Clinical Correlations of Programmed Cell Death Ligand 1 Status in Liquid and Standard Biopsies in Breast Cancer

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BACKGROUND: Data regarding the prognostic value of programmed cell death ligand 1 (PD-L1) expression on circulating tumor cells (CTCs) are lacking. However, CTCs could represent an alternative approach to serial biopsies, allowing real-time monitoring of cancer phenotype.

METHODS: We evaluated, in a dedicated prospective clinical trial, the clinicopathological correlations and prognostic value of PD-L1$^{(+)}$-CTCs in 72 patients with metastatic breast cancer (MBC).

RESULTS: Eighteen of 56 patients with available archival tissue presented at least one positive (≥1%) PD-L1 tumor sample. Baseline CTCs and PD-L1$^{(+)}$-CTCs were detected in 57 (79.2%) and 26 (36.1%) patients. No significant correlation was found between PD-L1 tumors and CTC expression. In univariate analysis, triple negative (TN) phenotype, number of metastatic treatments, >2 metastatic sites, ≥5 CTCs and PD-L1$^{(+)}$-CTCs were significantly associated with progression-free survival, while tissue PD-L1 expression was not. In multivariate analysis, TN phenotype, number of metastatic treatments and of metastatic sites were the only 3 variables independently associated with progression-free survival. Progesterone receptor negativity, TN phenotype, >2 metastatic sites and ≥5 CTCs were significantly associated with overall survival in univariate analysis. In multivariable analysis, TN phenotype and >2 metastatic sites were the only 2 independent variables.

CONCLUSIONS: Unlike PD-L1$^{(+)}$-tumor, PD-L1$^{(+)}$-CTCs correlate to survival in MBC. Reappraisal of the role of PD-L1 expression by tumor tissue and by CTCs under anti-PD-1/PD-L1 treatment is necessary to evaluate its predictive value and potential role as a stratifying factor in strategies and trials for MBC patients with MBC.

CLINICAL TRIAL REGISTRATION: NCT02866149

Introduction

The programmed cell death 1/ programmed cell death ligand 1 (PD-1/PD-L1) pathway is part of a negative feedback loop regulating the cellular immune response, found upregulated in many tumors. Inhibition of this pathway with PD-1 or PD-L1 antibodies was shown to induce dramatic responses and therapeutic effects in many tumors *(1)*. PD-L1 binding to PD-1 triggers a strong inhibitory signal in the T-cell, inducing reduction of cytokine production and arrest of T-cell proliferation *(2–5)*. This activation allows the PD-L1$^{(+)}$ cancer cells to evade the immune recognition *(6)*.

PD-1 and PD-L1 have been validated as therapeutic targets in various cancer types, and promising results have been reported in metastatic breast cancer (MBC) *(5, 7, 8)*. The clinical value of tumor and/or stromal PD-L1 expression as theranostic marker has been reported with conflicting results *(9–11)*. In the large

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phase III trial IMpassion 130 evaluating the addition of atezolizumab, an anti-PD-L1 antibody, to nab-paclitaxel, any benefit seems restricted to the PD-L1 positive subgroup *(12)*. Many issues also remain regarding the PD-L1 evaluation process in cancer, the cellular compartment to be tested, thresholds selected or antibody used. In addition, recent data point the spatial heterogeneity of PD-L1 expression in tumors, between the primary breast cancer and associated lymph nodes metastases *(13)*, or in renal cell carcinomas, between the primary tumor and distant metastases *(14)*, with a relative enrichment in PD-L1$^{(+)}$ cancer cells during tumor progression. These data highlight the need for a better understanding of PD-L1 expression during cancer evolution and for an accurate monitoring of its status in the metastatic setting.

An alternative approach to serial biopsies is the detection and characterization of circulating tumor cells (CTCs) in the peripheral blood *(15)*, allowing real-time monitoring of the cancer phenotype. Because CTCs isolated from the peripheral blood are a pool of cells derived from the primary tumor and/or of different metastatic sites, this approach could provide a comprehensive real-time picture of the whole tumor burden in a given patient *(16)*. Most studies have tested primary tumor tissues, precluding the possible evolution of cancer cells and micro-environment PD-L1 expression during the metastatic process *(17)*.

