating T and B cells and their organization in TLS. Stromal TIL scores once again show that overall TIL distribution forms a continuum where tumors can be stratified into TIL^{neg}, TIL^{int} and TIL^{hi} (Fig. 2B; the thresholds are defined in Material and Methods) as previously described [10,14]. This TNBC cohort included 85% TIL^{pos} (= TIL^{int} plus TIL^{hi}) tumors. Our previous flow cytometric analysis of fresh tumor tissues, in a cohort that included all four BC subtypes, identified 75% as TIL^{pos} based on thresholds set using normal breast tissue controls. The higher percentage identified for the TNBC cohort here is due to a significant increase in the number of TIL^{pos} tumors in the *BRCA*^{mut} group (93%; Fig. 2C). The *BRCA*^{wt} group (76% TIL^{pos}) was, on the other hand, remarkably similar to our previous study of all BC subtypes.

The TIL scores for *BRCA*^{wt} and *BRCA*^{mut} both form a continuum, as we previously determined for a BC cohort that included all four subtypes [10]. Examination of TIL as a categorical variable also detected an important difference in the portion of TIL^{neg} tumors in the *BRCA*^{mut} group (7%) compared to the *BRCA*^{wt} group (24%; $P = 0.0376$, Table 2, Fig. 2C), principally due to a significantly higher number of TIL^{int} in the former. Our TNBC cohort included seven medullary-like TNBC, known

