Peer Review File

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Reviewer A

Comment 1: To monitor the effectiveness of treatment both CTC and cfDNA are valuable to assess treatment effectiveness. The decline in both CTC and cfDNA after treatment would indicate effectiveness. Was any mutational work performed on tumor tissue? If a comparison of cfDNA pre to post, the susceptible tumor mutations can be identified. In addition follow-up therapies can be selected for the remaining mutations. If a significant number of CTCs post treatment are present tailored therapies can be selected to match the residual mutations on the CTCs not affected by the first treatment. Were cfDNA comparisons made between pre and post treatment and then with at progression results? It was not clear if only total cfDNA was compared or where specific mutations followed.

Reply 1: In the present study only the CTCs enumeration and the cfDNA levels were interrogated since the aim of the study was to analyze easy to implement markers to predict the patients' evolution. Of course, the reviewer appreciation regarding the value of cfDNA and CTCs to go insight the molecular patterns or changes associated with the therapy response is of great value but this represents a different approach that implies a more complex methodology to identify the molecular actors behind the response to chemotherapy.

Changes in the text: No changes have been made in the text.

Comment 2: Each cancer sheds CTCs in varying amounts SCLC has thousands of CTC, compared to the number seen in colon which are usually low in number, breast cancers can be all over the range in numbers with inflammatory breast cancer having larger numbers. If a breast cancer patient is susceptible to paclitaxel and responds, her CTC numbers will drop within a week to almost nothing. Prostate cancer will show CTCs early and more prostate cancer patients have CTCs with response to therapies and progression much easier to follow.

In summary to be able to follow any tumor type the starting point is to know which mutations are present in the tumor prior to any treatment. Quantity of cfDNA and the numbers of CTCs can easily be followed; however, the power of these technologies following chemotherapies is in the ability to choose treatments targeting specific mutations in the remaining CTCs and the composite of mutations in residual cfDNA.

This paper was written well, references were adequate. Driver mutations RB1 and TP53 were followed and the three timepoints were chosen well. The one piece I feel was missing was to also follow CTCs at the other time points.

Reply 2: Thank you for comment this point. CTCs analyses during therapy could apport more information about treatment response. Thus, we analyzed CTCs using CellSearch system at time of progression disease in 14 SCLC patients, however we decided not include these results due to the information found was not enough relevant and the sample size was small. Our preliminary results showed that the majority of those patients with an increase in the number of CTCs at progression disease (n=5/6), experienced progression disease or stable disease in the first 3 months after start therapy (See Figure). A wide range of CTCs between patients was

found (some patients presented an increase of 6 CTCs, and others an increase of 100 CTCs), so results should be interpreted with caution. In addition, CTCs analyses at time of progression disease will not allow to anticipate the treatment response. In contrast, we think than CTCs analysis at intermediate time points (for example at 3 weeks), could be more interesting.

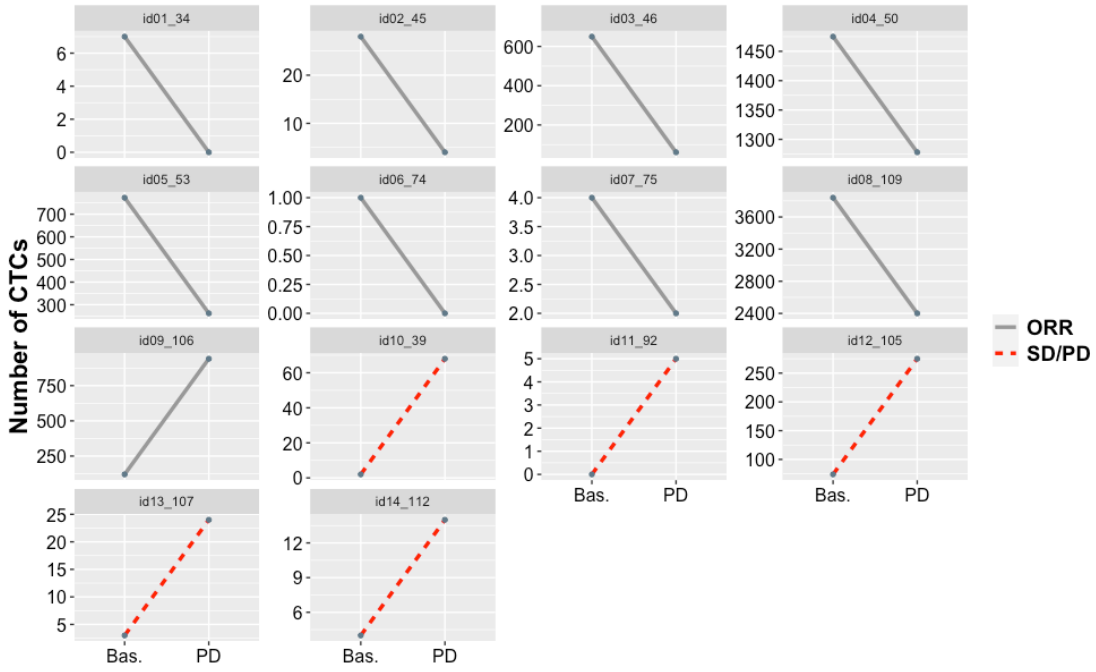

Figure. *Changes into CTCs number for each patient between baseline and at time of progression disease. Abbreviations: Bas., baseline; PD, progression disease; ORR, objective response rate.*

Changes in the text: According reviewer comment, we have added the lack of CTCs analysis during therapy as a limitation. (See page 19, lines 455-458).

