
This is the **accepted version** of the journal article:

Nuez-Zaragoza, Elisa [et al.]. «Utility of the combination of high fluorescence cells and tumor markers for the diagnosis of malignant pleural effusions».

Clinical Biochemistry, Vol. 136, (March 2025). DOI 10.1016/j.clinbiochem.2025.110888

This version is available at <https://ddd.uab.cat/record/310366>

under the terms of the  ^{IN}COPYRIGHT license.

TITLE PAGE

TITLE: UTILITY OF THE COMBINATION OF HIGH FLUORESCENCE CELLS AND TUMOR MARKERS FOR THE DIAGNOSIS OF MALIGNANT PLEURAL EFFUSIONS

Elisa Nuez-Zaragoza^a, Indira Bhambi-Blanco^a, Mònica Vidal-Pla^a, Isabel Aparicio-Calvente^a, M.Rosa Escoda-Giralt^b, Joana Gallardo-Campos^b, JC Ferreres^b, Luis Frisancho^c, Laia Mas-Maresma^d, Patricia Aguilera-Fernández^d, Sonia Marco-Continente^e, Marina Sierra-Boada^f, Pablo Andreu-Cobo^f, Miquel Gallego^g, Jaume Trape^h, Vicente Aguaderoⁱ.

^aClinical Laboratory Department, Parc Taulí Hospital Universitari, Institut d'Investigació i Innovació Parc Taulí (I3PT-CERCA), Universitat Autònoma de Barcelona, Sabadell, Barcelona, Spain; ^b Pathology Department, Parc Taulí Hospital Universitari, Institut d'Investigació i Innovació Parc Taulí (I3PT-CERCA), Universitat Autònoma de Barcelona, Sabadell, Barcelona, Spain; ^c Digestive System Department, Parc Taulí Hospital Universitari, Institut d'Investigació i Innovació Parc Taulí (I3PT-CERCA), Universitat Autònoma de Barcelona, Sabadell, Barcelona, Spain; ^d Internal Medicine Department, Parc Taulí Hospital Universitari, Institut d'Investigació i Innovació Parc Taulí (I3PT-CERCA), Universitat Autònoma de Barcelona, Sabadell, Barcelona; ^e Intensive Care Department, Parc Taulí Hospital Universitari, Institut d'Investigació i Innovació Parc Taulí (I3PT-CERCA), Universitat Autònoma de Barcelona, Sabadell, Barcelona; ^f Oncology Department, Parc Taulí Hospital Universitari, Institut d'Investigació i Innovació Parc Taulí (I3PT-CERCA), Universitat Autònoma de Barcelona, Sabadell, Barcelona; ^g Respiratory Department, Parc Taulí Hospital Universitari, Institut d'Investigació i Innovació Parc Taulí (I3PT-CERCA),

Universitat Autònoma de Barcelona, Sabadell, Barcelona; ^h Laboratory Medicine
Department, ALTHAIA Xarxa Assitencial Universitària de Manresa, Universitat de Vic -
Universitat Central de Catalunya, Faculty of Medicine. Manresa, Spain; ⁱ Laboratory
Department, CLILAB, Sant Joan Despí-Moisès Broggi Hospital, Sant Joan Despí, Spain

Corresponding author:

Elisa Nuez-Zaragoza, M.D.

Clinical Laboratory Department, Parc Taulí Hospital Universitari, Institut d'Investigació i Innovació
Parc Taulí (I3PT-CERCA), Universitat Autònoma de Barcelona, Sabadell, Barcelona, Spain E-mail:
enuez.zaragoza@gmail.com

MANUSCRIPT

ABSTRACT

Background: New diagnostic tools have emerged to assist the traditional diagnosis of malignant pleural effusion (MPE), such as high fluorescence cells (HFc) and tumor markers (TMs), determined by clinical laboratory automated pleural fluid workup. This study aimed to evaluate the diagnostic ability of the combination of HFc and TMs for diagnosing MPE.

Methods: We recruited hospitalized patients with pleural effusion at Parc Taulí University Hospital. We collected and analyzed pleural fluid and serum samples in the clinical laboratory, and we sent a sample of pleural fluid to the Pathology Department for cytology workup. We determined the pleural fluid cell count by Sysmex XN-10 and assessed TMs (CEA, CA19.9, and CA15.3) using the ECLIA Cobas e801 Roche in both pleural fluid and serum samples. We established the final MPE diagnosis based on positive cytology and/or positive pleural biopsy. We classified patients based on these final diagnoses and conducted a comparison between variables, along with multivariate logistic regression.

Results: The study included 316 pleural effusions from 221 patients recruited. Multivariate logistic regression indicated the most significant predictor variables for MPE were CA15.3 in serum, CEA ratio, and HFc. We calculated two different models: one excluding HFc and one including it, with the latter displaying superior diagnostic ability (area under the curve 0.91). This model could identify 100% of MPE cases with 30% specificity at low cut-offs, and higher values could help identify 60% of MPE cases with 100% specificity.

Conclusions: Per our findings, this model has high diagnostic performance and could serve as a swift, automated, dependable, non-invasive tool for MPE detection.

List of Key Words: High fluorescence cells, Tumor markers, malignant pleural effusion, CEA, CA 15.3, pleural fluid

1. INTRODUCTION

The prevalence of pleural effusion of neoplastic origin ranges from 15–30%, making the biochemical and cytological study of pleural fluid (PF) an essential tool for diagnosing malignant pleural effusion (MPE) [1-10]. Laboratories receive PFs from various pathological situations and must be capable of detecting and differentiating a suggestive MPE from other etiologies. The presence of MPE in cancer patients typically indicates a poor prognosis, with an overall survival rate of less than a year [11-12]. Therefore, a prompt diagnosis of MPE is crucial for the therapeutic and prognostic implications of the patient's cancer disease [2,4,13].

The first step in a PF workup is to differentiate between transudate and exudate [1,10,12]. Transudates are primarily due to heart failure and liver cirrhosis, while exudates can have various causes, such as pneumonia, tuberculosis, viral pleuropericarditis, or cancer. This makes the diagnostic algorithm more complex [1]. MPE are, in 95% of cases, exudates produced by abnormal neoplastic cell proliferation in the pleural cavity [11]. The remainder are paraneoplastic effusions, which do not result from direct pleural damage by the tumor, but they affect other processes that regulate the formation and/or reabsorption of serous fluid [11-12].

