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REVIEW ARTICLE

# Diagnostic performance of *SHOX2* and *RASSF1A* gene methylation assays in malignant pleural effusion: A systematic review and meta-analysis

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## Abstract

**Background:** Malignant pleural effusion (MPE) is a common complication of advanced malignancies, requiring differentiation from benign pleural effusion for appropriate management. Cytology and biopsy have limitations, necessitating more sensitive, less invasive diagnostic techniques. The objective of this study was to evaluate the diagnostic accuracy of methylated *SHOX2* (short-stature homeobox 2) and *RASSF1A* (Ras association domain family member 1A) genes in detecting MPE. **Methods:** A systematic review and meta-analysis included studies that compared benign pleural effusion and MPE cohorts using methylation of *SHOX2* and *RASSF1A* genes in pleural fluid as the index test and cytology/histopathology as the reference standard. A random-effects model was used to calculate sensitivity, specificity,

The first three authors contributed equally to this article.

predictive values, and diagnostic odds ratios. Subgroup analysis assessed performance in lung-predominant versus nonlung-predominant MPE.

**Results:** Four studies with a total of 534 participants were included. The pooled sensitivity and specificity were 85% (95% confidence interval [CI], 53%–96%; heterogeneity [$I^2$] = 0.00%) and 92% (95% CI, 88%–95%; $I^2$ = 24.8%), respectively. The positive and negative predictive values were 93% (95% CI, 85%–97%; $I^2$ = 61.5%) and 84% (95% CI, 53%–96%; $I^2$ = 0.00%), respectively. The diagnostic odds ratio was 22.78 (95% CI, 11.00–47.17; $I^2$ = 25.8%). Subgroup analysis showed a slight decrease in sensitivity (70%; 95% CI, 64%–76%; $I^2$ = 0.00%) and specificity (91%; 95% CI, 86%–94%; $I^2$ = 26.1%) when excluding the study with a lung cancer-predominant population.

**Conclusions:** The combined analysis of *SHOX2* and *RASSF1A* methylation demonstrated promising diagnostic accuracy for MPE detection, outperforming cytology. This less invasive method could reduce reliance on more invasive procedures, although further research is needed to confirm its efficacy across diverse populations and cancer types.

**KEYWORDS**
diagnostic accuracy, malignant pleural effusion, methylation biomarkers, Ras association domain family member 1A (*RASSF1A*), short-stature homeobox 2 (*SHOX2*)

## INTRODUCTION

Pleural effusion refers to the abnormal accumulation of fluid within the pleural cavity. This fluid buildup compresses the lungs and can manifest clinically as symptoms such as dyspnea, a nonproductive cough, and pleuritic chest pain.[1] Pleural effusions are a prevalent clinical condition, with greater than 50y identifiable etiologies, including disorders of the pleura or lungs, systemic illnesses, organ dysfunction, and adverse effects of medications.[2] This condition can be broadly classified into malignant pleural effusion (MPE) and benign pleural effusion (BPE), depending on its etiology.[3] MPE is most commonly associated with advanced malignancies, particularly lung and breast carcinomas, which account for the majority of cases.[4]

In contrast, BPE arises secondary to nonmalignant conditions, such as congestive heart failure, pneumonia, or pulmonary embolism.[5] Accurately differentiating MPE from BPE is crucial because treatments and prognoses vary. MPE management typically emphasizes palliative care, including drainage procedures like thoracentesis, indwelling pleural catheter insertion, or pleurodesis to prevent fluid recurrence.[6] Conversely, BPE is managed by treating the underlying condition and generally requires less invasive interventions.[7]

Cytology of pleural fluid is the test of choice for detecting malignant cells in pleural effusion and typically serves as the initial step in diagnosing MPE. However, its sensitivity varies between 40% and 80%, depending on multiple factors, including the volume of fluid analyzed, the tumor's primary location, and the examiner's expertise.[8] For cases in which cytology is inconclusive, thoracoscopic pleural biopsy offers a more sensitive alternative, although this procedure is more invasive, is not widely available, and carries associated risks and costs.[9] Tumor biomarkers have emerged as valuable tools for assisting in diagnosing MPE. A study evaluating the diagnostic value of tumor markers such as carcinoembryonic antigen, cancer antigen 125, carbohydrate antigen 15-3, and cytokeratin 19 fragments indicated that the use of a tumor marker panel increased the sensitivity of cytology by 18%, making it a useful tool to complement cytology.[10] Whereas markers like carcinoembryonic antigen and cytokeratin 19 fragments demonstrate high specificity, their sensitivity is relatively low. This limitation reduces their effectiveness in ruling out malignancy when used alone. Consequently, a combined approach using multiple tumor markers can enhance diagnostic accuracy. However, a definitive diagnosis still requires confirmation through cytologic analysis or biopsy.[11] These limitations highlight the ongoing need to develop more sensitive, accurate, and less invasive diagnostic techniques for detecting MPE. One emerging approach involves analyzing DNA methylation changes, a hallmark of many cancers. Promoter CpG island hypermethylation, which silences tumor suppressor genes, has been associated with various cancers, including lung cancer.[12,13] Two genes of particular interest are short-stature homeobox 2 (*SHOX2*) and RAS association domain family member 1A (*RASSF1A*). *SHOX2*, located on chromosome 3, is part of the human short-stature homeobox gene family; whereas *RASSF1A*, a known tumor suppressor, belongs to the C-terminal *RASSF* family.[14,15] Evidence based on populations from different nationalities suggests that aberrant hypermethylation of CpG islands in these genes could be a biomarker for diagnosing and prognosing various cancers that can metastasize to the pleura, including colorectal, breast, and lung cancers.[8,16–18]

Given the strong association between methylation of the *SHOX2* and *RASSF1A* genes and lung cancer and recognizing that MPE frequently results from malignancies such as lung cancer, there is significant potential to use these epigenetic markers as diagnostic tools for MPE. The objective of the current study was to evaluate the diagnostic accuracy of combined *SHOX2* and *RASSF1A* gene methylation in detecting MPE, comparing these biomarkers with conventional cytologic and histologic methods.

