Article

Prediction of response to Atezolizumab plus nab-Paclitaxel in unresectable locally advanced Triple Negative Breast Cancer (TNBC): the Clinical usefulness of PD-L1 mRNA expression in plasma-derived exosomes

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Abstract: Patients diagnosed with unresectable locally advanced Triple Negative Breast Cancer (TNBC) usually have poor outcome for its aggressive clinical behaviour. Atezolizumab plus nanoparticle albumin-bound (nab)-Paclitaxel prolonged progression-free survival (PFS) and overall survival (OS) among patients with unresectable locally advanced TNBC but its use is hampered by the lack of reliable predictors of tumor response. Seventy-seven consecutive patients with unresectable locally advanced TNBC treated with Atezolizumab plus nab-Paclitaxel were studied by blood draws at baseline, 28 days and 56 days after initiation of treatment. Exosomal PD-L1 mRNA in plasma was determined using Bio-Rad QX100 digital droplet PCR system and exoRNeasy kit and objective responses were defined following the RECIST criteria v.1.1. The study evaluates whether PD-L1 mRNA copies per ml in plasma-derived exosomes may predict response to anti-PD-L1 antibodies early in the course of therapy. Our data showed patients with unresectable locally advanced TNBC and higher levels of PD-L1 mRNA expression in plasma-derived exosomes at baseline demonstrated greater response to atezolizumab plus nab-paclitaxel. Furthermore, the levels of mRNA decreased with successful treatment while the copy number increased in patients experiencing disease progression following atezolizumab plus nab-paclitaxel. For the first time, our data showed the usefulness of assessment of exosomal PD-L1 as non-invasive real-time biopsy in patients diagnosed with TNBC suggesting exosomal PD-L1 is significantly associated with outcome and response to Atezolizumab plus nab-Paclitaxel.

Keywords: Exosomal PD-L1 mRNA; extracellular vesicles; Triple Negative Breast Cancer; Immunotherapy; PD-L1 axis; Atezolizumab – nab-paclitaxel; Predictive biomarkers; Liquid biopsy

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1. Introduction

Triple-negative breast cancers (TNBC), defined by a lack of both estrogen (ER) and progesterone (PgR) receptors as well as no amplification of the human epidermal growth factor receptor 2 (HER2) gene, represent a highly malignant group of tumors accounting for nearly 20% of all breast cancers and it is associated with impaired clincal outcome compared with other types of breast cancers [1-3]. The absence of defined molecular targets is responsible for the limited current systemic treatment options available for this disease, including cytotoxic chemotherapy with poor response [4-6]. Furthermore, the development of resistance to chemotherapy continues to be the major cause of treatment failure in TNBC, having an impact on the survival of these patients [7]. The median overall survival (OS) of TNBC is about 18 months with treatment while the OS of metastatic TNBC is about 12 months [8].

A growing understanding of tumour biology, of alterations in molecular processes involved and cancer microenvironment has allowed the developing of new therapeutic approaches to treatment of TNBC. TNBC subtype, compared to luminal subtype, is considered to be highly immunogenic, with a higher enrichment by tumour-infiltrating lymphocytes (TILs) and higher levels of programmed cell death ligand 1 (PD-L1) expression [9]. For these reasons, TNBC may be more likely than other subtypes to benefit from immunotherapy [10,11]. Immune checkpoint blockade therapy, extensively used in different types of solid tumors having improved the clinical outcome notably in cancers poorly responsive to chemotherapy, are currently being evaluated as an emerging treatment option for TNBC. In the light of these observation, the randomized phase III trial IMpassion130 (NCT02425891) showed atezolizumab, a monoclonal antibody targeting PD-L1, combined with nab-paclitaxel exhibited a PFS benefit that was statistically significant, at 7.2 months over 5.5 months with placebo for patients with unresectable locally advanced TNBC or metastatic TNBC whose tumours express PD-L1 (≥1%) (HR, 0.80; 95% CI, 0.69-0.92; *P* =.002) [12]. Paclitaxel is used in its nanoparticle albumin–bound(nab) form in order not to require steroid premedicationand to avoid their potential immunosuppressive effects [13]. There were clinically meaningful improvements in OS as well, even though it was not formally tested in that study. Despite correlation between PD-L1 expression and clinical outcome has been investigated in several malignancies,the usefulness of PD-L1 expression levels in predicting response to atezolizumab plus nab-paclitaxel in TNBC remains uncertain [14].

