Peer Review File

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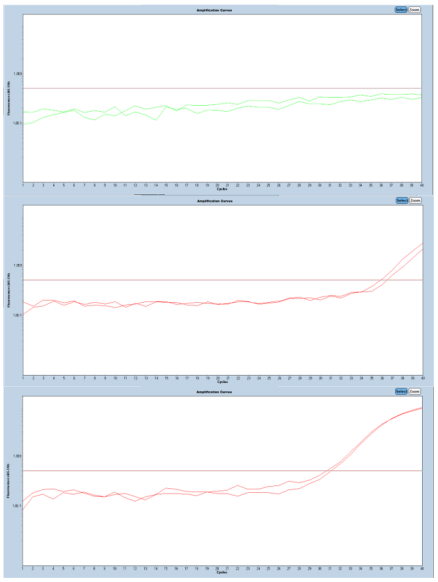
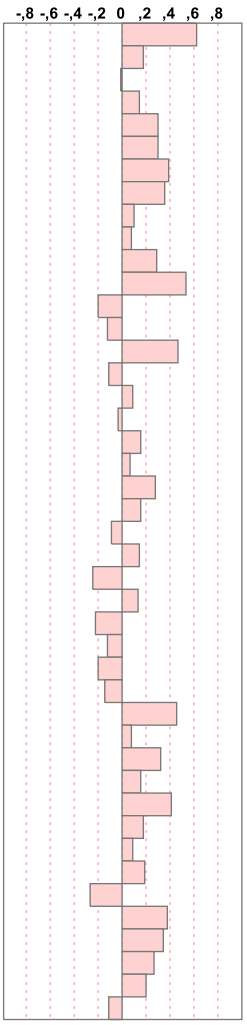
Reviewers' comments	Authors' reply
Reviewer 1	
The PDL-1 testing was performed using ZR3 clone. instead of 22C3. It will be of value if the authours can provide information on whether ZR3 has been validated against 22C3. What will be the concordance with 22C3?	Some more information about ZR3 and its validation is now given in the methods part: ***ll 203-208*** >> The antibody ZR3 had been validated against 22C3 at our department and concordance with 22C3 was very high. An example is given in **figure 1**. In addition, after passing two German external quality assessments for PD-L1 staining the pathological department received approval with ZR3. In contrast to FDA companion diagnostic with 22C3 for use of pembrolizumab is not prescribed by the EMA in Europe. <<
In this section, there was no information provided as per the definitions of high and low level of mRNA expression of the plasma markers: PDL1, PD1, CLTA4, CD3, and CD8. The cutpoints should be determined a priori.	Thank you for this note! The definition of high and low was determined by the median for all markers as objective cut-off points. However, these cut-off should be optimized in the future for each gene. ***ll 247-248*** The following statement was added in the methods part: >> Objective cut-off points for high and low expression were determined by the median expression of each marker. <<
Throughout the manuscript, the authours used the term predictive and prognostic factors interchangeably. Based on the current study design, the change in CD8 and CD3 were only predictive biomarkers for various clinical outcome. Prognostic factors are baseline characteristics that predict the outcome regardless of therapy. The authours really need to have a detail review of the manuscript to ensure the right term is being used.	You are completely right! As already used in the headline term predictive is correct in this context of these analyses and prognostic was changed into predictive throughout the manuscript.