Comment 3: The authors did not state whether all the standard chemotherapy treatments were the same nor what the standard was; how many received immunotherapy with chemotherapy and whether that addition made any significant impact on cfDNA levels.

Reply 3: Thank you for your comment. 33 patients received chemotherapy based on carboplatin and etoposide, 9 carboplatin, etoposide and atezolizumab, and 4 carboplatin, etoposide and durvalumab. The use of chemotherapy was predominant given that at the time we included the first patients in the study, the use of immunotherapy was not approved for this indication in our country.

A study comparing DNA levels between the group that received chemotherapy exclusively and those that received combined treatment was performed however, no significant differences were found, probably for the small cohort size.

Changes in the text: According reviewer suggestion, we have added this information in page 11, lines 244-248.

Comment 4: Including the therapies received and comparing only "like" groups (chemo. alone

vs chemo and immunotherapy) would greatly enhance this work. This would not constitute a major revision and would greatly substantiate the conclusions drawn if no differences were seen.

Reply 4: Thank you for the comment. Effectively, comparison between homogenous groups could improve the conclusion of our study. Thus, we performed a study comparing DNA levels between the group that received chemotherapy exclusively and those that received combined treatment however, the sample size of each subgroup in our study was small, where only the 28% of the SCLC patients received chemotherapy + immunotherapy (9/46 atezolizumab; 4/46 durvalumab). Despite of, no significant differences were found. We have included this point as a limitation in the discussion section.

Changes in the text: We have added this issue as a limitation in discussion section (see page 19, lines 458-461)

Comment 5: What criteria did the healthy controls have to meet? All should have some level of cfDNA but done should have CTCs. This needs to be included.

Reply 5: Thank you for your comment. We included 20 healthy controls matched for age without previous tumor disease or suspect of cancer.

Due to cfDNA include DNA released from all cell types present into the blood (including normal cells), healthy controls could present variable cfDNA concentration. Thus, we quantified cfDNA levels in healthy controls using our qPCR assay and compared these values with those from SCLC patients to understand how specific is the increment of cfDNA levels in our cohort of patients. Our results showed that healthy controls presented significantly lower cfDNA levels than SCLC cancer patients (p-value= $1.5x10^{-11}$), evidencing that cfDNA levels were mainly increased as a result of the malignant disease in SCLC patients.

In contrast, CTCs are tumor cells originated from the primary or metastatic sites that are able to enter the circulation and disseminate to distant sites. Due to the tumoral origin of CTCs, healthy controls not should present CTCs into the circulation, as previously reported many authors (For example, Andree KC et al. Mol Oncol. 2016; doi:10.1016/j.molonc.2015.12.002). Taking into account that absence of CTCs in healthy people is well stablished, we didn't analyze CTCs in our control cohort.

Changes in the text: In order to clarify the tumoral origin of CTCs, we include some modification in the Introduction section (see page 5, line 97-99).

Reviewer B

Comment 1: Authors should explain the cohorts (favorable and unfavorable groups) to make the stratification clearer.

"Favorable group (patients 304 with low cfDNA levels at baseline and at 3 weeks + patients with high cfDNA levels 305 at baseline but low levels at 3 weeks; n=18) and unfavorable group (patients with low 306 cfDNA levels at baseline but high cfDNA levels at 3 weeks + patients with high 307 cfDNA at baseline and at 3 weeks; n=22)".

Reply 1: Thank you for the comment. Due to favorable/unfavorable classification results the

same than when we only analyze the levels of cfDNA at 3 weeks, now we have simplified the classification and we reported these results as "cfDNA levels at 3 weeks". We apologized for the confusion with the previous classification.

Changes in the text: According reviewer suggestion, we have simplified the classification in results section (see page 13; lines 307-3011) and figure 3.

Comment 2: Did the authors check for CTC levels post treatment? I was curious to know whether the CTC levels complement the changes in cf/ctDNA levels?

Reply 2: Thank you for comment this point. Effectively, CTCs analyses during therapy could apport more information. Thus, we analyzed CTCs using CellSearch system at time of progression disease in 14 SCLC patients, however we decided not include these results due to the information found was not enough relevant and the sample size was small. Our preliminary results showed that the majority of those patients with an increase in the number of CTCs at progression disease (n=5/6), experienced progression disease or stable disease in the first 3 months after start therapy (See Figure). A wide range of CTCs between patients was found (some patients presented an increase of 6 CTCs, and others an increase of 100 CTCs), so results should be interpreted with caution. In addition, the increase/decrease of CTCs count was not associated with an increase/decrease of cfDNA levels. We think than CTCs analysis at intermediate time points (for example at 3 weeks) in a larger cohort could be arise more robust conclusions.