## MATERIALS AND METHODS

### Eligibility criteria

This review was conducted in accordance with the PRISMA (Preferred Reporting Items for a Systematic Review and Meta-Analysis) of Diagnostic Test Accuracy Studies checklist.[19] We included studies that met the following criteria: patients with benign pleural effusion (BPE) and malignant pleural effusion (MPE) as the target cohorts for comparison; methylation of *SHOX2* and *RASSF1A* genes in pleural fluid as the index test using reverse transcriptase polymerase chain reaction (PCR); cytology and/or histopathology examination as the reference standard; and sufficient information to construct a two-by-two contingency table for extracting true positive, false positive, false negative, and true negative rates, which are essential for calculating various pooled diagnostic accuracy metrics as outcomes. We excluded studies that violated any inclusion criterion, secondary research articles, case reports, case series, studies targeting nonhuman populations, studies with overlapping participants, and book extracts. We did not apply any restrictions on population in our inclusion criteria.

### Search strategy and selection process

We thoroughly searched five electronic databases: PubMed, Embase, Scopus, the Cochrane Library, and the Web of Science. Our search terms focused primarily on the *SHOX2* and *RASSF1A* genes, deliberately excluding *pleural effusion* to ensure a broad screening of relevant articles and to avoid missing any studies, because most research on these genes targets patients who have pleural effusion. The online Supporting Information details the complete search strategy (see Table S1). We used Covidence systematic review software to screen the initially retrieved studies. After Covidence automatically removed duplicates, nine reviewers (A.O.K., M.B., D.A., G.K., H.F., I.C., K.M.G., M.E.A., and M.S.A.) conducted a dual review of the titles, abstracts, and full texts. Discrepancies among reviewers were resolved through discussion until a consensus was reached. If consensus could not be achieved, a third independent reviewer was consulted for additional feedback.

### Data extraction

Two reviewers independently extracted data of interest from the eligible articles using a standardized data-extraction form (such as Excel; Microsoft Corporation). The extracted items were categorized into: (1) study details, including study identification and design, study start and end dates, country, and setting; (2) target population characteristics, including total sample size and cohort number, age, and male-to-female ratio for both the MPE and BPE cohorts, and DNA analysis method; (3) index and reference standard tests used along with their cutoff values; and (4) patients with BPE and MPE according to the reference standard (Table 1).[20–23] Furthermore, we extracted precalculated sensitivity, specificity, and positive and negative likelihood ratio values for each study included in the meta-analysis. These individual results were then compared with the pooled results to assess the overall diagnostic accuracy and variability across studies. For cases in which outcome data were incomplete or inconsistently reported, authors of the original studies were contacted for clarification. No imputation was performed for missing values. For reliability purposes, an independent reviewer (M.S.A.) reviewed the gathered data by comparing the extracted data with the information from the included studies. Any

**TABLE 1** Basic demographics of included studies.

| Study ID | Country | Design | Sample size, no. | Recruitment | | MPE/BPE, no./total no. | Age: Mean ± SD, years | Male/female, no. | MPE ≥90% because of lung cancer | Mesothelioma, no. |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Start date | End date | | | | | |
| Zhang 2023[20] | China | Cross-sectional | 95 | August 2020 | July 2021 | 50/45 | 57.90 ± 15.20 | 58/37 | Yes | 1 |
| Zhong 2023[21] | China | Cross-sectional | 214 | June 2021 | September 2022 | 104/110 | 61.19 ± 14.28 | 149/65 | No | Unknown |
| Chen 2023[22] | China | Retrospective Cohort | 68 | March 2020 | December 2021 | 35/33 | 72.05 ± 14.22 | 39/29 | No | 0 |
| Liang 2022[23] | China | Cross-sectional | 148 | October 2019 | May 2021 | 100/48 | 56.10 ± 20.42 | 80/68 | No | 5 |

Abbreviations: BPE, benign pleural effusion; MPE, malignant pleural effusion; SD, standard deviation.

discrepancies were resolved through discussion to an agreement point.

## Risk-of-bias assessment

We used the QUADAS-2 tool (Quality Assessment of Diagnostic Accuracy Studies) recommended by the Cochrane Collaboration to assess the risk of bias in studies that tested the accuracy of diagnostic tests.[24] Four key domains, including patient selection, an index test, reference standard, and flow and timing, were assessed in terms of the risk of bias, and the first three were concerned with applicability. Each domain was flagged as having a *low*, *high*, or *unclear* risk of bias. Judgments of each signaling question, domain, and overall risk of bias were estimated in light of the QUADAS-2 complete guidance document rules. For each study, two reviewers independently assessed the risk of bias. Two reviewers (K.M.G. and M.S.A.) revised the judgments made and resolved any conflicts. We used Robvis (Risk-Of-Bias VISualization) for the graphical visualization of QUADAS elements.[25] We used this assessment to ensure consistent results of our meta-analysis.