The authours should have a statement that none of the mRNA markers at baseline predict the outcome of	In the table 3 you can see that none of the baseline expressions was predictive. This was added in the text. In addition, table 3 was divided into 3A and 3B according to

patients.	the suggestions of reviewer 4. ***ll 625-630*** >> Relative gene expressions divided by their median in low and high expression revealed no significant difference for either PFS or OS in log rank test **(Table 3A). <<**
The current study found the change of CD8 mRNA level was predictive of outcome. Is this finding clinically important? This predictive biomarker is useful in the sense that we know ahead of the CT scan whether the patient is responding or not. This may be helpful to change therapy before the CT scans especially if the patients may not have symptomatic progression. It may also be useful to use in conjunction with CT post cycle 3 to determine if the patients have psuedoprogression or atypical response. But this marker will not improve the selection of PDL-1 high NSCLC patients who are most likely to respond to pembrolizumab prior to cycle 1.	In our opinion these markers (CD3 and CD8) are helpful for the treating physician and therefore, is clinically relevant. Of course, it would be best to have a marker which can predict response and outcome before starting a therapy. However, the only approved predictive marker to date is the PD-L1 TPS score. But even negatively tested patients can benefit from an ICI therapy as shown for 2nd line studies with nivolumab as well for NSCLC squamous and adeno-carcinoma histologies. The 5-year survival rate for this cohort was 22% in contrast to 0% of patients treated with docectaxel [Borghaei et al., JCO 2021]. In addition, after one cycle of therapy most patients will not have a harm of pembrolizumab. Therefore, it might be enough time to add chemotherapy for those patients how might not benefit from the monotherapy alone.
Reviewer 2	
Although this concept in itself is worthy of investigation, the way this concise data is now represented seems a bit overenthusiastic. They present data from a retrospective cohort of only 45 patients and show that increase mRNA expression of CD8 (which I presume is CD8A?) in the first 3 weeks after start of pembrolizumab is associated with improved PFS and OS in a multivariate analysis. In the introduction a long list of previously investigated biomarkers are presented together with their flaws or impracticalities. However, in no way the authors prove that their approach of mRNA dynamics is any better compared to the current biomarker data. They do however speculate quite elaborately on what clinical implications their approach may bring. The authors should either bring more data (see line 363/364: prospective, validate, more patients) or remove this elaborate suggestions from this paper.	In contrast to reviewer 2 we believe that this early dynamic change of markers is clinically relevant and should be published. Of course, the cohort is rather small. But the data are even more interesting in the way that such a small cohort leads to these clear and independent predictive results shown here. In addition, this liquid biopsy testing might be much more easier to establish and is less costly as other marker profiles, e.g. TMB. This study was already prospective with blood samples collected during therapy. The reviewer is right, that these data are preliminary and somehow hypothesis generating. Of course, they need to be confirmed by another cohort of NSCLC patients treated with ICI monotherapy and in patients treated by ICI+CTx which is done by us as a following project and will be published than as an additional paper. Anyway, the results obtained by us are in line with more experimental obtained data on CD3 and CD8 as discussed. In addition, we present not only the data as they are but put them into a clinical frame. Physicians need not only to have potential markers but solutions how these markers could lead to more benefit for their NSCLC patients in the

	future. Therefore, the authors have the opinion, that this outlook of clinical implications is necessary for the reader to understand the meaning of these results. The CD8 marker used is CD8A and the CD3 marker is CD3Z. This was added in the methods part and was already described in the discussion: ***ll 225*** >>The marker measured by us was CD3Z, a sequence being part of the T-cell receptor (TCR) (28). <<
Also, how should we interpret their argument in line 330/331 in regards to future applications for dynamics in mRNA?	The dynamic change was measured after three weeks because this is the earliest relevant clinical time point before patients receiving their 2nd cycle of treatment. Other time points will also be evaluated in the next project which is related to progressive disease. Those date will be published in another manuscript.
They seem to use predictive and prognostic as it is the same thing, for example lies 265-269, but this is not so.	You are completely right! As already used in the headline term predictive is correct in this context of these analyses and prognostic was changed into predictive throughout the manuscript (see also reply to reviewer 1).