We previously published a validated and robust method for PD-L1$^{(+)}$-CTC detection *(18)*, allowing the detection of this biomarker in liquid biopsies. However, few data are currently available regarding the correlation between the primary tumor tissue, as usually determined in clinical practice, and CTC PD-L1 expression in the same patients, as well as the prognostic value of the determination of PD-L1 expression on CTCs. We present here the clinico-pathological correlations and prognostic value of the expression of PD-L1$^{(+)}$ on CTCs in a cohort of patients with MBC treated with conventional therapies.

Materials and Methods

PATIENTS

Patients with histologically-proven MBC (all lines and subtypes were eligible) were enrolled in the monocentric prospective **A**na**L**ysis of **CI**rculati**N**g tumor m**A**rkers in blood (**ALCINA**) study (NCT02866149). All patients gave their written informed consent before study entry. The study was approved by a local ethics committee and conducted in accordance with the Good Clinical Practice and Declaration of Helsinki. In addition, survival data (not previously reported) from the 16 patients included in our previous study *(18)*, the BMS_PD-L1_onco clinical trial (NCT10660776), assessing PD-L1 as a biomarker in oncology and hematology, were collected.

CTC DETECTION AND CTC PD-L1 EXPRESSION

Peripheral blood from 72 patients with MBC was collected before the start of treatment. Blood was drawn from the arm vein of each patient in CellSave® tubes (10 mL) used specifically for CTC detection. Blood samples (7.5 mL) were used for CTC detection on the FDA-cleared CellSearch® system in the laboratory dedicated to these analyses. The CellTracks® Autoprep was used on the samples within 72 h.

PD-L1 expression on CTCs was developed by our group [18] using the CellSearch® CXC kit and an anti-human B7H1/PD-L1 PE-conjugated antibody (Cat N° FAB1561P, R&D Systems). In this study, we optimized the detection of PD-L1$^{(+)}$-CTCs using the IVD CellSearch® CTC Kit with the anti-human B7H1/PD-L1 A488-conjugated monoclonal antibody (Cat N° FAB1561G, R&D Systems). This antibody was used at a final concentration of 17 μg/mL using the CellSearch® Analyzer II. Briefly, fixed CTCs were first positively enriched via the epithelial cell adhesion molecule (EpCAM) expression and defined as EpCAM isolated intact cells stained positive for cytokeratins (CK8, 18, 19), positive or negative for B7H1 (PD- L1), and negative for CD45 (the exclusion marker specific of normal leukocytes): CTCs were identified as EpCAM$^{(+)}$DAPI$^{(+)}$CK$^{(+)}$CD45$^{(-)}$. The classical cut-off of 5 CTCs was used as threshold *(19, 20)*, as well as the median (10 CTCs, exploratory analysis).

PD-L1 EXPRESSION ON TUMOR TISSUE

Tumor samples were provided by the ICM biological resources center (Biobank number BB-0033-00059), following the ethics and legal national French regulations for the patients' written informed consent. Whole primary tumor sections and, if available, recurrent tumor, nodal or distant metastatic tissue samples, were used to assess PD-L1 expression by immunohistochemistry (IHC). IHC was performed on 3-μm thin sections of formalin-fixed paraffin-embedded samples using the Autostainer AS48 (Dako). Sections were submitted to PTLink pretreatment (Dako), allowing simultaneous deparaffinization and antigen retrieval. Heat-induced antigen retrieval was performed for 20 min in low pH target retrieval Buffer (Dako) at 95 °C. Following antigen retrieval, endogenous peroxidase was quenched using Flex Peroxidase Block (Dako) for 5 min at room temperature. Slides were then incubated for 30 min with anti-human PD-L1 antibodies (mouse monoclonal, clone 22C3, 1:50, Dako). A mouse linker (Dako) was used to amplify the signal. After 2 rinses in wash buffer (Dako), incubation of the slides with a horseradish peroxidase-labeled polymer coupled to secondary anti-mouse and anti-rabbit antibodies was performed for 30 min, followed by 3,3'-diaminobenzidine for 10 min as substrate and DAB enhancer for 5 min.