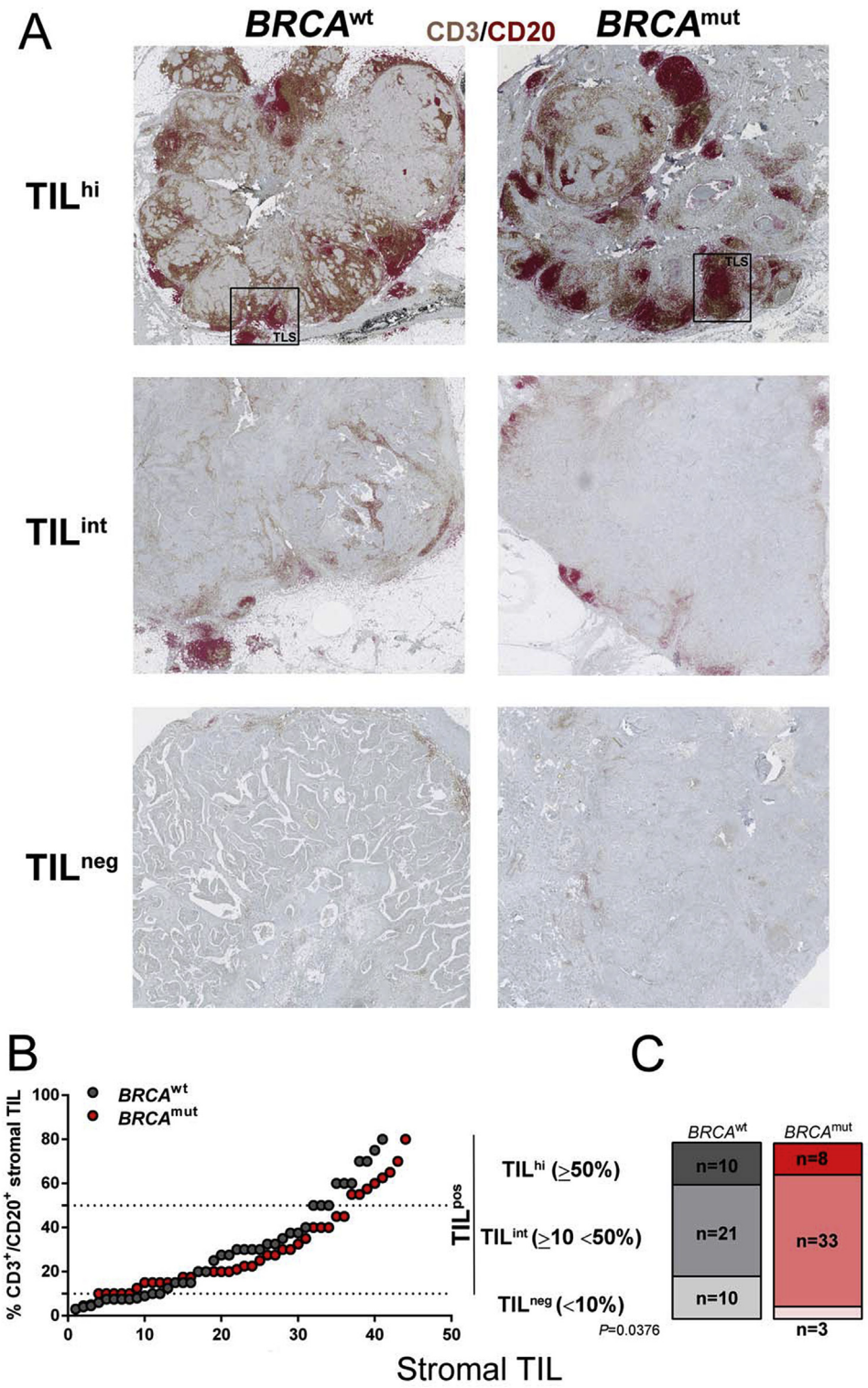

Fig. 2. The location and organization of TIL in TIL^{neg}, TIL^{int} and TIL^{hi} TNBC from the *BRCA*^{wt} and *BRCA*^{mut} groups. (A) Immunohistochemical staining of TIL in representative tumors from the three density groups: TIL^{hi}: ≥50% stromal TIL, TIL^{int}: ≥10% and < 50% stromal TIL, TIL^{neg}: < 10% stromal TIL. CD3 (brown) is a pan T cell marker and CD20 (red) is a pan B cell marker; 5x magnification. (B) Distribution of stromal TIL scores in the *BRCA*^{wt} and *BRCA*^{mut} groups. (C) Prevalence of TIL^{hi}, TIL^{int} and TIL^{neg} tumors in the *BRCA*^{wt} and *BRCA*^{mut} groups. The *P* value was calculated using the Chi-square test.

to be more highly infiltrated [36], which were equally distributed between the groups (3 *BRCA*^{wt} and 4 *BRCA*^{mut}; Table 2). Five of these tumors were graded TIL^{hi} with 2/3 (67%) in the *BRCA*^{wt} group and 3/4 (75%) in the *BRCA*^{mut} group. No other statistically significant differences were identified between the *BRCA*^{wt} and *BRCA*^{mut} groups, neither in TIL subpopulations and their location nor their organization in TLS (Table 2). In addition, differences in I-DFS and OS were not detected when patients were stratified on TIL infiltration levels or TLS positivity, for either the entire cohort or the individual *BRCA*^{wt} and *BRCA*^{mut} groups (Table S5).

As expected, the immune infiltrate in our TNBC cohort was principally composed of T cells, with a higher percentage of CD3^{+} T cell TIL

Table 2
Immune infiltration parameters of TNBC patients grouped by germline *BRCA* gene status.