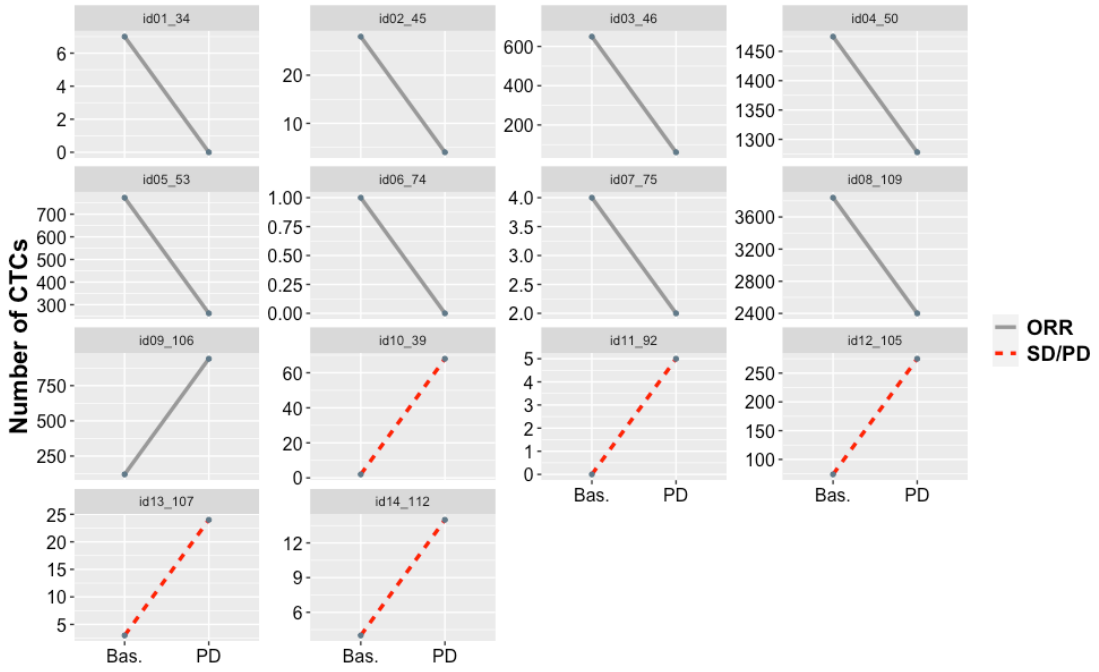

***Figure**. Changes into CTCs number for each patient between baseline and at progression disease. Abbreviations: Bas, baseline; PD, progression disease; ORR, objective response rate.*

Changes in the text: We have added the lack of CTCs analysis during therapy as a limitation. (See page 19, lines 457-458).

Comment 3: Table 1: In characteristics, under the Age parameter – below is written as bellow

Reply 3: Thank you for the comment and sorry for the mistake. We have arranged the mistake.

Changes in the text: We have corrected the word “below” (see Table 1)

Comment 4: Authors should mention the type of immunotherapy and chemotherapy administered to the patients

Reply 4: Thank you for your question. 33 patients received chemotherapy with carboplatin and etoposide, 9 carboplatin, etoposide and atezolizumab, and 4 carboplatin, etoposide and durvalumab. The use of chemotherapy was predominant given that at the time we included the first patients in the study the use of immunotherapy was not approved for this indication in our country.

Changes in the text: According reviewer suggestion, we have added this information in page 11, lines 244-248.

Comment 5: In all the figures with survival curves, in the section where “number at risk” are mentioned, PFS and Time in days are barely visible. Increase the font size to make it readable.

Reply 5: We appreciate the reviewer comment and we have improved the size and quality of all figures with survival curves.

Changes in the text: Figure 3, figure 4C-D and supplementary figure 2C.

Comment 6: Supplementary figure 2: The axis labels and the font size are too small and difficult to read. Please fix these.

Reply 6: Again, we appreciate the reviewer comment and we have improved the font size of axis labels.

Changes in the text: Supplementary figure 2.

Reviewer C

This is an excellent study investigating the various aspects of liquid biopsy in small cell lung cancer. Specific comments below:

Comment 1: Introduction - a bit of literature around CTCs and why different methodologies are applied should be included (see https://www.sciencedirect.com/science/article/abs/pii/S016777992200035X https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8813755/

Reply 1: Thank you for the comment. Articles proposed summarized different liquid biopsy biomarkers investigated in lung cancer and different platforms that can be employed in order to isolate CTCs. According reviewer suggestion, now we have included these references in our introduction.

Changes in the text: We included some information about CTCs isolation platforms in Introduction section and according reviewer suggestion, we have added 2 references (see page 5, lines 105-108 and references 14,30).

Comment 2: sample processing - 96 hours seems a bit of a larger window than recommended

- usually ctDNA should be processed within a few hours of sample collection - the reasons for 96 hours was this because they were cellsave tubes? The cell save is usually for CTC workflows?

