



High sensitivity flow cytometry immunophenotyping increases the diagnostic yield of malignant pleural effusions

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Abstract

Diagnosing malignant pleural effusions (MPE) is challenging when patients lack a history of cancer and cytopathology does not detect malignant cells in pleural effusions (PE). We investigated whether a systematic analysis of PE by flow cytometry immunophenotyping (FCI) had any impact on the diagnostic yield of MPE. Over 7 years, 570 samples from patients with clinical suspicion of MPE were submitted for the FCI study. To screen for epithelial malignancies, a 3-color FCI high sensitivity assay was used. The FCI results, qualified as “malignant” (FCI+) or “non-malignant” (FCI-), were compared to integrated definitive diagnosis established by clinicians based on all available information. MPE was finally diagnosed in 182 samples and FCI detected 141/182 (77.5%). Morphology further confirmed FCI findings by cytopathology detection of malignant cells in PE (n=91) or histopathology (n=29). Imaging tests and clinical history supported the diagnosis in the remaining samples. The median percentage of malignant cells was 6.5% for lymphoma and 0.23% for MPE secondary to epithelial cell malignancies. FCI identified a significantly lower percentage of EpCAM⁺ cells in cytopathology-negative MPE than in cytopathology-positive cases (0.02% vs. 1%; p<0.0001). Interestingly, 29/52 MPE (55.8%) where FCI alerted of the presence of malignant cells were new diagnosis of cancer. Overall, FCI correctly diagnosed 456/522 samples (87.4%) suitable for comparison with cytopathology. These findings show that high sensitivity FCI significantly increases the diagnostic yield of MPE. Early detection of FCI+ cases accelerates the diagnostic pathway of unsuspected MPE, thus supporting its implementation in clinical diagnostic work-up as a diagnostic tool.

Keywords Malignant pleural effusions · Immunophenotype · Flow cytometry · EpCAM · Lymphoma

Abbreviations

MPE Malignant pleural effusions
PE Pleural effusions
FCI Flow cytometry immunophenotyping

EpCAM Epithelial cell adhesion molecule
PET Positron emission tomography
mAb Monoclonal antibodies
FITC Fluorescein isothiocyanate
PerCP Peridinin Chlorophyll Protein Complex
APC Allophycocyanin
EpCAM Epithelial cell adhesion molecule
LOD Limit of detection
LLOQ Lower limit of quantification
IQR Interquartile range
M Male
F Female
PEL Primary effusion lymphoma
NHL Non-Hodgkin lymphoma
SSC Squamous cell carcinoma
NSCLC Non-small cell lung cancer
NET Neuroendocrine tumor
PPV Positive predictive value

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NPV Negative predictive value

Introduction

Malignant pleural effusions (MPE) are usually a late manifestation of a disseminated disease in a patient with a known history of cancer. However, between 10 and 15% of MPE are the first sign of an underlying tumor, making this diagnosis particularly challenging. Reported sensitivity of pleural fluid cytopathology ranges from 45 to 60%, influenced by the experience of the cytopathologist, volume of samples received or type of tumor [1]. To date, biomarkers tested for MPE have not reached enough evidence to support their routine measurement in clinical practice [2]. Clinical guidelines recommend the use of flow cytometry immunophenotyping (FCI) for very selected pleural effusions (PE) with high suspicion of involvement by lymphoma [3]. However, many authors have also used FCI to detect infiltration of non-hematological neoplasms in body fluids [4–11]. Considering that lymphoma accounts for less than 10% of MPE, FCI would offer an opportunity as part of the diagnostic algorithms for PE given its function to detect epithelial cell malignancies through the identification of the epithelial cell adhesion molecule (EpCAM) [12]. This prospective 7-year study is the result of investigating whether implementing a systematic FCI evaluation of PE samples with clinical suspicion of malignancy would have an impact on the diagnostic yield of MPE.

Materials and methods

Study design

Between 2015 and 2022, 1,750 PE samples were recorded at our hospital. Patients with clinical suspicion of malignancy were submitted to a pleural tap to study PE using biochemistry, cytopathology and cell culture. A PE sample was also systematically sent for FCI study.

Data from patients included: age at evaluation, gender, tumor history, imaging diagnostic tests (radiography, computerized tomography, PET), cytopathology and/or histopathology data. Cytopathological analysis was performed by conventional microscopy and findings were reported according to the international system for reporting serous fluid cytology [13]. All PE samples were examined using smears. Immunohistochemistry of cell blocks was only performed for samples with a high percentage of malignant cells.

The final diagnosis of PE was established by clinicians on the basis of all available information, and this integrated diagnosis was considered as the “gold standard”. Three clinical categories of PE were established: MPE, paramalignant effusions and PE negative for malignancy [14].

MPE was established when tumor cells were detected, either in PE by cytology or in a tissue biopsy sample. In the present study, samples reported by cytology as “malignant” or “suspicious for malignancy” were considered MPE. In turn, some cases with a negative cytopathology result were finally diagnosed as MPE on the basis of an integrated diagnosis, including histology, imaging tests and clinical impression of malignancy.

The term “paramalignant effusions” was reserved for those effusions thought to be related to an active primary neoplastic disease but without demonstration of malignant cells in PE or pleural tissue.