Counterstaining was performed using Flex Hematoxylin (Dako), followed by slide wash (tap water, 5 min). Finally, the slides were mounted after dehydration. A slide of normal tonsil was used as positive control in each IHC run.

Sections were analyzed by two trained observers, blinded to the clinic-pathological characteristics and patient outcomes at the time of scoring. For each sample, tumor compartment was assessed, without considering the staining of stromal cells. On tumor cells, only the membrane staining was considered. The percentage of marked tumor cells and staining intensity was reported for each sample, scored using a 0–3 scale [none (0), weak (1+), moderate (2+), and strong staining (3+)]. In case of discordance between the two observers, slides were reviewed to reach a consensus. PD-L1 threshold positivity was set at 1% (1+). In case of multiple samples evaluation for a given patient, the patient was classified as positive if at least one sample was positive.

STATISTICAL ANALYSES

Descriptive analyses were performed using medians and ranges for continuous variables, and frequencies and percentages for categorical variables. Continuous parameters were compared using the Wilcoxon or Kruskal-Wallis test, and categorical parameters using chi-square test (or Fisher's exact test, if appropriate).

Median follow-up was calculated using the reverse Kaplan-Meier method. Progression-free survival (PFS) and overall survival (OS) were estimated using the Kaplan-Meier method and compared using the Log-rank test. PFS was defined as the time between the date of blood sampling and the date of the first progression or death from any cause. OS was defined as the time between the date of blood sample and the date of death from any cause. Multivariable analyses were performed using the Cox proportional hazard model. Variables affected with a P-value < 0.10 were considered in the multivariate model. Hazard ratios (HR) are given with their 95% confidence interval (95% CI). All tests were two-sided, and P-values ≤ 0.05 were considered statistically significant. All statistical analyses were performed with STATA 13.0 software (StatCorp). The follow-up cut-off date was March 21, 2018.

Results

PATIENT DEMOGRAPHICS

Seventy-two patients were included in this prospective study (Table 1). Median age was 65 years (range: 35–87). Tumors were classified for hormone receptor (HR), human epidermal growth factor receptor 2 (HER2), or triple negative (TN) status. Fifty-one patients (70.8%) were $HR^{(+)}/HER2^{(-)}$, 13 (18.1%) $HER2^{(+)}$, and 8 (11.1%) triple negative (TN). Forty patients (55.6%) were treated with at least 4 metastatic lines and 42 (58.3%) presented with more than 2 metastatic sites. None of the patients received anti-PD-1/PD-L1 targeted therapy.

CORRELATION BETWEEN TISSUE AND CTC PD-L1 EXPRESSION

There was no available tumor tissue for 16 patients. At least one archival tissue was available for 56 patients (47 primary tumors, 10 local relapses, 8 lymph node metastases, and 28 distant metastases), with multiple samples available for 22 patients (94 analyzed samples, Supplemental Table 1). Eighteen of the 56 patients (32.1%) had at least one sample with a positive (≥1%) PD-L1 expression.

Baseline CTCs were detected in 57 patients (79.2%); 41 (56.9%) patients presented with ≥5 CTCs. PD-L1$^{(+)}$-CTCs were detected in 26 (36.1%) cases (Supplemental Table 1; Fig. 1). There was no statistically significant correlation between the presence of at least one PD-L1$^{(+)}$-CTC and the total number of CTCs detected neither in the global population (Supplemental Fig. 1, Spearman's rho = 0.27), nor in the population with at least one detectable CTC (Supplemental Fig. 2, Spearman's rho = 0.24).

We evaluated the correlations between CTC PD-L1 expression in the population of patients with available tissue (n = 56). No statistically significant correlation was found between PD-L1 tumor tissue expression and the presence of PD-L1$^{(+)}$-CTCs ($P = 0.589$, Supplemental Table 2). No difference was observed in the distribution of the number of PD-L1 CTC when dichotomizing the population in two groups (patients with positive and negative PD-L1 tumors ($P = 0.589$; Supplemental Table 1; Supplemental Fig. 3). In addition, we evaluated this correlation in the population of patients with available tissue and at least one detectable CTC (n = 36) and found no correlation in this subpopulation ($P = 0.6$).