The PF cellularity workup has been modernized with hematological autoanalyzers partially substituting optical microscopy for cell counting and differentiation [3-6,14]. The Sysmex XN-10 hematology analyzer is based on flow cytometry and nucleic acid fluorescence staining. It has a biological fluid module that determines the total nucleated cell count (TBC) including leukocyte count (WBC) (with differentiation into mononuclear (MN) and polymorphonuclear (PMN) cells), as well as high fluorescence cells (HFc). The HFc group, a research parameter, consists of large cells with high nucleic acid content, such as mesothelial cells, macrophages or histiocytes, plasma cells, and neoplastic cells. This suggests the potential use of HFc as a marker of malignancy in body fluid analysis [2-7,14].

Most of the studies published to date concur that HFc is valuable as a screening parameter for identifying malignant cells [2,3,7], with cytological examination combined with an immunohistochemical workup serving as the gold standard for diagnosing MPE [4-6,12]. Cytological examination sensitivity ranges from 40% to 87% depending on the literature [11-15], so in addition to cytology, a pleural biopsy must be conducted for the accurate diagnosis of MPE [12-15].

On the other hand, the use of tumor markers (TMs) in pleural effusions is only moderately established in clinical laboratories due to ongoing discrepancies in the literature concerning which TM or combinations are most useful for diagnosing MPE [8-10,13,16]. Variability also exists in the cut-off points to be used, though the fluid/serum ratio can be a helpful parameter for standardization between laboratories, eliminating false positives due to TM extravasation from plasma [8-10,16]. Multiple neoplasms can cause MPE, with lung cancer, mesothelioma, breast cancer, and hematological neoplasms being the most common. The diagnostic ability of TM depends largely on the type of tumor and its TM expression pattern. Of these, carcinoembryonic antigen (CEA), primarily synthesized in adenocarcinomas, mucin antigen CA15.3, overexpressed in lung, breast cancers and lymphomas, and carbohydrate antigen CA19.9, predominantly produced in digestive tract cancers, appear to hold the most diagnostic significance in MPE [8-10,16]. These TM are typically not synthesized by the mesothelium and high levels typically indicate local synthesis, indirectly reflecting the presence of tumor cell infiltration and reflecting a metastatic stage [13].

The scarcity and lack of consensus in the literature concerning the utility of measuring HFc and TM as predictors of MPE have prompted this study. The study aims to evaluate the diagnostic capability of combining both parameters to diagnose MPE. The objective is to enhance diagnostic performance in the clinical laboratory.

2. MATERIALS AND METHODS

2.1 Study design

This was a prospective, experimental, unicentric study evaluating laboratory diagnostic tests conducted at Parc Taulí University Hospital. The study received approval from the hospital's Research Ethics Committee (Ref.: 2021/5058). All patients provided written informed consent to participate, and all good research practices were upheld.

2.2 Participants

The study incorporated adult outpatient care and hospitalized patients from May 2021 to November 2022 in various departments of our hospital: Emergencies, Internal Medicine, Intensive Care Unit, Digestive System, Pneumology, and Oncology. Each patient had clinically diagnosed pleural effusion and underwent PF extraction for routine diagnostic purposes. Patients were anonymized for enrollment in the study, and data from each patient (age, sex, cause of pleural effusion) were recorded. The only patient-related exclusion criterion applied was the patient's refusal to participate in the study.

2.3 Body fluid and serum workup

2.3.1 Sample collection

PF samples and serum samples were immediately collected and analyzed in the Hospital's Clinical Laboratory after extraction. All samples were obtained for routine diagnostic purposes, and no additional body fluids or serum were collected for this study.

PF was collected in sterile containers without additives and divided into two aliquots. One aliquot, with a minimum volume of 1 mL, was sent to the Urgent Care Laboratory for cell count, differentiation, and biochemical analysis, while the other, requiring a minimum of 10 mL, was sent from the laboratory to the Pathology Department for cytological workup. In instances where the Pathology Department received the sample directly from the extraction, no additional aliquot was sent from the laboratory. In both laboratories, the sample was processed immediately after collection.

2.3.2 Cell count and differentiation

Cell counts were determined using a Sysmex XN-10 Body Fluid Mode (Sysmex, Kobe, Japan) in the Urgent Care Laboratory. The following parameters, obtained by cytometry with fluorescence staining, were recorded: WBC, PMN% (neutrophils, eosinophils, and basophils), MN% (lymphocytes, macrophages, and plasma cells), and HFc (mesothelial cells, macrophages, plasma cells, and malignant cells). HFc was recorded in both absolute count (HFc/ μ L) and relative (%) terms from the TBC.

The count for red blood cells (RBC) was determined through electrical impedance and was similarly recorded.

2.3.3 Cytological workup

All the cases were blinded to the Pathology Department, which conducted the cytological workup utilizing Papanicolaou and Diff-Quick staining for an initial morphology examination. Immunocytochemistry techniques were employed to confirm MPE if suggestive neoplastic cells were present. Patients were categorized into two groups: MPE and non-MPE. The MPE group was defined by positive cytology and/or a positive pleural biopsy (performed by image-guided pleural biopsy or medical thoracoscopy). The final diagnosis was confirmed by consulting the patient's medical history six months after sample collection. In cases of multiple diagnoses, we always recorded the one primarily suspected by clinicians, ensuring the diagnosis of cancer.

2.3.4 Biochemical analysis

After cell count and differentiation, the PF was centrifuged for 5 minutes at 1500 rpm. The supernatant was then used for biochemical and TMs analyses. Meanwhile, blood samples were centrifuged at 3500 rpm for 10 minutes to obtain serum.

Glucose, lactate dehydrogenase (LDH), and total proteins were measured using Cobas c501 (Roche Diagnostics, Mannheim, Germany) in both PF and serum. The ratio of body fluid/serum LDH and total proteins was then calculated in order to classify the samples as either transudate or exudate per Light's criteria [1]. According to Light's criteria, a pleural effusion is classified

as exudative if it meets one or more of the following conditions, while a transudate meets none: a pleural fluid/serum protein ratio greater than 0.5, a pleural fluid/serum LDH ratio greater than 0.6, or a pleural LDH concentration exceeding two-thirds of the upper normal limit for serum LDH.