Reply 2: Thank you for comment this point. It is true that the most common method to isolate plasma is within a few hours after blood collection when use EDTA tubes. However, CellSave Preservative tubes allow to maintain the blood samples for up 96 hours at room temperature, due to the preservative reagents stabilizes the blood cells avoiding haemolyses (Rothwell et al. Mol Oncol 10.1016/j.molonc.2015.11.006, 2016; van Dessel et al. Mol Oncol 10.1002/1878-0261.12037, 2017). CellSave tubes have been specifically developed for CTC stabilization in view of CellSearch enrichment, labelling and counting. However, several works have been reported that CellSave tubes also can be employed for cfDNA isolation and posterior sequencing analyses (Mohan et al. J Thorac Oncol 10.1016/j.jtho.2019.10.007, 2019; Moding et al. Nat Cancer 10.1038/s43018-019-0011-0, 2020).

In the same line, our group has employed CellSave tubes for posterior circulating tumor DNA (ctDNA) analyses, obtaining a good detection rate in cancer patients (PMID: 32098121). In addition, in order to check that the CellSave tubes can be employed to isolate cfDNA in our cohort, we analysed its integrity with the TapeStation 4700 (Agilent, Santa Clara, CA, USA) and the High Sensitivity DNA ScreenTape® (Agilent, Santa Clara, CA, USA). We confirm that in terms of integrity, CellSave tubes don't interfere in the cfDNA isolation, presenting a size around 120 bp (see Figure).

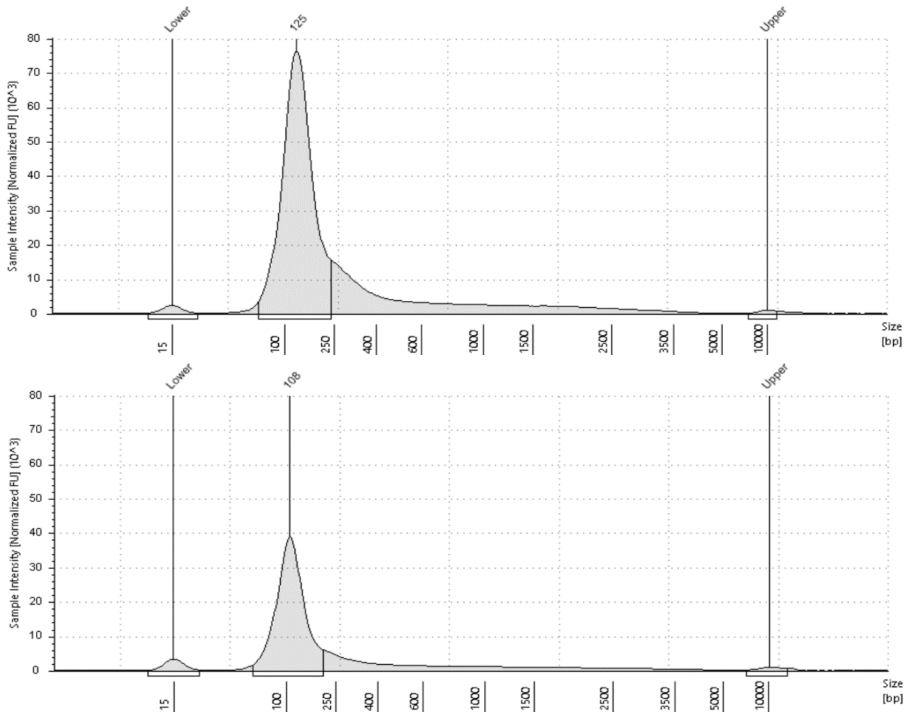

Figure. cfDNA profile of samples extracted with CellSave tubes analyzed with the Agilent TapeSation system.

Changes in the text: Now, we have added one reference that indicates that CellSave Preservative tubes allow to maintain the blood samples for up 96 hours for posterior analyses (see page 7, lines 159-160, reference 31).

Comment 3: usually the first few ml of blood is excluded to prevent fibroblasts/endothelial

cells from the puncture going directly into the cellsave tube - was this the case?

Reply 3: Thank you for your interest comment. Since we are looking for epithelial CTCs (CK positive CTCs) both fibroblast and endothelial cells are discarded during the analysis. However, it is true the recommendation to discard the first volumes of blood because in a very low frequency any epithelial cell with no tumoral origin can appear in healthy people. However, we consider of low benefit this additional step that can induce other type of bias in our study.

Changes in the text: Not applicable.

Comment 4: were the 20 healthy controls matched for age/gender/co-morbidities?

Reply 4: Thank you for comment this point. Demographic characteristics of healthy controls are presented in the next table. Healthy controls were matched for age (mean age 60.1, range 37-78) however females were more present than males (n=13 females versus 7 males). Nevertheless, a comparison between both groups was performed and no significant differences between them were found (Wilcoxon p-value=0.68) (see Figure/Table).

