Peer Review File

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Reviewer:

Kocher and colleagues examine an important question of metabolomic differences in patients with advanced non-small cell lung cancer who have response versus progressive disease at first follow up scan on treatment with immune checkpoint inhibitors.

Major comments:

Reviewer comment 1)

It is unclear whether tryptophan level differences noted between responders and those who progressed were at baseline or at the time of follow up scan. Does Figure 5A represent baseline levels or levels at the time of follow up scan?

- The authors write:

"In the whole cohort of 23 patients median Trp levels, Kyn levels as well as Kyn/Trp were comparable at baseline and at first restaging. Similar observations with regard to Trp and Kyn/Trp were made when dividing patients in patients into cancer progression versus disease control."

Here they are saying there were no differences in baseline tryptophan levels. What would be the utility of a biomarker which predicts progression at the time of actual progression?

- Figure 5B is invalid if there were no differences in baseline tryptophan levels

Author comment 1: Figure 5A displays Trp levels at baseline when patients were divided according to therapy response upon ICI. It revealed that patients primary refractory upon ICI are characterized by lower Trp levels than patients achieving controlled disease. Figure 5B shows the PFS Kaplan-Meier with stratification of patients according to baseline Trp levels. The optimized Trp cut-off was established by ROC analysis. It revealed that there was a trend towards better PFS in patients showing baseline Trp levels ≥49.3 µmol/L.

In the revised version we now have adapted the respective paragraph in the results section. (Page 9, line 20):

"Similarly subgroup analysis according to therapeutic response (i.e. controlled disease and primary refractory) showed comparable levels of Trp, Kyn and Kyn/Trp between baseline and first follow up. Most importantly, when comparing baseline Trp levels in patients with controlled disease and primary refractory disease it revealed that patients achieving disease

control are characterized by significantly higher Trp levels (median 63.8 µmol/L vs. 45.0 µmol/L, p = 0.007; **Figure 5a**)."

Moreover we adapted Figure Legend 5 (page 15, line 22):

"**Figure 5:** a) Median baseline Trp levels in patients with controlled disease (partial response and stable disease) compared to patients with primary refractory disease (median Trp levels controlled disease vs. primary refractory 63.8 µmol/L vs. 45.0 µmol/L, p = 0.007). b) Kaplan-Meier plot showing progression free survival in the whole cohort with stratification according to Trp levels at baseline (the cut-off at Trp 49.3µmol/L was established according to ROC analysis). A trend towards improved PFS was observed in patients with Trp levels ≥49.3µmol/L (PFS ≥49.3µmol/L vs. <49.3µmol/L: 164 days vs. 61 days (HR 0.47 [0.19 – 1.18], p = 0.095)."

Review comment 2)

Did all patients receive follow up scans at the same time interval? The time interval should be specified. Was it 8 weeks or 12 weeks or something else? What was the median follow up duration of the patients?

Author comment 2: All patients received follow-up scans every twelve weeks or earlier in cases of clinical suspicion of tumor progression. Median follow-up duration was 327 days (Range: 34 – 668 days). We have now included this information in the manuscript (page 6, line6 and page 8, line 6):

"All patients received follow-up scans every twelve weeks or earlier in case of clinical suspicion of tumor progression."

"Median follow-up duration of the cohort was 327 days (range: 34-668 days)."

Reviewer comment 3)

Please show metabolic profile for those who had disease control at the first follow up scan. Data for only 9 patients with primary resistant disease is shown right now.

Author comment 3: No figure was generated for the metabolic profile of patients with disease control at first follow-up since significant metabolic alterations were only observed for histidine, glutamate and phenylanine. No significant alterations in important metabolites (including IDO and BCAA) were observed in this subset of patients. Thus, an additional figure would not have added important additional information. However, we now state in the manuscript, that no significant alterations in important metabolites (including IDO and BCAA) were observed in this subset of patients.

We now have included this information in the manuscript (page 9, line 11 and page 11, line 13-15):

"Patients responding to therapy showed higher levels of histidine and phenylalanine, whereas glutamate levels were decreased compared to baseline levels. No significant alterations in important metabolites (including IDO and BCAA) were observed in this subset of patients."

"However, these metabolites were not altered in the controlled disease group, including patients with partial remission or stable disease at first re-evaluation."

Reviewer comment 4)

Figure 2 and Figure 4 report fold change concentrations. Please explain in text clearly what these fold changes represent. What is the numerator and what is the denominator? Please show variance as well in the graphs (e.g. standard deviation).

Author comment 4: Figure 2 displays significant metabolic differences between lung cancer patients and healthy controls. In figure 2, fold-changes express the ratio of mean levels of a certain metabolite in the cohort of NSCLC patients divided by the mean level of the same metabolite in the healthy controls. In figure 4, the ratio of significant metabolic changes in the subcohort of primary refractory NSCLC patients at timepoint of cancer progression divided by baseline levels is presented. Therefore, no variances can be presented.

To improve comprehensibility of the figures we have adapted Figure 2 and figure 4 and provide information on the parameter „fold changes in the figure legends (page 15 line 16, page 15, line 22).

"**Figure 2:** Metabolites significantly altered in patients with NSCLC before treatment start and healthy controls. Fold-changes express the ratio of mean levels of a certain metabolite in the cohort of NSCLC patients divided by the mean level of the same metabolite in the healthy controls."

"**Figure 4:** Metabolites significantly altered in patients with NSCLC before treatment start and progressing after the first follow up scan. Fold-changes express the ratio of a certain metabolite in the subcohort of primary refractory NSCLC patients at timepoint of cancer progression divided by baseline levels."

Reviewer comment 5)

Please include details of healthy controls (wherever relevant) and details of prior therapy for patients with advanced non-small cell lung cancer in Table 1

Author comment 5) All patients received as first-line treatment a platinum-doublet and were than treated – as already mentioned in the material and methods section – in the second-line with a checkpoint inhibitor. However, we have now stated in the "Results section" that all patients had a prior therapy with platinum (page 8, line 3). Furthermore, a

pool of 20 healthy donors were included in this study. We have added this information in the "Material and Methods section" accordingly (Page 6, line 7).

Reviewer comment 6)

Limitations should be clearly acknowledged: small sample size, not adjusted for confounding factors, exploratory analysis at best, all patients treated in second line setting (immune checkpoint inhibitors are now used in first line)

Author comment 6: We have added the limitations in our study in the "Discussion section" (page 12, line 8).

Minor comments:

1) Restaging is not the right term. Staging is done once at the time of diagnosis. Follow up scan would be a better term.

   → we changed "Restaging" to "Follow up"

2) Line 86: typographical error- adaptation

   → done

3) PFS is generally defined as duration till disease progression or death. If that was the case, please modify that in the method.

   → correctly. We have changed it accordingy

4) Figure 1 legend: Is it plot or blot?

   → done

5) Please make legends of Figure 2 and 4 clear to indicate what are fold changes calculated for (e.g patients with NSCLC/ healthy controls)

   → Figure legends were changed accordingly

6) Figure 2 quality is poor. Cannot read what's written inside blue bars.

   → We have adapted Figure 2.

7) Figure 7 legend specifies mean, but the text mentions median. Please select one measure of central tendency.

   → A figure 7 does not exist. However, we used as statistically adequate median