Peer Review File

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Reviewer A

1. The study does not include a direct comparison with pleural fluid carcinoembryonic antigen (CEA), which is an established and widely used tumor marker. Without such a comparison, it is difficult to assess the relative utility or added value of CA242. In particular, since your previous work has reported the AUC for CEA, it would greatly strengthen the current manuscript if decision curve analysis (DCA) were also performed for CEA and presented alongside the CA242 DCA curve.

Reply: The DCA curve for CEA has been published in our previous work (*Transl Lung Cancer Res.* 2024;13(5):1061-68). We feel that the concern about duplicate publication and copyright infringement may be raised if the DCA curve of CEA appears in this article. Nonetheless, we cited this article in our paper.

2. The figure legends, particularly for Figure 4, are overly simplistic and lack sufficient explanatory detail. For instance, the legend for Figure 4 fails to clearly describe the interpretation of the decision curve, threshold probabilities, or the implications for clinical utility. This limits reader comprehension and should be revised to provide adequate context and interpretation.

Reply: We have added a legend to help readers better understand (Lines155-157). In addition, we have discussed the DCA in the discussion section.

Reviewer B

• Line 35:

There is no description of complications in Reference 5.

Reply: We have updated the references (Lines35-36).

• Line 38:

The meaning of the sentence "Specifically, it helps assess a patient's risk of MPE before pleural biopsy, thereby making pleural biopsy more targeted" is unclear. Please describe it in more detail.

Reply: We have described it in more detail. The revised version is as follows: Specifically, elevated tumor marker levels indicate a high probability of MPE, and thus encourage pleural biopsy, or a repeated biopsy and a repeated effusion cytology when the diagnosis cannot be made after initial assessments (Lines38-42).

• Line 67:

It is stated that "participants with a history of pleural effusion within the last three months and whose corresponding aetiology was clear" were excluded, but could they have been used as positive or negative controls?

Reply: They are not used as a negative or positive control.

• Line 68:

Were patients under 18 years excluded?

Reply: We have excluded patients under the age of 18 years (Line69).

• Line 68:

What is the definition of a sufficient volume of pleural effusion?

Reply: After sending samples for cytology, biochemical testing, cell counting and classification, molecular diagnostics, and microbiological testing of pleural effusion, if there is any remaining specimen available for research, we define it as a sufficient volume of pleural effusion.

• Line 73:

There is an extra period.

Reply: We have deleted it.

• Line 72:

What were the conditions for centrifugation?

Reply: The condition for centrifugation is 1200g, 10min (Line72).

• Line 82:

The statement "malignancy-related clinical features (e.g., imaging findings of pleural nodules/thickening or pulmonary masses or bloody/exudative effusion with rapid progression)" does not sufficiently confirm the diagnosis of malignancy. It is inadequate as a gold standard and should be noted as a limitation.

Reply: We have added this limitation in the discussion section (Lines206-212).

• Line 91:

Please provide the rationale for setting the ADA cut-off value at 35.

Reply: The cut-off of 35 U/L is the most widely used threshold in previous works (Ann Transl Med. 2016;4(15):282). We have added a reference there.

• Line 93:

Shouldn't the definition of PPE also include clinical improvement?

Reply: We have revised it as follows: For patients lacking microbiological evidence but

presenting high-probability tuberculous features (e.g., lymphocyte-predominant exudate with adenosine deaminase >35 U/L), clinical diagnosis was supported by positive *M. tuberculosis* nucleic acid amplification testing and IMPROVED response to antituberculosis therapy (Lines90-94).

• Line 101:

Please provide a clear description of the Roche Cobas electrochemiluminescence system.

Reply: The platform for CA242 determination is MAGLUMI 2000 (Shenzhen New Industries Biomedical Engineering Company, China), rather than Roche Cobas. I apologize for the typo. We have added the analytical performance of MAGLUMI 2000 CA242 assay in the revised manuscript (Lines100-104).

• Line 117:

Why were patients excluded due to a history of malignancy?

Reply: These four patients have a long history of cancer, and the diagnosis of MPE can be easily made by history, signs, symptoms, and imaging.

• Line 145:

What are the 95% confidence intervals for sensitivity and specificity when using a cut-off value of 20 KU/L? Also, "KU" should be written in full the first time it appears.

Reply: The 95% confidence intervals of sensitivity and specificity have been added. The unit of CA242 should be U/mL, rather than KU/L. I have revised it throughout the manuscript (Lines147-148).

• Result:

Would it be possible to present a supplementary table summarizing the cancer type and diagnostic method for each individual case? The overall picture of the cohort is unclear. Additionally, presenting the distribution of CA242 according to pathological subtypes (e.g., adenocarcinoma, squamous cell carcinoma) may enhance diagnostic sensitivity. Is there any evidence that CA242 is released from tumors? It would be helpful to include data such as blood levels of CA242 or immunohistochemical staining of tumor tissue, if available.

Reply: (1) The characteristics of the participants have been presented in our previous work (*Transl Lung Cancer Res.* 2024;13(5):1061-68). We feel that the concern about duplicate publication and copyright infringement may be raised if the table appears in this article. Nonetheless, we cited this article in our paper. (2) Identifying the cell origin of CA242 is a complex topic, and it is not the focus of this study. Theoretically, effusion 242 can be derived from the passive effusion of serum CA242, as well released by local tumor cells metastasized to the pleura or other cells in the pleural cavity. (3) We did not collect the serum specimen and

pleural tissue of the participant. This is a limitation of our study, and we have addressed this limitation in the revised manuscript (Lines206-208).