Characteristics	**cfDNA, mean (GE/ml)**	***p*-value**
Age, *(Median, Mean age ± SD, range)* 64, 60.1 ± 11.7, 37-78		
Below median, n=9 (45%)	918.2	0.97
Above median, n=11 (55%)	925.5	
Gender		
Male, n=7 (35%)	985.7	0.82
Female, n=13 (65%)	888.0	

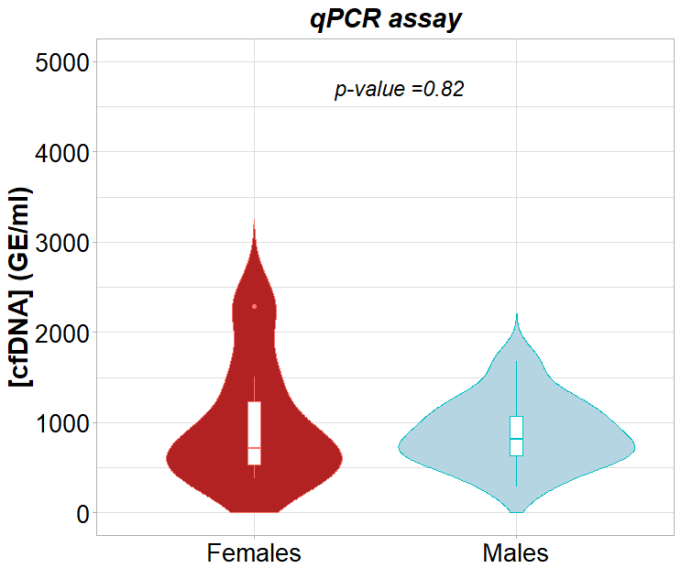

Figure. *cfDNA levels in healthy controls regarding their sex. No significant differences between females and males were found. Abbreviations: GE, genome equivalents.*

Changes in the text: Not applicable.

Comment 5: Were any ctc clusters found? These have been reported extensively in lung cancer
Reply 5: We appreciate the comment. Effectively, CTC clusters detected in blood samples have showed a negative predictive value in several cancer types including lung cancer (Murlidhar V, et al. 2017; Cancer Res; doi:10.1158/0008-5472.CAN-16-2072). In our SCLC cohort, we found CTC clusters (≥2 CTCs or CTC with white blood cells) in all patients with >10 CTCs/7.5mL of blood. Thus, we found the same prognostic impact of having CTCs clusters than having >10 CTCs/7.5mL CTCs indicating not additional value in our cohort of patients.
Changes in the text: We have added details about the number of clusters found in our patient cohort (see page 14, lines 330-332).

Comment 6: My concern in this manuscript is what the collective ctDNA call is - yes it's total, but is it tumour specific? Does this matter? In GBM, similar findings have been shown is this total ctdna a proxy for mutational load/burden?
Reply 6: Your comment is a very important point in our work. Our strategy in the present study was to establish if the global content of cfDNA can provide clinicians an additional prognostic tool. We know that in many patients the fraction of ctDNA is lower than 1%, therefore the impact on the total cfDNA seems to be low. However, several studies have demonstrated that it increases in accordance with the tumor burden. Therefore, although cfDNA content is not tumor specific, it can be assumed that total cfDNA in cancer patients originates from tumor cells, but also from cells present in the tumor microenvironment or from cells involved in the antitumor response. Therefore, our results are in line with the fact that cfDNA can be used as a surrogate of ctDNA, but always taking into account that they are different entities that can provide us different information.
Changes in the text: We have included in the discussion a comment regarding this important point (line 386-393).

Reviewer D

Comment 1: I propose that the title includes the notion of CTC, as they were investigated together with cfDNA.
Reply 1: Thank you for your suggestion. Now, we have modified the manuscript title as "Analysis of Plasma cell-free DNA and CTCs as prognostic biomarkers in small cell lung cancer patients".
Changes in the text: We have changed article title (see Page 1).

Comment 2: In the abstract, it should be clear that the cohort of interest is represented by advanced SCLC candidate to first-line therapy. Authors should also specify, in the abstract and in the methods, that first-line therapy included chemotherapy or chemo-immunotherapy.
A quick reference in the abstract to the type of cfDNA analysis (quantitative, not qualitative) should be considered.
Reply 2: Thanks for these comments. True, in our cohort all patients received first-line therapy and now we indicated it in abstract and methods sections. In order to highlight the type of cfDNA analysis performed in our study, we have added the term "cfDNA quantitative analysis"

in the abstract.
Changes in the text: According your suggestion, we have added more information about our cohort in the abstract (see page 3, lines 45-46) and in the methods section (see page 6, line 126-127). Information regarding cfDNA analysis employed have been also included (see page 3, line 57).

Comment 3: Line 111: Authors should put a parenthesis to close the sentence.
Reply 3: Done.
Changes in the text: See page 6, line 118.

Comment 4: In results, Authors should write how many patients were treated with chemotherapy and how many with chemo-immunotherapy (the information is present in Table 1).
Reply 4: Thank you for your kind comment. Now, we also explain how many patients were treated with each regimen of therapy in the text.
Changes in the text: According your suggestion, we added the number of patients treated with each therapy in the text (see page 11, lines 244-248).