The levels of CEA, CA15.3, and CA19.9 TMs were determined in both PF and serum by employing an electrochemiluminescence immunoassay with Cobas e801 (Roche Diagnostics, Mannheim, Germany). All samples were stored at -80°C and thawed and analyzed in batches. The TMs in PF and serum were always tested simultaneously, and the ratio of body fluid/serum was calculated.

2.4 Statistical analysis

To calculate the sample size, an estimation of the incidence of MPE in our population was conducted to achieve a significant number of MPE. Beginning with a reference population of 400 individuals (the number of PFs received by the laboratory annually), we determined that a random sample of 310 individuals would be sufficient to estimate, with 95% confidence, a population percentage (MPE) anticipated to be approximately 15%.

To conduct the statistical analysis, patients were organized based on their final diagnosis for variable comparison. Normality for quantitative variables was verified using the Kolmogorov-Smirnov test. Nonparametric quantitative variables were articulated as medians and interquartile ranges (IQR, P25–P75). The Mann-Whitney U test was conducted to compare medians between MPE/non-MPE patient groups. Qualitative variables were expressed as percentages, and Pearson's chi-squared test was applied to compare percentages between MPE/non-MPE patient groups.

Multivariate logistic regressions were performed using a selection of variables, aiming to identify independent variables and fit the relevant clinical covariables. Predictor variables were selected using the backward-step method. Additionally, we calculated the Wald statistic and odds ratio (OR) for the chosen variables. Cook's distance assessment and the Bonferroni outliers test detected outlier cases.

We evaluated the diagnostic feasibility of algorithms using the area under the curve (AUC), accuracy, Akaike Information Criterion (AIC), and Nagelkerke's R². For the comparison of

different AUCs, the nonparametric DeLong test was applied.

We considered a $p < 0.05$ alpha error level as significant. R-Commander 3.6.1 program and SPSS Statistics version 25.0 were used to perform all these analyses.

3. RESULTS

Initially, a total of 341 PFs were included in the study. However, 25 were excluded for various reasons: a patient's refusal to participate in the study, an insufficient sample for cytological study, or an inconclusive final diagnosis. Finally, a cohort of 316 pleural effusions and 302 serum from 221 patients was included (a diagram is available in Supplementary Figure 1). This included 136 males (61.5%) and 85 females (38.5%), with a median age of 72 years.

Table 1 shows the different causes of the patient's pleural effusions as well as fluid type, classified according to Light's criteria. Neoplastic origin being the main cause, followed by cardiac insufficiency, parapneumonic/empyema, and cirrhosis. The most prevalent neoplasia was lung cancer (52.9%), followed by hematological neoplasms. The majority of the collected samples were exudates (249, 78.7%), while transudates were primarily associated with cardiac insufficiency and cirrhosis. The fluid type could not be determined in ten samples due to the inability to collect serum.

PF were divided into two groups, leading to 244 cases of benign pleural effusion, non-MPE (77.2%), and 72 cases of MPE (22.8%). The MPE group was confirmed through positive cytology or a pleural biopsy workup, with 18 cases testing negative for cytology (Table 2). Table 2 displays the analyzed differences between the non-MPE and MPE groups for both qualitative variables (assessed with Pearson's chi-squared test) and quantitative variables (evaluated using the Mann-Whitney U test), demonstrating several statistically significant differences between the two groups.

The statistically significant differences observed in cellularity were attributed to the WBC count, the percentage of MN and the RBC count. HFc displayed significant differences between the MPE and non-MPE groups expressed as both percentage and absolute count (Table 2). The relationship between HFc and cytology in the MPE group was also examined. Significant differences were obtained between the two variables, with a median of 30 HFc (IQR: 42.3) for the negative cytology group and a median of 89 HFc (IQR: 350.1) for the positive cytology

group. A figure illustrating the median absolute HFc count with IQR across the different groups is provided in the Supplementary Material (Supplementary Figure 2).

The determinations of TMs in PF and serum, as well as the calculated ratio, showed a high capacity to differentiate between MPE and non-MPE. Only serum CA19.9 did not allow for discrimination between the two study groups.

To increase the diagnostic yield of variables separated from bivariate analysis, we combined the significant ones into multivariate diagnostic algorithms. This multivariate analysis led to the creation of a binary logistic regression model incorporating the most notable variables as indicated by Wald and OR statistics: CA15.3 serum and CEA ratio. To evaluate HFc's influence in the model, we created another version that included this variable. In both models, CA15.3 serum demonstrated the highest significance, and the second most influential variable was CEA ratio. HFc demonstrated an OR of 1.6 in the model that included it. Both models were based on 301 PF and serum samples, with 67 MPE, and are detailed in Table 3.

The diagnostic feasibility of the algorithm's results is presented in Table 4. The model incorporating HFc demonstrated superior performance compared to the model excluding HFc. In both models, a cut-off point was determined using the Youden Index. For the model without HFc, the cut-off was set at -0.77, achieving a sensitivity of 69% and a specificity of 95%. Conversely, the model with HFc, having a cut-off of -0.53, yielded a higher sensitivity (74%), maintaining the same specificity of 95%.

Two additional cut-off points were calculated in the model with HFc to obtain a higher specificity or sensitivity, available in Table 4.

The AUCs of the two models were evaluated and are depicted in Figure. 1. Significantly, the AUC of the model with HFc was found to be notably higher than the model without HFc, as verified by the DeLong test ($p < 0.05$).

Finally, we evaluated and compared the diagnostic capabilities of the HFc model with the individual analytes on which the model is based. The HFc model showed superior performance, as illustrated in Figure. 2.

4. DISCUSSION

MPE is produced by primary tumors of the pleura and in tumor metastasis, and its diagnosis significantly influences the subsequent management of the neoplastic condition [12,13]. MPE diagnosis has therapeutic implications and an impact on disease prognosis, primarily for the patient's life expectancy. The median survival period is often less than a year [1,4,7,11].

PF cytology remains the gold standard for diagnosing MPE despite its sensitivity having a considerable range, mostly dependent on the type of primary tumor [11,15]. A recent meta-analysis, which includes 6,057 patients with MPE, reports an overall sensitivity of 58.2% [15]. This limited diagnostic sensitivity for cytology necessitates complementary tests for confirmation, which are based on invasive tests such as pleural biopsies [12,15,17]. New diagnostic tools should be developed to assist in MPE diagnosis, with a preference for less invasive approaches like fluid biomarkers [18,17].