• Discussion:

How about citing other studies that have used decision curve analysis (DCA)? Although the diagnostic sensitivity of CA242 is slightly lower than that of pleural CEA, please discuss how clinical evidence supporting the use of CA242 can be further established in the future.

Reply: We have added two references to help readers better understand DCA. Previously, we have investigated the diagnostic accuracy of CEA and its potential net benefit (Transl Lung Cancer Res. 2024;13(5):1061-68). To avoid the concerns of duplicate publication and copyright infringement, we did not present the DCA curve of CEA in our manuscript. However, we have cited our previous work to help readers better understand our work.

Reviewer C

1. Some state epidemiological claims (i.e. proportion of MPE caused by lung vs breast cancer) but could be more specific by stating actual prevalence

Reply: The tumor type of MPE varies across different countries and regions. According a large sample size study (n>3000), lung and breast cancers account for 53% of MPE. We have revised accordingly (Lines27-28).

2. Some sentences are overly long and contain multiple clauses which can reduce clarity for the reader. Some phrases are repetitive i.e. “primarily focused primarily” and “to diagnose MPE” repeated in quick succession.

Reply: We have carefully checked our manuscript to enhance it readability.

3. far below the expectations of clinicians and patients[6][9, 10].- please review reference formatting

Reply: We have modified the citation format.

4. A single prior study suggested the potential diagnostic utility of CA242 for MPE.- need to reference this

Reply: We have added it (Lines53-55).

5. "More targeted" is a bit vague- targeted how? patient selection? site selection?

Reply: We have revised it (lines38-42). We have revised as follows: Specifically, elevated tumor marker levels indicate a high probability of MPE, and thus encourage pleural biopsy, or a repeated biopsy and a repeated effusion cytology when the diagnosis cannot be made after initial assessments.

6. "Consequently, patients with negative cytology results still require pleural biopsy"- could mislead readers into thinking biopsy is mandatory in all cytology negative cases

Reply: We have revised it. Indeed, for patients with a high risk of MPE and negative cytology, pleural biopsy should be used to confirm the diagnosis (Clin Chem Lab Med 2025;63(3):515-20) (Line34).

7. "The detection of tumour markers in pleural effusion can aid in the diagnosis of MPE[6]. Its clinical significance lies in improving diagnostic efficiency"- in diagnostics, it's more common to use terms like diagnostic accuracy. Could you clarify

Reply: I believe the word efficiency is correct, just like the statement in our previous work (Clin Chem Lab Med 2025;63(3):515-20). The efficiency means: the CA242 value can help the pulmonologist to estimate the risk of MPE and promote the establishment of an efficient diagnostic pathway.

8. "A single prior study suggested the potential diagnostic utility of CA242 for MPE. Nevertheless, its findings were limited by its small sample size, single-centre retrospective design, and insufficient inclusion of confounding aetiologies such as HF and parapneumonic pleural effusion (PPE), which may compromise accuracy.[15]."

The authors could elaborate a bit further about what they actually found on this study (i.e. what were the results) to help justify reasons for repeating the test on a larger scale

Reply: I have revised it accordingly (Lines53-55).

9. "This study received approval from the ethics committees at the the Affiliated Hospital of Inner Mongolia Medical University (No: 2018011)."- typographical error

Reply: Done.

10. It is a bit hard to follow the DCA description and figure 4. It would help to report the threshold probability range over which CA242 provided net benefit compared to "treat all" and "treat none" strategies. Correlating these thresholds to realistic clinical decision points for suspected MPE would help readers understand the practical significance of the findings.

Reply: We have revised the discussion section accordingly. Particularly, we added some statements on the principle of DCA.

11. Although the specificity of the >20 KU/L cut-off is high, the low sensitivity (30%) should be discussed further, as this means the marker would miss a lot of MPE cases at this threshold. A negative result at this level would not remove the need for further diagnostic procedures such as pleural biopsy. The authors should comment on whether,

in light of this, CA242 at this cut off is clinically meaningful beyond serving as a confirmatory marker in selected patients.

Reply: Given that the hazards of MPE misdiagnosis are more significant than those of missed diagnosis, a threshold with 100% specificity is of important clinical value because pleural biopsy can be avoided at this threshold if the patient is not amenable to active oncologic treatment (Clin Chem Lab Med 2025;63(3):515-20). We agree that many MPE patients will be missed if CA242 is used alone, and have discussed this in the revised version (Lines198-200).

12. The AUC for CA242 was 0.71, which the authors note is slightly worse than more established markers like CEA (AUC 0.89 in their previous work). They acknowledge this as a limitation but I think the overall language about the utility of CA242 from a diagnostic accuracy perspective should be a bit more cautious. The authors could also explore whether CA242 could still be useful when combined with other tumour markers.

Reply: AUC is a diagnostic metric that reflects the global diagnostic performance of an index test. However, it has limitations such as a lack of straightforward clinical interpretation. A given threshold with high sensitivity or specificity is more clinically meaningful because the target disease can be ruled in or ruled out at this threshold. Therefore, we emphasized the clinical value of CA242 at the threshold of 20 U/mL. Investigating the diagnostic accuracy of tumor marker combinations is an interesting topic, and we will do this work in the near future.

13. There is a mismatch between the methods (which state that patients were recruited prospectively) and the discussion (which describes the study as retrospective) that needs clarification.

Response: We have revised it.