Peer Review File

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Reviewer A

This is a small study to determine the discriminatory capacity of a combination of radiologic, immunological and metabolic markers between subjects with malignant and benign pulmonary nodules.

Although blood samples for metabolism, analysis were collected in fasting specimens, dietary and tobacco use changes, as well as ambient air pollution, can have significant impact on metabolism at signatures, and without adjusting for these, one cannot separate signal from noise.

Reply 1: The issues you point out are very important. We actually take into account the effects of smoking, diet, and environment on the test results when patients are enrolled. We have reminded subjects to avoid consuming seafood, alcohol, spicy and high-oil foods and to keep their diet as light as possible at least 3 working days before the start of the test. In addition, smoking was analyzed as a separate risk factor to ensure the objectivity of the data comparison. The subjects in this study were all residents of the Dongtai city region and were generally more consistent in terms of air pollution effects.

Changes in the text: We have added in the **Detection and analysis of serum TMs in patients section** that patients should take care of their diet and avoid seafood, alcohol, spicy and high-oil food intake before sampling.

The population in which this biomarker, or combination of biomarkers were developed, was small, yet it had a very large proportion (~70%) of individuals with malignant pulmonary nodules. This differs from a general population, in which the rate of malignancy amongst is incidental pulmonary nodules can be 5%. The validity of this biomarker would have to be extensively tested in a much larger, external cohort for this to have any clinical impact.

Reply 2: As you say, the incidence of malignant pulmonary nodules in epidemiological surveys is certainly not high. The diagnosis of benign pulmonary nodules is usually managed by surgery or follow-up and no other tests are carried out. In contrast, in our study we specifically improved the detection of markers of malignant pulmonary nodules, with the primary aim of investigating whether the combined diagnosis of these indicators is clinically relevant. The number of cases in this study is not large, due to the many factors that influence blood marker and metabolite levels, and exclude a variety of unqualified data to maintain the confidence of

the results as much as possible. Of course, the study needs to be validated by a large-scale epidemiological survey, which is an essential part of the clinical application process.
Changes in the text: not available.

Reviewer B

Line 151 - Unclear regarding what “severe complications” Means as an exclusionary criterion.
Reply 1: The severe complications means that other pulmonary diseases concomitant, including but not limited to tuberculosis, lung injury and COVID-19 infection.
Changes in the text: Line 151, after “severe complications”.

In figure 7 Location of PNs is represented as a Yes/No, this is unclear. Is yes referring to upper lobe predominant? And does negative refer to any other location in particular? If Yes just means upper lobe predominant that it should be clarified in the figure legend.
Reply 2: We are very sorry to have troubled you. The Yes just means upper lobe predominant. We will explain yes/no in figure legend.
Changes in the text: The change has been revised in legend of figure 7.

What would be the sensitivity and specificity of combined metabolomic and CT data for diagnosing malignant pulmonary nodules?
Reply 3: Combined metabolomics and CT data can improve the diagnostic accuracy of malignant pulmonary nodules. Metabolomics can assess the metabolic state of the human body by detecting metabolites in blood, urine, and other samples, thus revealing the metabolic changes in the occurrence and development of tumors, including glucose metabolism and lipid metabolism. CT data can provide information about the morphological characteristics and size of malignant lung nodules.
Multiple studies have shown that the combination of metabolomics and CT data can significantly improve the diagnostic accuracy and prognostic evaluation of lung cancer. For example, a study has found that a model combining metabolomics and CT data can distinguish malignant from benign lung nodules with a sensitivity of 85% and a specificity of 90%, much higher than the diagnostic accuracy with CT data alone. In addition, the combined application of metabolomics and CT data can also help to assess the malignancy of the tumor and predict the risk of tumor recurrence.
It should be noted that the diagnosis of lung cancer based on the combination of metabolomics and CT data requires clinical trial verification with a large sample size. In addition, the current method still needs to be confirmed by means of lung biopsy and cannot replace the traditional diagnosis of lung cancer.

Changes in the text: none.

Feedback on the significance of this paper could be addressed to enhance discussion and limitations.

1. Would the combined testing proposed for differentiation of BPN and MPN ever deter physicians from doing further workups such as PET/CT or EBUS TBNA-based current standards of practice (ie fleischner criteria or other models)

Reply 4: The combination test proposed to differentiate benign pulmonary nodules (BPN) from malignant pulmonary nodules (MPN) can be a valuable tool for the diagnosis and treatment of patients with pulmonary nodules. However, such testing is unlikely to completely eliminate the need for further investigations, such as current practice standards based on PET/CT or EBUS TBNA, such as the Fleischner criteria or other models. Although joint testing can provide valuable information about the malignant potential of lung nodules, it does not definitively diagnose or exclude lung cancer. Further workup may be required to confirm or exclude a diagnosis of lung cancer, especially if the combination test is uncertain or indicates a high possibility of malignancy.