With the advent of novel techniques, such as liquid biopsies, various tumor components released into blood stream can be analyzed,including exosomes, nano-sized extracellular vesicles (EVs), with a diameter ranging from 40 to 150 nm and a lipid bilayer membrane carring proteins, DNA and RNA of tumour cells from which they are generated [15-16]. In this scenario, exosomes seem to reflect the phenotypic state of cells from which they are originated [17]. Despite theirestablished role in multiple diseases, only fewstudies have focused on their role on TNBC [4,18]. Citing the paucity of reliable indicators of response in TNBC, we conducted this study to evaluate whether PD-L1 mRNA copies per ml in plasma-derived exosomes may predict the response to anti-PD-L1 antibodies early in the course of therapy in unresectable locally advanced TNBC.

2. Results

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2.1. Patient Characteristics

A total of 77 consecutive patients, all of them Caucasian, diagnosed with unresectable locally advanced histologically documented TNBC treated with atezolizumab in combination with nab-paclitaxel were enrolled in the study between March 2019 and December 2019. They were assessable for the analysis of PD-L1 mRNA and LMr. Patients who did not complete blood draw were excluded from the analysis.

The main baseline clinicopathological characteristics of the 77 eligible patients are presented in Table A1 (Appendix A).

All patients were women and the median age was 49 years (range, 37-68 years). Sixty-six patients (86%) had an Eastern Cooperative Oncology Group Performance Status (ECOG PS) of 0 and 11 patients (14%) had an ECOG PS of 1-2. Sixty-nine (90%) patients had an invasive ductal carcinoma; 55 patients had Ki-67 > 20 and 67 patients (87%) had positive nodal status before enrollment. We studied Human epidermal growth factor receptor 2 (HER2) status using both immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) using the FDA-approved Vysis PathVysion HER-2/neu DNA Probe Kit (Dako Cytomation, Denmark): 32 patients belonged to the group of score 0 or 1+ while 45 patients belonged to the group of score 2+, .

All patients were assessable for the analysis and about 200 blood draws were collected at baseline, and at 28 and 56 days after initiation of treatment.

2.2. PD-L+ mRNA copies at baseline corresponded to response

The 28 patients achieving complete response (CR) and partial response (PR) had a significantly higher number of PD-L1 mRNA copies per ml compared to 49 patients showing stable disease (SD) or progressive disease (PD); the mean value (standard error of mean [s.e.m.] was 785.6 (±121.1) copies/ml compared to 114.7 (±31.4), respectively ($p < 0.001$).

The mRNA copy level decreased with treatment. Patients showing CR and PR demonstrated mean PD-L1 copies/ml of 747.6±121.1 at baseline that was reduced to 175.4 copies/ml at 2 months post treatment ($p = 0.001$). Patients with SD had mRNA levels that remained relatively constant from baseline to 2 months post treatment; mean (± s.e.m.) values were 270 (±71.1) and 217.5 (±17.3) copies per ml, respectively ($p = 0.614$). Levels of PD-L1 mRNA copies increased with treatment in patients showing PD from 124.1 (±31.2) at baseline to 494.3 (±46.2) copies per ml at 2 months post treatment ($p < 0.001$).

Response	Patients with: PD-L1mRNA copies/mL Baseline	PD-L1mRNA copies/ mL Two months later	*p-value*
CR+PR	747.7±121.1	175.4±11.3	**p=0.001**
SD	270±71.1	217.5±17.3	p=0.614
PD	124.1±31.2 vs	494.3±46.2	**p<0.001**

