Peer Review File

Article Information: http://dx.doi.org/10.21037/tlcr-21-156

Reviewer A

The authors have written a very clear manuscript regarding the prognostic significance of baseline blood biomarkers for patients with advanced NSCLC with high PD-L1 TPS receiving first line pembrolizumab.

There are a few important analyses missing:

1. Median OS and PFS for whole cohort?

Thank you for this observation.

According with your suggestion we have added median OS and PFS for whole cohort (see page 10, lines 229-230):

"*For the whole cohort included in the analysis median OS and PFS were 10,64 months (95% CI: 4,25-17,05) and 5,52 months (95% CI: 2,96-8,08) respectively*."

2. Median follow-up?

Thank you for the valuable comment.

We have added median follow-up (see page 9, lines 210-211):

"*Median follow-up for the cohort was 6,93 (range, 0.20–26,19) months…*"

3. Any patients lost to follow-up?

We have added data about patients lost follow-up (see page 9, line 212):

"…and only one patient was lost to follow-up."

4. Median number of cycles delivered? .

We have added median number of cycles delivered (see page 9, line 210):

"*Median number of cycles of pembrolizumab administered was 7 (range, 1-28).*"

5. For each blood biomarker, I think it is important to look for differences in baseline characteristics.

Thank you for bringing this point.

According with your suggestion we have added the most relevant data about the relation of patient's characteristics and biomarkers in Table 3:

"*Table 3. Associations between the NLR, MLR and PLR and baseline characteristics.*"

In addition, we have briefly described the results in the text (see page 9, lines 213-216):

"*The relation of blood biomarkers and baseline clinicopathologic characteristics are shown in Table 3. Low baseline NLR was significantly associated with low baseline MLR ($p<0.001$) and PLR ($p<0.001$), likewise there was an association between low baseline MLR and PLR ($p<0.001$).*"

Table 3. Associations between NLR, MLR and PLR and baseline characteristics.

Characteristics	NLR			MLR			PLR		
	<5.6 n (%)	≥5.6 n (%)	p-value	<0.54 n (%)	≥0.54 n (%)	p-value	<198 n (%)	≥198 n (%)	p-value
Age									
<75	27 (64.3)	15 (35.7)	1.00*	23 (54.8)	19 (45.2)	0.72*	23 (54.8)	19 (45.2)	0.72*
≥75	6 (66.7)	3 (33.3)		4 (44.4)	5 (55.6)		4 (44.4)	5 (55.6)	
Gender									
Male	23 (62.2)	14 (37.8)	0.74*	18 (48.6)	19 (51.4)	0.32†	21 (56.8)	16 (43.2)	0.37†
Female	10 (71.4)	4 (28.6)		9 (64.3)	5 (35.7)		6 (42.9)	8 (87.1)	
Smoking Status									
Never or +10years former smokers	8 (53.3)	7 (46.7)	0.27†	7 (46.7)	8 (53.3)	0.56†	7 (46.7)	8 (53.3)	0.56†
Current or -10 years former smoker	25 (69.4)	11 (30.6)		20 (55.6)	16 (44.4)		20 (55.6)	16 (44.4)	
ECOG									
0-1	27 (69.2)	12 (30.8)	0.30*	22 (56.4)	17 (43.6)	0.37†	22 (56.4)	17 (43.6)	0.37†
≥2	6 (50.0)	6 (50.0)		5 (41.7)	7 (58.3)		5 (41.7)	7 (58.3)	
Histology									
Adenocarcinoma	21 (65.6)	11 (34.4)	0.86†	17 (53.1)	15 (46.9)	0.97†	17 (53.1)	15 (46.9)	0.97†
Non-adenocar.	12 (63.2)	7 (36.8)		10 (52.6)	9 (47.4)		10 (52.6)	9 (47.4)	
NLR									
<5.6				26 (78.8)	7 (21.2)	<0.001†	25 (78.8)	8 (24.2	<0.001†
≥5.6				1 (5.6)	17 (94.4)		2 (11.1)	16 (88.9)	
MLR									
<0.54	26 (96.3)	1 (3.7)	<0.001†				22 (81.5)	5 (18.5)	<0.001†
≥0.54	7 (29.2)	17 (70.8)					5 (20.8)	19 (79.2)	
PLR									
<198	25 (92.6)	2 (7.4)	<0.001†	22 (81.5)	5 (18.5)	<0.001†			
≥198	8 (33.3)	16 (66.7)		5 (20.8)	19 (79.2)				

*P value for Fisher`s exact test; †, P value for Chi-squared test. ECOG, Eastern Cooperative Oncology Group; NLR, neutrophil-to-lymphocyte ratio; MLR, monocyte-to-lymphocyte ratio; PLR, platelets-to-lymphocyte ratio.