	BRCA^{wt}[a]	*BRCA*^{mut}[b]	Test
	N = 41[c] (48%)[d]	*N* = 44[c] (52%)[d]	*P*- value[e]
% Stromal TIL			**U-Mann Whitney**[f]
Median (min/max)	27.5 (3–80)	21.75 (3–80)	0.88[e]
Mean (+/− SD)	29.8 (22.6)	28.94 (19.59)	
% Intratumoral TIL			**U-Mann Whitney**[f]
Median (min/max)	6.5 (1–40)	5.25 (1–30)	0.69[e]
Mean (+/− SD)	9.4 (9.88)	9.1 (7.4)	
% Global TIL			**U-Mann Whitney**[f]
Median (min/max)	17.5 (2–60)	15 (3–55)	0.88[e]
Mean (+/− SD)	19.48 (15.3)	19 (13.8)	
TIL status			**Chi-square**[f]
TIL^{neg}	10[c] (24%)[d]	3[c] (7%)[d]	**0.037**[e]
TIL^{int}	21[c] (52%)[d]	33[c] (75%)[d]	
TIL^{hi}	10[c] (24%)[d]	8[c] (18%)[d]	
TLS			**U-Mann Whitney**[f]
Median (min/max)	4 (0–30)	4.75 (0–27.5)	0.81[e]
Mean (+/− SD)	6.91 (8.36)	5.92 (6.21)	
TLS presence			**Chi-square**[f]
No	13[c] (32%)[d]	9[c] (20%)[d]	0.32[e]
Yes	28[c] (68%)[d]	35[c] (80%)[d]	
% CD3$^+$			**U-Mann Whitney**[f]
Median (min/max)	10 (1.5–48.75)	10.13 (1.9–35)	0.62[e]
Mean (+/− SD)	13.05 (11.33)	12.55 (8.25)	
% CD4$^+$			**U-Mann Whitney**[f]
Median (min/max)	6.5 (0.5–30)	5.37 (1.25–25)	0.75[e]
Mean (+/− SD)	7.68 (6.13)	6.83 (4.97)	
% CD8$^+$			**U-Mann Whitney**[f]
Median (min/max)	3 (0.5–30)	4.37 (0.65–20)	0.15[e]
Mean (+/− SD)	5.39 (5.98)	5.75 (4.52)	
% CD20$^+$			**U-Mann Whitney**[f]
Median (min/max)	5.5 (0.25–25.5)	4.1 (0.3–23.8)	0.93[e]
Mean (+/− SD)	6.46 (5.61)	6.48 (5.77)	
Global PD-1$^+$			**Chi-square**[f]
Negative	29[c] (71%)[d]	30[c] (68%)[d]	0.82[e]
Positive	12[c] (29%)[d]	14[c] (32%)[d]	
Global PD-L1$^+$			**Chi-square**[f]
Negative	20[c] (49%)[d]	20[c] (45%)[d]	0.83[e]
Positive	21[c] (51%)[d]	24[c] (55%)[d]	
% PD-1$^+$ TIL			**U-Mann Whitney**[f]
Median (min/max)*	5 (7.5–10)	5 (5–35)	0.99[e]
Mean (+/− std)*	7.5 (3.5)	9.7 (9.7)	
% PD-1$^+$ TLS			**U-Mann Whitney**[f]
Median (min/max)*	10 (1–25)	10 (2–35)	0.80[e]
Mean (+/− SD)*	10.5 (7.2)	11.7 (9.7)	
% PD-L1$^+$ IC			**U-Mann Whitney**[f]
Median (min/max)*	7.5 (3–25)	7.5 (5–35)	0.99[e]
Mean (+/− SD)*	9.1 (7)	10.2 (8)	
% PD-L1$^+$ TC			**U-Mann Whitney**[f]
Median (min/max)*	5 (4–30)	7.5 (3–35)	0.69[e]
Mean (+/− SD)*	9 (8)	10.1 (9.5)	
% PD-L1$^+$ SC			**U-Mann Whitney**[f]
Median (min/max)*	7.5 (0.5–22.5)	5 (0.5–30)	0.70[e]
Mean (+/− SD)*	10 (8.7)	7.9 (6.6)	

*median and mean values were calculated in PD-1 or PD-L1 positive cases, respectively.
TNBC: triple negative breast cancer.
wt: wild-type.
mut: mutated.
TIL: tumor-infiltrating lymphocytes.
TLS: tertiary lymphoid structures.
SD: standard deviation.
TIL^{neg}: < 10% stromal TIL.
TIL^{int}: > 10% and < 50% stromal TIL.
TIL^{hi}: > 50% stromal TIL.
IC: immune cells.
TC: tumor cells.
SC: stromal cells.
[a] *BRCA*^{wt}: wild-type germline *BRCA1* or *BRCA2* genes.
[b] *BRCA*^{mut}: mutated germline *BRCA1* or *BRCA2* genes.
[c] Number of patients.
[d] Percentage of patients.
[e] *P* - value; significance < 0.05.
[f] Statistical test.