PE was considered “negative for malignancy” when morphology and the whole clinical information reasonably ruled out a neoplasm after a follow-up of at least 6 months. Particularly, in patients with a previous history of tumor, PE was classified as negative for malignancy when there was no data of tumor progression and the development of PE could be explained by a specific etiology such as: congestive heart failure, infection, surgery, renal or hepatic disease, and so on.

FCI results were either reported as “non-malignant”, “malignant”, or “suboptimal sample”. The results were then compared with the final diagnosis of PE established by clinicians. An overview of the design of the study is summarized in Fig. 1. All clinical procedures followed the standards set by guidelines of the latest Declaration of Helsinki.

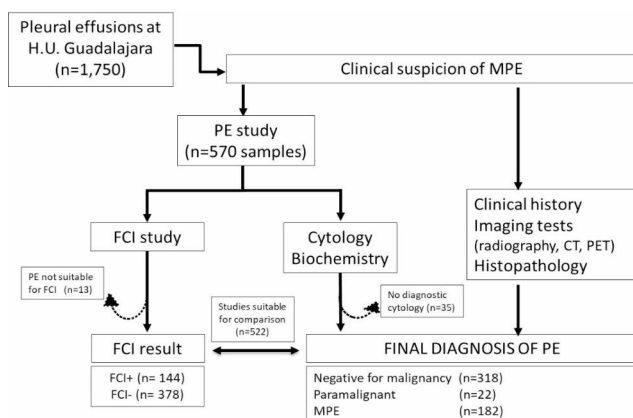


Fig. 1 Overview of the study design. PE: Pleural effusion; FCI: flow cytometry immunophenotyping; MPE: malignant pleural effusion; FCI+: PE classified as malignant by flow cytometry immunophenotyping; FCI-: PE classified as non-malignant by flow cytometry immunophenotyping. Numbers in parentheses refer to PE samples

FCI studies

Fresh PE samples were processed within 24 h from reception, and most within the first 3 h after collection. After cell count and centrifugation, fluid was removed to get a cell-rich sample. Whenever it was possible, the cell pellet was resuspended in phosphate buffered saline at a final concentration of $6\text{--}8 \times 10^6$ cells/200 microliters. For cases with low cellcount, all cells were stained to cover the acquisition of the largest possible number of events in the flow cytometer.

The most likely clinical diagnosis determined the panel of monoclonal antibodies (mAb) to be used. For most samples, the following 3-color “epithelial cell” tube was used: anti-EpCAM FITC (clone Ber-EP4, DAKO), anti-CD45 PerCP (clone 2D1, BDB), and anti-CD200 APC (clone OX104, e-Bioscience). For samples with suspicion of hematological malignancy, an 8-color combination of mAb was used [15]. In all cases, a direct immunofluorescence stain-and-then-lyse technique was applied [5].

Cells were acquired in a FACSCanto II flow cytometer (Becton Dickinson, San Jose, CA) using the FACSDiva software (Becton Dickinson). Two different types of calibration beads (BD FACSDiva™ CS&T IVD Beads, and 8-peak Rainbow beads, Spherotech, Lake Forest, IL), were daily used to set up the flow cytometer and to perform daily quality control. To minimize sample contamination, a careful cleaning of the fluidic system was performed prior to acquisition. The parameter “time” was used to discard problems during the acquisition due to clogs. To screen for epithelial malignancies, we acquired the whole sample volume or at least 5×10^6 events. For samples with suspicion of lymphoma, 1×10^6 events were acquired.

Data analysis was performed with the Infinicyt software program (Cytognos SL, Salamanca, Spain). Figure 2 summarizes the strategy used to identify epithelial cells. In brief, mesothelial cells were used as an internal negative control to set the cut-off for EpCAM and CD45 expression. Lymphocytes were used as an internal positive control of CD45. For detecting epithelial cells the gating strategy was focused on CD45^{neg} events where epithelial cells, if present, would overlap the CD45^{neg} expression of mesothelial cells. In hematological malignancies, FCI was set to search for abnormal patterns of antigen expression in leukocytes [16]. Neoplastic cells were referred to total cellularity (leukocytes and non-hematological cells) [5].

The limit of detection (LOD) and lower limit of quantification (LLOQ) defined the level of sensitivity of the FCI assay [17]. In brief, 30 clustered events were the cutoff to classify a rare population as detectable, and 50 events were the minimum value to define a population as quantifiable. Therefore, LOD and LLOQ were not fixed values and varied according to whole collected events. FCI qualified a

sample as malignant when the percentage of EpCAM⁺ cells or hematological malignant cells was above the LLOQ. All FCI results were blinded to cytopathologist analyses.

Statistics

Sensitivity, specificity, positive and negative predictive values of FCI were calculated for PE samples that integrated diagnosis classified as MPE or negative for malignancy; paramalignant cases were excluded. Relationships between categorical variables were performed using χ^2 test or Fisher’s exact test. Statistical analyses were carried out using GraphPad Prism Viewer 9.4.1 software. All p-values represented were two-sided and p-values < 0.05 were considered statistically significant.

