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PLEURAL DISEASE

Cytotoxic Natural Killer Subpopulations as a Prognostic Factor of Malignant Pleural Effusion

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Abstract

Background Malignant pleural effusion (MPE) is a sign of advanced disease of poor prognosis. As natural killer (NK) cells are involved in the first line of tumour defence, we aimed to validate a new diagnostic and prognostic indicator for MPE based on NK subpopulations of pleural fluid (PF) and peripheral blood (PB).

Methods NK subpopulations were determined in PF and PB in 71 patients with malignant, paramalignant or benign pleural effusion. The receiver operating characteristic (ROC) curves, Kaplan–Meier, multivariable Cox model and decision trees created with the CHAID (Chi-square automatic interaction detector) methodology were employed.

Results We demonstrated that the PF/PB ratios of the CD56 bright CD16− and CD56 dim CD16− NK subpopulations were higher ($p=0.013$ and $p=0.003$, respectively) in MPEs and paramalignant pleural effusions (PPEs) than in benign ones, with an AUC of 0.757 and 0.741, respectively. The PF/PB ratio of CD16+ NK and CD57+ NK obtained a higher hazard ratio (HR) in the crude Cox's regression analysis. In the adjusted Cox's regression analysis, the PF/PB ratio of CD16+ NK gave the highest HR (HR 6.1 [1.76–21.1]) ($p=0.004$). In the decision tree created for the MPE prognosis, we observed that the main predictor variable among the studied clinical, radiological, and analytical variables was lung mass, and that 92.9% of the patients who survived had a PF/PB ratio of the CD56 dim CD16+ NK subpopulation ≤ 0.43.

Conclusions Our data suggest that both the PF/PB ratios of cytotoxic subpopulations CD57+ NK and CD16+ NK are useful as a prognostic factor of MPE. Other subpopulations (CD56 bright CD16− and CD56 dim CD16− NK) could help to diagnose MPE.

Keywords Biomarker · Cancer · Diagnosis · Flow cytometry · Natural killer cells · Pleural effusion · Prognosis

Abbreviations

MPE	Malignant pleural effusion
NK	Natural killer
PF	Pleural fluid
PB	Peripheral blood
ROC	Receiver operating characteristic

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CHAID	Chi-square automatic interaction detector
CT	Computed tomography
PPE	Paramalignant pleural effusion
CEIC	Committee of Ethics and Clinical Trials
PBS	Phosphate-buffered saline
SD	Standard deviation
AUC	ROC area under the curve
CI	Confidence intervals
BPE	Benign pleural effusion
HR	Hazard ratio

Background

Malignant pleural effusion (MPE) represents 15–35% of all pleural effusions [1]. Its appearance is a sign of advanced disease as it implies that the pleural cavity is affected by this malignant process, and its prognosis is poor [2]. MPEs are characterised by a high percentage of mononuclear cells involved in defence mechanisms, where natural killer (NK) cells are one of the main components that participate in tumour defence [3]. Nevertheless, total NK (CD3− CD56+) quantification performed in pleural effusions in former studies [4–8] has led to contradictory results. The literature contains no data on the prognostic value of NK cells in MPEs, nor has an association between NK subtype and survival been established.

Based on the functions performed by NK, subpopulations have been identified according to the intensity of the expression of surface antigens CD56 and CD16. Given their good capacity for producing proinflammatory and anti-inflammatory cytokines [9], CD56 bright NK cells are regulators, while CD56 dim NK cells are cytotoxic due to their high lytic activity. They are efficient mediators of antibody-dependent cell cytotoxicity if they are also characterised by a high CD16 expression. CD57 expression, considered a marker of NK cells maturity [10], has been correlated with both CD56 dim NK and a higher CD16 expression [11], and, therefore, identifies cells with a high cytotoxic potential [12]. Nowadays, information is limited on the phenotypes and functions of the NK cells present in the MPEs of patients with primary lung tumours and tumours with lung or pleural metastases of distinct origins. In theory, as neoplastic cells are present in pleural fluid (PF) or tissue, cytotoxic activity should increase in MPEs compared to PF of other aetiologies.

Given all of this, the hypotheses posed for study in this work were the following:

- The determination of NK subpopulations, especially those with a cytotoxic function (CD56 dim CD16− NK, CD56 dim CD16+ NK, CD16+ NK and CD57+ NK) in pleural effusions, could help to establish both the neoplastic nature of the effusion and the prognosis of MPE patients.
- As the analysis of PF is done in relation to peripheral blood (PB), we aimed to evaluate whether determining the PF/PB ratio of NK populations, and not only isolated NK populations in PF or PB, could help provide the diagnosis and prognosis of MPE.