6. I have significant concerns about the 6 week analysis and I think there is more than enough information in the manuscript to exclude it entirely. First, since all patients receiving pembrolizumab the blood biomarkers are prognositc, but the authors seem to be describing a predictive biomarker based on changes in blood biomarker ratios at the 6 week mark. Second, the blood biomarkers at the 6 week mark are a time dependent covariate (not known at baseline.)

We totally agree with this point.

According with your comment we have modified our text excluding longitudinal (after 6 week of treatment) analysis, focusing on the baseline study. Therefore, the main sections of the manuscript that we have removed or modified are:

1. Abstract.
    - Page 2, lines 42-43: *"...biomarkers (at baseline and 6 weeks after starting*

treatment) and….".

- Page 2, lines 51-52: "*We did not identify any relationship between survival and biomarkers over time.".*
- Page 3, lines 57-59: "*In contrast, we did not observe a relationship between survival outcomes and changes in biomarkers during treatment*.".

2. Introduction.
   - Page 6, lines 129-138: "*In this way, it is also suggested that changes in immune biomarkers are not sufficiently significant until 6 weeks of treatment…*".
3. Patients and methods. Clinical outcome variables and statistical analysis.
   - Page 8, lines 174-178: "*To calculate changes in NLR, MLR and PLR (ΔNLR, ΔMLR and ΔPLR), we used haematological….*"
4. Results.
   - Page 12-14, lines 281-312, we have removed the entire section titled: "*Immune blood biomarker changes during treatment with pembrolizumab*"
5. Discussion.
   - Page 17, lines 394-420: "*Due to the aforementioned heterogeneity of the cut-off values used to stratify patients and the fact that the immune system is not static…*"
   - Page 20, lines 472-473: "*On the other hand, we did not find a relationship between survival and biomarkers over time*."

Additionally, we have modified table 1 and removed figure 3 and S1 and table 5.

Table 1. Definition of the different blood-derived biomarkers analysed.

Parameter	Description	Cut-off value
NLR	ANC/ALC	5,6
MLR	AMC/ALC	0,54
PLR	Platelets/ALC	198
NMLR	(ANC+AMC)/ALC	6.3
RBB SCORE	A 1-point was assigned for each NMLR or WBC count beyond the cut-off	0. NMLR<6.3 and WBC≤11.5x10^9/L 1. NMLRC≥6.3 or WBC>11.5x10^9/L 2. NMLRC≥6.3 and WBC>11.5x10^9/L

NLR, neutrophil-to-lymphocyte ratio; ANC, absolute neutrophil count; ALC, absolute lymphocyte count; MLR, monocyte-to-lymphocyte ratio; AMC, absolute monocyte count; PLR, platelets-to-lymphocyte ratio; NMLR, neutrophil-monocyte-to-lymphocyte ratio; WBC, White blood cell count; RBB, risk blood biomarker.