Also, unfortunately no data is presented regarding best ORR and their markers, although this clinical information was certainly obtained.	These data were obtained. However, they were not reported in the first version of the manuscript as they are not so robust outcome parameters as PFS and OS. The data are no given as **suppl Tables 1, 2A and 2B.** In addition, the response data are described in the results part: ***ll 295-298*** >> After a median follow-up of 27.4 months an ORR to 1st line therapy was observed in 66.7% of patients with a median duration of response (DOR) of 34.1 months, (95%CI: 20.6-47.6 months). Disease control rate was 68.9% with 22% showing a progressive disease (PD) (**suppl. Table 1**).>> >>Relative gene expressions divided by their median in low and high expression revealed no significant difference for ORR, PFS or OS in log rank test **(Table 3A and suppl. Table 2A).<<** ***ll 336-338*** >> ORR and PFS were significantly improved by patients with increased whole blood expression of CD3 and CD8 within 3 weeks after start of pembrolizumab therapy (***Table 3B and suppl. Table 2B***).<<
Reviewer 3	
The technique should be better	The technique has been developed for diagnostic

described. It is not clear if this is a validated diagnostic method or if this analysis is used here for the first time. Is this a commercial kit or a homemade assay? In the latter case please provide the primer sequences. How was the method validated? Are spiking experiments used? Maybe a test series with blood samples including different amounts of lymphocytes can show a correlation. Do blood cell counts of the patients' blood samples correlate with CD3 mRNA expression?	purposes and led to the market launch of several blood based and tissue based diagnostic test systems (Siemens Versant HCV test, Endopredict® and Mammatyper® breast cancer tests). The immune cell and checkpoint RT-qPCR test for CD3, CD8, PD-1, PD-L1 and CTLA-4 are commercially available (Stratifyer Molecular Pathology GmbH, Cologne; Catalogue no. MP121, MP768,MP675, MP676, MP671). The technical performance of the objective quantitation of target gens has been published previously (Laible et al BMC cancer 2016). The superiority of the quantitative assessment of immune marker determination as exemplified for PD-L1 over IHC assessment has been published for lung cancer and bladder cancer (see Erber et al Anti-Cancer Res 2017, Eckstein et al. Oncotarget 2018). The primer probe sets are no in detail described in the methods part: ***ll 225-229*** >>The mRNA levels of **CD3Z, CD8A**, PD-1, PD-L1. CTLA-4 and the reference genes Calmodulin2 (CALM2) and Beta-2 microglobulin (B2M) were determined by a one-step RT-qPCR using the SuperScript III RT-qPCR system (Invitrogen, Waltham, MA, USA) and gene specific primer-probe combinations (Assay number MP317, MP769, MP675, MP676, MP501 and MP810, respectively; STRATIFYER Molecular Pathology GmbH, Cologne, Germany). << Absence of significant correlation: see below.
Was the mRNA extracted from whole blood lysates, serum, or plasma? What are the differences? It seems that different samples were used (line 154). It is particularly important that plasma and blood were not mixed when the dynamic changes of mRNA levels were calculated. Please provide a list of sample types. Why only one housekeeping gene was used for normalization?	The mRNA was extracted from whole blood collected in 5 ml EDTA tubes and extracted by a commercially available extraction kit (XTRAKT whole blood kit (Stratifyer Molecular Pathology GmbH, Cologne; Catalog No. XTK-5.0). Only extrations from whole blood were analyzed by quantitative RT-qPCR by triplicate assessment with matched samples of each patient being measured in identical PCR runs side by side to reduce any technical variability when intraindividually comparing before and post whole blood samples. The housekeeping gene was selected out of a group of housekeeping genes to provide most stable results doing inter- and intraday results. The term plasma is incorrect and was deleted in line 154.
It would be nice to see some raw RT-PCR curves. Please also show PCR curve with a negative control (adding instead of plasma/blood 100µl of water).	Here are some raw RT-PCR curves of one and the same patient including a negative control (green).