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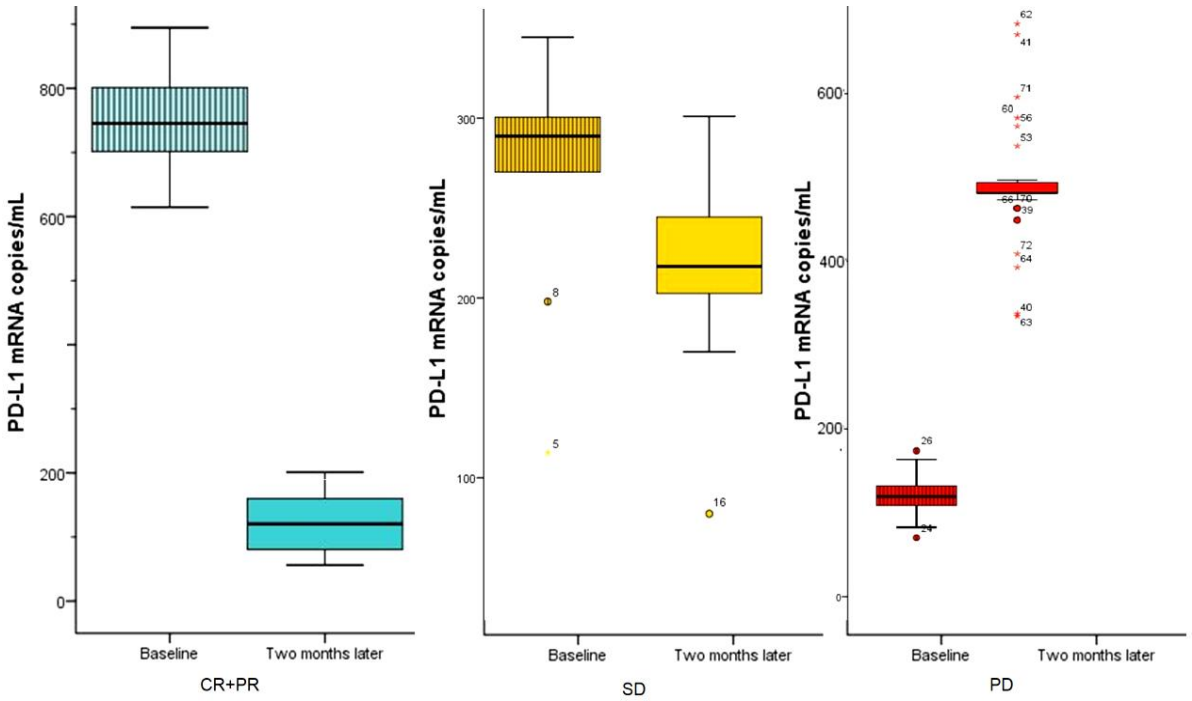

Figure 1 and Table 1: PD-L1 mRNA copies per mL at baseline and two months after the start of Atezolizumab plus nab-Paclitaxel treatment. CR:complete response; PR:partial response;SD:stable disease; PD:progressive disease.

2.3. Increased PD-L1 mRNA with treatment was associated with shorter survival

Patients having an increase of PD-L1 mRNA copies per ml following treatment with atezolizumab plus nab-paclitaxel demonstrated significantly shorter progression-free survival ($p = 0.007$). Overall survival (OS) was 5 months in these patients (range, 2 to 7 months, 95% confidence interval 1.1-6.1) as compared to OS that was more than doubled (range, 8-15 months) in patients not showing an increase in mRNA with treatment ($p = 0.001$). Two patients died, one due to PD (mRNA PD-L1 copies per ml increased from 90 to 360), and the other because of acute myocardial infarction, while on CR (PD-L1 mRNA decreased from 2000 to 1000 copies per ml), as showed in Figure 2.