In this study, we evaluated various parameters in PF and serum to develop a predictive model using the most relevant variables for aiding MPE diagnosis. We analyzed PFs from hospitalized patients consecutively recruited from different units, yielding a heterogeneous sample with diverse pathologies. MPE accounted for 22.9% of total PFs, consistent with the prevalence found in other studies [1,17]. The most prevalent conditions producing MPE were lung cancer, followed by hematological neoplasms, mesothelioma, and breast cancer, respectively. Out of all MPE diagnoses, the cytology workup showed a sensitivity of 75%; the rest of the MPE cases were diagnosed by biopsy.

Pleural effusions were categorized according to Light's criteria into exudates and transudates. It was observed that in the MPE group, there were two transudates: one lung cancer with negative cytology workup, and one breast cancer metastasis with positive cytology.

A cellular study conducted with Sysmex XN-10 provided an HFc count, showcasing its potential as a variable because of the statistical differences between the MPE and non-MPE groups. Other authors have described HFc's capability for malignant effusion detection [2,4,14,19]. Labaere et al. [4] determined that the absolute numbers of HFc yielded a more precise prediction of malignant cells in body effusions, achieving an AUC of 0.77 for the absolute count versus 0.72 for the percentage in the pleural fluid subgroup. Similarly, Larruzea et al. [2] reported similar conclusions with an AUC of 0.84 for absolute HFc counts and 0.75 for the percentage. However, these findings remain controversial. For example, Wu et al. [6] and Wong-Arteta [7] determined a slightly lower diagnostic yield for the HFc absolute count. In our case, our results align with those of Labaere et al. [4] and Larruzea et al. [2]. The HFc

absolute count is one of the more determinant variables for our predictive model, with an AUC of 0.74 (Figure. 2), slightly lower than reported.

One of the reasons our study demonstrates a lesser AUC could be our focus on PF. Unlike other studies that combine PF with other body fluids, we chose to concentrate on this area. Another possible reason could be the heterogeneity of our samples. They were prospectively incorporated from all hospital areas. Samples that did not have a requested cytological workup were sent to the Pathology laboratory. This procedure helped us avoid bias due to clinical suspicion of MPE.

In our study, we observed that HFc correlates with cytology results in the MPE group, as expected. We found that with positive cytology, the amount of HFc is significantly higher than in those with negative cytology. However, some MPE samples with positive cytology did not exhibit an elevated HFc count. This could be due to an extremely low number of tumor cells in some samples, thereby evading detection by the hematology analyzer, as reported in other studies [3,20]. Additionally, the morphological characteristics of the neoplastic cells can determine their position in the scattergram, and they could be placed in the regions of MN (smaller cells with less fluorescence, such as lymphocytes, monocytes, etc.) instead of the region of HFc [3]. On the other hand, some tumors do not exfoliate tumor cells into the pleural space but affect the pleura. Therefore, in these cases, cytology will always be negative, as should HFc corresponding to tumoral cells.

All this misdiagnosis suggests that HFc alone does not have sufficient diagnostic capacity to differentiate between MPE and non-MPE, and additional tests should be conducted for accurate diagnosis.

The utilization of TM for predicting MPE is indeed contentious in existing literature [13,21]. As noted in our study, we find higher concentrations of TM in patients with malignant fluids compared to those with benign fluids [20]. This was significantly pronounced in all determinations of CA 15.3 and CEA, additionally to CA19.9 in PF and ratio.

Various studies report discrepancies in cut-offs, which complicate interpretation and harmonization for diagnostic use in routine clinical laboratories [13,16,18]. Furthermore, comparing the cohorts described in the literature presents significant challenges [16].

Previously, other combinations of TM have been examined, as evidenced in the meta-analysis by Yung et al [8], where the results of 20 studies on TM in PF were evaluated. Higher

diagnostic accuracy was demonstrated with combinations of TM rather than singular TM determination. This had an overall specificity greater than 90% with a variable sensitivity ranging from 58% to 95%. The combination of CEA+CA 15.3+CA 19-9 showed a specificity of 97% and a sensitivity of 71%.

Other authors propose different strategies for interpreting TM results, combining various cut-off values to maximize sensitivity and specificity in detecting false-positive TMs samples [13]. They describe an algorithm that combines reactive markers like adenosine deaminase, C-reactive protein, and PMN% with several TMs (CA-72.4, CA19.9, CEA, and CA15.3). With their algorithm, they achieved a sensitivity of 64% at the high TMs cut-off point and 77% sensitivity in patients with all reactive markers negative, using the TMs low cut-off point and fluid/serum ratio. The specificities achieved were 100%, allowing differentiation between MPE and non-MPE [13].

Following the strategy of combining different variables of value to detect cases of MPE, we derived a model with the following parameters through a preliminary logistic regression study: CA15.3 serum, CEA ratio, and HFc. In this model, HFc is the variable with the least predictive power. However, it adds significant sensitivity compared to the model based solely on TMs without compromising specificity. As illustrated in Figure. 2, the diagnostic yield (AUC) of the model surpasses that of the independent variables on which it is based.

Our results demonstrate that the amalgamation of HFc, CEA ratio, and CA15.3 in serum can detect 100% of MPE cases with a specificity of 30% using low cut-offs. Conversely, higher cut-offs allows for the identification of 60% MPE patients, with a specificity of 100%.

Our models performed exceptionally well in diagnosing MPE, rendering it a potentially valid test to supplement other diagnostic techniques, such as imaging. It is important to bear in mind, however, that treatment and management decisions should be based on positive cytological or biopsy results from the pleura.

Our predictive model has the advantage of being quick, as it can be executed on the same day as PF extraction. Additionally, it is efficient, affordable, and can easily be adopted by many clinical laboratories without requiring expert analyzers or additional materials. For patients with a positive algorithm result but negative diagnostics from imaging scans or cytology workups, physicians should instigate regular follow-up, monitoring, and recurrent reassessment to avert missing potential MPE diagnoses. In our study, we applied the predictive

model to 67 MPE samples (with PF and serum collection). Of these samples, 16 (23.9%) had negative cytology, but of those, six (9%) had a positive algorithm result (>0.29), five of which were >0.65 . Hence, our research presents a potential new opportunity in an area with limited alternatives.