(median 10%, interquartile range (IQR: 5.25–16.69%) than CD20$^+$ B cell TIL (median 4.9%, IQR: 2.37–9.4; $P < 0.0001$), consistent with our previous study of all BC subtypes [10]. TLS were identified in 75% of the TNBC cohort with a median of 4.5 TLS per cm^2 of tumor tissue (IQR: 1.06–13.23%), which is higher than the 60% of TLS^{pos} tumors in the all BC subtype cohort and likely due to higher TIL in the former [10]. The major T cell TIL subpopulations, CD4$^+$ and CD8$^+$ T cells, were also scored (Fig. 3) with a higher CD4/CD8 ratio detected (median: 6%, IQR: 3.2–10% and median: 3.75%, IQR: 2.125–6.625%, respectively; $P = 0.0073$; Table 2). This again is similar to our previous findings in the all BC subtype cohort [10]. No statistically significant differences in CD4$^+$ T cell TIL, CD8$^+$ T cell TIL or CD20$^+$ B cell TIL were observed between the *BRCA*^{wt} and *BRCA*^{mut} groups (Table 2). Thus, the only significant differences detected between the *BRCA*^{wt} and *BRCA*^{mut} groups were the increases in TIL^{pos} tumors in the latter group.

3.4. PD-1 and PD-L1 expression in TNBC

Expression of the immune checkpoint molecules PD-1 and PD-L1 in the tumor microenvironment was assessed using a dual IHC stain (Fig. 4) to score positive TC, SC and IC. The overall TNBC cohort included 31% PD-1$^+$ and 53% PD-L1$^+$ tumors where one or more positive cell types were identified (Table 2). PD-1 expression was principally expressed on IC within TLS (where labeling was also more intense) compared to TIL in the stroma or tumor bed ($P = 0.0003$) as previously noted in studies of two independent BC cohorts (all subtypes) [8,10]. PD-1 expression was positively correlated with stromal TIL (r = 0.389, $P = 0.0002$) and the overall number of TLS (r = 0.384, $P = 0.0003$). PD-1 expression was also more positively correlated with CD20$^+$ B cell TIL (r = 0.425, $P < 0.0001$) than CD3$^+$ T cell TIL (r = 0.327, $P = 0.0023$). Correlations between PD-1 expression and the percentage of CD4$^+$ (r = 0.3398, $P = 0.0015$) and CD8$^+$ (r = 0.2452, $P = 0.0237$) TIL were similar.

PD-L1 expression on any cell type was positively correlated with stromal TIL (r = 0.6352, $P < 0.0001$) but not with a TLS presence or overall TLS scores, despite a trend towards a higher prevalence of PD-L1 positive tumors within the TLS^{pos} group ($P = 0.07$). PD-L1 expression was also positively correlated with the percentage of T cell TIL [CD3$^+$: r = 0.6448, $P < 0.0001$; CD4$^+$ and CD8$^+$ T cell TIL (r = 0.6083, $P < 0.0001$; $P < 0.001$ and r = 0.5724, $P < 0.0001$, respectively)] and B cell TIL (r = 0.5629, $P < 0.0001$). A positive correlation between PD-1 and PD-L1 expression was detected (r = 0.4814, $P < 0.0001$) with 26% of tumors expressing both markers. No differences in PD-1 and PD-L1 expression were found between the *BRCA*^{wt} and *BRCA*^{mut} groups (Table 2). Furthermore, no differences in I-DFS and OS were observed in the overall TNBC cohort based on PD-1 and/or PD-L1 expression (Table S5).

4. Discussion

The remarkable benefit that a limited number of cancer patients achieve after treatment with immunotherapy underscores the importance of identifying the composition and balance in clinically relevant immune responses. As an important first step, an in-depth characterization of the cellular and molecular interactions in BC immune infiltrates and their functional organization is needed. The present study adds to this knowledge by examining details of the immune infiltrate and its organization in a cohort of TNBC patients with known germline *BRCA* mutational status, the most frequent inherited mutations detected in BC.