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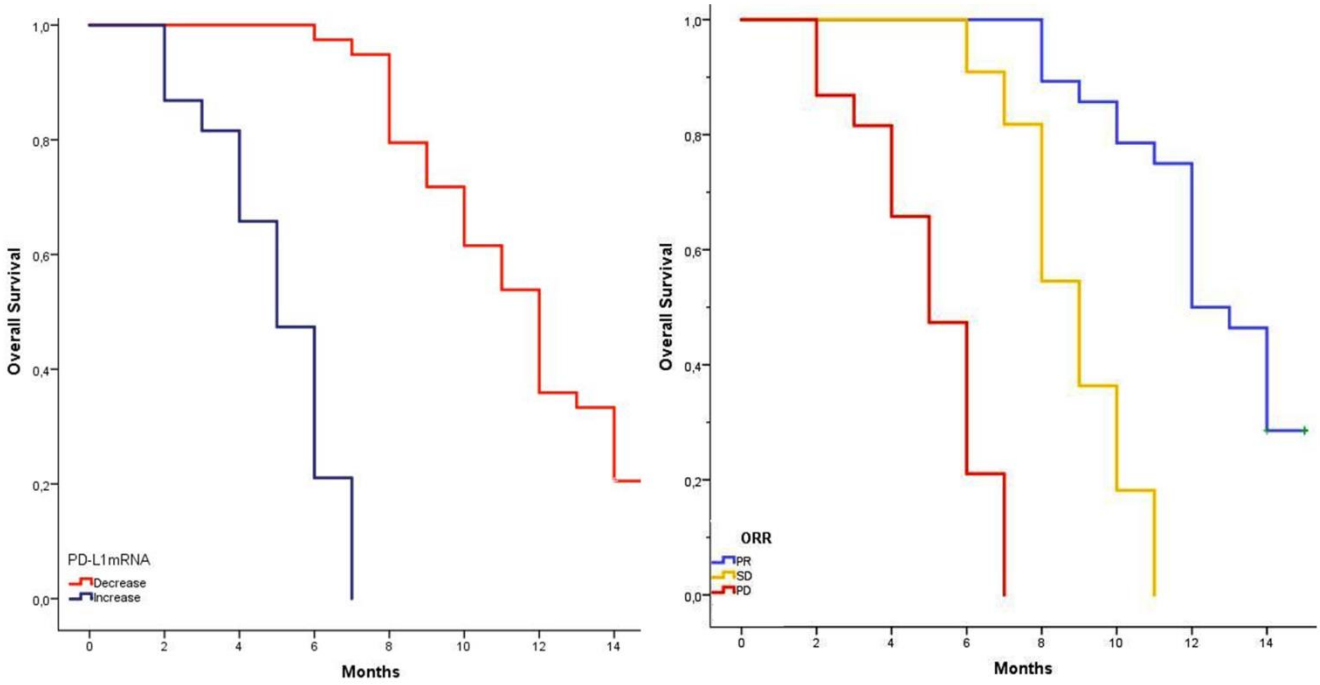

Figure 2. Increase of PD-L1 mRNA copies per ml was significantly associated with worse and shorter OS: median OS in patients with increase of PD-L1 mRNA copies/ml was 5 months (range 2-7 months, 95%CI 1.1-6.1); on the other side median OS in patients with decrease of PD-L1 mRNA copies/ml was more than double (range 8-15 months) (*p<0.001*). CR:complete response; PR:partial response;SD:stable disease; PD:progressive disease.

2.4. Exploratory analyses of the LMr during treatment

We collected data on pre-treatment LMr and LMr after 2 cycles of Atezolizumab – nab-Paclitaxel. The median value of baseline LMr was 4.150 (range: 1.2-20; IQR: 2.47-8.25). A time-dependent receiver operating characteristic (ROC) curve was performed. The area under the curve (AUC) was 99.7. Overall, sensitivity and specificity were 96.6% and 100%. The assessment of the best cut-off was carried out maximized the sum of specificity and sensitivity. We used 5.0 as cut-off value.

Baseline LMr was significantly higher in patients with higher number of PD-L1 mRNA copies per ml at baseline. LMr at baseline (median 5.86; range, 1.90 – 10.65) was significantly increased after two treatment cycles (median, 7.01; range, 2.12 – 11.91; p = 0.0010, Figure) only in patients responders to treatment (Figure 3A). Although there was no significant difference in lymphocyte count at baseline and after two cycles (median, 1620 [range, 1000-2140] vs. 1624 [998-2170]; p = 0.0724; Figure 3B), monocyte count was significantly increased after two cycles (median, 230 [range, 125-775] vs. 765 [range, 415-1275]; p< 0.001; Figure 3C) in patients with no-response to treatment (Figure 3). During PD-L1 blockade, 2 months after starting treatment, monocytes progressively raised in non-responders and declined in clinical benefit group (p=0.001)

Relationship between PD-L1 mRNA and peripheral blood markers is summarized in Table 2.