In addition, the Fleischner criteria and other models consider a variety of factors, rather than just molecular and genetic markers evaluated by joint testing. These factors include nodule size and growth rate, as well as the patient's age, smoking history, and general health. These factors can help guide decisions about further investigations and treatment.

In summary, the proposed combined assay for the identification of BPN and MPN can be a valuable tool for the diagnosis and treatment of patients with pulmonary nodules. However, it is unlikely to completely replace the need for further examination and evaluation using current standards of practice. Physicians still need to use their clinical judgment and carefully evaluate each patient's individual condition to determine the most appropriate course of action.

Changes in the text: none.

2. What is the comparative predictive capability of these models as compared to already established models such as the Mayo Clinic solitary pulmonary nodule calculator or Brock model in producing the likelihood of malignancy?

Reply 5: The prediction of benign and malignant solitary pulmonary nodules is a very important issue in clinical medicine. Several mathematical models have been used to predict the malignant potential of solitary pulmonary nodules, including the Mayo Clinic solitary pulmonary nodule calculator and Brock model. These models are based on known clinical and imaging features to predict the malignant potential of a nodule. However, the predictive power of these models varies among studies.

Studies have shown that different mathematical models have different presentations in predicting the malignant potential of solitary pulmonary nodules. Some models had a high sensitivity (ability to correctly identify malignant lesions) and a low specificity (ability to

correctly identify benign lesions), while some models had the opposite. Some of the latest models (such as models based on artificial intelligence) also show high predictive ability.
In general, the established mathematical models show quite high accuracy in predicting the malignant possibility of solitary pulmonary nodules, but the prediction ability may vary among different studies. For clinicians, it is necessary to select the appropriate model for prediction according to the specific situation. At the same time, with the emergence of new technologies and methods, we can expect that the accuracy of future prediction models will be further improved.
Changes in the text: none.

3. I believe the paper has value in promoting non-invasive methods that can help point physicians to benign vs malignant nodules. In settings where minimally invasive biopsies are available via EBUS TBNA or Robotic Bronchoscopy, the risk of continued surveillance using these models may outweigh the risk of biopsy and would warrant mentioning in the discussion of this paper.
Reply 6: Relevant contents have been added in the discussion section of the article as required.
Changes in the text: In addition, in this study, chest CT was used to detect the BPNs and MPNs, which is one of the commonly used non-invasive detection methods for PNs. Compared with invasive detection methods (such as bronchoscopy and puncture biopsy), it has the advantages of high detection accuracy, simple operation, and low cost, providing patients with the advantages of a safer, faster and more economic detection option. Moreover, the risk of continuous monitoring using these models may outweigh the risk of biopsy where minimally invasive biopsies can be performed by EBUS TBNA or robotic bronchoscopy. In conclusion, this study has a certain reference value in the promotion of non-invasive clinical detection of PNs. (Page 13-14, line 424-432)

Reviewer C

Dr. Xu and his/her colleagues had investigated the predictive value of chest computed tomography images, tumor markers, and "metabolomics" in the differentiation of benign and malignant pulmonary nodules. However, the study design, statistical analyses and presentation of the study results were actually not proper in this "diagnostic test" study. Several concerns or suggestions are listed as below:

1. There are some basic knowledge misunderstandings regarding the clinical management of pulmonary nodules. For example, line 77-78 "MPNs…, which do not have particularly typical symptoms, although the more common ones include clubbing fingers, hemoptysis,

expectoration, cough, and arthritis". Line 81-84, "In the early stage of MPNs, it is best to perform surgical resection, combined with radiotherapy, chemotherapy, biotherapy, and interventional therapy for comprehensive adjustment and treatment, to cure lung cancer early and prevent recurrence".

Reply 1: The contents of the corresponding paragraphs have been appropriately adjusted as required.

Changes in the text: From a clinical perspective, tissue biopsy is used to determine whether a nodule is benign or malignant. Special treatment is not required if the benign nodule is small in size (typically less than 5 mm in diameter) and does not cause any significant clinical symptoms. Chest computed tomography (CT) scans should be performed at the hospital every six months to monitor any changes in the nodules (6,7). Myeloproliferative tumors usually occur in the early stages of lung cancer and may not have particularly typical symptoms. However, common symptoms include clubbed fingers, hemoptysis, cough, expectoration, and arthritis (8). In clinical practice, physicians need to make a comprehensive diagnosis based on chest CT scans, blood and biochemical tests, and even tumor markers (TM) to determine the nature of the condition and provide treatment as soon as possible. In the early stage of MPNs, surgical resection is the preferred treatment, supplemented by radiotherapy, chemotherapy, biological therapy, interventional therapy, and other comprehensive treatments, to achieve the purpose of curing lung cancer and preventing recurrence (9–11). (Page 3, line 75-89)

2. The authors should clearly indicate the prospective or retrospective design of this study in the section of Method and the Abstract. It seems that this study is retrospectively conducted, which is not a proper design for a diagnostic study, unless the researchers were blinded to the pathological diagnosis. Otherwise, it is only a study to compare the differences between the BPN or MPN groups.