Results

Study cohort and final diagnosis of PE

Based on clinical suspicion of malignancy, 570 out of the 1,750 PE samples (32.6%) collected in our hospital in this 7-year study were sent for FCI evaluation. Only samples with a proper FCI and cytopathology result were considered for analyses. Samples not suitable for comparison included those PE not sent for cytopathological analysis ($n = 18$), samples classified by cytopathology as “nondiagnostic” ($n = 9$) or “atypia of undetermined significance” [13] ($n = 8$), and suboptimal samples not adequate for FCI evaluation ($n = 13$) (Supplementary Information 1). At the end, the study included 522 out of the 570 samples (91.6%) and involved 467 patients. At the time of the study, no history of malignancy had been reported in most of them ($n = 285/467$; 61%) (Table 1).

Integrated diagnosis classified 318/522 samples (60.9%) as negative for malignancy, 22 as paramalignant (4.2%), and 182 as MPE (34.9%) (Fig. 1). Lymphoma was the only hematological malignancy causing MPE (24/182 cases; 13.2%), mesothelioma was involved in 12 MPE (6.6%), and epithelial cell neoplasms in 140 (76.9%) (Table 2). Lung and pleural tumors were responsible for most MPE (110 vs. 48 from other localizations), and for most new diagnosis of cancer ($n = 76$). Gastrointestinal tract tumors ($n = 14$) and breast adenocarcinoma ($n = 11$) were the most frequent non-lung tumors developing MPE (Table 3).

Correlation of FCI findings and definitive diagnosis

Median sample volume received for FCI study was 15 ml (IQR1:10; IQR3: 20), and median cell count was 1 cell/ 1×10^{-3} L (IQR1:0.4; IQR3: 2.7). FCI results were