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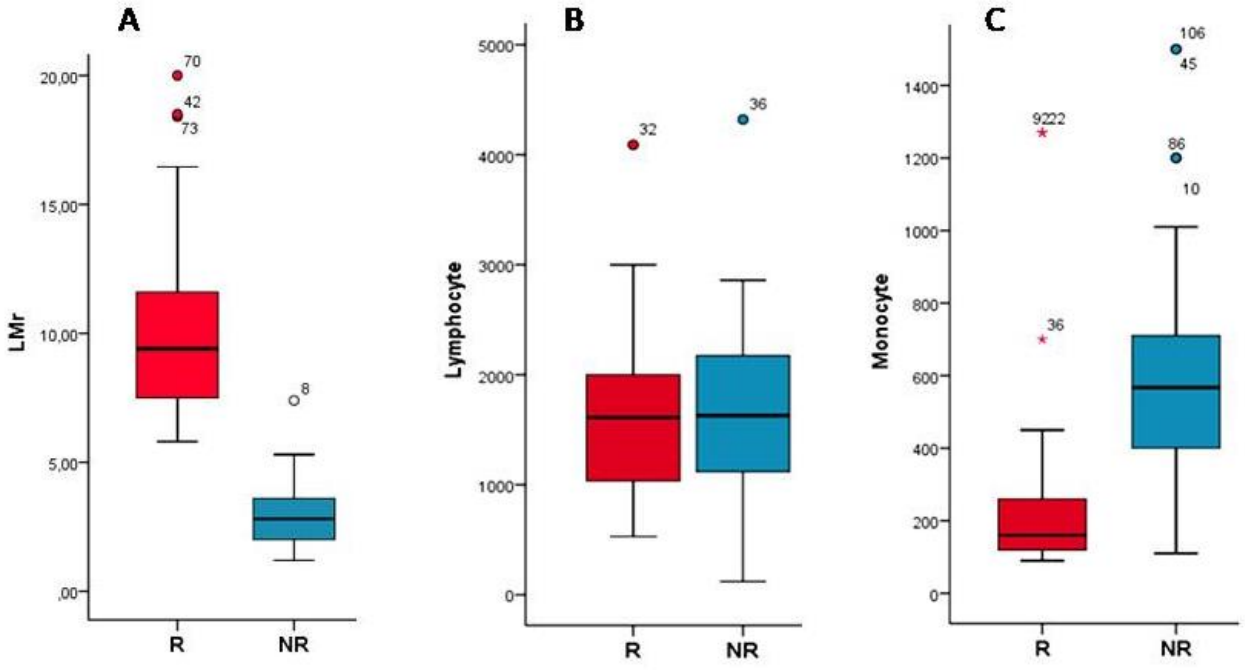

Figure 3. Differences in baseline peripheral blood biomarkers between responders (R) achieving complete or partial response and no-responders (NR) to Atezolizumab-nab-Paclitaxel as indicated by (a) LMr, (b) Monocyte count and (c) Lymphocyte count.

Table 2. Relationship between responders/non-responder patients and peripheral blood markers.

Variables	Patients with:		
	Responders	**Non-Responders**	*p-value*
Leucocytes	4880 [3060-6258]	5920 [4230 – 7390]	0.103
Neutrophil	2300 [1310 – 3500]	2980 [1540 – 4585]	0.025
Lymphocyte	1630 [1165 – 2140]	1610 [1000 – 2000]	0.263
Monocyte	210 [125.5 – 355]	600 [415 – 775]	**<0.001**
LMr	8.10 [6.10 -10.65]	2.70 [1.90 – 3.45]	**<0.001**

3. Discussion and Conclusions

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Despite the specific role and the expression of Programmed Death-1 (PD-1)/ Programmed Death-Ligand 1 (PD-L1) signaling axis and immune cell subpopulations still need to be elucidated, immunotherapy and especially immune checkpoint blockade therapy has improved clinical outcome of several tumours, including breast cancer.