DNA damage occurs in normal cells every day but these events are managed via different repair pathways charged with maintaining

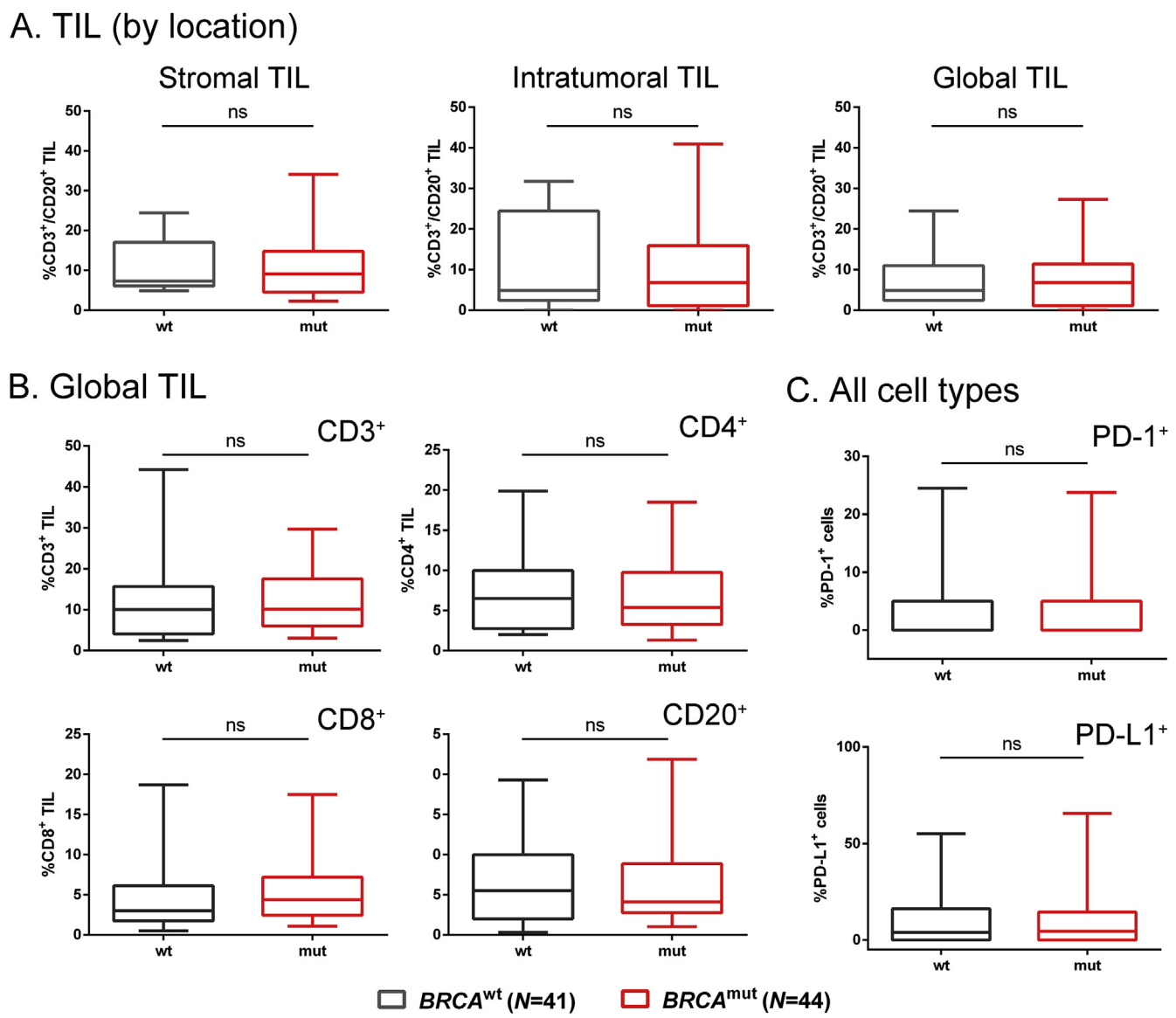

Fig. 3. Parameters of TIL infiltration in the *BRCA*^{wt} and *BRCA*^{mut} groups. (A) Stromal, intratumoral and global TIL scores for the *BRCA*^{wt} (N = 41) and *BRCA*^{mut} (N = 44) tumors in our TNBC cohort. TIL are scored as a percentage of the defined area (stromal, intratumoral or global) infiltrated with CD3$^+$ (T cells) plus CD20$^+$ (B cells) IHC stained cells. (B) Global TIL scores for TIL subpopulations in the *BRCA*^{wt} and *BRCA*^{mut} tumors. Global TIL are scored as a percentage of the stromal plus intratumoral area infiltrated with TIL subpopulations, including CD3$^+$ T cells, CD4$^+$ helper T cells, CD8$^+$ cytotoxic T cells and CD20$^+$ B cells. (C) PD-1 (scored on TIL) and PD-L1 (scored on any cell type: tumor, stromal or immune cells) expression in the tumor microenvironments of *BRCA*^{wt} and *BRCA*^{mut} tumors. The Mann-Whitney U test (non-parametric) was used to compare distribution of the continuous variables.

genomic integrity. DNA repair pathway defects are frequent in cancer with malignant cells shown to possess hundreds to thousands of somatic mutations [37]. Mismatch repair defects are found in multiple cancer types, including BC [38], with recent large-scale genomic analyses showing that a subset of patients within most tumor types carry a high mutational burden [39,40]. Higher mutational burdens increase the chance that at least one mutation is expressed as a neoantigen. Studies on the mutational burden in human cancer have highlighted an important