## Effect measures

We used R software version 4.4.2 (R Core Team), with the *mada* package, to allow calculation of the following diagnostic accuracy tests using a random-effects model: pooled sensitivity and specificity, positive predictive value (PPV), negative predictive value (NPV), diagnostic odds ratio with their respective 95% confidence intervals (CIs), as well as the bivariate summary receiver operating characteristic curve with a summary point. All 95% CIs for pooled diagnostic estimates were derived using the Clopper–Pearson method with the bivariate random-effects model, accounting for both within-study and between-study variance. The heterogeneity was assessed through visual inspection of results, the Cochrane Q statistic test and its corresponding $p$ value, and prediction intervals. Testing for heterogeneity testing was considered significant if the $\chi^2$ test $p$ value was $< .1$. The degree of inconsistency was estimated by using the $I^2$ value, with $I^2 < 25\%$ indicating low inconsistency across studies, $I^2 = 25\%–50\%$ indicating moderate inconsistency across studies, and $I^2 > 50\%$ indicating substantial inconsistency across studies. In addition, we conducted a subgroup analysis to evaluate the performance of our index test model in identifying lung-predominant versus nonlung-predominant MPE. This analysis categorized studies into those with ≤10% nonlung cancer MPE and those with >10% nonlung cancer MPE.

## Subgroup analysis

The subgroup analysis identified three studies from the latter group and excluded one study with a lung cancer–predominant population from the analysis. Such an approach was chosen because the literature lacks studies that specifically compare lung cancer versus nonlung cancer as sources of MPE. We determined that this method was the most effective way to evaluate our index model in identifying MPE, regardless of the source of metastasis.

# RESULTS

## PRISMA flow diagram

We initially identified 2458 studies. After removing 311 duplicates, we screened the remaining 2147 studies according to titles and abstracts, which led to 2138 studies being excluded. We assessed nine studies for eligibility using full text, and we excluded five because of criteria mismatches. Three studies (Du et al. 2024, Gao et al. 2022, and Tsou et al. 2007) did not take the samples from the pleural fluid.[20,26,27] One study (Liu et al. 2024) did not classify patients according to the pleural effusion type (benign vs. malignant).[28] We excluded the 2024 study by Zhang et al. because of overlapping participants.[29] Ultimately, four studies were selected for the final analysis. Figure 1 illustrates this structured process.[21–23,30]

## Basic characteristics

All included studies were conducted in China and collectively enrolled 534 participants, of whom 289 had MPE. Despite a shared national setting, these cohorts were nonrandom and derived from distinct hospital-based populations with differing demographic and clinical profiles. Recruitment periods spanned from October 2019 to September 2022. The reported mean participant ages ranged from 56.10 to 72.05 years, with various degrees of age dispersion across cohorts. In three studies, the male-to-female ratio was approximately balanced,[22,23,30] whereas the cohort in the 2023 article by Zhong et al. showed a predominance of male participants (149 males vs. 65 females).[21] The etiology of MPE also differed: three studies included participants with mixed malignancy origins, whereas Zhang et al. (2023) reported that ≥90% of MPE cases were from lung cancer (Table 1).

## Systematic review and diagnostic metrics

All studies used pleural effusion cytology or pleural tissue biopsy as the reference standard, with the combination of *SHOX2* and *RASSF1A* gene methylation as the index test using a consistent cutoff <32 for the *SHOX2* cycle threshold (Ct*SOX2*) and <35 for Ct*RASSF1A*, all using methylation-specific real-time PCR. However, there were some differences in terms of DNA extraction and processing. Within this context, there were differences in terms of the amount of extracted