The IMPassion130 is the only phase 3 trial demonstrating benefit from the combination of atezolizumab and nab-paclitaxel compared to nab-paclitaxel alone in patients with mTNBC and PD-L1 immune cell positivity with a statistically significantly prolonged both of PFS (7.2 versus 5.5 months; HR= 0.62, 95% CI 0.49-0.78, p<0.001) and OS (21.3 vs 17.6 months; HR=0.71, 95% CI 0.54-0.94) [12]. Based on this study results, Federal Food and Drug Administration (FDA) and European Medicines Agency (EMA) have approved the use of the first immune checkpoint inhibitor in patients diagnosed with breast cancer. TNBC subtype is considered to be highly heterogeneous and more immunogenic as compared to luminal subtype [19,20]. Moreover, in recent times many studies focused on the correlation between cancer and inflammation, arguing the existence of a dynamic crosstalk responsible for tumor initiation, promotion and metastasis: immune cells that infiltrate tumor and the microenvironment with the resultant inflammatory response, seem to play decisive roles in responses to therapy [21].

A low lymphocyte-to-monocyte ratio (LMr) was first reported to be related to poor prognosis in hematologic malignancies but results in predicting the prognosis of solid tumours are controversial [22]. Hsu et al highlighted patients with lower LMr (≤4.8) had more aggressive tumor behaviour with worse long-term survival (HR: 1.36, 95% CI: 1.08–1.69) [23]. While many studies have shown that lymphocytes could play a considerable role in tumor defence inducing apoptotic cell death through cytotoxicity and inhibiting the proliferation and metastasis of tumor cells, other experimental researches have shown that monocytes could be considered as pro-tumor cells [24,25]. Monocytes seem to facilitate the progression and dissemination of tumor cells producing cytokines and chemokines, including vascular endothelium growth factor- A (VEGF-A) and matrix metalloproteinase 9 (MMP9), responsible for the release of VEGF from the extracellular matrix thereby promoting angiogenesis [26,27]. Moreover tumor cells supported by cytokines and chemokines in microenvironment induce the differentiation of monocytes into tumor-associated macrophages that stimulate the metastatic spread of tumor cells [28].

In the era of precision medicine, while a single needle biopsy may vastly underrepresent molecular heterogeneity and not capture heterogeneity of resistance, missing alterations that might drive treatment failure, liquid biopsy may prove to become a powerful tool for the evaluation of immune-treatment response in TNBC, thanks to its advantages of being a non-invasive, high sensitivitive and specific and real-time monitoring method [29]. Liquid biopsy may offer the ability to monitor emergence of resistance mechanisms in real-time and adjust therapy accordingly. However, its inclusion in both diagnostic and therapeutic decision-making processes raises many questions and the absence of standardized protocols for using liquid biopsy in clinical setting highlights an urgent needing of further directions and remarks that its application in practice could still be a little far from reality. Despite this, the analysis of exosomes shows its superiority in terms of mirroring the original cell metabolic landscape and stability in circulation in almost all body fluids,more than any another liquid biopsy's biomarkers. In fact, focusing on RNA, exosomes architecture itself protects circulating RNA and microRNA from RNase catalytic function [30]. Despite the lack of studies, exosomal RNA is widely considered an ideal biomarker for breast cancer; however, crucial contents of exosomes still need to be fully elucidated [31].

Exosomes are involved in immune escape and cell-to-cell communication, leading to interactions between different cell types, such as endothelial cells and immune cells,

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especially monocytes, modulating their cellular function. Our data showed two months after the start of treatment with atezolizumab plus nab-paclitaxel, PD-L1 levels in plasma-derived exosomes significantly decreased in patients responding to treatment. A decrease of PD-L1 mRNA copies per ml associated with a high LMr and a lower monocyte count, in responders versus non-responders may be considered as an indirect marker for immune reaction against cancer cells, indicating that treatment can induce an inflamed tumor microenvironment which can further sensitize patients to PD-1 blockade. To confirm this hypothesis, larger cohorts of patients will be required.

4. Materials and Methods

4.1 Patients

Patients with unresectable locally advanced histologically documented TNBC who were treated with atezolizumab (840 mg i.v. on days 1 and 15) plus nab-paclitaxel (100 mg per square meter of body-surface area, i.v. on days 1,8 and 15 every 28-day cycle) were prospectively enrolled. Patients were eligible for the study if they had biopsy-proved advanced or metastatic TNBC and were treatment naïve. All patients, 18 years of age or older, had radiographic evidence of disease and despite this study collected clinical data with no risk to the participants, signed written informed consent was obtained for collection of plasma before any study-related procedure.