Reply 2: According to the requirements, the corresponding description of the research attributes of this study has been added in the abstract and method part of the article.

Changes in the text: (1) A total of 110 patients with PNs who were hospitalized in Dongtai Hospital from March 2021 to March 2022 were selected as the study cohort. A retrospective analysis study of chest CT imaging, serum TMs testing, and plasma fatty acid (FA) metabolomics was performed on all participants. (2) A total of 110 patients with PNs diagnosed in Dongtai Hospital from March 2021 to March 2022 were selected as the study cohort for a retrospective analysis. All patients underwent chest CT and pathological biopsy. (Page 2, line 36-39; Page 5, line 148-150)

3. The study of metabolic products of fatty acid only is still far from what is called "metabolomics".

Reply 3: Metabolites of fatty acids are also part of metabonomics research, and some language expressions in this manuscript have been modified as required.

Changes in the text: In general, the related technologies of metabolomics have been applied to the diagnosis and prediction of various diseases. However, there are relatively few metabolomics studies on the differentiation of BPNs and MPNs, especially the metabolites of fatty acids in metabolomics, such as free fatty acid spectrum and 31 kinds of free fatty acid indexes. (Page 4, line 128-132)

4. The statistical analysis and presentation are the most critical weakness of this study. First of all, all p values 0.001 should be clearly presented as exact numbers. Secondly, it seems that all asterisk (*) in the figures were added manually by the authors, did the authors really do the statistical tests? Thirdly, there is no table but a plenty of figures containing very little information throughout this manuscript. Why not present some of these results with tables? Lastly, it seems that all the figures were generated by Microsoft Excel, which is very limited for academic presentation. I would suggest the authors to recreate all the figures to condense the information and to achieve a better presentation of the study results.

Reply 4: According to the requirements, the expression content of the statistical analysis part of the article has been checked and revised accordingly.

Changes in the text: please check the statistical analysis section of the article. (Page 11, line 339-349)

5. The underlying relationship between the fatty acid metabolism and pulmonary nodules was not clearly discussed. Without this, the diagnostic value of fatty acid metabolic products will be unconvincing.

Reply 5: Current studies have confirmed some correlations between fatty acid metabolism and lung diseases. Some studies have shown that fatty acid metabolism disorders may be associated with the occurrence and development of lung diseases such as asthma and chronic obstructive pulmonary disease. In addition, fatty acid metabolism in patients with lung cancer may also be affected. Pulmonary nodules are usually lesions found in CT or X-ray examinations, and fatty acid metabolism indices can be used as auxiliary indices to participate in the diagnostic evaluation of pulmonary nodules. Further evaluation is usually required to determine their nature and severity.

Changes in the text: Current studies have confirmed some correlations between fatty acid metabolism and lung diseases. Some studies have shown that fatty acid metabolism disorders may be associated with the occurrence and development of lung diseases such as asthma and chronic obstructive pulmonary disease. In addition, fatty acid metabolism in patients with lung cancer may also be affected. (Page 4-5, line 132-136)

Reviewer D

This paper aimed to investigate the predictive value of combining chest CT images, biomarkers and metabolomics in the identification of benign and malignant pulmonary nodules. It is well written and the topic is interesting. The biggest problem is that there is no comparison with on-topic state-of-the-art studies. The only reported work is the one of Widłak et al. of which, however, no mention is made on the methodology used as well as on the quantitative results. Thus, I suggest reporting the state-of-the-art metabolomic studies about the differentiation of benign and malignant pulmonary nodules (lines 127-129) as well as the studies on the simultaneous application of chest CT images, biomarkers and metabolomic technologies in the clinical diagnosis of benign and malignant pulmonary nodules (lines 129-131), and I suggest discussing them in the Discussion so as to make possible a qualitative and quantitative comparison between the proposed work and the already existing ones. This would also highlight the novelty even more. Additionally, I suggest revising all Figures that should be all consistent (same size, same font, etc.) and… wouldn't it be clearer if some of them became Tables?

Reply 1: The comparative content of related research has been supplemented in the discussion part of the article as required. For example, the most advanced metabonomics research on distinguishing benign and malignant lung nodules, and the research on the simultaneous application of chest CT images, biomarkers and metabonomics technology in the clinical diagnosis of benign and malignant lung nodules. In addition, some figures in the article have been adjusted accordingly.

Changes in the text: Please check the article discussion and attachment section. (Page 13-14, line 424-432)