association between immune activities including responsiveness to immunotherapy and hypermutated tumors (reviewed in Refs. [27,41]). This link was initially demonstrated for MSI^{hi} colorectal carcinomas, which were shown to be more sensitive to PD-1/PD-L1 pathway inhibition [42]. Studies into the relationship between mismatch repair defects and responses to immunotherapy across multiple cancer types are underway, with initial results supporting an important relationship.

The *BRCA* genes function to repair DNA double-strand breaks through homologous recombination, thereby acting as tumor suppressor genes with a known association between *BRCA* germline gene mutation and hereditary BC and OC [43,44]. Loss of *BRCA* gene function is correlated with genomic instability and increased copy number aberrations in these tumors. Loss of the *BRCA* DNA repair pathway has been repeatedly associated with immunogenicity and TIL density in OC [32,33,45–47] and in germline *BRCA1* mutated *versus* sporadic TNBC [48]. First, the *BRCA* gene mutations were correlated with increased immune infiltration in TNBC and OC [45,46,48] and *TP53* gene mutations in OC [46]. Next, a study showed that *BRCA*^{mut}, compared to homologous recombination proficient OC, had significant neoantigen increases that were associated with higher TIL and PD-1/PD-L1 expression [32]. Finally, two recent OC studies found that *BRCA* and *TP53* gene mutational status were correlated with PD-1/PD-L1 expression [33] and immunogenicity [47]. Overall, these data identify an important link between *BRCA* gene mutation, immune infiltration and the response to immunotherapy in OC. In BC, information on these