Patients received treatment until progression, according to the Response Evaluation Criteria in Solid Tumors (RECIST), version 1.1 or an unacceptable level of toxic effects occurred. Complete or partial responses (CR, PR), stable disease (SD) and progressive disease (PD) were defined following RECIST (v. 1.1) criteria.

4.2 Assessment of exosomal PD-L1

Blood draws were obtained from all patients at baseline after study enrolment and at 28 and 56 days after initiation of treatment. A blood sample of 7 ml, collected in Ethylenediaminetetraacetic acid (EDTA) tube, was centrifuged for 10 min at 1900 g within 2 hours and exosomal RNA from prefiltered plasma was isolated through exoRNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Triplicate digital droplet PCR analyses were performed per plasma sample. The levels of exosomal PD-L1 mRNA in plasma was determined using the Bio-Rad QX100 ddPCR system and exoRNeasy kit (Bio-Rad, Hercules, CA, USA). The number of mRNA copies per ml, mean of the triplicate, was determined thanks to the ratio of positive (fluorescence intensity threshold higher than 4000) vs negative droplets.

4.3 Measurements of Lymphocyte-Monocyte ratio (LMr) and patient outcomes

Venous blood samples for complete blood count were collected in EDTA tubes at baseline, the same day just before the start of treatment and every two cycles of treatment (the same day just before the start of following cycle). Lymphocyte and monocyte counts were determined automatically with haematology analyzer. Lymphocyte-to-Monocyte ratio (LMr) was obtained for each patient by the absolute lymphocyte count (cells/mm^3) divided by the absolute monocyte count (cells/mm^3) derived from the complete blood count of the patients. Using 5.0 as cut-off value, we divided patients into different groups: LMr-low (<5.0) and LMr-high (≥5.0).

4.4 Statistical analysis

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Continuous variables are presented as mean ± SD or median ± IQR, depending on the shape of the distribution curve. Categorical variables are summarized with counts and percentages and were compared by X2 or Fisher's exact tests. The significance of differences between paired samples (PD-L1 levels at time 0 vs 28 and 56 days) was assessed by paired t-test. Using unpaired t-test the differences between unpaired groups (time 0 CR+PR vs SD+PD) were assessed. Progression Free Survival (PFS) and Overall Survival (OS) were estimated through Kaplan–Meier estimates and effects of predictors were assessed through log-rank tests and univariate Cox regression. To evaluate the relationship between LMr level and PFS, a time-dependent a Receiver Operating Characteristic (ROC) curve was performed. Changes in peripheral blood markers at baseline and after two cycles of treatment were calculated using the Wilcoxon signed rank test. Statistical significance was set at $p<0.05$ and all analyses were performed by using R version 3.5.1.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: title, Table S1: title, Video S1: title.

Author Contributions: Conceptualization, L.R. and G.N.; methodology, L.R. and G.P.S.; software, L.D.B.; validation, L.D.B. and R.L.; formal analysis, L.R., P.C. and S.V.; investigation, L.R. and P.C..; resources, P.C. and F.M.R.; data curation, L.R.; writing—original draft preparation, L.R. and P.C.; writing—review and editing, L.G. and F.M.R.; visualization, F.M.R., P.C., S.V. and L.R.; supervision, G.N. and G.P.S.; project administration, L.R. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

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Table A1. Baseline patients' characteristics

Characteristics	Total (N=77)
Age, median (range), y	49 (37-68)
Menopausal status, No. (%)	
Pre-	12 (*16*)
Post-	61 (*79*)
Unknown	4 (*5*)
ECOG PS, No. (%)	
0	66 (*86*)
1-2	11 (*14*)
Histology, No. (%)	
Invasive ductal carcinoma	69 (*90*)
Others	8 (*10*)
Nodal status before enrollment	
Negative	10 (*13*)
Positive	67 (*87*)
Ki-67 status, No. (%)	
< 20%	22 (*29*)
>20%	55 (*71*)
TP53, No. (%)	
Negative	35 (*45*)
Positive	42 (*55*)
HER2 status	
- or 1+	32 (*41*)
2+	45 (*59*)

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