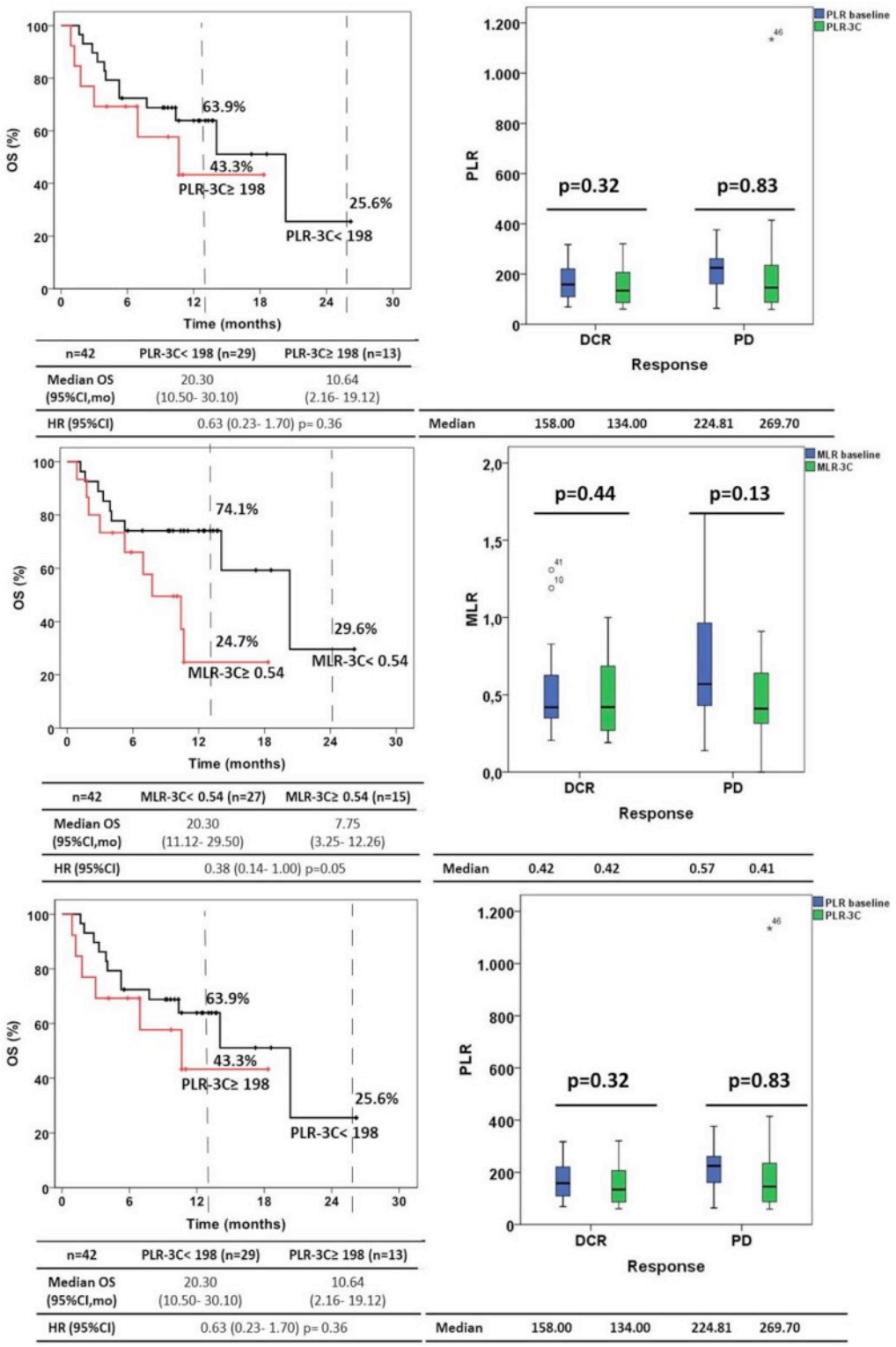
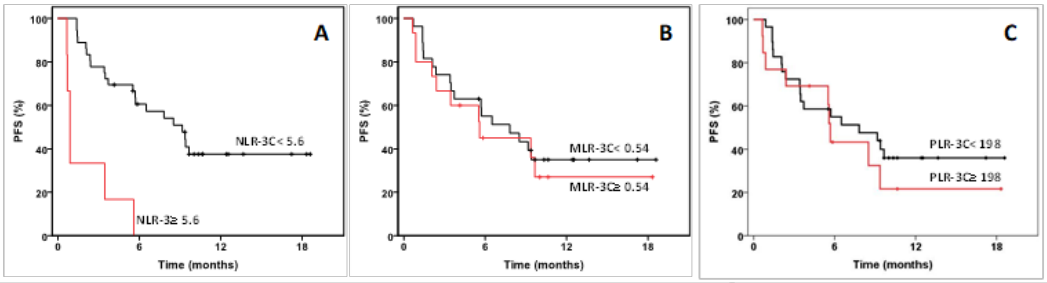