PROGRESSION-FREE SURVIVAL

At the time of analysis, after a median follow-up of 17.2 months (95% CI: 16.6–19.6), 60 patients (83.3%) had progressed and 39 (54.2%) were dead.

Median PFS was 5.1 months (95% CI: 3.5–8.2). In univariate analysis, TN phenotype ($P = 0.02$), more than 3 previous metastatic treatment lines ($P < 0.001$), more than 2 metastatic sites ($P = 0.01$), CTC count using the classically used ≥5 CTCs threshold ($P = 0.05$) and detection of PD-L1$^{(+)}$-CTCs ($P = 0.03$) (Fig. 2A) were significantly associated with PFS, while PD-L1 expression in biopsies ($P = 0.34$) was not (Table 1).

In multivariable analysis (Table 2), TN phenotype (HR = 3.37; 95% CI: 1.36—8.37, $P = 0.01$), more than 3 previous metastatic treatment lines (HR = 2.47; 95%

Table 1. Characteristics of the population and univariate correlations (N = 72).

Variables	N (%)	1-year PFS rate (%)	*P*	1-year OS rate (%)	*P*
Age			0.979		0.801
<65 years old	36 (50.0)	25.0		54.5	
≥65 years old	36 (50.0)	31.6		68.3	
ER			0.849		0.371
Negative	14 (19.4)	28.6		55.6	
Positive	58 (80.6)	28.1		62.5	
PR			0.148		**0.036**
Negative	31 (43.1)	16.2		51.5	
Positive	41 (56.9)	36.4		68.1	
HER2			0.143		0.301
Negative	59 (81.9)	24.1		58.0	
Positive	13 (18.1)	46.2		76.2	
Triple negative			**0.019**		**0.018**
No	64 (88.9)	31.6		66.0	
Yes	8 (11.1)	0		25.0	
HR/HER2 status			**0.039**		0.085
$HR^-/HER2^-$	8 (11.1)	0.0		25.0	
$HR^-/HER2^+$	5 (6.9)	60.0		100.0	
$HR^+/ HER2^-$	51 (70.8)	28.0		63.3	
$HR^+/ HER2^+$	8 (11.1)	37.5		62.5	
Number of metastatic lines			**< 0.001**		0.169
≤3	32 (44.4)	51.7		70.7	
>3	40 (55.6)	10.0		54.0	
Number of metastatic sites			**0.014**		**0.028**
≤2	30 (41.7)	40.0		69.7	
>2	42 (58.3)	19.7		55.2	
Primary tumor PD-L1 expression (N=47)			0.463		0.858
Negative	37 (78.7)	33.5		60.1	
Positive	10 (21.3)	40.0		70.0	
PD-L1 expression on any tumor sample (primary or metastases, N=56)			0.480		0.303
All negative samples	38 (67.9)	29.8		66.8	
At least one positive sample	18 (32.1)	33.3		60.6	
Baseline CTC count			0.053		0.051
<5	31 (43.1)	37.0		76.2	
≥5	41 (56.9)	22.0		50.1	
Baseline CTC count			0.072		**0.043**
<10	36 (50.0)	34.5		76.8	
≥10	36 (50.0)	22.2		45.9	
Baseline $PD\text{-}L1^{(+)}$-CTC count			**0.026**		0.091
Negative	46 (63.9)	35.6		68.5	
Positive	26 (36.1)	15.4		48.3	

ER, estrogen receptor; HR, hormone receptors; PR, progesterone receptor; CTC, circulating tumor cells; PFS, progression-free survival; OS, overall survival.

CI: 1.38—4.42, $P = 0.002$) and more than 2 metastatic sites (HR = 2.13; 95% CI: 1.13–4.02, $P = 0.02$) were the only 3 variables independently associated with PFS.