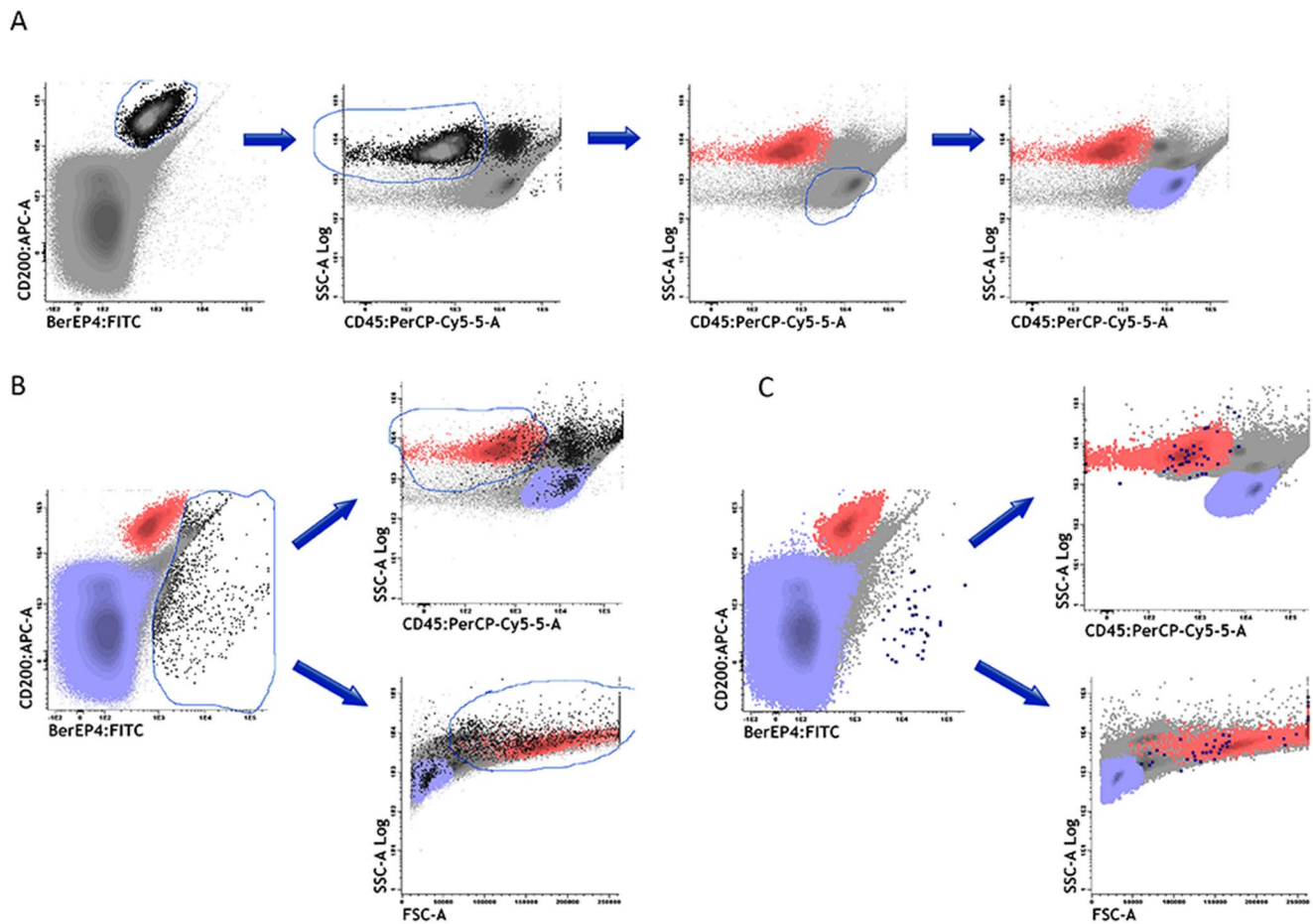


Fig. 2 FCI strategy used to identify epithelial cells. The analysis was performed in 2 steps. Panel A shows the sequential steps for the identification of mesothelial cells (MC, depicted in pink) and lymphocytes (depicted in violet). Both populations were used as internal negative controls for EpCAM expression. Panel B describes the sequential steps for definition of EpCAM⁺ cells using the monoclonal antibody anti-EpCAM FITC (clone Ber-EP4); after a wide selection of Ber-EP4 positive events, 2 additional gates were performed to include only CD45

negative cells. MC established the cutoff for any CD45 negative population, and lymphocytes were markers of positive CD45 expression. Finally, only those Ber-EP4 positive events with the same or higher forward and side scatter pattern than lymphocytes were selected. Panel C depicts the result of the whole analysis after discarding all the events that did not fulfill the requirements of a Ber-EP4 positive population. In this specific case, the epithelial cell population (depicted in blue) was negative for CD200 expression

reported independently of cytopathology, and median time between the reception of the PE and FCI reporting was 12 h. Besides, whenever FCI detected malignant cells, the result was reported to clinicians within 3 h after sample reception. Overall, FCI correctly diagnosed 415/467 patients (88.9%) and 456/522 samples (87.4%). Interestingly, FCI also identified 95.7% of MPE without a simultaneous cytological study and those classified by cytology as “acellular” (Supplementary Information 1).

Malignant FCI cases

FCI identified 144 samples as malignant (FCI+) and 141/144 (97.9%) were diagnosed as MPE (77.5% of all MPE). In contrast, 3 patients where FCI detected low percentages of EpCAM⁺ cells were finally considered as PE negative

for malignancy. One patient (0.04% EpCAM⁺ cells) was diagnosed with pulmonary tuberculosis. Another patient (0.014% EpCAM⁺ cells) presented an advanced alcoholic hepatitis with no data of malignancy in pleural biopsy. Interestingly, this patient was diagnosed of gastric adenocarcinoma without metastatic dissemination two months later. The third patient (0.011% EpCAM⁺ cells) was receiving therapy for a disseminated thyroid papillary cancer, and needed a pericardial drainage for a concomitant pericardial effusion. After a ten-month follow-up, neither PE nor progression of the disease was documented in this third patient.

For the remaining 141 FCI+ cases, the diagnosis of MPE was corroborated by other techniques. In most cases (n = 89), cytopathology detected malignant cells in the same pleural tap, and a second pleural tap was necessary to detect malignancy in 2 patients. Histopathology (n = 29), imaging

Table 1 Baseline characteristics of patients included in the study

	Patients	Samples
N	467	522
Gender (M : F)	301 : 166	
Median age (IQR1-IQR3)	72 years (61–82)	
N° of studies performed		
One	421	421
Two	40	80
More than two	6	21
Known history of cancer		
No	285	315
Yes	182	207
N° of previous malignancies		
Three*	1	1
Two	11	13
Two epithelial cell malignancies	8	9
Melanoma & epithelial cell malignancy	1	1
Hematological & epithelial cell malignancy	1	1
Pheochromocytoma & epithelial cell malignancy	1	2
One	170	193
Epithelial cell malignancy	139	150
Hematological disease	23	34
Melanoma	2	2
Thymic epithelial tumors	2	3
Sarcoma	3	3
Hepatocarcinoma	1	1

FCI: flow cytometry immunophenotyping; M: male; F: female; IQR: interquartile range

* Three simultaneous hematological neoplasms: Bowel B-NHL, chronic myelomonocytic leukemia and Mycosis Fungoides

tests (n = 11) or consistent clinical history (n = 6) confirmed FCI+ results. Finally, a low-grade lymphoma was detected in the remaining 4 samples from two patients.

Regarding diagnosis, FCI detected 20/24 lymphoma (83.3%), and 121/158 (76.6%) solid neoplasms, most of which (117/121; 96.7%) were epithelial cell neoplasms.

There was no association between FCI+ cases, previous history of cancer or primary tumor localization (Table 3). Of note, 29/52 MPE (55.8%) which were only detected by FCI were new diagnosis of cancer; 4 were NHL and 20/25 solid tumors were located in the lung (Table 3). FCI detected malignant cells in all histological lung subtypes, and statistically significant differences were observed among them (Fig. 3). As expected mesothelioma was the tumor with a fewer number of FCI+ cases, followed by squamous cell carcinoma (SSC). Median percentage of EpCAM+ cells detected by FCI was 0.23% (IQR1: 0.031%- IQR3: 3.75%). In 21/117 samples (18%) the percentage of EpCAM+ cells was ≤ 0.01%. As shown in Fig. 4, the intensity of EpCAM expression was heterogeneous among histological subtypes and also within individual tumors.