Figure 3. Kaplan-Meier curve for OS after 2 cycles of treatment stratified by NLR (**A**), MLR (**C**) and PLR (**E**) and effects of pembrolizumab treatment on the values of NLR (**B**), MLR (**D**) and PLR (**F**). Initial values (blue) before starting treatment, final values (green) before three treatment cycles (6 weeks). NLR, neutrophil-to-lymphocyte ratio; MLR, monocyte-to-lymphocyte ratio; PLR, platelet-to-lymphocyte ratio; DCR, disease control rate; PD, progression disease; OS, overall survival; CI, confidence interval; mo, months; HR, hazard ratio.



n= 42	NLR-3C< 5.6 (n=36)	NLR-3C≥5.6 (n=6)
Median PFS (95%CI,mo)	9.16 (5.44-12.89)	0.89 (0.66-1.11)
HR (95%CI)	0.14 (0.05-0.37) p<0.001	

n= 42	MLR-3C<0,54 (n=27)	MLR-3C≥0,54 (n=15)
Median PFS (95%CI,mo)	7.82 (3.30-12.34)	5.58 (1.90-9.27)
HR (95%CI)	0.81 (0.37-1.78) p=0.61	

n= 42	PLR-3C<198 (n=29)	PLR-3C≥198 (n=13)
Median PFS (95%CI,mo)	7.82 (0.98-14.66)	5.68 (5.41-5.95)
HR (95%CI)	0.73 (0.32-1.64) p=0.45	

Figure S1. Kaplan-Meier curves and HR for PFS of NSCLC patients before three cycles (-3C) of Pembrolizumab. **A** patients stratified by NLR-3C, **B** by MLR-3C and **C** by PLR-3C. NLR, neutrophil-to-lymphocyte ratio; MLR, monocyte-to-lymphocyte ratio; PLR, platelets -to-lymphocyte ratio; PFS, progression free survival; HR, hazard ratio; CI, confidence interval; mo, months.

Table 5. Univariate analysis of survival outcomes (PFS and OS) with variation of blood biomarkers.

	PFS			OS		
Variable	HR	95% CI	p value	HR	95% CI	p-value
ΔNLR	0.65	0.30-1.40	0.27	0.49	0.19- 1.29	0.15
ΔMLR	0.99	0.46-2.16	0.99	0.88	0.34- 2.30	0.80
ΔPLR	0.91	0.41-2.04	0.83	0.56	0.21- 1.50	0.25

ΔNLR, neutrophil-to-lymphocyte ratio variation; ΔMLR, monocyte-to-lymphocyte ratio variation; ΔPLR, platelets-to-lymphocyte ratio variation; PFS, progression free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval.

7. Multiple blood biomarkers are tested. A Pearson coefficient (or other statistical analysis) might be useful to determine how different the data sets are for each blood biomarker.

According with your recommendation we have briefly described the results of the correlation between biomarkers (see page 9-10, lines 216-219):

"*Moreover, using continuous variables, a strong correlation between NLR and PLR (Spearman's r=0.75, p<0.001), NLR and MLR (Spearman's r=0.83, p<0.001) and PLR and MLR (Spearman's r=0.76, p<0.001) was found.*"

Reviewer B

The authors have explored whether the biomarker scores NLR, PLR and MLR had prognostic value in a cohort of 51 patients treated with immune checkpoint inhibitors. Furthermore, they have created a new score, which is a combination of NLR, MLR and WBC. Applying regular blood test as prognostic markers is an interesting thought and could easily be implemented in clinics. Unfortunately, the study have some major flaws that should be taken to consideration and I cannot recommend this study to be accepted.