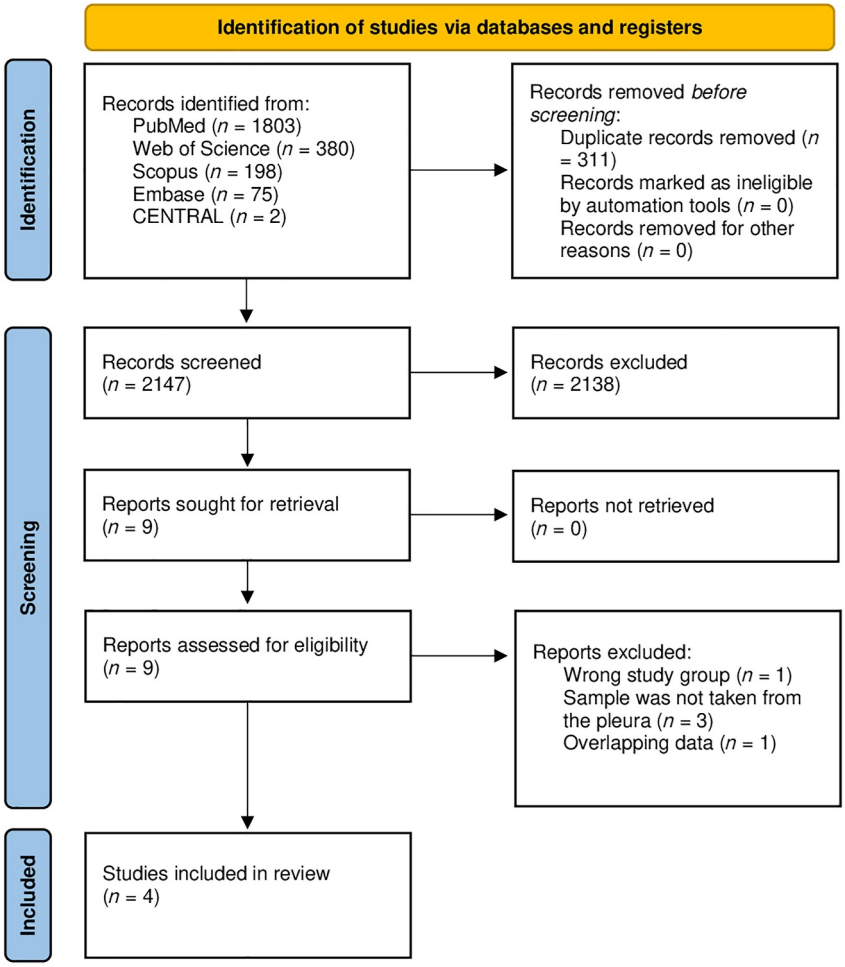


**FIGURE 1** PRISMA flow diagram. PRISMA indicates Preferred Reporting Items for a Systematic Review and Meta-Analysis.

DNA, ranging from 5 to 10 milliliters. In addition, the extracted DNA after centrifugation was different. Although the 2023 study by Zhang et al. used cell-free DNA, the other included studies used cellular precipitates. Details regarding DNA extraction are described in Table S2. Furthermore, all studies reported sensitivity and specificity, and three reported PPV and NPV. Sensitivity ranged from 66.3% in Zhong et al. (2023) to 96% in Zhang et al. (2023). Specificity was notably high across all studies, with Zhang et al. achieving 100%. The PPV was >80% in the three studies that reported it, with Zhang et al. reaching 100% and Liang et al. reporting 97.4%. The NPV was highest in Zhang et al. at 95.7%, whereas Liang et al. reported the lowest at 63.9%. Table 2 provides a comprehensive overview of the diagnostic metrics.[20–23]

## Quality assessment

The studies predominantly exhibited a low risk of bias across the majority of domains, as illustrated in Figure 2.[20–23] Minor concerns arose in the index test and reference standard domains. These were caused primarily by the potential lack of blinding and insufficient detail on cutoff threshold application across studies.

## Diagnostic accuracy

### Diagnostic parameters of all studies

The pooled sensitivity and specificity were 85% (95% CI, 53%–96%; $I^2$ = 0.00%) and 92% (95% CI, 88%–95%; $I^2$ = 24.8%), respectively (Figure 3A,B, respectively).[20–23] Furthermore, the PPV and NPV were 93% (95% CI, 85%–97%; $I^2$ = 61.5%) and 84% (95% CI, 53%–96%; $I^2$ = 0.00%; Figure 3C,D, respectively). The estimated diagnostic odds ratio was 22.78 (95% CI, 11%–47.17; $I^2$ = 25.8%; Figure 3E).

The summary receiver operating characteristic curve analysis (Figure 4) revealed that the summary point positioned favorably in the upper left quadrant of the receiver operating characteristic space, indicating good overall diagnostic accuracy. The relatively narrow confidence ellipse suggests good precision in the pooled estimate, whereas the wider prediction ellipse indicates the expected range for future study results. The clustering of study points near the summary point demonstrates reasonable consistency across studies, although some heterogeneity is evident from the spread of individual results. These findings suggest that *SHOX2* and *RASSF1A* methylation testing has promising diagnostic potential for detecting MPE, with favorable sensitivity and specificity characteristics.

**TABLE 2** Diagnostic metrics for biomarker analysis in included studies.

| Study ID | Reference standard | Index test | Cutoff Ct*SHOX2* | Cutoff Ct*RASSF1A* | DNA analysis method | Sensitivity, % | Specificity, % | PPV, % | NPV, % |
|---|---|---|---|---|---|---|---|---|---|
| Zhang 2023[20] | Pleural effusion cytology or pleural tissue biopsy | *SHOX2* + *RASSF1A* methylation | <32 | <35 | MS-PCR | 96.0 | 100.0 | 100.0 | 95.7 |
| Zhong 2023[21] | Pleural effusion cytology or pleural tissue biopsy | *SHOX2* + *RASSF1A* methylation | <32 | <35 | MS-PCR | 66.3 | 90.9 | NA | NA |
| Chen 2023[22] | Pleural effusion cytology or pleural tissue biopsy | *SHOX2* + *RASSF1A* methylation | <32 | <35 | MS-PCR | 71.4 | 84.8 | 83.3 | 73.7 |
| Liang 2022[23] | Pleural tissue biopsy | *SHOX2* + *RASSF1A* methylation | <32 | <35 | MS-PCR | 74.0 | 96.0 | 97.4 | 63.9 |

Abbreviations: BPE, benign pleural effusion; Ct, cycle threshold; MPE, malignant pleural effusion; MS-PCR, methylation-specific real-time polymerase chain reaction analysis; NA, not applicable/available; NPV, negative predictive value; PPV, positive predictive value; *RASSF1A*, Ras association domain family member 1A; *SHOX2*, short-stature homeobox 2.