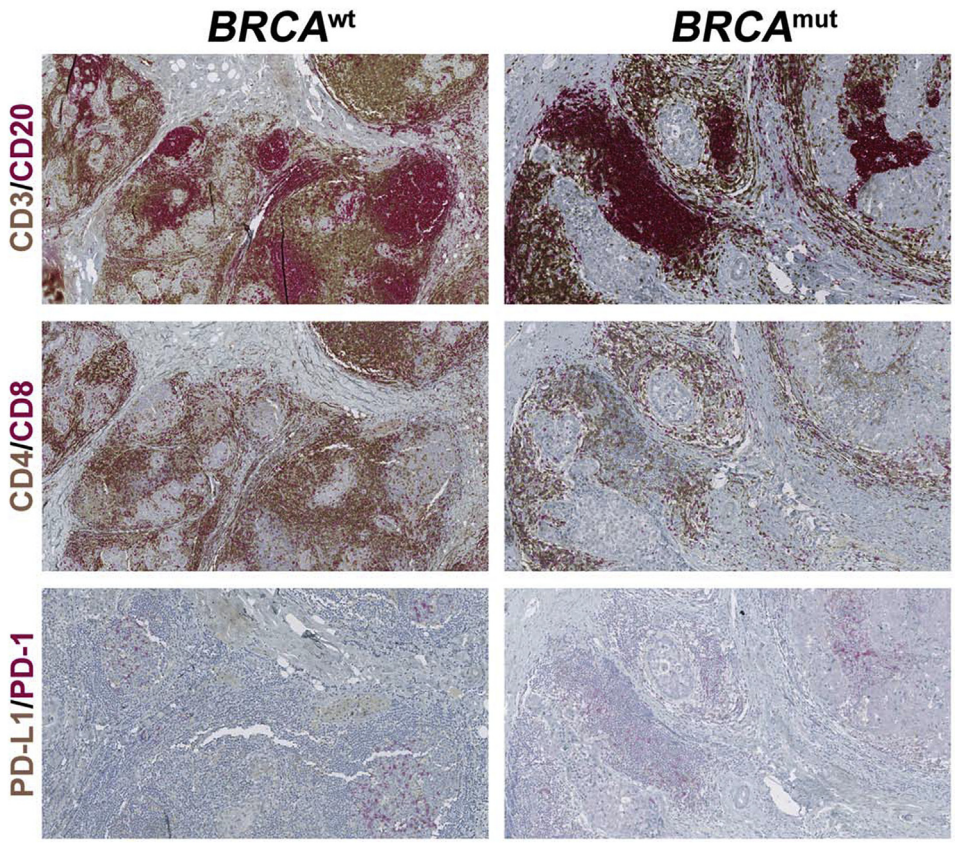

Fig. 4. Immune marker expression in the *BRCA*^{wt} and *BRCA*^{mut} TNBC groups. Representative examples of dual immunohistochemistry stains for T plus B cells (CD3/CD20), helper plus cytotoxic T lymphocytes (CD4/CD8) and PD-L1 plus PD-1 (PD-L1/PD-1) in TIL^{hi} tumors from the *BRCA*^{wt} and *BRCA*^{mut} groups.

associations is limited [48].

The current study asked whether germline *BRCA* gene mutational status was directly correlated with features of the immune infiltrate in a cohort of TNBC patients with a personal or familial history of BC or OC. Unexpectedly, similar scores for TIL density and location (stroma or intratumoral), lymphocyte subpopulation distribution (helper and cytotoxic T cells and B cells), PD-1 and PD-L1 expression on tumor, stromal or immune cells and lymphoid cell organization (TLS) characterized both the *BRCA*^{wt} and *BRCA*^{mut} groups in this cohort. Many studies have established that the frequency of TIL^{hi} tumors increases from Luminal A to Luminal B to HER2+ to TNBC [12] suggesting that our TNBC cohort should have been skewed toward higher infiltration. Similar to our previous data across all BC subtypes [10], a continuum of TIL densities was detected in both the *BRCA*^{wt} and *BRCA*^{mut} groups. An important notable difference was the significantly smaller number of TIL^{neg} tumors in the *BRCA*^{mut} group, suggesting these tumors may have a higher TIL set point than their wild-type counterparts in this TNBC cohort. The significant increase in TIL^{pos} tumors observed in the *BRCA*^{mut} group supports this view. These data also suggest that a basal level of immune infiltration, higher than that detected in *BRCA*^{wt}, is triggered in TIL^{pos} *BRCA*^{mut} BC; however, this concept has not been investigated. This increase in TIL associated with tumors in the *BRCA*^{mut} group supports the notion that there is higher immunogenicity associated with *BRCA* gene mutations.