Median percentage of lymphoma in MPE was 6.5% (IQR1: 0.2%- IQR3: 63.1%), and high-grade lymphoma presented the highest percentage of infiltration (median 38.5%; IQR1: 3.3%- IQR3: 78.3%). These patients started therapy after diagnosis compared to only 6/8 patients diagnosed with low-grade lymphoma.

Non-malignant FCI cases

FCI classified 378 samples as non-malignant (FCI-): 22 were finally classified as paramalignant, and 315 (83.3%) correlated with the clinical category of “PE negative for malignancy”. One hundred and five samples (33.3%) were from 88 patients with previous history of cancer. The remaining 41 FCI- cases, which belonged to 38 patients, were finally classified as MPE. An explanation for FCI- results could be obtained in 18/38 patients (47.4%): histopathology identified 1 synovial sarcoma and 10 mesothelioma, 3 patients had a percentage of EpCAM+ cells below the LOD, 1 clear renal carcinoma presented a negative expression of EpCAM according to histopathology, and 2 MPE secondary

Table 2 Diagnostic yield of FCI and cytopathology in samples diagnosed as MPE secondary to different tumors

	FCI+ (n = 141)		FCI- (n = 41)		P
	FCI+/Cy+ (48.9%)	FCI+/Cy- (28.6%)	FCI-/Cy+ (2.7%)	FCI-/Cy- (19.8%)	
Epithelial cell tumors	140	80	37	22	< 0.001
Mesothelioma	12	0	1 ^a	8	1
Other tumors	6	2 ^b	1 ^a	3	0.39
Non-Hodgkin lymphoma (NHL)	24	7	13	3	1
High-grade NHL	12	6	2	3	0.22
Low-grade NHL	12	1	11	0	1
Total	182	89	52	36	< 0.001

^aOne solitary fibrous tumor and one mesothelioma presented low EpCAM expression

^bFCI detected 0.06% and 4% of CD45-/EpCAM-/CD200- small size cells in 2 patients with history of melanoma

FCI+: PE classified as “malignant” by flow cytometry immunophenotyping

FCI-: PE classified as “non-malignant” by flow cytometry immunophenotyping

Cy+: PE classified as “malignant” by cytology

Cy-: PE classified as “non-malignant” by cytology

Table 3 Characteristics of samples classified as MPE secondary to solid tumors

Tumor localization	No previous history of cancer			Previous history of cancer			TOTAL (samples)	TOTAL (%)
	FCI+	FCI-	TOTAL	FCI+	FCI-	TOTAL		
Lung	53	11	64	29	4	33	97	61.4
Adenocarcinoma	33‡	1	34	15†		15	49	31.0
Squamous cell carcinoma	3‡	4	7	4	3†	7	14	8.9
Non-small cell cancer not otherwise specified	5‡	1	6	2†		2	8	5.1
Small cell carcinoma (NET)	5‡	2	7	6††		6	13	8.2
Non-small cell NET				1†		1	1	0.6
Carcinoid tumor				1		1	1	0.6
No histopathology	5	2	7				7	4.4
Undifferentiated tumor	1		1		1	1	2	1.3
Carcinomatoid sarcoma/synovial sarcoma	1	1	2				2	1.3
Pleura	2	10	12		1	1	13	8.2
Mesothelioma	1‡	10	11		1†	1	12	7.6
Solitary fibrous tumor	1‡						1	0.6
Other tumor localizations	11	2	13	26	9	35	48	30.4
Thyroid				2		2	2	1.3
Thymoma					2	2	2	1.3
Breast cancer				10	1	11	11	7.0
Unknown origin	2		2		1	1	3	1.9
Melanoma				2		2	2	1.3
Mouth/Gastric/esophagus/bowel adenocarcinoma	2‡	1	3	8	3	11	14	8.9
Pancreatic/Gallbladder/ampulla of Vater cancer	3‡		3		1	1	4	2.5
Gynecologic cancer	4‡		4	1		1	5	3.2
Kidney cancer		1	1		1	1	2	1.3
Prostate/urothelial adenocarcinoma				3*		3	3	1.9
TOTAL	66	23	89	55	14	69	158	

MPE: malignant pleural effusion

FCI+: PE classified as “malignant” by flow cytometry immunophenotyping;

FCI-: PE classified as “non-malignant” by flow cytometry immunophenotyping;

NET: neuroendocrine tumor

* 1 patient simultaneously presented a prostate tumor and an urothelial tumor

† Number of patients who had a previous history of neoplasm and presented a MPE secondary to a different tumor

‡ New diagnosis of cancer only detected by FCI (FCI+/Cy-) included: 5 lung adenocarcinoma, 7 non-small cell cancer not otherwise specified, 3 squamous cell carcinoma, 3 small cell cancer, 1 mesothelioma, 1 solitary fibrous tumor, 1 gastric adenocarcinoma, 2 pancreatic cancer, 1 gallbladder cancer and 1 ovarian carcinoma

to diffuse large B-cell lymphoma lacked B-cells. These last two patients were finally diagnosed by pleural biopsy and imaging techniques. Another patient which was initially evaluated to discard epithelial cell neoplasms was finally diagnosed with primary effusion lymphoma (PEL). Seventeen days later a second FCI study focused on lymphocytes detected lymphoma. In the remaining 21 patients, the diagnosis of MPE was established on the basis of biopsy (n = 10) or PET/TC images (n = 7), but 4 cases presented a very bad

performance and clinicians concluded progression of thymoma (n = 1) and high suspicion of malignancy (n = 3).