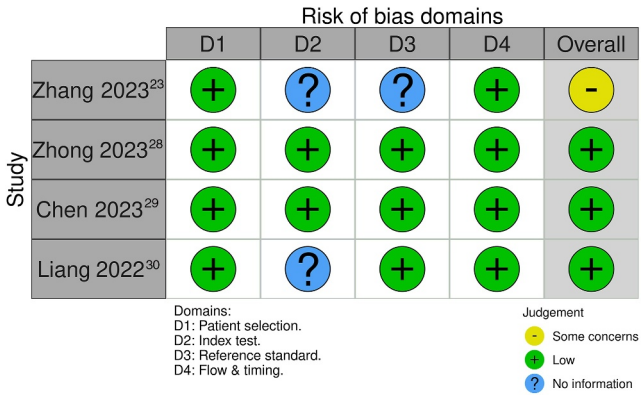


**FIGURE 2** Quality Assessment of Diagnostic Accuracy Studies (QUADAS-2).

### Subgroup analysis

Three studies in which at least 10% of participants had MPE originating from cancers other than lung cancer were aggregated for analysis. In this context, after excluding the study conducted by Zhang et al. in which more than 90% of participants had MPE from lung cancer,[30] we observed a decrease in both sensitivity, which was 70% (95% CI, 64%–76%; $I^2 = 0.00\%$), and specificity, which was 91% (95% CI, 86%–94%; $I^2 = 26.1\%$; Figure 5A,B, respectively).[21–23] In addition, we observed a decrease in both PPV, which was 91% (95% CI, 81%–96%; $I^2 = 64.8\%$), and NPV, which was 71% (95% CI, 65%–76%; $I^2 = 20\%$; Figure 5C,D, respectively). We noticed no change in the diagnostic odds ratio after removing the study by Zhang et al. (Figure 5E).

### Heterogeneity

The analysis revealed no significant variation in sensitivity (Q statistic = 1.45; degrees of freedom [df] = 3; $p = .69$) with a variance of true effect sizes ($\tau^2$) of 2.05, a prediction interval (PI) ranging from 0.02 to 0.99, and $I^2 = 0.0\%$, indicating minimal inconsistency. Specificity demonstrated limited variability (Q statistic = 3.99; df = 3; $p = .26$) with no observed variance ($\tau^2 = 0$) and a PI of 0.84–0.96, accompanied by a moderate heterogeneity measure ($I^2 = 24.8\%$). In the subgroup analysis ($n = 3$), sensitivity exhibited consistent effect sizes (Q statistic = 1.45; df = 2; $p = .48$) with no variance ($\tau^2 = 0$) and a PI of 0.56–0.81, reflecting negligible heterogeneity ($I^2 = 0.0\%$). Similarly, specificity in the subgroup showed minor variability (Q statistic = 2.71; df = 2; $p = .2583$) with no variance ($\tau^2 = 0$) and a PI of 0.77–0.96, alongside a modest heterogeneity estimate ($I^2 = 26.1\%$).

## DISCUSSION

We evaluated the diagnostic accuracy of combining *SHOX2* and *RASSF1A* methylation for the detection of MPE. The results revealed that the combined analysis achieved a diagnostic sensitivity of 85% (95% CI, 53%–96%) and specificity of 92% (95% CI, 88%–95%), significantly outperforming the sensitivity of single-gene methylation markers and cytology.[21,23,30] These results were consistent, with generally no to modest variations between studies, and demonstrated higher performance of these biomarkers, generally around 80%, in all diagnostic metrics such as sensitivity, specificity, PPV, and NPV. The diagnostic odds ratio was as high as 20, which is generally considered strong when it is above 1. The consistent results of pooled diagnostic metrics can be explained by the augmented effect of the combination of two related tumor suppressor genes located within the same chromosome.

Furthermore, all studies used the same cutoff point for the methylation of each gene for each patient using reverse transcriptase PCR. For CtSHOX2, it is <32, and, for CtRASSF1A, it is <35. This adds a layer of methodological similarity across the included studies. Although all studies were conducted in China, they represent nonrandom, hospital-based cohorts drawn from distinct institutions with differing demographic and clinical characteristics. There was variation in sample size, recruitment periods, mean age (ranging from 56.10 to 72.05 years), sex distribution, and primary cancer types, including one study in which ≥90% of patients had lung cancer and other studies in which patients had more mixed malignancy types.