BC has a relatively low number of nonsynonymous mutations compared to melanoma and lung cancer, the prototypic highly mutated and immunogenic tumors [6]. Analysis of 35,409 BC using a 25-gene hereditary cancer panel found that 9.3% of tumors carried pathogenic mutations with ~50% arising in genes other than *BRCA1* and *BRCA2* [6]. The prevalence of *BRCA1* mutations and other BC-associated genes, including *ATM*, *BARD1*, *BRIP1*, *CHEK2*, *PALB2* and *RAD51C*, were significantly enriched in the TNBC subgroup ($N = 4797$) of this study [6]. A sequencing study of 560 breast tumor genomes found numerous somatic mutations, including driver and recurrent mutations [38]. Interestingly, they also found 90 of these tumors had alterations in the *BRCA* genes associated with loss of DNA double-strand break repair functions. Considering these data, we suggest that the frequency of mutations in our *BRCA*^{wt} group could be similar to the *BRCA*^{mut} group, although this hypothesis needs to be tested in a future study that includes a TNBC group with no personal or family history of BC or OC.

Pathogenic germline *BRCA* gene mutations could increase the likelihood of immunogenic somatic mutations being generated due to their essential role in repairing double-strand DNA breaks [5]. Compared to other mutations in BC, including other DNA repair pathway defects, *BRCA*^{mut} may directly or indirectly provoke mutations that elicit stronger anti-tumor memory immune responses. This hypothesis is supported by numerous studies suggesting that patients with *BRCA* gene mutations have better clinical outcomes than those with sporadic BC or OC [49–51]. In our TNBC cohort, *BRCA*^{mut} patients showed a trend towards longer OS compared to the *BRCA*^{wt} group although this did not achieve statistical significance, which could be attributed to the small number of patients and/or events. Several recently published clinical studies have, however, shown there are no statistically significant differences in survival between *BRCA*^{wt} and *BRCA*^{mut} BC [50–52]. Additional support for this view comes from a recent OC study showing that *BRCA1*-mutated tumors have a potent immunogenic phenotype that is independent from tumor mutation burden [47].

Our TNBC cohort was composed of patients with a personal or family history of BC or OC, arguing that the *BRCA*^{wt} group could be different from sporadic BC. The *BRCA*^{wt} group potentially includes patients with 'BRCAness' or other homologous recombination DNA repair defects [44]. The continuum of TIL we detected in the *BRCA*^{wt} group was, however, remarkably similar to that previously found across all BC subtypes [10]. Further, the set point was not increased in the

BRCA^{wt} group, as observed in the *BRCA*^{mut} group. These data suggest that while *BRCA* and other DNA repair defects may be drivers of tumorigenesis and thereby increase the frequency of mutations, because these mutations are randomly generated their potential as a neoantigen is completely arbitrary. Once a mutation does create a neoantigen, however, then these tumors have acquired a well-established mechanism for activating immune responses. Overall, our data support the view that in TNBC the generation of mutations due to DNA repair defects is largely independent from immune status with the level of TIL and number of TLS being an indirect effect of this process and occurring at random.

Declaration of interests

Ethical approval and consent to participate

This study was approved by the Institut Jules Bordet's ethics committee (EC2038).

Consent for publication

All of the authors agree with the contents of this manuscript and consent to its publication.

Availability of data and materials

All of the relevant data analyzed in this study are included in the published article and online supplementary data. Individual patient data have not been made public but if necessary reasonable requests to the corresponding author will be considered. Patient information will only be released without identifiers.

Competing interests

The authors declare no financial or non-financial competing interests with the data presented in this manuscript. All authors declare that there are no financial and personal relationships with other people or organizations that could inappropriately influence (bias) this work.

Author contributions

Cinzia Solinas, Diane Marcoux, Daphné t'Kint de Roodenbeke and Karen Willard-Gallo participated in the conception and design of the study.

Cinzia Solinas, Soizic Garaud, Anaïs Boisson and Karen Willard-Gallo contributed in the development of the methodology.

Acquisition of the data was conducted by Cinzia Solinas, Diane Marcoux, Alexandre de Wind and Gert Van den Eyden.

Analysis and interpretation of data was performed by Cinzia Solinas, Diane Marcoux, Daphné t'Kint de Roodenbeke, Joel Rodrigues Vitória and Karen Willard-Gallo.

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The manuscript was written by Cinzia Solinas, Diane Marcoux, Daphné t'Kint de Roodenbeke and Karen Willard-Gallo.

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Appendix A. Supplementary data

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