Here are my comments

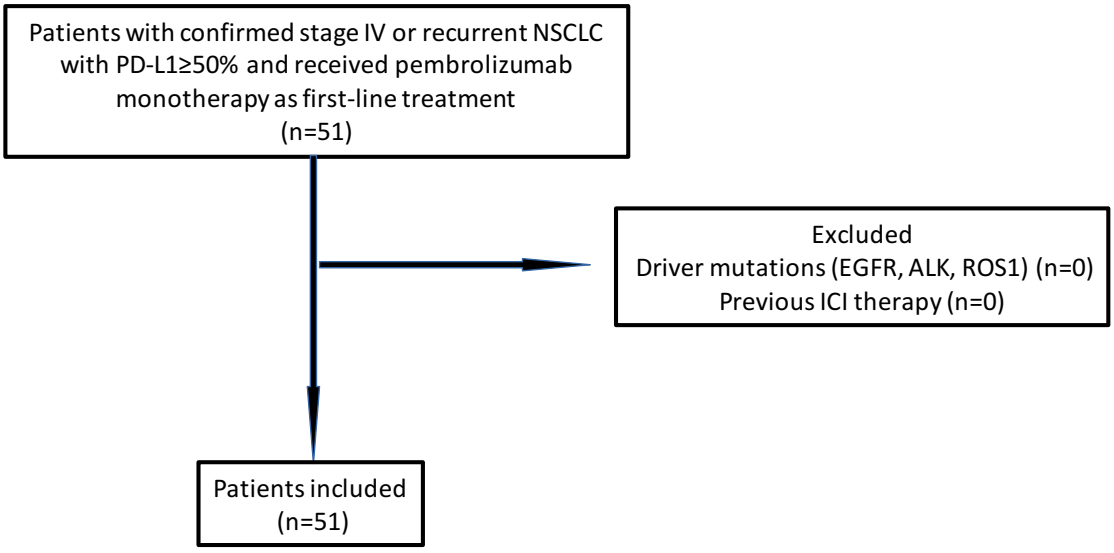
1) It is unclear how the patients were selected for this study. In and exclusion criteria should be stated clearly. A flowchart could improve the transparency of the in and exclusion process.

Thank you for this valuable comment. We did not accurately describe the inclusion and exclusion criteria making some unnecessary misunderstanding. In order to clarify that question we have modified the paragraph of the inclusion and exclusion criteria (see page 7, lines 150-154):

"The inclusion criteria were: confirmed stage IV or recurrent NSCLC, PD-L1≥50% and use of pembrolizumab monotherapy as first-line treatment. Exclusion criteria were: previous ICI therapy and any driver mutations (EGFR, ALK, ROS1) (Figure S1). "

We have also included a flowchart in the supplementary information:

Figure S1. Patient selection process.



There were no excluded patients and, therefore, the final sample size was 51 patients.

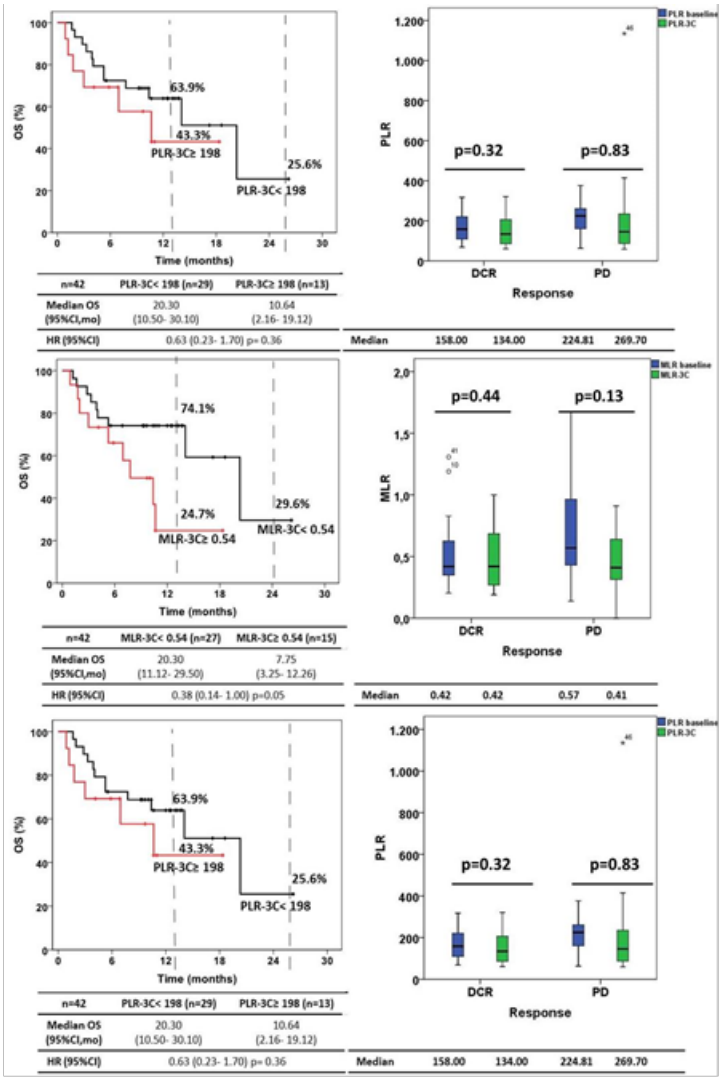
2) A total number of 51 patients only allows a limited number of statistical test. In this study numerous test are being performed, however, multiple testing is not taken into consideration. Furthermore, prior to initiating such a study, a power calculation should be performed to ensure the strength of the study. Has a power calculation been performed?