We have carefully considered the limitations of the study, and we believe they do not undermine its reliability or detract from the positive results. While this was a single-center study, the patients we included exhibited clinical diversity and varying diagnostic characteristics. Although the number of samples recruited was substantial, as determined by sample size estimation, the proportion of MPE was small compared to the total number of samples recruited; ultimately, we had 72 MPE samples and 67 included in the algorithm. This limitation is not uncommon as prospective studies typically require a large number of controls to gain the necessary quantity of cases. Further studies of the designed algorithm should be conducted with an increased patient sample and validated in independent cohorts. Moreover, the number of TMs analyzed was limited compared to other series reported in the literature. Therefore, future studies should aim to combine HFc with more TMs and develop more precise algorithms for different primary neoplasms.

On the other hand, we also controlled for the most common biases in this type of study, such as selection bias and overfitting, which indicate the presence of well-identified non-neoplastic diseases in the groups.

In conclusion, MPE diagnosis can occasionally have limitations and may need to rely on complementary invasive diagnostic techniques. The determination of TM and HFc in PF presents an economical, immediate, and reproducible method that could assist in clinical decision-making when investigating pleural effusion diagnosis. Our model has high sensitivity and specificity, potentially serving as a quick, automated, reliable, and non-invasive tool for cancer detection and preclinical diagnosis in patients with MPE.

ACKNOWLEDGMENTS

We would like to express our gratitude to the heads of department for all the support provided during this project, Dra. M^aLuz Muñoz and Dr. Eugenio Berlanga. Special thanks to the laboratory team from Servei Laboratoris Clínics at the Hospital Universitary Parc Taulí.

This work has been carried out within the framework of the PhD program in Biochemistry, Molecular Biology, and Biomedicine at the Universidad Autònoma de Barcelona (UAB). We thank Dra. Francesca Canalias for her assistance and support during this PhD research program.

REFERENCES

- [1] Villena Garrido V, Cases Viedma E, Fernández Villar A, de Pablo Gafas A, Pérez Rodríguez E, Porcel Pérez JM, et al. Recommendations of diagnosis and treatment of pleural effusion. Update. Archivos de Bronconeumología [Internet]. 2014 Jun 1;50(6):235–49.
- [2] Larruzea, V. Aguadero, Orellana R, Berlanga E. High-fluorescent cells: A marker of malignancy in the analysis of body fluid samples. International journal of laboratory hematology. 2018 Mar 2;40(3).
- [3] Cho Y -U, Chi H -S, Park SH, Jang S, Kim Y -J, Park C -J. Body fluid cellular analysis using the Sysmex XN-2000 automatic hematology analyzer: focusing on malignant samples. International journal of laboratory hematology. 2014 Sep 12;37(3):346–56.
- [4] Labaere D, Boeckx N, Geerts I, Moens M, Van den Driessche M. Detection of malignant cells in serous body fluids by counting high-fluorescent cells on the Sysmex XN-2000 hematology analyzer. International Journal of Laboratory Hematology. 2015 Jun 13;37(5):715–22.

- [5] Xu W, Yu Q, Xie L, Chen B, Zhang L. Evaluation of Sysmex XN-1000 hematology analyzer for cell count and screening of malignant cells of serous cavity effusion. *Medicine* [Internet]. 2017 Jul 7;96(27). Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5502180/>
- [6] Wu W, Zhao C, Shen T, Tong X, Chen W. The diagnostic ability of high-fluorescent cells combined with carcinoembryonic antigen for malignant pleural effusion. *International journal of laboratory hematology*. 2019 Apr 24;41(4):509–12.
- [7] Jhonatan Wong-Arteta, Gil-Rodríguez E, Cabezon-Vicente R, Edurne Bereciartua-Urbieto, Bujanda L. High fluorescence cell count in pleural fluids for malignant effusion screening. *Clinica chimica acta*. 2019 Dec 1;499:115–7.
- [8] Yang Y, Liu YL, Shi HZ. Diagnostic Accuracy of Combinations of Tumor Markers for Malignant Pleural Effusion: An Updated Meta-Analysis. *Respiration*. 2017;94(1):62–9.
- [9] Gu Y, Zhai K, Shi HZ. Clinical Value of Tumor Markers for Determining Cause of Pleural Effusion. *Chinese Medical Journal*. 2016 Feb;129(3):253–8.
- [10] Volarić D, Flego V, Žauhar G, Bulat-Kardum L. Diagnostic value of tumour markers in pleural effusions. *Biochemia Medica*. 2017 Dec 27;28(1).
- [11] Susana Herrera Lara, Fernández-Fabrellas E, Gustavo Juan Samper, Josefa Marco Buades, Rafael Andreu Lapiedra, Amparo Pinilla Moreno, et al. Predicting Malignant and Paramalignant Pleural Effusions by Combining Clinical, Radiological and Pleural Fluid Analytical Parameters. *Lung*. 2017 Jun 27;195(5):653–60.

[12] HEFFNER JE. Diagnosis and management of malignant pleural effusions. *Respirology*. 2007 Oct 22;0(0):071023220449004

[13] Jaume Trapé, Bérnago S, González-García L, González-Fernández C. Lung cancer tumor markers in serous effusions and other body fluids. *Tumor biology*. 2024 Mar 19;46(s1):S99–110.

[14] Alcaide Martín MJ, Altimira Queral L, Sahuquillo Frías L, Valiña Amado L, Merino A, García de Guadiana-Romualdo L. Automated cell count in body fluids: a review. *Advances in Laboratory Medicine / Avances en Medicina de Laboratorio*. 2021 Mar 15;2(2):149–61.

[15] Kassirian S, Hinton SN, Cuninghame S, Chaudhary R, Iansavitchene A, Amjadi K, et al. Diagnostic sensitivity of pleural fluid cytology in malignant pleural effusions: systematic review and meta-analysis. *Thorax* [Internet]. 2023 Jan 1 [cited 2023 Mar 9];78(1):32–40. Available from: <https://thorax.bmj.com/content/78/1/32>

[16] Trapé J, Sant F, Franquesa J, Montesinos J, Arnau A, Sala M, et al. Evaluation of two strategies for the interpretation of tumour markers in pleural effusions. *Respiratory Research* [Internet]. 2017 May 25 [cited 2023 Sep 1];18(1). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5445332/pdf/12931_2017_Article_582.pdf

[17] Porcel JM. Biomarkers in the diagnosis of pleural diseases: a 2018 update. *Therapeutic Advances in Respiratory Disease*. 2018 Jan;12:175346661880866.