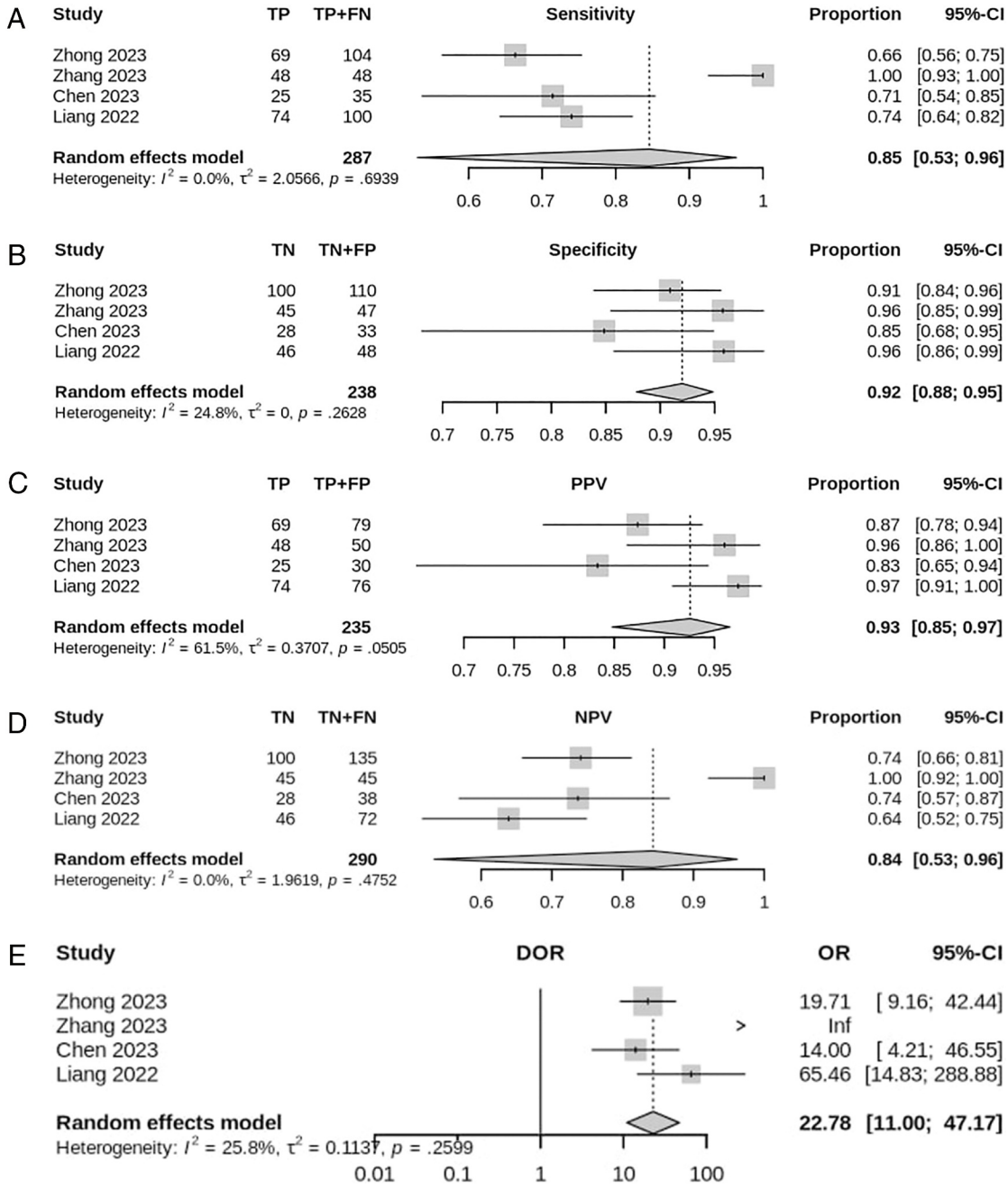


**FIGURE 3** (A–E) Pooled diagnostic performance metrics of combined *SHOX2* and *RASSF1A* methylation in detecting malignant pleural effusion. (A) Sensitivity, (B) specificity, (C) PPV, (D) NPV, and (E) DOR. Each plot represents a meta-analytic estimate summarizing the accuracy of this combined methylation marker in differentiating malignant from benign pleural effusion. CI indicates confidence interval; DOR, diagnostic odds ratio; FN, false negative, Inf, infinite number; OR, odds ratio; NPV, negative predictive value; PPV, positive predictive value; *RASSF1A*, Ras association domain family member 1A; *SHOX2*, short-stature homeobox 2; TN, true negative; TP, true positive.[20–23]

Therefore, it is inaccurate to assume population uniformity. These differences may account in part for the variations in PPV and NPV, which are inherently influenced by disease prevalence and case mix. Another point is that the sensitivity and specificity of isolated studies were also high and close to the pooled results for the entire population. This makes our findings more solid and reliable.

Our secondary analysis included only studies with a mixed population caused by nonlung cancer MPE after excluding the 2024 study by Zhang et al.,[29] which focused on lung cancer-related MPE. We observed a slight decrease in diagnostic performance compared with the main meta-analysis, with sensitivity decreasing to 70% (95% CI, 64%–76%) and specificity decreasing to 91% (95% CI, 86%–94%). Although the CIS for these results were wide, particularly for sensitivity, this decline in accuracy still may reflect that *SHOX2* and *RASSF1A* are sensitive to some types of MPE but may not perform as well for others.