Comment 5: I would reinforce, both in results and in discussion, that cfDNA and CTC mirror disease burden.
Reply 5: Thank you for comment this point. Now, we have reinforced the association between our circulating biomarkers and disease burden.
Changes in the text: See page 12, lines 268-269; page 14, lines 324-325; page 19, lines 449.

Comment 6: In table 1, please convert the p value of liver metastases 2.20x10-4 into 0.0002 (or $p < 0.001$).
Reply 6: Thank you for your comment. We changed the nomenclature of the p value.
Changes in the text: According your comment, we converted the p value to 0.0002 in table 1. (See tables, Table 1).

Reviewer E

Major comments:
Comment 1: The study includes patients treated with different treatment modalities (chemo and chemo/IO). What criteria was used to decide treatment, may this affect the results. Treatment type was not included in the multivariate analysis. At minimum, this should be discussed and acknowledge as a limitation.
Reply 1: Thank you for your question. 33 patients received chemotherapy with carboplatin and etoposide, 9 carboplatin, etoposide and atezolizumab, and 4 carboplatin, etoposide and durvalumab. The use of chemotherapy was predominant given that at the time we included the first patients in the study the use of immunotherapy was not approved for this indication in our country.
The sample size of our cohort is relatively small and only the 28% of the SCLC patients

received chemotherapy + immunotherapy (9/46 atezolizumab; 4/46 durvalumab). We thought not include more artifacts. We have included this point as a limitation in the discussion section.

Changes in the text: According reviewer suggestion, we have added information about treatment regimens in page 11, lines 244-248 and a limitation in discussion section (see page 19, lines 460-462).

Comment 2: The model requires validation in an independent cohort, ideally more homogenous relative to treatment type. This should be acknowledged in the discussion more explicitly.

Reply 2: Thank you for comment this point. Our cohort includes 33 SCLC patients treated with chemotherapy (carboplatin and etoposide) and 13 SCLC patients (28.26%) treated with chemotherapy in combination with immunotherapy, being a limitation now explained in discussion section. In addition, a validation study in an independent cohort is the next step to confirm our results. Now, we have added both issues in discussion section.

Changes in the text: According reviewer suggestion, we have added a new paragraph of limitations in discussion section (see page 19, lines 460-463).

Comment 3: Please also comment on how you see for this prognostic model to be used clinically.

Reply 3: We thought that the most relevant of the prognostic model proposed, including cfDNA levels, is that could allow to stratify SCLC patients and detect those who could benefit from the actual therapies. In practical terms, in those SCLC patients with high cfDNA levels and some clinical characteristics, the choice of a more aggressive therapy or the intensification of clinical visits would be considered. In addition, cfDNA analyses are a cost-effective method to classified SCLC patients in comparison with ctDNA analyses and they could be used into clinic routine easily. However, we recognize that a validation study in a larger cohort of SCLC patients is needed to reach robust findings.

Changes in the text: See page 16, lines 374-377; page 18, lines 432-434.

Comment 4: In a technical/practical level having different threshold for PFS and OS seems harder for future standardization. Consider using the same threshold.

Reply 4: Thank you for this specific comment, we are fully aware of this need. Effectively, we thought that the ultimate objective must be unify the threshold for PFS and OS. However, for this purpose the future validation studies must use sample sizes of SCLC patients sufficiently powerful to estimate this threshold, and to evaluate all the potential confounders effects.

Changes in the text: Not applicable.

Comment 5: The survival analysis relative to high-cfDNA at PD, with time of OS calculated from commencement of treatment seems inappropriate, as it included time that has passed before the measurement is made. A more appropriate analysis would be to measure the time to death from progression (when cfDNA was measured).

Reply 5: Thank you for comment this point. Effectively, analysis taking into account the time to death from progression to investigated the overall survival of patients regarding the levels of cfDNA at progression disease is more appropriated. Now, we have modified the analyses, obtained a similar association. Patients with high cfDNA levels at progression disease time point present lower overall survival than patients with low levels (p-value<0.001)
Changes in the text: According reviewer comment we have included the definition of overall survival for progression disease analyses in the "methods" section (see page 7, lines 151-154). In addition, we have changed some results regarding prognostic value of cfDNA at progression disease (see page 14; lines 314-316), Figure 3E and Table 2.

Minor:
Comment 6: 6. Abstract: 'Both biomarkers were associated with patients' outcomes and a 49 prognostic model was developed' – sounds like a conclusion, I suggest rewording.
Reply 6: Thank you for your comment. Now, we have reworded the sentence.
Changes in the text: See page 3, lines 47-49.

Comment 7: Lines 117 and 119: It should be 'newly diagnosed'.
Reply 7: We have modified the expression as advised. Thank you for the comment.
Changes in the text: Now, lines 125 and 127.