Methods

Subjects

This prospective observational cohort study conducted in two recruitment years (2013–2015) included 73 patients who presented pleural effusion of unknown aetiology. The final sample included 71 patients because two patients were excluded as no cellularity was obtained in PF.

After diagnosing pleural effusion by a chest X-ray and obtaining signed consent, the following were obtained: medical history, physical examination and initial radiological findings, such as images suggestive of malignancy in a chest computed tomography (CT). A thoracocentesis was done with all the patients before starting any treatment type. On the same day, PB was drawn. In both samples, NK and NK subpopulations were separately quantified as percentages by flow cytometry.

These patients were classified into three different groups according to their pleural effusion diagnosis: malignant, paramalignant and benign.

- *MPE* was diagnosed if the presence of tumour cells in the pleural cavity [13] was confirmed by a cytological study of PF, or in pleural tissue obtained by blind pleural biopsy, thoracoscopy or thoracotomy.
- *Paramalignant pleural effusion (PPE)* [14] is pleural effusion that is thought to be due to a tumour process, but with no demonstrated pleural infiltration by the tumour, nor any tumour cells detected in PF or pleural tissue.
- Pleural effusion is considered *benign* if the tumour aetiology for PF has been reasonably ruled out by imaging techniques/previous examinations/medical history and patient follow-up.

Patients were followed up and their clinical status tracked until their death or the end of the study period.

All the participating patients received written information about the nature and purposes of the study, and gave informed consent. Ethical aspects were approved on 29 February 2012 by the Committee of Ethics and Clinical Trials (CEIC) of the Dr. Peset University Hospital in Valencia (Spain), with CEIC code: 10/12. All the patients who offered to participate were accepted.

Measuring Natural Killer Cells

In both PF and PB, 100 µl were incubated with 10 µl of the following monoclonal antibodies: CD45, CD19, CD3, CD56, CD16 and CD57, for 30 min, and 0.5 ml of Opti-Lyse® and 2 ml of phosphate-buffered saline (PBS) were added. This mixture was centrifuged at 300×*g* (~ 1600 r.p.m.) in a Microcen 21®, supernatants were decanted, and cells were resuspended in 1 ml of PBS and were placed inside a Navios® flow cytometer (Beckman-Coulter). A blind analysis of the diagnosis was run with the Kaluza 1.3 software (Beckman-Coulter). The sensitivity of the technique was 10^{-2}–10^{-3}. After studying the expression of CD45 first, NK cells (CD3− CD56+) were studied compared to the 100% total lymphocytes. According to the intensity of the expression of antigens CD56 and CD16, the following subpopulations were differentiated: CD56 bright (++) CD16− NK, CD56 bright (++) CD16+ NK, CD56 dim (+) CD16− NK, CD56 dim (+) CD16+ NK and CD16+ (CD56+/++ CD16+) NK. CD57+ (CD56+/++ CD57+) NK were also determined and the percentage quantification of all the NK subpopulations was done compared to the percentage of the total NK cells.

Statistical Analysis

The parametric continuums variables are expressed as mean ± standard deviation (SD) and the non-parametric continuums as medians (minimum and maximum). The percentages of the NK and NK subpopulations and their PF/PB ratios were compared using the Student's *t* test or the Mann–Whitney *U* test. A one-way ANOVA or the Kruskal–Wallis test was applied when comparing more than two groups. A Chi-square test was used for the qualitative variables expressed as absolute values and percentages. The diagnostic efficacy of the PF/PB ratio, with differences to discriminate between malignant–paramalignant and benign pleural effusions (BPEs), was determined by a receiver operating characteristic (ROC) curve analysis with ROC area under the curve (AUC). The Wilcoxon signed-rank test or the Student's *t* test for the paired samples was used to see if there were any significant differences between the percentages obtained for the NK and NK subpopulations present in PF and PB. Kaplan–Meier was employed in a survival analysis, and the log-rank test was run to study the survival difference between groups. Cox's regression model was used to identify the MPE patients' prognostic factors. A crude analysis, for instance, for each factor without adjusting, of the NK and NK subpopulations present in PF and PB, and also of the PF/PB ratio, was carried out. Next by following a step-by-step methodology, in which the variables were selected by sequential exclusion, the hazard ratios (HR) were calculated with the 95% confidence intervals (CI) by an adjusted Cox's regression analysis. A decision tree by the CHAID (Chi-square automatic interaction detector) method was employed to study the implication of the clinical, radiological and analytical variables in the MPE prognosis. The CHAID algorithm builds a decision tree by the repeated partitions of each subset into two child nodes or more, beginning with the full database [15]. To determine the best split in each node, the categories of each predictor were merged into pairs until statistically significant differences were no longer observed within each component of the pair compared to the target variable. Thus, the algorithm not only identified the main interactions, but also built the subgroups defined by the different sets of independent variables. In short, CHAID allows interactions to be automatically detected by Chi square by selecting the independent (predictor) variable in each step that presents the strongest interaction with the dependent variable. The procedure automatically excludes any variable whose contribution to the final model is not significant, and a decision tree is obtained. A p value < 0.05 was considered significant with a 95% CI. The IBM SPSS Statistics statistical package for Windows (version 21.0. Armonk, New York: IBM Corp., USA) was employed.