[18] Jaume Trapé, Molina R, Sant F. Clinical Evaluation of the Simultaneous Determination of Tumor Markers in Fluid and Serum and Their Ratio in the Differential Diagnosis of Serous Effusions. *Tumor biology*. 2004 Jan 1;25(5-6):276–81.

[19] Ai T, Tabe Y, Takemura H, Kimura K, Takahashi T, Yang H, et al. Novel flowcytometry-based approach of malignant cell detection in body fluids using an automated hematology analyzer. PLOS ONE. 2018 Feb 9;13(2):e0190886–6.

[20] Sun J, Ding S, Zhu L, Liu Y, Jiang Q, Song S, et al. Improving performance of recently introduced flow cytometry-based approach of malignant cell screening in serous cavity effusion. International journal of laboratory hematology. 2020 Jun 23;42(5):612–8.

[21] Topolcan, Holubec L, V Polivkova, S Svobodova, Pesek M, Treska V, et al. Tumor markers in pleural effusions. PubMed. 2007 Jul 1;27:1921–4

FUNDING SOURCES

This work was supported by the Fundació Institut d'Investigació i Innovació Parc Taulí (I3PT), under a grant received in the '23 Convocatòria de les Beques Taulí de Recerca i Innovació', and the Fundación José Luis Castaño-SEQC under the PhD program grant 'Ayudas para Profesionales Jóvenes para Tesis Doctoral 2023'.

AUTHOR CONTRIBUTIONS

Conceptualization: E Nuez-Zaragoza

Data curation: E Nuez-Zaragoza, I Bhambi-Blanco, I Aparicio-Calvente, M Vidal-Pla, V Aguadero

Funding acquisition: E Nuez-Zaragoza

Investigation: E Nuez-Zaragoza, I Bhambi-Blanco, I Aparicio-Calvente, M.R Escoda-Giralt, J Gallardo-Campos, JC Ferreres, L Frisancho, L Mas-Maresma, P Aguilera-Fernández, S Marco-Continente, M Sierra-Boada, P Andreu-Cobo, M Gallego.

Methodology: E Nuez-Zaragoza, I Bhambi-Blanco, I Aparicio-Calvente, M Vidal-Pla, V Aguadero

Visualization: E Nuez-Zaragoza

Project administration: E Nuez-Zaragoza

Supervision: V Aguadero, J Trapé

Writing – original draft: E Nuez-Zaragoza

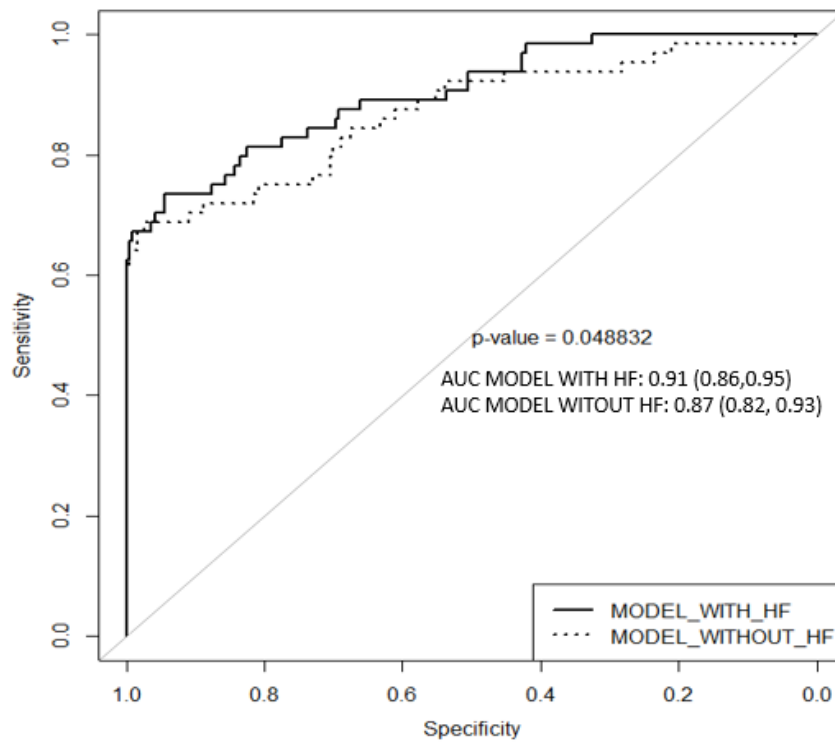
Writing – review & editing: I Bhambi-Blanco, I Aparicio-Calvente, M Vidal-Pla, V Aguadero, J Trapé

CONFLICTS OF INTEREST

The authors state no conflict of interest.

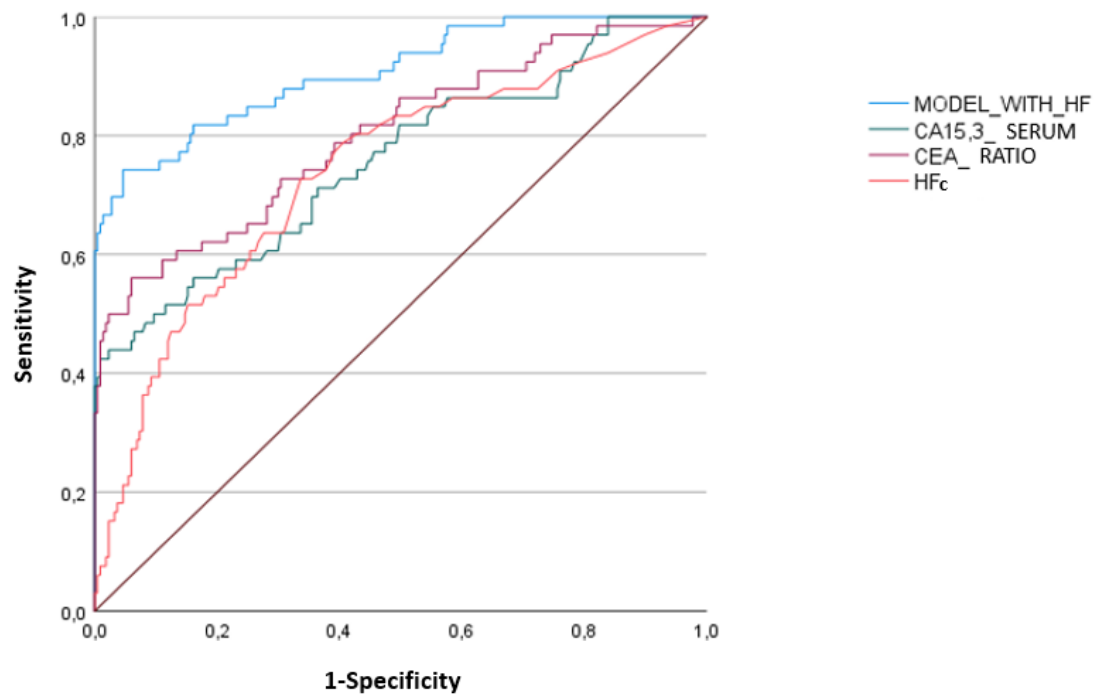
FIGURES

Figure. 1. Paired ROC curves comparison: model with HFc vs. model without HFc. P-value (Delong Test).