Thanks you for bringing this point. We are in agreement with this comment and in fact the small number of patients is acknowledged in the paragraph about limitations of the study (see page 20, lines 457-459):

"*First, the small number of patients in our cohort might not be sufficient to support the stability of the results*."

Nevertheless, despite the low number of patients evaluated we believe that the results obtained could be very interesting as a starting point to continue further studies with larger series. On the other hand, according with your suggestion about numerous tests are being performed, we have removed some of them (all those related to the longitudinal study), such as Wilcoxon test, the Kaplan-Meier method and log-rank test, hazard ratios (HRs) and 95% confidence intervals (CIs). The results we have removed are:

- Page 12-14, lines 281-312, we have removed the entire section titled: "*Immune blood biomarker changes during treatment with pembrolizumab*"
- We have removed figures 3 and S1 and table 5.



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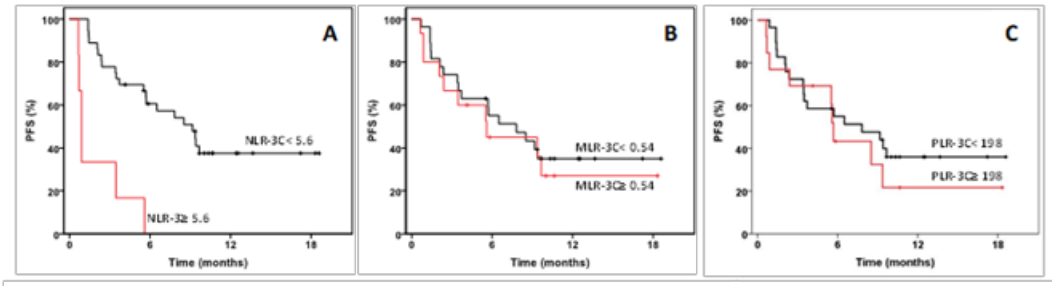

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n= 42	MLR-3C<0,54 (n=27)	MLR-3C≥0,54 (n=15)
Median PFS (95%CI,mo)	7.82 (3.30-12.34)	5.58 (1.90-9.27)
HR (95%CI)	0.81 (0.37-1.78) p=0.61	

n= 42	PLR-3C<198 (n=29)	PLR-3C≥198 (n=13)
Median PFS (95%CI,mo)	7.82 (0.98-14.66)	5.68 (5.41-5.95)
HR (95%CI)	0.73 (0.32-1.64) p=0.45	

Figure S1. Kaplan-Meier curves and HR for PFS of NSCLC patients before three cycles (-3C) of Pembrolizumab. **A** patients stratified by NLR-3C, **B** by MLR-3C and **C** by PLR-3C. NLR, neutrophil-to-lymphocyte ratio; MLR, monocyte-to-lymphocyte ratio; PLR, platelets -to-lymphocyte ratio; PFS, progression free survival; HR, hazard ratio; CI, confidence interval; mo, months.

Table 5. Univariate analysis of survival outcomes (PFS and OS) with variation of blood biomarkers.

	PFS			OS		
Variable	HR	95% CI	p value	HR	95% CI	p-value
ΔNLR	0.65	0.30-1.40	0.27	0.49	0.19- 1.29	0.15
ΔMLR	0.99	0.46-2.16	0.99	0.88	0.34- 2.30	0.80
ΔPLR	0.91	0.41-2.04	0.83	0.56	0.21- 1.50	0.25

ΔNLR, neutrophil-to-lymphocyte ratio variation; ΔMLR, monocyte-to-lymphocyte ratio variation; ΔPLR, platelets-to-lymphocyte ratio variation; PFS, progression free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval.