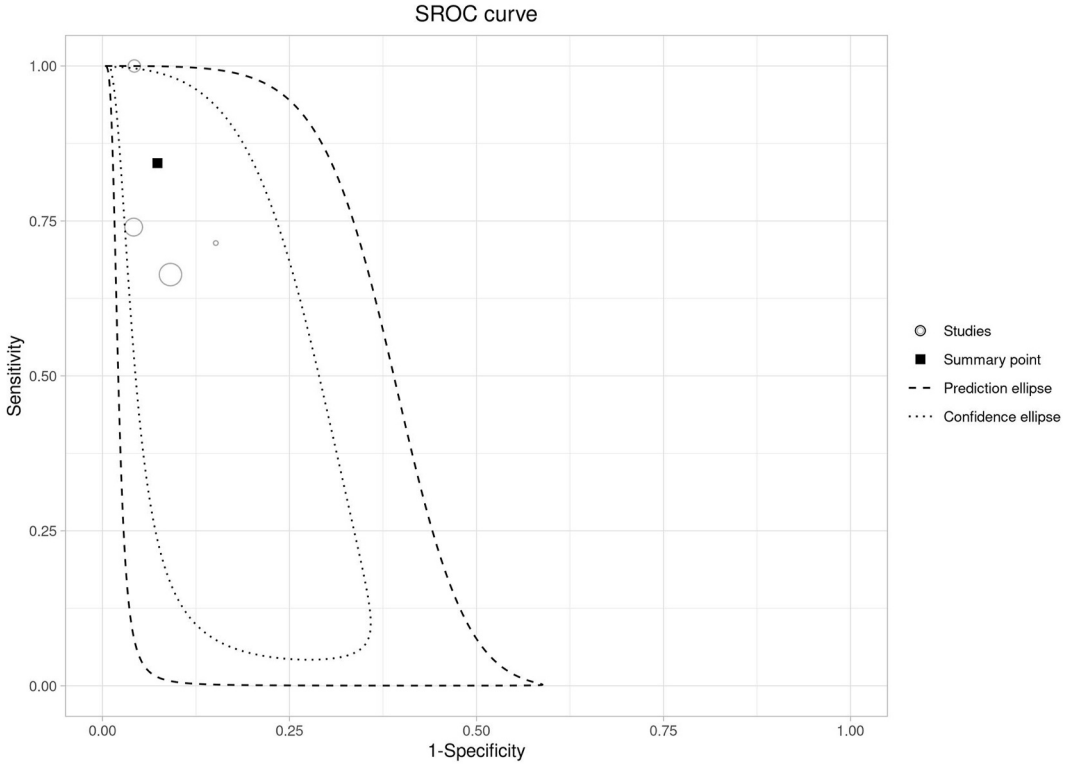


**FIGURE 4** SROC curve (summary receiver operating characteristic curve).

MPE, whether originating from the lung or metastasizing from other body parts, significantly complicates the primary disease, dramatically alters the plan for treating the primary disease, and causes significant morbidity to the patient. In contrast, BPE arises from nonmalignant causes and can often be resolved promptly and appropriately by treating the underlying cause. Differentiating between MPE and BPE is critical because it directly influences treatment strategies and prognosis.[31] Currently, the gold-standard methods for diagnosing MPE are cytopathologic examination of pleural effusion and thoracoscopic pleural biopsy.[32] Although effective, these techniques have notable limitations, including low sensitivity, sampling errors, trauma risks, surgical complications, and the necessity for specialized surgical staff and resources.[33] These challenges underscore the urgent need for more objective, sensitive, and noninvasive diagnostic tools for MPE.

Tumor biomarkers have been widely explored as a diagnostic aid for MPE. Although numerous tumor markers have been investigated, their sensitivity and specificity are often limited by the heterogeneity of tumor pathologic types. Consequently, relying solely on tumor marker levels in pleural effusion remains insufficient for definitive diagnosis.

DNA methylation, an epigenetic modification commonly observed in tumor cells, particularly within promoter regions of genes, has emerged as a promising biomarker for cancer detection and characterization.[34] Among the methylation genes, *SHOX2* and *RASSF1A* were found to be methylated in various cancers, including lung cancer, breast cancer, colorectal cancer, and hepatocellular carcinoma.[35–38] Consequently, they were used as potential biomarkers of these neoplasms.

Schmidt et al. were the first to report that the detection of *SHOX2* methylation in bronchial aspirates can be used as a biomarker to differentiate benign from malignant lung lesions with a sensitivity of 68% and a specificity of 95%. Later, this biomarker was used to diagnose MPE caused by various cancers.[39] Similarly, *RASSF1A*, a tumor suppressor gene involved in the Ras signaling pathway and associated with cell proliferation and apoptosis, has been implicated in several malignancies.[40–42] In 2012, Fujii et al. reported that abnormal *RASSF1A* promoter methylation in pleural effusion could effectively differentiate between benign and malignant conditions, including malignant mesothelioma, lung cancer, and asbestos pleurisy.[43] These findings highlight the diagnostic potential of DNA methylation markers in MPE because this epigenetic modification can be present in various cancer cells, which can metastasize to the pleura.

The high diagnostic performance, demonstrated by strong sensitivity and specificity, underscores the potential of combined *SHOX2* and *RASSF1A* methylation analysis as a promising approach for clinical diagnostics. These findings suggest that this combined analysis could serve as a valuable adjunct to cytology, having higher sensitivity but lower specificity compared with cytology. However, we acknowledge that methodological limitations in the primary studies may have introduced potential biases. For instance, the consistent use of methylation-specific real-time PCR and fixed cutoff values improves comparability but does not eliminate concerns related to sample handling or patient selection variability.