Abbreviations: HF, High fluorescence cells.

Figure. 2. ROC curves of Tumor Markers and High fluorescence cells model versus individual variables. AUC values: Model with HFc: 0.91 (CI 0.86-0.95), CA 15.3: 0.76 (CI 0.69-0.84), CEA R: 0.80 (CI 0.73-0.86), HFc: 0.74 (CI 0.67-0.81).



Abbreviations: CI, Confidence Interval; HFc, High fluorescence cells.

13 **TABLES**14 **Table 1.** Etiologies and fluid type classified according to Light's criteria of pleural effusions.

Etiology		Fluid type				
		Transudates (N)	Exudates (N)	NC	N	
Neoplasia						72 (22.9%)
	Lung cancer	1	35	2	38 (52.78%)	
	Lymphoma and Multiple Myeloma	0	8	1	9 (12.5%)	
	Mesothelioma	0	7	0	7 (9.72%)	
	Breast cancer	1	4	0	5 (6.94%)	
	Pancreatic cancer	0	3	0	3 (4.17%)	
	Esophageal cancer	0	3	0	3 (4.17%)	
	Colon cancer	0	3	0	3 (4.17%)	
	Ovarian cancer	0	2	0	2 (2.78%)	
	Prostatic cancer	0	1	0	1 (1.39%)	
	Neuroendocrine	0	1	0	1 (1.39%)	
Cardiac Insufficiency		26	34	2		62 (19.6%)
Parapneumonic and empyema		0	61	1		62 (19.6%)
Cirrhosis		19	11	0		30 (9.5%)
Not known etiology		4	13	1		18 (5.7%)
Post-surgery		1	13	0		14 (4.4%)
Post traumatic		0	12	0		12 (3.8%)
Tuberculosis		1	9	0		10 (3.2%)
Pleuropericarditis		1	7	0		8 (2.5%)
Pulmonary thromboembolism		0	5	0		5 (1.6%)
Hepatic abscessus and cholangitis		0	5	0		5 (1.6%)
Asbestosis		0	3	2		5 (1.6%)
Pleural fibrosis		0	3	1		4 (1.3%)
Autoimmune disease		2	2	0		4 (1.3%)
Pancreatitis		1	1	0		2 (0.6%)
Paramalignant		0	2	0		2 (0.6%)
Atelectasia		0	1	0		1 (0.3%)
Total		57	249	10		316

Abbreviations: N, number of samples; NC, non classifiable by Light's criteria.

Table 2. Epidemiological characteristics and bivariate analysis of the variables studied of pleural fluid groups.

	Pleural Fluid		<i>p</i> *
	NON-MPE	MPE	
N	244	72	
Qualitative variables.			
Sex (M/F)	151/93	42/30	0,646
Fluid Type (Exudate / Trasudate)	182/55	67/2	<0.001
Cytology (Positive / Negative)	0/244	54/18	
Quantitative variables. Median (IQR)			
Age (years)	72 (19)	70 (17)	0.129
WBC (10 ⁹ cells/L)	0.588 (1.931)	1.535 (2.753)	<0.001
RBC (10 ¹² cells/L)	0.004 (0.0224)	0.0119 (0.0409)	0.002
MN (%)	74 (44)	88 (32)	<0.001
HFc (cells/μL)	14 (36)	57 (191)	<0.001
HFc (%)	2 (6)	4 (8)	<0.001
GLU PF (mmol/L)	6.38 (3.05)	5.55 (2.05)	<0.001
LDH PF (μkat/L)	2.83 (5.52)	5.42 (8.7)	<0.001
LDH BS (μkat/L)	3.33 (1.53)	3.75 (2.03)	0.006
TP PF (g/L)	36 (18)	41 (13)	0.002
TP BS (g/L)	64 (12)	65 (11)	0.199
CA 15.3 PF (U/mL)	7.38 (7.91)	26.9 (139.8)	<0.001
CA 15.3 BS (U/mL)	17.75 (12.68)	33.5 (73.85)	<0.001
CA 15.3 RATIO	0.46 (0.47)	0.88 (1.11)	<0.001
CEA PF (μg/L)	0.97 (1.36)	9.67 (73.87)	<0.001
CEA BS (μg/L)	2.27 (2.50)	2.99 (11.62)	0.006
CEA RATIO	0.48 (0.41)	1.12 (5.18)	<0.001
CA 19.9 PF (U/mL)	3.03 (3.21)	7.14 (91.98)	<0.001
CA 19.9 BS (U/mL)	11.75 (18.77)	13.85 (67.58)	0.081
CA 19.9 RATIO	0.29 (0.38)	0.59 (0.84)	<0.001

* *p* values indicate differences between malignant and non-malignant pleural effusions. *P* < 0.05 was considered statistically significant (by Chi-Square Test for Qualitative variables, U-Mann-Whitney for quantitative variables).

Abbreviations: BS, Blood serum; F, Female; GLU, Glucose; HFc, High fluorescence cells; IQR, Interquartile range; LDH, lactate dehydrogenase; M, Male; MN, Mononuclear cells;

24 MPE, Malignant pleural effusion; N, Number of samples; PF, Pleural fluid; TP, total proteins;
 25 RBC, Red blood cells; WBC, Leukocytes.

26 **Table 3.** Multivariate Logistic Regression models for malignant pleural effusion diagnosis.