OVERALL SURVIVAL

Median OS was 19.8 months (95% CI: 11.7–37.5). Progesterone receptor negativity ($P = 0.04$), TN phenotype ($P = 0.02$), more than 2 metastatic sites ($P = 0.03$),

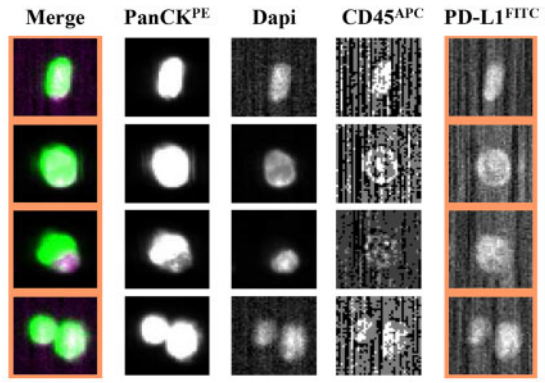

Fig. 1. Representative photos of programmed cell death ligand 1 positive circulating tumor cells (PD-L1$^{(+)}$- CTCs) detected in metastatic breast cancer using the IVD CellSearch® CTC Kit in the CellSearch® system. CTCs are identified as EpCAM$^{(+)}$CK$^{(+)}$Dapi$^{(+)}$CD45$^{(-)}$. The antibody used in the 4th channel is the anti-human B7-H1/PD-L1 A488-conjugated monoclonal antibody at a final concentration of 17 μg/mL. At the bottom of the figure, a cluster of two CTCs can be observed. EpCAM, epithelial cell adhesion molecule; PE, phycoerythrin; APC, allophycocyanin; A488, AlexaFluor; PanCK, panel of cytokeratins 8, 18 & 19.

Table 2. Multivariable analysis on progression-free and overall survival (N = 72).

Progression-free Survival			
Variables	Hazard ratio	95% CI	*P*
Triple negative phenotype	3.37	1.36–8.37	0.014
More than 3 previous metastatic lines	2.47	1.38–4.42	0.002
More than 2 metastatic sites	2.13	1.13–4.02	0.015
Overall Survival			
Variables	Hazard ratio	95% CI	*P*
Triple negative phenotype	8.38	2.57–27.25	<0.001
More than 2 metastatic sites	3.58	1.43–8.98	0.002
≥5 CTCs	1.95	0.93–4.06	0.068

95% CI: 95% confidence interval; CTC: circulating tumor cells. Variables included in the multivariate analysis: overall survival: PR status, triple negative status, number of metastatic sites, baseline CTC count, baseline PD-L1$^{(+)}$-CTC count; progression-free survival: triple negative status, HR/HER2 status, number of metastatic lines, number of metastatic sites, baseline CTC count, baseline PD-L1$^{(+)}$-CTC count.