We have added others multiple tests in order to clarify the results, such as the relationships between the biomarkers and the clinicopathological characteristics using Chi-squared test or Fisher´s exact test and the correlation between biomarkers using Spearman's coefficient.

- Relation of patient`s characteristics and biomarkers:

"*Table 3. Associations between the NLR, MLR and PLR and baseline characteristics.*"

In addition, we have briefly described the results in the text (see page 9, lines 213-216):

"*The relation of blood biomarkers and baseline clinicopathologic characteristics are shown in Table 3. Low baseline NLR was significantly associated with low baseline MLR ($p<0.001$) and PLR ($p<0.001$), likewise there was an association between low baseline MLR and PLR ($p<0.001$).*"

Table 3. Associations between NLR, MLR and PLR and baseline characteristics.

Characteristics	NLR			MLR			PLR		
	<5.6 n (%)	≥5.6 n (%)	p-value	<0.54 n (%)	≥0.54 n (%)	p-value	<198 n (%)	≥198 n (%)	p-value
Age									
<75	27 (64.3)	15 (35.7)	1.00*	23 (54.8)	19 (45.2)	0.72*	23 (54.8)	19 (45.2)	0.72*
≥75	6 (66.7)	3 (33.3)		4 (44.4)	5 (55.6)		4 (44.4)	5 (55.6)	
Gender									
Male	23 (62.2)	14 (37.8)	0.74*	18 (48.6)	19 (51.4)	0.32†	21 (56.8)	16 (43.2)	0.37†
Female	10 (71.4)	4 (28.6)		9 (64.3)	5 (35.7)		6 (42.9)	8 (87.1)	
Smoking Status									
Never or +10years former smokers	8 (53.3)	7 (46.7)	0.27†	7 (46.7)	8 (53.3)	0.56†	7 (46.7)	8 (53.3)	0.56†
Current or -10 years former smoker	25 (69.4)	11 (30.6)		20 (55.6)	16 (44.4)		20 (55.6)	16 (44.4)	
ECOG									
0-1	27 (69.2)	12 (30.8)	0.30*	22 (56.4)	17 (43.6)	0.37†	22 (56.4)	17 (43.6)	0.37†
≥2	6 (50.0)	6 (50.0)		5 (41.7)	7 (58.3)		5 (41.7)	7 (58.3)	
Histology									
Adenocarcinoma	21 (65.6)	11 (34.4)	0.86†	17 (53.1)	15 (46.9)	0.97†	17 (53.1)	15 (46.9)	0.97†
Non-adenocar.	12 (63.2)	7 (36.8)		10 (52.6)	9 (47.4)		10 (52.6)	9 (47.4)	
NLR									
<5.6				26 (78.8)	7 (21.2)	<0.001†	25 (78.8)	8 (24.2	<0.001†
≥5.6				1 (5.6)	17 (94.4)		2 (11.1)	16 (88.9)	
MLR									
<0.54	26 (96.3)	1 (3.7)	<0.001†				22 (81.5)	5 (18.5)	<0.001†
≥0.54	7 (29.2)	17 (70.8)					5 (20.8)	19 (79.2)	
PLR									
<198	25 (92.6)	2 (7.4)	<0.001†	22 (81.5)	5 (18.5)	<0.001†			
≥198	8 (33.3)	16 (66.7)		5 (20.8)	19 (79.2)				

*P value for Fisher`s exact test; †, P value for Chi-squared test. ECOG, Eastern Cooperative Oncology Group; NLR, neutrophil-to-lymphocyte ratio; MLR, monocyte-to-lymphocyte ratio; PLR, platelets-to-lymphocyte ratio.

- Correlation between biomarkers (see page 9-10, lines 216-219):

"*Moreover, using continuous variables, a strong correlation between NLR and PLR (Spearman's r=0.75, p<0.001), NLR and MLR (Spearman's r=0.83, p<0.001) and PLR and MLR (Spearman's r=0.76, p<0.001) was found.*".

Finally, concerning the power of the study, we did not calculate the power since our sample could not be increased and the study was not designed to detect a specified effect size. However, we have calculated the power after the study, of all tests carried out, the one with the lowest power with respect to OS was for MLR, it was 75%.