Results

Demographics

This study analysed 71 patients who presented pleural effusion of unknown aetiology at the Dr. Peset University Hospital in Valencia (Spain) from 2013 to 2015. Three groups were formed according to the final pleural effusion diagnosis made: malignant ($n = 31$; 43.7%), paramalignant ($n = 15$; 21.1%) and benign ($n = 25$; 35.2%). The mean age of the study population was 69.1 years, and no inter-group differences were observed. The male gender clearly predominated between the malignant and paramalignant effusion cases. The most frequent primary tumour location was the lung, regardless of whether pleural effusion had been classified as malignant (51.6%) or paramalignant (73.3%). Adenocarcinoma was the most frequently found histology among the MPEs (Table 1). Table 2 offers the biochemical and cellular characteristics of the studied effusions.

Distribution of the NK and NK Subpopulations in Pleural Fluid and Peripheral Blood

The percentage of NK was higher in PB than in PF in all the groups. For the NK subpopulations, we observed that CD56 bright NK as a whole and the NK subpopulation CD56 dim CD16−, predominated in PF in all the groups, while the presence of the other subpopulations was greater in PB.

Table 1 Characteristics of the patients with malignant, paramalignant and benign pleural effusions

	Malignant ($n=31$)		Paramalignant ($n=15$)		Benign ($n=25$)		p Value
Age (years)	69.2 ± 8.9		69.8 ± 11.1		68.7 ± 12.2		0.949
95% CI	65.9–72.4		63.6–76		63.6–73.7		
Gender							0.133
Male	19 (61.3%)		12 (80%)		12 (48%)		
Female	12 (38.7%)		3 (20%)		13 (52%)		
Origin	Lung	15 (48%)	Lung	11 (73.3%)			
	Lung and breast	1 (3.2%)	Breast	1 (6.7%)			
	Lymphoma	4 (13%)	Ovary	1 (6.7%)			
	Mesothelioma	2 (6.5%)	Pancreas	1 (6.7%)			
	Breast	2 (6.5%)	Lymphoma	1 (6.7%)			
	Ovary	2 (6.5%)					
	Peritoneal	1 (3.2%)					
	Pancreas	1 (3.2%)					
	Unknown	3 (9.7%)					
Diagnosis	Adenocarcinoma	22 (71%)			Non-specific	12 (48%)	
	Lymphoma	4 (13%)			CHF	4 (16%)	
	Mesothelioma	2 (6.5%)			Infectious	3 (12%)	
	Epidermoid	1 (3.2%)			TBC	2 (8%)	
	Microcytic	1 (3.2%)			Exp. to asbestos	2 (8%)	
	Myxoid sarcoma	1 (3.2%)			Cirrhosis	1 (4%)	
					RA	1 (4%)	

Data expressed in absolute values and percentages or mean ± SD. Chi-square test or ANOVA

CI confidence interval, *CHF* congestive heart failure, *TBC* tuberculosis, *Exp*. exposure, *RA* rheumatoid arthritis

Table 2 Biochemical and cellular characteristics of the studied effusions

	Malignant ($n=31$)	Paramalignant ($n=15$)	Benign ($n=25$)	p Value
pH	7.31 (6.42–7.48)***	7.41 (7.23–7.48)***	7.36 (6.35–7.50)	0.105
Pleural glucose (mg/dl)	91 ± 41.4*	128.2 ± 28.7*	114.2 ± 53.8	0.021*
Pleural LDH (UI/l)	346 (108–6270)	274 (119–1045)	214 (90–10225)	0.628
Proteins (g/dl)	4.2 (2.5–5.2)	4.2 (2.6–5.9)	3.7 (1.2–5.8)	0.194
Albumin (g/dl)	2.4 ± 0.6	2.2 ± 0.4	1.9 ± 0.6	0.018*
ADA (UI/l)	21.6 (5–133)	22.5 (13–140)	19 (10–188)	0.584
Leukocytes (× 10e9/l)	1.8 (0.3–86.7)	0.9 (0–4.8)	1.6 (0.1–90.1)	0.629
Lymphocytes %	47.9 ± 28.3	59.1 ± 23.2	53.4 ± 23	0.381
Neutrophils %	52.1 ± 28.3	40.9 ± 23.2	45.4 ± 22.7	0.352
Hematocrit %	0.4 (0–3.1)	0.2 (0–3.4)	0.3 (0–2.8)	0.436