		CA15.3 BS		Ratio CEA		HFc	
		WE	O R	WE	OR	WE	O R
MODEL WITH HFc	LOGIT (P)= -8,482+1,886*LnCA153_BS+1,49*LnCEA_RATIO+0,476*LnHFc	20,9	6,6	26,2	4,4	15,2	1,6
MODEL WITHOUT HFc	LOGIT (P)= -6,446+1,745*LnCA153_BS+1,487*LnCEA_RATIO	23,8	5,7	29,2	4,4		

27

28 Abbreviations: BS, Blood serum; HFc, High fluorescence cells; Ln, logarithm neperien; OR,
 29 Odds ratio; P, Probability of MPE; WE, Wald Statistic.

30

31 **Table 4.** Performance of the algorithms obtained in the multivariate study.

	R2 NAGELKERK E	AI C	Accurac y (%)	AUC (95% CI)	Cut-off point	S (%)	Sp (%)	PPV (%)	NPV (%)
MODEL WITHOUT HF	0,58	181	91,0	0,87 (0,82-0,93)	-0.77*	69	95	79	91
MODEL WITH HF	0,64	159	90,8	0,91 (0,86-0,95)	-0.53*	74	95	83	92
					-3.47	100	33	31	100
					-3.14	95	42	34	97
					0.29	64	99	98 [†]	90
					0.65	60	100	100[‡]	89

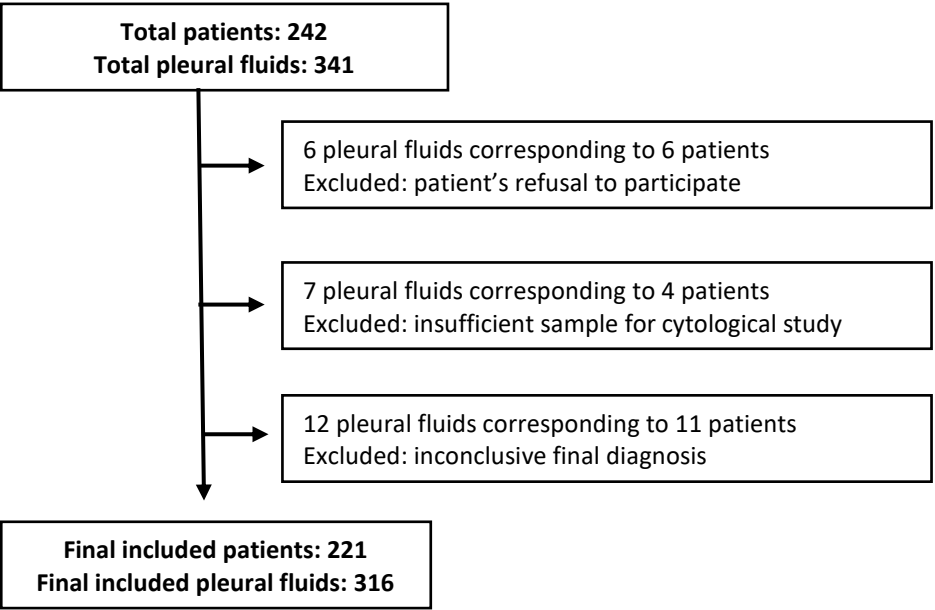
32 * Youden's Index Roc Curve.

33 [†]42 MPE samples detected.

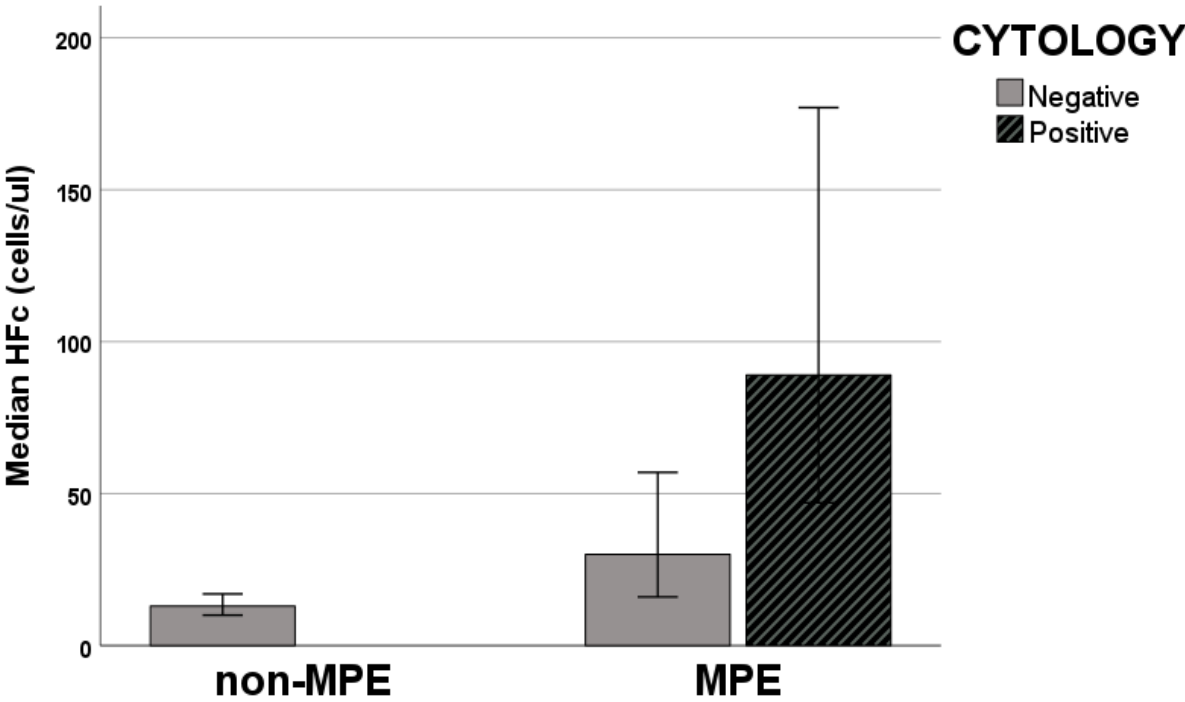
34 [‡] 40 MPE samples detected.

35 Abbreviations: AIC, Akaike information criterion; AUC, Area under curve; CI, Confidence
 36 interval; S, Sensitivity; Sp, Specificity;.

Supplementary Figure 1. The figure illustrates the flow of patients and fluids throughout the study, detailing the exclusion criteria along with the corresponding number of patients and fluids for each category.



Supplementary Figure 2. Graph representing median absolute HFc count (cells/ μ L) with its interquartile ranges for non-MPE and MPE. MPE is subdivided in cytology negative group and cytology positive group.



61

62

63

64