Comment 8: Line 146: Please present a reference showing that different times from processing of CellSave tubes up to 96 hours do not affect cfDNA levels.
Reply 8: Thank you for the comment. When EDTA tubes are employed for blood collection, plasma isolation should be performed within a few hours. Nevertheless, CellSave Preservative tubes allow to maintain the blood samples for up 96 hours at room temperature, due to the preservative reagents stabilize the blood cells avoiding hemolysis. Several works have reported these data (Rothwell et al. Mol Oncol 10.1016/j.molonc.2015.11.006, 2016; van Dessel et al. Mol Oncol 10.1002/1878-0261.12037, 2017).
Changes in the text: According reviewer suggestion, now we have added a reference showing that different processing times do not affect cfDNA levels when CellSave preservatives tubes are employed (see page 7, line 159, reference 37).

Comment 9: Please clarify in methods and figure legends the statistical analysis done. For example, Figure 2B and C – is that a Mann-Whitney test p-value?
Reply 9: Thanks for your kind comment. Yes, in Figure 2B and 2C we employed the Mann–Whitney–Wilcoxon U-Test to compare cfDNA levels between SCLC patients and healthy controls. Now, we have added more information about statistical analyses performed in each figure legend.
Changes in the text: See figure legends (page 30).

Comment 10: Similarly, in Supplementary Figure 1, the comparison indicates Wilcoxon test, which is a paired test, but the number of samples varies between groups. Please clarify and/or correct the figure.
Reply 10: Thank you for comment this issue. Two-sample location problem is one of the most

encountered problems in statistical practice, and sometimes this problem involve observations are paired and some are not. This problem is also known as paired two-sample problems with missing data, and this is our case. Often it is difficult for some invasive procedures to collect data from an individual at both conditions we are interested in comparing, or even impossible (event of death). Existing rank-based two-sample comparison procedures for partially paired data do not make efficient use of all available data (PMID: 28968816), but the Wilcoxon test is a robust tool where missing values are ignored.
Changes in the text: Not applicable.

Comment 11: What is hG/mL? and why all the figures are reported then as genome equivalents then?
Reply 11: Thank you for the comment**.** hG/mL refers to human genome equivalents per mL of plasma. We apologize for the confusion. We unified both terms around all the manuscript.
Changes in the text: We have changed the terms "hG/mL" for GE/mL (see page 12, line 284).

Comment 12: Lines 298-313: explain what threshold was used for high cfDNA at 3 weeks – same as above?
Reply 12: Thank you for comment this point. The threshold employed to determine high cfDNA levels at 3 weeks was 7.879 (log cfDNA levels) both PFS and OS analysis.
Changes in the text: According reviewer suggestion, we added the data for the cfDNA threshold at 3 weeks (see page 13, lines 304-305).

Comment 13: Note that the favourable/unfavourable classification of the changes in cfDNA by 3 weeks, will result the same if just use high and low just at 3 weeks without considering baseline. Or explain better if that is not the case.
Reply 13: Thank you for your kind comment. Effectively, favorable/unfavourable classification results the same than when we only analyze the levels of cfDNA at 3 weeks. The confusion was as a result to try all the possible combinations between the changes between cfDNA at baseline and at 3 weeks. Sorry for the mistake. Now, we have simplified and reported these results as "cfDNA levels at 3 weeks".
Changes in the text: According reviewer suggestion, we have modified some terms in result section (see page 13; lines 307-311) and figure 3.

Comment 14: I would suggest describing the multivariate analysis at the end rather than for each marker. For example, the description of the multivariate regression in lines 284-287, do not make sense prior description of the other variable in the model (ctDNA change and CTC).
Reply 14: Thanks for comment this point. According your suggestion, now we describe the multivariate analysis in the section "Multivariate analyses and prognostic model for PFS and OS".
Changes in the text: See page 14-15, lines 335-345.

Comment 15: 15. It is unclear how sex was introduced in the model (Figure 4) as it was not included in the multivariate analysis in Table 2. Please explain in the statistics how model was built, forward/backward/stepwise – and what criteria for inclusion.

Reply 15: Certainly, we have no detailed this issue. We applied backward stepwise model selection based on the AIC on our data. We then compare models using AIC, with a smaller AIC value indicating the better model. Now, we have added more details in this regard, in the methodology section.
Changes in the text: According reviewer suggestion, we have more details about the development of our model (see page 10, lines 234-235).

Comment 16: Lines 348-351: the paragraph should be reworded. The 'progression model' is not the one that 'quintuples' the risk, but the model indicates an increased risk in the unfavourable category 5-times higher than …
Reply 16: We apologize the mistake and thank you for your comment. We reworded the paragraph in order to clarify the result.
Changes in the text: According reviewer suggestion, we modified the paragraph (see page 15, lines 359-362).

Comment 17: Line 385: I think you mean 'no' instead of 'any'.
Reply 17: Thank you for the comment. Now we use "no" instead of "any".
Changes in the text: Line 402.

Comment 18: Lines: 436 and 444 – Finally used again. Also consider rewording the lats part as it sounds too colloquial. 'Thinking about…'
Reply 18: We agree with the comment. We have changed the paragraph in order to improve it. Thank you. (See page 19, lines 458-459).
Changes in the text: According reviewer suggestion, we have changed some expressions (see page 20, lines 463, 467).