Correlation of FCI and cytology findings

Since FCI and cytopathology were the primary tools to evaluate PE, we aimed to compare both techniques. FCI results significantly correlated with morphological results diagnosis: 462/522 studies (88.5%) were concordant, while 60/522 (11.5%) were discordant ($p < 0.0001$). MPE was

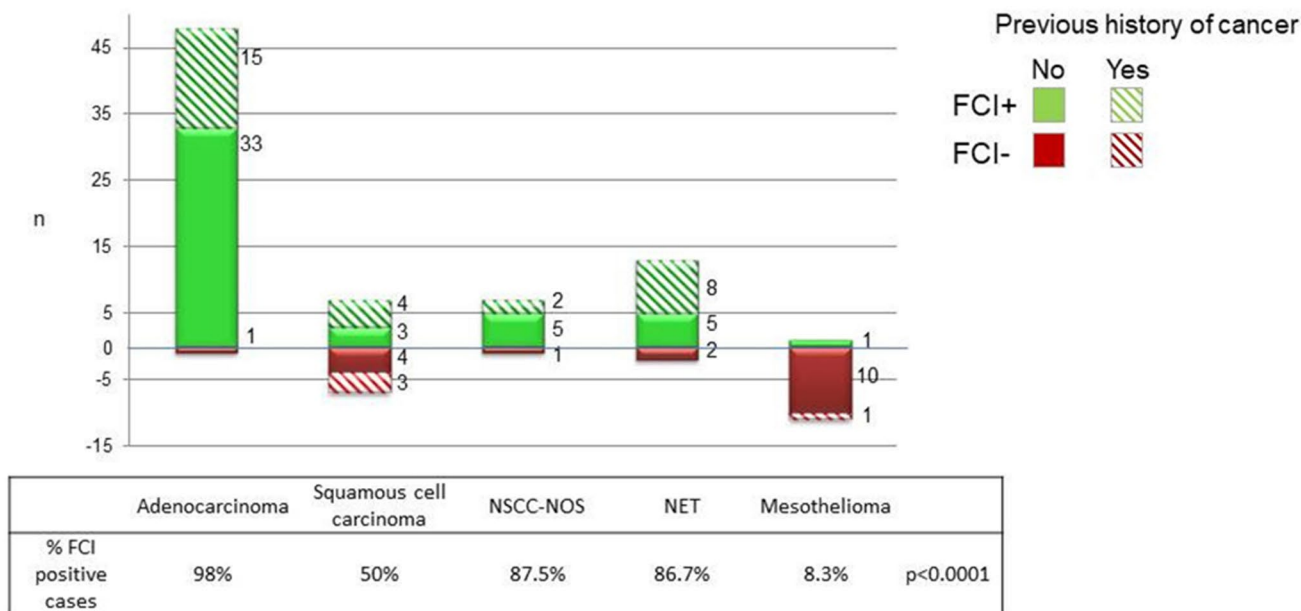
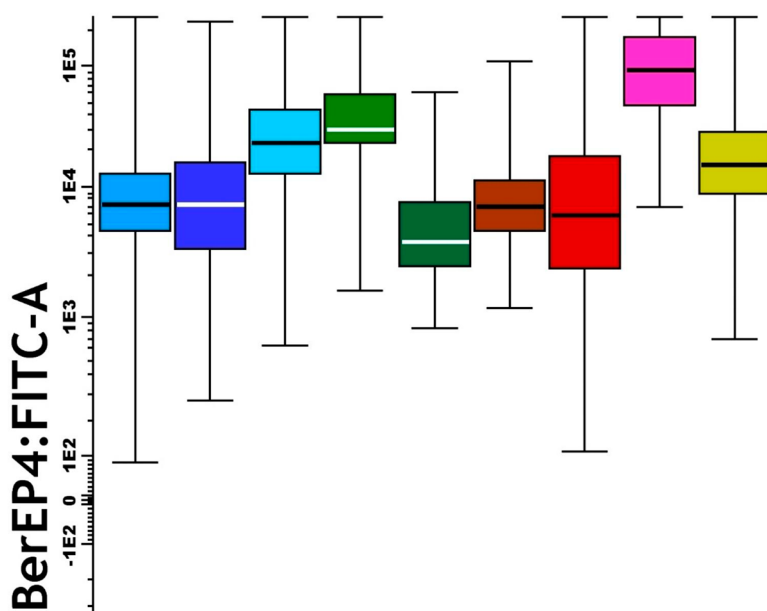


Fig. 3 Description of FCI results in MPE secondary to mesothelioma and different histological lung subtypes. Green boxes reflect MPE detected by FCI (FCI+). Negative data (depicted in red) represent the number of cases not detected by FCI (FCI-). Dashed bars include cases

with a known previous history of cancer. NSCC-NOS: non-small cell cancer not otherwise specified. NET: neuroendocrine tumors included 13 small-cell carcinoma, 1 non-small cell NET and 1 atypical carcinoid tumor