3) For each score, the optimal cut-off was established. This was based on ROC-curves where outcome was used to define the exposure. In other words, the cut offs are data dependent. Data dependent cut offs will increase the change of finding an association tremendously. This increase will be excaggarated when several scores with optimal cut-offs are combined, which has been performed in this study. Therefore, according to REMARK, when defining cut-offs in

biomarkers, this cut-off should always be validated in an independent cohort.

Thank you very much for your appreciation. We agree with this observation, in fact this is a point widely discussed in the bibliography as we noted in the discussion (see page 17, lines 382-393):

"*One of the most controversial and limiting issues in the application of blood biomarkers as prognostic factors for use in clinical practice is identifying the optimal cut-off value […]. Despite these discrepancies, the conclusion was similar in all studies: high baseline ratios in patients with NSCLC treated with ICIs are correlated with poor survival outcomes.*"

This limitation is also acknowledged in the paragraph about limitations of the study (see page 20, lines 460-462):

"*However, this is a retrospective study, and it has several limitations that must be taken into account when interpreting the conclusions […]. Third, we utilized an ROC curve-derived optimum cut-off of variables as the grouping criterion since a standard cut-off value of NLR, MLR, PLR and NMLR has not been defined clearly in NSCLC; therefore, a unified cut-off value is needed*."

To solve the problem of the cut-off value authors usually use ROC curves and choose the value with the highest sensitivity and specificity. In this sense, our purpose is not to establish a validated cut-off point, but rather to confirm the trend of previous studies regarding these biomarkers, so that in the future a unified cut-off point can be reached. Regarding your comment, unfortunately we did not have a validation cohort and therefore we have not included this information. Nevertheless, we have added in the manuscript similar cut-off point obtained from ROC curves from other studies (see page 17, lines 387-389):

"Our optimal cut-off values of 5.6, 198 and 0.54 for NLR, PLR and MLR, respectively, were determined using ROC curves. These values are similar to the cut-off values tested in other studies (41–44)."

Furthermore, in accordance with your comment we have also added a paragraph in the study limitations section (see page 20, line 462-465):

"*The statistical significance of the prognostic effect of our biomarkers was preceded by a determination of the cut-off point of the same sample; therefore, the results may be magnified. This cut-off point should be validated by an independent cohort*."

4) New prognostic markers are needed to optimise the outcome in NSCLC. However, the prognostic marker needs to add value to the already established markers as TNM, histology, PS etc. This could be evaluated by fx c-statistics

Thank you for this observation. The C-statistic is a measure of goodness of fit and it is similar to the area under the Receiver Operating Characteristic (ROC) curve. In our study, we have performed a binary logistic regression. We made two models, the first using histology and PS as independent variables and OS as dependent variable, and the second adding to model one our biomarkers. However, in these models neither the PS nor histology variables are significant, obtaining poor models (partly caused by the small sample size). As the prognostic probabilities are obtained from the logistic regression models and these are not significant, the ROC curve and the AUC would not have validity. Therefore, we decided not to include this information in the manuscript.

5) The introduction could benefit of a more focused approach - what is the main story the authors want to tell?

Thank you for this valuable comment. According with your suggestion and in order to focus the main story of the article, we have eliminated the longitudinal analysis, focusing on the baseline study. In this sense, we think that now the article has benefited from a more focused approach and the aim of the study is now more evident. Regarding the introduction, we have eliminated:

- Page 6, line 129-130: "*In this way, it is also suggested that changes in immune biomarkers are not sufficiently significant until 6 weeks of treatment…*".

We have focused the main objective of our study:

- Page 6, lines 131-143: "*Taking into account everything already exposed, the main objective of our study was to analyse the relationship between several baseline peripheral blood biomarkers, including NLR, PLR and MLR, and the effectiveness of the first-line pembrolizumab in NSCLC patients with PD-L1 expression greater than or equal to 50% of tumor cells. In addition, we determined a risk score termed risk blood biomarker (RBB) calculated from the combination of neutrophil-monocyte-to-lymphocyte ratio (NMLR) and white blood cell count (WBC) that enables the stratification of patients by clinical outcome (i.e., treatment response and survival). This score could be a very useful prognostic tool in daily clinical practice in advanced NSCLC patients who are suitable for first-line pembrolizumab."*