Reviewer F

Comment 1: Authors need to give more details about the patient selection criteria. It is not clear why the patient group is strongly slanted towards male since this does not represent the demographic distribution of SCLC. Were all patients from 2017 and 2021 enrolled? Were any SCLC patients within this time frame excluded from the study? if yes, how and why was this done? Would this introduce a bias into the patient selection? Could this skew the results?
Reply 1: Thank you for your kind comment. Indeed, the percentage of men in our study is higher than in other trials: 64%, 71% and 66% of the patients in the experimental arm of the clinical trials Impower 110, Caspian and Keynote 604, respectively, were men. However, we enrolled all new diagnosed SCLC patients that signed informed consent without any patient selection or exclusion on the basis of gender.
Changes in the text: Not applicable.

Comment 2: Also assay failures are not uncommon in these types of studies. Was the failure rate zero? Did some patients' results have to be excluded due to assay failure?
Reply 2: Thank you for the comment. In the cohort of patients of the present study we don´t

have failed results for both CTCs count and cfDNA analysis. Thus, the failure rate in our CTCs and cfDNA quantification assays was zero.
Changes in the text: Not applicable.

Comment 3: It is known that in some cancers, not all patients shed tumour ctDNA into the bloodstream. is this also true in SCLC and if so, would cfDNA be elevated independent of ctDNA shedding in SCLC patients. If this is the case, would cfDNA still have utility as a prognostic marker in such patients? While larger studies may address these questions, I think the authors do need to address or discuss this in their manuscript
Reply 3: We appreciate the important reviewer comment. The source of cfDNA is an intriguing question in cancer and in other pathological conditions. According to previous studies, the fraction of ctDNA varies from 0.1–89% of cfDNA but it increases in accordance with the tumor burden. Therefore, although cfDNA content is not tumor specific, it can be assumed that total cfDNA in cancer patients originates from tumor cells, cells in the tumor microenvironment or from cells involved in the antitumor response. Therefore, cfDNA can be uses as a surrogate of ctDNA, but always taking into account that they are different entities that can provide us different information.
Changes in the text: We have included in the discussion a comment regarding this point (line 386-393).

Grammatical errors
Comment 4: line 444 Finally, a validation study of our prognostic model in a larger cohort of patients can arise more robust conclusions.
line 449 In addition, a prognostic model employing cfDNA levels and some clinical characteristics (ECOG and gender) allow us to stratify patients
line 357 Recently, the management of SCLC has changed and new therapies, such as immunotherapy among others, are been investigated and approved for clinical use.
Reply 4: We appreciate the reviewer comment. The manuscript has been revised in-depth and we have corrected the errors. Thank you.
Changes in the text: See lines 369, 463,467.

Reviewer G

Comment 1: The introduction (in particular lines 87-92 but also generally speaking) is not satisfactorily describing the common knowledge of usage of cfDNA as a diagnostic marker in cancer and in particular lung cancer. The authors need to include more up to date reference to the current knowledge. For one example, but not limited to this, the authors themselves have presented a very related study addressing NSCLC. It is urgently needed that the readers are actual and briefly informed about relevant current knowledge and that the presented novelty of the particular study thereby can be evaluated.
Reply 1: We appreciate the reviewer comment and we have included more information regarding previous studies that showed the interest of cfDNA/ctDNA analysis in lung cancer, including our previous work focused on NSCLC patients treated with Pembrolizumab.

Changes in the text: We have included new references in the introduction (see lines 89-96).

Comment 2: It is a technique note not highly relevant to the conclusions of the manuscript that there is included a comparison of two different cfDNA quantification strategies. This also lacks reference to previously presented work comparing cfDNA quantification. This part of the manuscript should be revised to only be very shortly presented and only as supplement material. Thus, Figure 2 should be supplementary, text in paragraph 242-259 accordingly shortened. The authors have themselves beforehand selected usage of the PCR methodology (reference 29 and 45) !. Instead it could be more interesting to integrate supplementary figure 1 as a main figure.
Reply 2: Thank you for the comment. Nowadays, a platform to quantify cfDNA samples is not yet well standardized. Thus, we decided to include a comparison of two different cfDNA quantification strategies and therefore, to evaluate the feasibility of qPCR method in our cohort. Nevertheless, it's true that the methodology employed has been previously reported and it's not enough relevant. According your suggestion, the result section regarding different cfDNA strategies has been summarized. In addition, we have included the supplementary figure 1 as a main figure and the figure 2A, 2B and 2C were moved to supplement material.
Changes in the text: The section "cfDNA levels are specially increased in SCLC patients" has been shortened (see page 11, lines 260-263). In addition, figure 2 and supplementary figure 1 have been modified.

Comment 3: Some reference issues appear e.g is reference 29 and 45 the same?
Reply 3: Sorry for the mistake. Effectively, both are the same reference. We have arranged it.
Changes in the text: We have unified references 29 and 45 (now it's the reference 26).

Minor points.
Comment 4: Only few typos (i.e. line 111 and 395) appear in this very well written and proofread manuscript.
Reply 4: Thank you for your comment. We have arranged the mistakes.
Changes in the text: See page 6, line 119 and page 17, line 412.