Fig. 4 Examples of EpCAM expression in different tumor subtypes plotted as median fluorescence intensity with boxplots. The line inside the box is the median value, the upper and lower box limits show the 75th and 25th percentiles, and whiskers indicate the minimum and maximal level of expression. Examples of 3 lung adenocarcinoma (boxplots 1–3 depicted in blue), 2 neuroendocrine tumors (boxplots 4–5, depicted in green), 1 squamous cell carcinoma (boxplot 6 depicted in brown), 1 gastric adenocarcinoma (boxplot 7 depicted in red), 1 breast adenocarcinoma (boxplot 8 depicted in pink), and 1 bowel carcinoma (boxplot 9 depicted in yellow)



identified by both techniques in 89/182 (48.9%) cases, but neither cytology nor FCI detected 36/182 (19.8%) MPE (Table 2). As detailed in Table 4, the diagnostic yield of the FCI study on its own was high, but when cytology and FCI were considered together, the sensitivity and negative predictive value of the PE study increased to 80% and 90%, respectively.

In an attempt to explain the differences between the FCI and cytopathology findings, we focused on epithelial cell

tumors, and compared MPE where both FCI and cytopathology were positive for malignancy ($n=80$) and MPE only detected by FCI ($n=37$) (Table 2; Fig. 5). There was no association with sample volume, cell count or median LOD and LLOQ, but statistically significant differences were observed in the median percentage of EpCAM⁺ cells detected by FCI in cytopathology-positive MPE (1%) and cytopathology-negative MPE (0.02%) $p < 0.001$. Interestingly, a significant higher number of MPE with $\leq 0.01\%$

Table 4 FCI diagnostic yield of PE samples, alone or in combination with cytopathology

	All neoplasias		Non-hematological malignancies		Hematological malignancies	
	FCI	FCI & Cytopathology	FCI	FCI & Cytopathology	FCI	FCI & Cytopathology
Sensitivity	77	80	77	79	83	88
Specificity	99	99	99	99	100	100
PPV	98	98	98	98	100	100
NPV	88	90	89	90	83	86

FCI: flow cytometry immunophenotyping; PPV: positive predictive value; NPV: negative predictive value

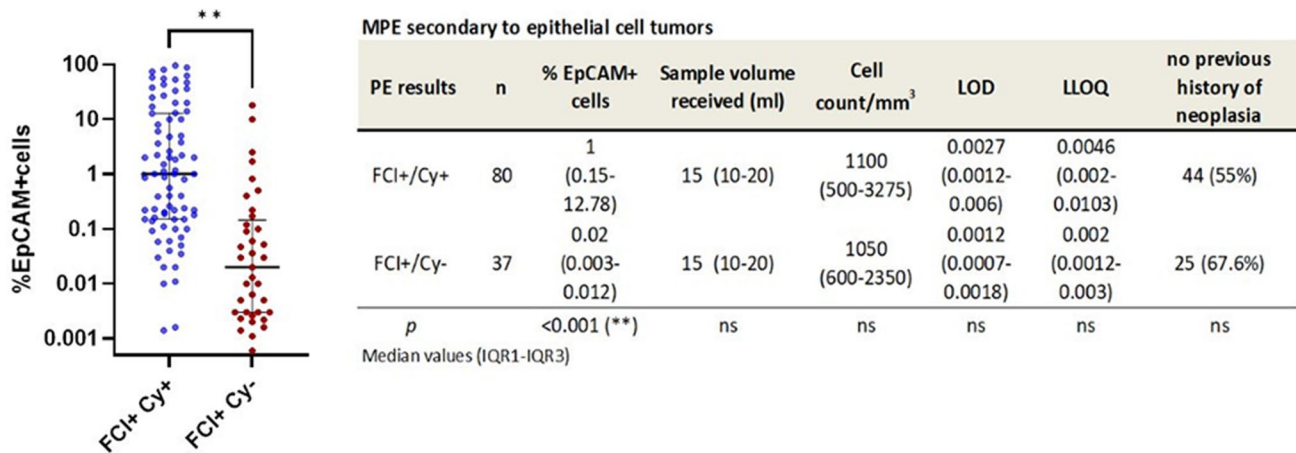


Fig. 5 Percentages of EpCAM⁺ cells detected by FCI in MPE samples classified as positive or negative for malignancy by cytopathology. The top and bottom lines represent the 75th and 25th percentiles, and the line in the middle represents the median value. FCI+: PE clas-

sified as “malignant” by flow cytometry immunophenotyping. Cy+: PE classified as “malignant” by cytology. Cy-: PE classified as “non-malignant” by cytology. LOD: limit of detection; LLOQ: lower limit of quantification. ns: statistically non-significant

EpCAM⁺ cells was found in the group of cases not detected by cytopathology (17/37 (46%) compared to the cytopathology positive group (4/80; 5%) ($p < 0.001$).