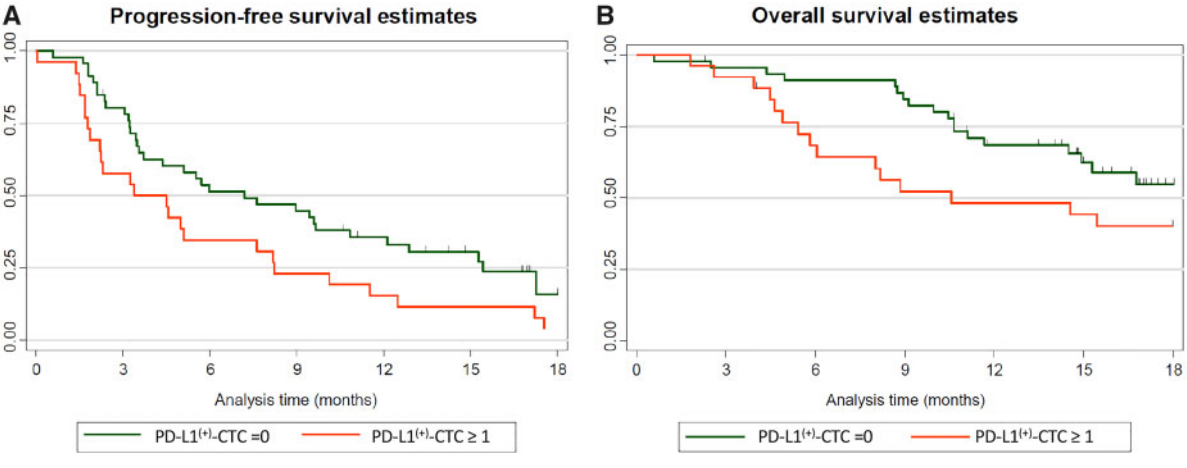

Fig. 2. Patients survival according to the detection or absence of programmed cell death ligand 1 positive circulating tumor cells (PD-L1$^{(+)}$-CTCs). a) Progression-survival according to the detection (orange) or absence (green) of PD-L1$^{(+)}$-CTCs. b) Overall survival according to the detection (orange) or absence (green) of PD-L1$^{(+)}$-CTCs.

and the CTC count using the classically used ≥5 CTCs threshold ($P = 0.05$) were significantly associated with OS in univariate analysis. There was an association with borderline significance between OS and PD-L1$^{(+)}$-CTCs detection ($P = 0.09$) (Fig. 2B; Table 1). TN phenotype (HR = 8.38; 95% CI: 2.57–27.25, $P < 0.001$) and more than 2 metastatic sites (HR = 3.58; 95% CI: 1.43–8.98, $P = 0.002$) were the only 2 variables independently associated with OS in multivariable analysis; additionally, an association with borderline significance was observed for the detection of more than 5 CTCs (HR = 1.95; 95% CI: 0.93–4.06, $P = 0.068$).

CTC-POSITIVE PATIENTS

As PD-L1$^{(+)}$- CTCs could only be detected in cases with CTCs we performed a subgroup analysis in the CTC$^{(+)}$-patients (n = 57). In univariate analysis, more than 3 previous metastatic treatment lines ($P < 0.001$) and more than 2 metastatic sites ($P = 0.03$) were significantly associated with PFS, while an association with borderline significance was observed for CTC count using the classically used ≥5 CTCs threshold ($P = 0.07$) and PR negativity ($P = 0.08$). In multivariable analysis, more than 3 previous metastatic treatment lines (HR = 2.35; 95% CI: 1.19–4.61, $P = 0.01$), and more than 2 metastatic sites (HR = 2.28; 95% CI: 1.05–4.93, $P = 0.04$) were the only 2 variables independently associated with PFS, while an association with borderline significance was observed for TN phenotype (HR = 2.86; 95% CI: 0.94–8.71, $P = 0.07$).

TN phenotype ($P = 0.02$) and more than 2 metastatic sites ($P = 0.05$) were the only 2 variables significantly associated with OS in univariate analysis. In multivariable analysis, TN phenotype (HR = 9.54; 95% CI: 2.47–36.76, $P < 0.001$) and more than 2 metastatic sites (HR = 4.29; 95% CI: 1.41–13.05, $P = 0.01$) remained the only 2 variables independently associated with OS.

Discussion

Therapies targeting the PD-1/PD-L1 interaction have led to major improvements in many solid tumors and are still extensively evaluated in MBC. However, the quest for the optimal theranostic marker is still ongoing in this setting. To date, there is no guideline for antibody selection, validated scoring system, or optimal type of cells for PD-L1 status determination (tumor cells, stroma, immune infiltrate) in breast cancer, leading to highly heterogeneous results regarding its prognostic and predictive values. PD-L1 expression using IHC varies from 6% to 92.4%, depending on the series, the methods of evaluation, and the threshold used *(21)*. Other issues lie in the heterogeneity of expression depending on breast cancer subtypes *(22)*, and in the compartment being evaluated, considering global tissue, tumor cell, or T-infiltrating lymphocyte PD-L1 expression. In a previous study focusing on nonmetastatic TNBC, we reported a 56.1% tumor cell PD-L1 expression rate, while T-infiltrating lymphocyte PD-L1 expression rate was 82.1% *(23)*.