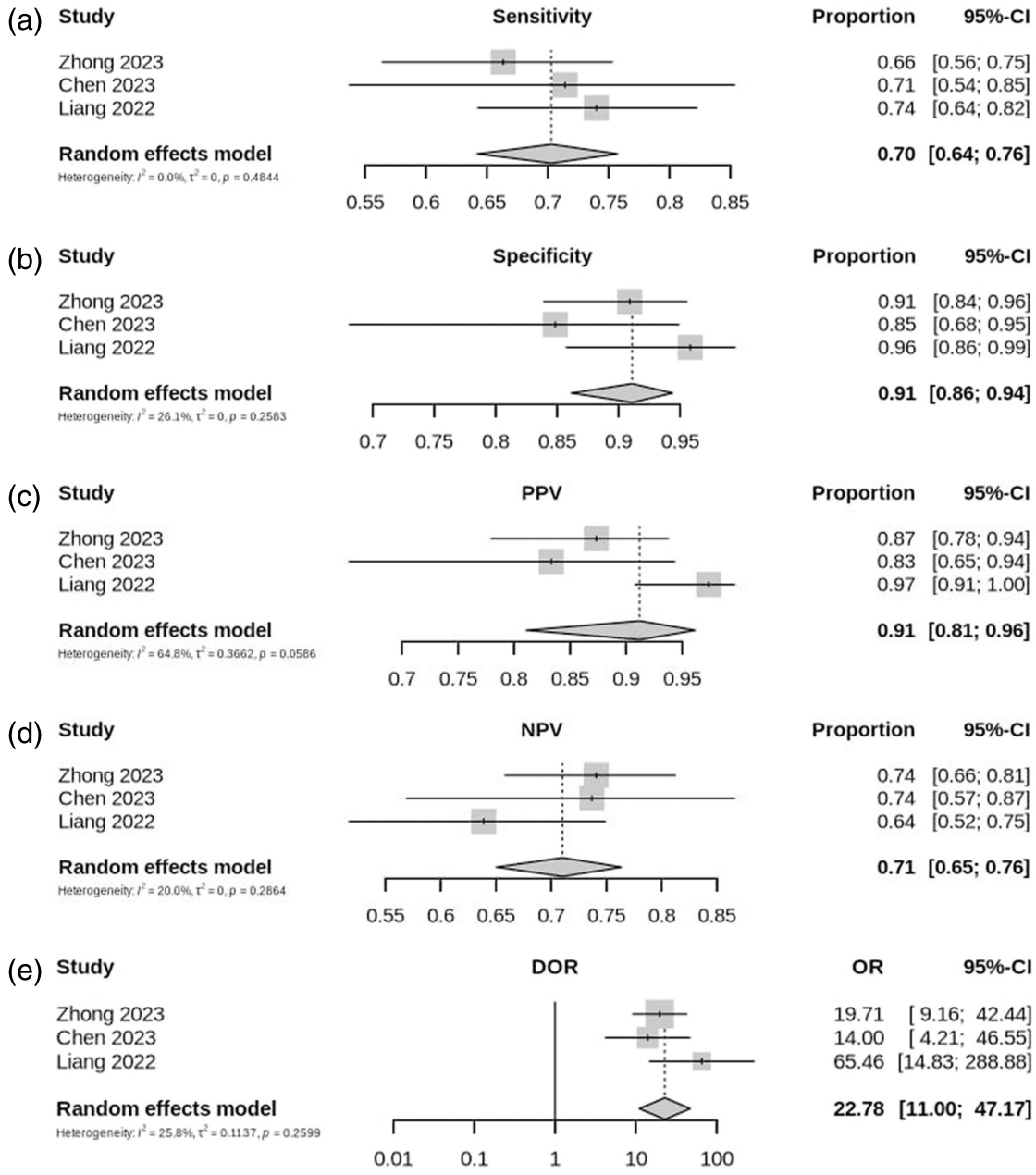


**FIGURE 5** (A–E) Subgroup analyses of pooled diagnostic accuracy estimates for combined *SHOX2* and *RASSF1A* methylation in detecting malignant pleural effusion. (A) Sensitivity, (B) specificity, (C) PPV, (D) NPV, and (E) DOR. These subgroup analyses assess the performance of the combined methylation markers across different study-level characteristics and clinical contexts to identify potential sources of heterogeneity. CI indicates confidence interval; DOR, diagnostic odds ratio; OR, odds ratio; NPV, negative predictive value; PPV, positive predictive value; *RASSF1A*, Ras association domain family member 1A; *SHOX2*, short-stature homeobox 2.[21–23]

Furthermore, the pooled PPV and NPV values should be interpreted cautiously because they are strongly influenced by disease prevalence and population characteristics. Given the heterogeneity in study populations and their nonrandom selection, these metrics may not be generalized to all clinical settings or regions. Although the statistical analyses were performed based on a small number of studies, we performed the recommended statistical analysis model with random effects, which is recommended even when the number of studies is small.[44] In addition, whereas the CIs were generally narrow for specificity, the wider range seen for sensitivity highlights the need for larger scale validation studies. Such studies could potentially reduce the reliance on more invasive diagnostic procedures, like biopsies, by providing a reliable, less invasive alternative.

In addition, evaluating the diagnostic performance of *SHOX2* and *RASSF1A* methylation in the context of MPE associated with different malignancies among different populations other than those in China is critical. Given the potential variation in molecular and biologic characteristics across various cancers, assessing the diagnostic accuracy of these markers in lung, breast, ovarian, colon, liver, and other types of cancer could yield valuable insights into their utility. Such research

would provide a clearer understanding of the consistency and reliability of this combined methylation analysis across different forms of MPE, further supporting its potential integration into clinical workflows for improved diagnostic accuracy and timely intervention.

## AUTHOR CONTRIBUTIONS

**Mohamed Smail Aissani:** Conceptualization, investigation, methodology, writing—original draft, writing—review and editing, and data curation. **Kyrillos Mahrous Gerges:** Conceptualization, writing—original draft, methodology, writing—review and editing, investigation, and data curation. **Ahmed Msherghi:** Conceptualization, investigation, writing—original draft, methodology, writing—review and editing, data curation, and formal analysis. **Hajer Farrara:** Investigation, writing—review and editing, and data curation. **Dawood Alatefi:** Data curation, writing—review and editing, and investigation. **Imane Chenfouh:** Investigation, writing—review and editing, and data curation. **Arwi Omar Kara:** Investigation, writing—review and editing, and data curation. **Maram Abuajamieh:** Data curation, writing—review and editing, and investigation. **Ghada Kareem:** Investigation, writing—review and editing, and data curation. **Mohammed Benhammou:** Data curation, writing—review and editing, and investigation. **Mohamed E. Ali:** Investigation, writing—review and editing, and data curation. **Max Wintermark:** Supervision, writing—review and editing, project administration, and investigation. **Muhammed Elhadi:** Conceptualization, investigation, writing—review and editing, writing—original draft, data curation, supervision, formal analysis, and project administration.

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## CONFLICT OF INTEREST STATEMENT

The authors declared no conflicts of interest.

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