	 CD8 Pat. 006 no template control CD8 Pat. 006 time point 0 (in duplicate) CD8 Pat. 006 time point 1 (in duplicate)
I suggest that the technique should be introduced in the introduction and also critically discussed in the discussion. Maybe it would be good to introduce some validation experiments at the beginning of the result parts of the manuscript as “assay” performance.	At the end of the introduction the commercial available kit is now mentioned and explained in the methods part: ***ll 157-159*** >> In this analysis we report on pre-treatment mRNA expressions and dynamic changes of CD3, CD8, PD-1, PD-L1 and CTLA-4 after the first application of pembrolizumab using a commercially available kit. << The technique is already described in detail in different publications implemented in different kinds of cancer (see comment and literature above). Therefore, to our opinion, it is not necessary to discuss it further as this is not a technical but a clinical paper.
How were the patients selected? For consecutive patients, in this time frame of three years, it would be too few patients. The characterization of patients should also include response to ICI (CR, PR, SD, PD).	The 52 patients were not selected and all consecutive patients treated at our thoracic oncology department were included. Including a median of 1.5 patients/months with NSCLC stage IV and PD-L1 >50% in this study is not such a bad quote for a single center. 7 patients were excluded as described in the methods part. Response data were obtained. See additional comment below.
Do the mRNA levels correlate with other blood/serum markers (e.g. leukocytes, CRP, LDH?).	This is a relevant point. We expanded our analysis to your suggestions and implemented the data in the results part: ***ll 319-326*** >> Comparing mRNA markers with general blood /serum marker (leucocytes, neutrophils, CRP or LDH) indicated that the quantitative assessment by molecular analysis of CD3, CD8, PD-1, PD-L1 and CTLA4 identifies a subset of immune cells, which does not relate to total amount of less characterized immune cell types. While no correlation of the mRNA levels of immune markers could be found with LDH, there were some modest positive Spearman correlations (r=0,26 to r=0,38) when

	comparing mRNA markers with lymphocyte counts, which did not reach statistical significance (p=0.07 to p=0.21) (data not shown). << Here are the data of the Spearmen correlation: **Non parametric Spearman Correlation** | Variable	Covariable	Spearman ρ	p-value | |---|---|---|---| | CD8 - Prä B | CD3Z - Prä B | 0,6239 | <,0001 * | | CD163 - Prä B | CD3Z - Prä B | 0,1760 | 0,2974 | | CD163 - Prä B | CD8 - Prä B | -0,0057 | 0,9733 | | CTLA4 - Prä B | CD3Z - Prä B | 0,1476 | 0,3833 | | CTLA4 - Prä B | CD8 - Prä B | 0,3053 | 0,0662 | | CTLA4 - Prä B | CD163 - Prä B | 0,3011 | 0,0702 | | PD1 - Prä B | CD3Z - Prä B | 0,3905 | 0,0169 * | | PD1 - Prä B | CD8 - Prä B | 0,3539 | 0,0316 * | | PD1 - Prä B | CD163 - Prä B | 0,0953 | 0,5747 | | PD1 - Prä B | CTLA4 - Prä B | 0,0777 | 0,6474 | | PDL1 - Prä B | CD3Z - Prä B | 0,2882 | 0,0837 | | PDL1 - Prä B | CD8 - Prä B | 0,5337 | 0,0007 * | | PDL1 - Prä B | CD163 - Prä B | -0,1991 | 0,2376 | | PDL1 - Prä B | CTLA4 - Prä B | -0,1242 | 0,4638 | | PDL1 - Prä B | PD1 - Prä B | 0,4656 | 0,0037 * | | CRP (mg/dL) | CD3Z - Prä B | -0,1064 | 0,5308 | | CRP (mg/dL) | CD8 - Prä B | 0,0913 | 0,5909 | | CRP (mg/dL) | CD163 - Prä B | -0,0361 | 0,8322 | | CRP (mg/dL) | CTLA4 - Prä B | 0,1569 | 0,3537 | | CRP (mg/dL) | PD1 - Prä B | 0,0691 | 0,6844 | | CRP (mg/dL) | PDL1 - Prä B | 0,2785 | 0,0951 | | LDH (U/L) | CD3Z - Prä B | 0,1526 | 0,3672 | | LDH (U/L) | CD8 - Prä B | -0,0899 | 0,5968 | | LDH (U/L) | CD163 - Prä B | 0,1439 | 0,3955 | | LDH (U/L) | CTLA4 - Prä B | -0,2411 | 0,1506 | | LDH (U/L) | PD1 - Prä B | 0,1378 | 0,4159 | | LDH (U/L) | PDL1 - Prä B | -0,2202 | 0,1903 | | LDH (U/L) | CRP (mg/dL) | -0,1265 | 0,4557 | | abs# Neutros | CD3Z - Prä B | -0,2012 | 0,3573 | | abs# Neutros | CD8 - Prä B | -0,1458 | 0,5068 | | abs# Neutros | CD163 - Prä B | 0,4607 | 0,0269 * | | abs# Neutros | CTLA4 - Prä B | 0,0761 | 0,7299 | | abs# Neutros | PD1 - Prä B | 0,3201 | 0,1364 | | abs# Neutros | PDL1 - Prä B | 0,1619 | 0,4604 | | abs# Neutros | CRP (mg/dL) | 0,4187 | 0,0468 * | | abs# Neutros | LDH (U/L) | 0,1834 | 0,4023 | | abs# Lymphos | CD3Z - Prä B | 0,0948 | 0,6670 | | abs# Lymphos | CD8 - Prä B | 0,1911 | 0,3824 | | abs# Lymphos | CD163 - Prä B | -0,2626 | 0,2261 | | abs# Lymphos | CTLA4 - Prä B | 0,3842 | 0,0703 | | abs# Lymphos | PD1 - Prä B | 0,3478 | 0,1039 | | abs# Lymphos | PDL1 - Prä B | 0,2694 | 0,2139 | | abs# Lymphos | CRP (mg/dL) | 0,2060 | 0,3456 | | abs# Lymphos | LDH (U/L) | -0,1082 | 0,6231 | 
The mRNA markers and the changes should be also correlated to response to therapy. It is not possible to differentiate if the change of markers is a general prognostic sign or if this is predictive for benefit of ICI. This unclarity should be discussed in the discussion part of the manuscript.	These data were obtained. However, they were not reported in the first version of the manuscript as they are not so robust outcome parameters as PFS and OS. The data are no given as **suppl Tables 1, 2A and 2B.** In addition, the response data are described in the results part: ***ll 295-298*** >> After a median follow-up of 27.4 months an ORR to 1st line therapy was observed in 66.7% of patients with a median duration of response (DOR) of 34.1 months, (95%CI: 20.6-47.6 months). Disease control rate was 68.9% with 22% showing a progressive disease (PD) (**suppl. Table 1**).>> ***ll 336-338*** >>Relative gene expressions divided by their median in

	low and high expression revealed no significant difference for ORR, PFS or OS in log rank test **(Table 3A and suppl. Table 2A).<<** >> ORR and PFS were significantly improved by patients with increased whole blood expression of CD3 and CD8 within 3 weeks after start of pembrolizumab therapy (***Table 3B and suppl. Table 2B***).<< See also comment to reviewer 1.
The mRNA changes indicate a strong prognostic impact. However, there were several tests made, to evaluate different markers. Were the statistics adjusted to multiple testing?	A preselected number of only 5 genes (CD3, CD8, PD-1, PD-L1 and CTLA4) were tested. As these markers are not independent from each other (e.g. immune cells expressing CD3 frequently also express CD8 or PD-1) a Bonferroni adjustment for multiple testing must not be done.
The discussion is focusing mainly on blood cell counts and PBMC counts in relation to ICI benefit. I think this should not be the main focus. Please include more technical aspects in the study.	We have found that the exact quantitation of specific subsets of immune cells pre and post ICI treatment is predictive for treatment outcome. Whole blood cell counts in clinical routine do not quantify these specific subsets (i.e. CD8 positive cells) and therefore are not predictive. A better approach would be CD8 counts from whole blood preparation by FACS analysis. However, this approach is technically more demanding due to variabilities in reagents, antibodies and instruments, while RT-qPCR assessment after nucleic acid extraction using commercially available kits is extremely robust and interobserver independent (Varga et al. Breast Cancer Research 2017).