Expression heterogeneity could also depend on the evaluated location, as suggested by Li et al. *(13)*. In this study, 25.7% of the primary tumor reported PD-L1 expression, a proportion that increased to 40.6% in metastatic lymph nodes tumor cells, while in 20.8% of the cases, negative PD-L1 expression in primary tumors was associated with positive PD-L1 paired lymph nodes *(13)*. PD-L1 positive cases, either on the primary tumor or on the lymph nodes, were associated with a worse prognosis than negative cases, both on the primary tumor and the lymph nodes. These results suggest that PD-L1 expression, either in tissue metastases or CTCs, could be a better biomarker for prognosis and for response than PD-L1 expression on the primary tumor cells. However, most of the current trials evaluating antibodies targeting the PD-1/PD-L1 checkpoint evaluate the biomarkers status either on a metastatic sample or primary tumor, without consideration of the biomarker evolution following previous treatments or microenvironment changes. Nevertheless, PD-L1 status, even in the primary tumor, appears as the major determinant of atezolizumab benefit in the Impassion 130 study *(12)*. Thus, a refinement in PD-L1 evaluation could have the potential to better select the population of interest for anti-PD-L1 treatments. Because most of the present indications for therapies targeting the PD-1/PD-L1 axis consider the tissue expression of PD-L1, most of the time on archival tissues, a better understanding of discrepancies between PD-L1 status over time could help refine the patient selection.

In the present study, using a 1% threshold, we found PD-L1 expression in 21.3% of the unselected MBC primary tumors, including all histological subtypes, with this percentage rising to 32.1% in the primary and metastatic samples. This rate is similar to previous reports in this setting, and close to the percentages found in the IMpassion 130 study *(12)*. In the IMPpassion 130 pivotal study, the analyzed tissue samples were representative tumor specimens from either formalin-fixed, paraffin-embedded archival or fresh pretreatment relapsed-disease tumor tissue, reflecting the heterogeneity seen in our cohort of patients. We found, as reported by Li et al. *(13)*, discrepancies between primary tumor and metastatic tissues in a proportion of our patients. However, contrarily to their report, we found no significant association between PD-L1 tissue expression and neither PFS or OS. This could be linked to the limited number of patients in our series, or the fact that, in order to encompass all the diversity of breast

cancer, we included all the different subtypes of breast cancers in our series, while Li et al. (*13*) focused on the triple negative breast cancer subtype. Another limitation lies in the predominance of $ER^{+}/HER2^{-}$ tumors, associated with a high percentage of nonmeasurable diseases, precluding, altogether with the sample size and the late stage setting, the use of objective response as a relevant endpoint.

Altogether, with classical MBC prognostic factors, we found in univariate analysis a significant correlation between PD-$L1^{(+)}$-CTCs detection and PFS. CTC evaluation appears of interest in this setting, as it probably faithfully reflects the PD-L1 status of the whole metastatic burden, and could represent an easy biomarker throughout the disease evolution. We were able to detect CTCs (whatever their PD-L1 status) in 79.2% of our patients, 57% of the patients presenting with 5 or more CTCs, a percentage in line with published MBC series and the published pooled analysis *(24)*. In univariate analysis, we found an association between baseline CTCs and survival, as classically described in MBC *(24–26)*. However, this association was not significant in multivariable analysis, probably due to the small sample size of our cohort.

Recently, Schott et al. *(27)* reported a very high number (94.5%) of breast cancer patients positive for PD-$L1^{(+)}$-CTCs. However, it is important to note that the authors detected a surprising increased number of CTCs in patients with breast cancer (median of 550/mL of blood, range: 50–8050) with their own technology. Moreover, the median PD-$L1^{(+)}$-CTCs rate was 68.9% (range: 0–100), thus these data must be taken with caution while comparing them with data obtained using the validated IVD CellSearch® system. Indeed, even if the CellSearch® system focuses only on the $EpCAM^{(+)}$-subpopulation of CTCs, CTCs with a very weak expression of EpCAM are still enriched efficiently. Moreover, the technology used by Schott et al. *(27)* must be validated in the future by other independent groups on independent cohorts of breast cancer patients to confirm and validate their data.