Discussion

Cytopathology plays a central role in the diagnosis of MPE, but a diagnosis is delayed when malignant cells are not detected in PE [1]. In line with previous studies [8, 18, 5] our large series confirms that by using FCI, the chances of detecting MPE in a single tap increases by 28.6%. The targets of this FCI approach are lymphoma and epithelial cell malignancies, but as described in one patient in our series, unusual mesotheliomas may show weak EpCAM expression detected by histology [19, 20] or FCI [5, 6]. A high level of expression of CD200 and CD81, expression of CD73, and absence of CD45 and EpCAM are normal features of mesothelial cells. A number of additional molecules already used by immunohistochemistry [11, 19, 21, 22] particularly, CD146 [23, 24] and desmin [8], might help FCI to discriminate between reactive mesothelial cells and mesothelioma.

There are two main advantages of using FCI to evaluate PE samples suspicious of malignancy. According to our study, a median PE volume of 15 ml is enough to get reliable

FCI results, which is lower than the recommended 50–75 ml for proper cytological evaluation [13]. The present finding would support considering FCI results in PE samples classified by cytopathologists as “non-diagnostic” [7, 8]. The second advantage is that the high sensitive FCI protocol reproduces the strategy to quantify measurable residual disease in hematological malignancies [25], and rescues MPE not detected by cytopathology. By staining and then analyzing a high number of cells, FCI may reach detection levels down to 1 malignant cell per 100,000 total cells. While other authors applied different cutoff levels of BerEP4 expression to separate benign from malignant effusions [7, 9], this high sensitive protocol would solve the limitation of cytology when malignant cells are scarce. Indeed, differences in the median number of EpCAM⁺ cells in morphological positive or negative MPE reveal that FCI can discover MPE with EpCAM⁺ cells in the range of 0.01%. Using a different technology, Thompson *et al* reported similar findings in PE samples from NSCLC patients [26].

From a practical point of view, FCI worked as a screening test and early detection of FCI+ cases accelerated the diagnostic pathway of unsuspected MPE. On the other side, high-sensitivity FCI may detect EpCAM⁺ cells in specific clinical situations not associated with malignancy. The cell-adhesion molecule EpCAM describes epithelial cells,

but the category of “malignant epithelial cells” depends on where they are detected. Serous cavity fluids lack epithelial cells, but disruption of epithelial integrity as seen in recent surgeries [9], inflammatory diseases, pneumonia, tuberculosis [10] or pericardial drainage, might release normal airway epithelial cells into PE. Benign epithelial cells have also been described in peritoneal washings from patients with advanced cirrhosis [11] and this might be the case of our patient diagnosed with an alcoholic hepatitis. Indolent clonal B-cell populations without clinical relevance can also be found in PE. These low percentages of clonal lymphocytes may be difficult to distinguish from MPE minimally infiltrated by low-grade NHL. An extended FCI study should help because clonal B-cells with immunophenotype of chronic lymphocytic leukemia or marginal-zone lymphoma may develop in patients with no need of further therapy, while identification of follicular lymphoma supports MPE.

Clinicians play an important role in the success of this approach because they choose which FCI study should be performed. Samples initially triaged to discard epithelial cell neoplasms and finally diagnosed with high-grade lymphoma explain some of our FCI-/Cy+ results. High sensitive FCI relies on the analysis of a very high number of cells, so no sample will be left to expand a lymphocytic study if finally needed. Some authors prefer to evaluate both malignancies in a single tube [10], but we separated them based on the low frequency of MPE secondary to lymphoma and the predominance of lymphocytes in many reactive PE [3, 27, 28]. Furthermore, the use of a 3-color epithelial tube [5, 7] while keeping costs low, also helps limiting background interference.

Using a single target of epithelial cells implies that tumors with weak or absent EpCAM expression, such as poorly differentiated tumors or epithelial-mesenchymal transition [29], might escape FCI detection. To improve the efficiency to detect frequent EpCAM-negative tumors such as SSC [30], FCI might include reagents directed against other epithelial cell antigens [31]. As previously described [4, 10] including CD56 (neural cell adhesion molecule) facilitates the identification of neuroendocrine tumors, and potential therapeutic targets, such as that recently described for PDL-1, could also be considered for the next FCI panels [32]. A recent FCI report includes a description of a design for an 8-color antibody panel to identify pediatric solid tumors [33]. Combining similar strategies with the high-sensitivity FCI approach would not only help to detect MPE, but also to locate its primary origin.

Conclusions

Clinical implementation of high sensitivity FCI as part of the diagnostic work-up of PE with suspicion of malignancy significantly increases the diagnostic yield of MPE. Even clinical laboratories with limited resources can easily perform the 3-color epithelial cell panel. By using FCI as a screening technique, implies that the early detection of FCI+ cases accelerates the diagnostic pathway of unsuspected MPE.

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Authors' contributions Dolores Subirá designed and conducted the research, analyzed and interpreted data, wrote the manuscript, and critically revised it. Jesús Fernández, Jorge Castelao and Luis Chara were responsible for clinical supervision. Fabiola Barriopedro and Ruth Martínez provided technical assistance and Fabiola Barriopedro collected data. Eugenia García supervised pathological data. The manuscript was written by Dolores Subirá. All authors read and approved the final manuscript.

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