Nürnberg is written consistently wrong, please check.	Nuernberg is in German language the same as Nürnberg and used when appropriate, e.g. name of hospital as it is called in German. Nuremberg is the English term and used when appropriate, e.g. name of town in the affiliation.
Reviewer 4	
Page3 Line 108 Design How much blood was collected at each point? How many times blood samples were taken? The study design was retrospective, and blood was collected prospectively. This is a somewhat puzzling statement. There is no mention of IRB approval or patient consent. I think this needs to be clearly stated.	These are important questions that were added in the manuscript. 5 ml of EDTA blood were taken during routine laboratory control. Blood was taken before start of ICI therapy and than every three weeks, every time before starting a new cycle. This a prospectively study as LB was planned and taken prospectively. In addition, clinical data were obtained and documented prospectively. Therefore, the term "retrospective" is somewhat misleading and was replaced

	by prospective. Due to this topic see also comments to reviewers 1 and 2 Of course, there was an IRB approval and written informed consent of the patients. This can already be found in the first version of the manuscript. ***ll 190-193*** >> This study was approved by the Ethics committee of the Friedrich-Alexander-University Erlangen-Nuremberg, Germany (numbers: 56_16B and 62_17B). Written informed consent for blood taking, collecting and analyzing for research purpose was obtained from every patient. <<
Page3 Line 127 anti-PD-L1 antibody The companion diagnostic for pembrolizumab is 22C3. This study was conducted in a patient-selected population using ZR3. Does the difference in antibody type affect the conclusions of this study? We believe that additional discussion is needed in this regard.	Some more information about ZR3 and its validation is now given in the methods part and a figure with the different IHC stainings was created: ***Ll 203-208*** >> The antibody ZR3 had been validated against 22C3 at our department and concordance with 22C3 was very high. An example is given in **figure 1**. In addition, after passing two German external quality assessments for PD-L1 staining the pathological department received approval with ZR3. In contrast to FDA companion diagnostic with 22C3 for use of pembrolizumab is not prescribed by the EMA in Europe. << The difference in antibody type did not effect the conclusions of this study as PD-L1 status was not an independent predictor in this analysis.
Page5 line244 In this section, only the mRNA high and low results of PD-L1 are described (Fig. 2). In line 246, there is a statement that "we examined five of those xxx" and that they searched for genes other than PD-L1, but there is no description of the results for genes other than PD-L1. If this part is deemed unimportant, the data should be deleted.	The 5 RNA markers examined were CD3, CD8, PD-1, PD-L1 and CTLA-4. The results are described in the results. It is now made more clear in the text: >> To identify predictive peripheral blood biomarkers for pembrolizumab treatment, we examined PD-1, PD-L1, CTLA-4, CD3 and CD8 before treatment. <<
Page6 Line255 This section analyzes the increase or decrease in gene expression. Table 3 cited in this section is confusing because it first lists high and low baseline gene expression. To distinguish between the data on baseline gene expression and	You are absolutely right. The table is confusing and we converted Table 3 in Table 3A and 3B.

the data on the increase/decrease of gene expression, it would be better to separate the tables. The table for baseline expression should be cited in the section above.	
There is no mention of PD-L1 and PD-1 in the rate of change of gene expression. Even if it is not a significant result, I think it should be mentioned since it is presented as the table's data.	We have adopted PD-1 and PD-L1 in the results section. Though assessment of dynamic changes of PD-1 and PD-L1 trended to be informative it did not reach statistical significance (see table 3). ***ll 345-346.*** >>In contrast, changes of PD-1 and PD-L1 were not predictive, neither for PFS nor OS (***Table 3B***).<<
Page15 Table 3 The mPFS of PD-L1 low and high are misaligned**.**	The Table 3 was corrected and divided in Table 3A and 3B. See comments above.