Interestingly, Ali et al. *(28)* showed that PD-L1 protein expression in breast cancer is rare, enriched in basal-like tumors and associated with infiltrating lymphocytes. The high frequency of PD-L1 expression we found contrasts with this previous work suggesting breast cancer cells may have low PD-L1 expression in the primary tumor. This discrepancy could be due to differences in the antibodies used among the studies. It could also be due to a small subset of more aggressive PD-$L1^{(+)}$ primary tumor cells but these minor clones might be difficult to identify because of the expression on lymphocytes and stromal cells. A last hypothesis could be that invasive and disseminating tumor cells could acquire PD-L1 expression during invasion. PD-L1 expression could then be induced and expressed when more aggressive cells disseminate.

The detection of PD-$L1^{(+)}$-CTCs as liquid biopsy appears to be an interesting method to evaluate the PD-L1 expression pattern in MBC, circumventing the heterogeneous expression of this biomarker between the primary tumor and the various metastatic sites. Its correlation with survival needs to be validated in a larger cohort.

In summary, in this heterogeneous cohort of patients with MBC, no statistically significant correlation was found between PD-L1 tumor tissue expression and the presence of PD-$L1^{(+)}$-CTCs. Our results suggest a correlation between the total CTC count and PD-L1 expression on CTCs, but not with PD-L1 expression in the tumor tissue. Patients with MBC harboring PD-$L1^{(+)}$-CTCs are a negative prognostic biomarker in MBC, because patients with MBC harboring PD-$L1^{(+)}$-CTCs have a shorter PFS; however, this result was not confirmed in multivariable analysis. A validation of these results in an independent population treated without anti PD-L1 therapies remains necessary to precise its prognostic value.

The reappraisal of the role of PD-L1 expression by tumor tissue and by CTCs under anti-PD-1/PD-L1 treatment is necessary in order to clearly evaluate the predictive value of this biomarker and its potential role as a stratifying factor in strategies and trials for patients with MBC.

Supplemental Material

Supplemental material is available at *Clinical Chemistry* online.

Nonstandard abbreviations: CTC, circulating tumor cell; ER, estrogen receptor; FITC, fluorescein isothiocyanate; HER-2, human EGF receptor 2; HR, hormone receptors; IHC, immunohistochemistry; IVD, in vitro diagnosis; MBC, metastatic breast cancer; NACT, neoadjuvant chemotherapy; NAHT, neoadjuvant hormone therapy; OS, overall survival; PanCK, panel of cytokeratins 8, 18, & 19; PD-1, programmed cell death 1; PD-L1, programmed cell death ligand 1; PE, phycoerythrin; PFS, progression-free survival

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W. Jacot contributed to the conception and design of the entire study, selected eligible patients, acquired data on clinical-pathological parameters, contributed to data interpretation, contributed to statistical analysis, and drafted the manuscript; M. Mazel was responsible for designing and optimizing the new PD-$L1^{(+)}$

CTC assay in the CellSearch® system, carried out the experiments for the CTC detection, and contributed to data interpretation; C. Mollevi supervised the statistical analysis, contributed to data interpretation, and assisted in drafting the manuscript; S. Pouderoux selected eligible patients, contributed to data interpretation, and assisted in drafting the manuscript; V. D'Hondt selected eligible patients, contributed to data interpretation, and assisted in drafting the manuscript; L. Cayrefourcq carried out the experiments for the CTC detection, contributed to data interpretation, and assisted in drafting the manuscript; C. Bourgier selected eligible patients, contributed to data interpretation, and assisted in drafting the manuscript; F. Boissiere-Michot was responsible for designing and optimizing PD-L1 IHC analyses, carried out most of the IHC experiments, contributed to data interpretation, and assisted in drafting the manuscript; F. Berrabah coordinated sample collection, acquired data on clinical-pathological parameters, and assisted in drafting the manuscript; E. Lopez-Crapez contributed to data interpretation and assisted in drafting the manuscript, F.-C. Bidard contributed to the conception and design of the entire study, assisted in drafting the manuscript, and contributed to the critical revision of the manuscript; M. Viala selected eligible patients, contributed to data interpretation, and assisted in drafting the manuscript; T. Maudelonde contributed to the conception and design of the study, drafted and revised the manuscript; S. Guiu selected eligible patients, contributed to data interpretation, and assisted in drafting the manuscript; C. Alix-Panabières contributed to the conception and design of the entire study, contributed to data interpretation, and drafted the manuscript.

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