Data expressed in absolute values and percentages, mean ± SD or median (minimum–maximum). Chi-square test, ANOVA or Kruskal–Wallis test

LDH lactate dehydrogenase, *ADA* adenosine deaminase

*$p < 0.05$;**$p < 0.01$;***$p < 0.001$

The PF/PB Ratio of the NK and NK Subpopulations in the Study Populations

We found neither inter-group differences, nor differences between malignant and benign, in any of the cells studied in PF. However, the PF/PB ratio of the CD56 bright CD16− and CD56 dim CD16− NK subpopulations were higher ($p = 0.013$ and $p = 0.003$, respectively) in MPEs and PPEs than in the benign ones (Table 3). When we analysed MPEs and PPEs together, and compared to the

Table 3 The PF/PB ratio of NK and NK subpopulations in the different patient groups

PF/PB ratio	Malignant ($n=31$)	Paramalignant ($n=15$)	Benign ($n=25$)	p Value
NK	0.3 (0–5.3)	0.4 (0.1–1.6)	0.7 (0–11.1)	0.502
95% CI	0.3–1.1	0.4–0.9	0.4–2.4	
CD56 bright NK	9 (1.7–193)	9.8 (1.2–38.5)	5 (0–144)	0.296
95% CI	11.4–48.2	5.8–20.9	0–38.6	
CD56 bright CD16−	17.5 (2.8–359.6)	19.4 (2.2–62.2)	4.9 (0–49.9)	0.013*
95% CI	8–68.7	11.1–34.1	3.2–18.5	
CD56 bright CD16+	3.3 (0.8–49)	3.8 (0.4–16.7)	5.5 (0.4–123)	0.836
95% CI	3.4–18.8	1.6–8.5	0–48.2	
CD56 dim NK	0.9 (0.4–1.2)	0.9 (0.5–1)	0.9 (0.4–1.1)	0.540
95% CI	0.8–0.9	0.8–0.9	0.8–0.9	
CD56 dim CD16−	4.7 (0.4–82.6)	6.1 (1.4–64.6)	2.2 (0.2–9.6)	0.003**
95% CI	4.3–16.5	4.3–26.2	1.6–3.6	
CD56 dim CD16+	0.5 (0–1)	0.4 (0.1–0.9)	0.7 (0–6.5)	0.100
95% CI	0.4–0.6	0.3–0.6	0.4–1.7	
CD16+ NK	0.6 ± 0.3	0.5 ± 0.3	0.7 ± 0.4	0.165
95% CI	0.5–0.7	0.3–0.7	0.5–0.9	
CD57+ NK	0.4 ± 0.3	0.3 ± 0.2	0.4 ± 0.4	0.220
95% CI	0.3–0.5	0.1–0.4	0.3–0.6	

Percentage data expressed as mean ± SD or median (minimum–maximum). ANOVA or Kruskal–Wallis test

NK natural killer, *CI* confidence interval

*$p<0.05$;**$p<0.01$

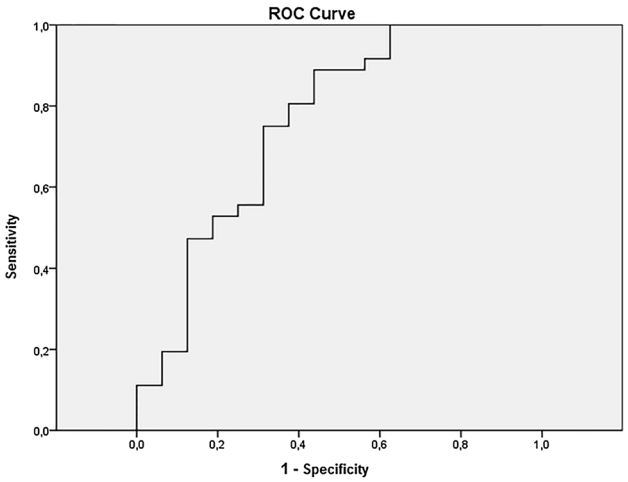

Fig. 1 ROC curve of the PF/PB ratio of the CD56 bright CD16− NK subpopulation to differentiate malignant and paramalignant pleural effusions from benign ones

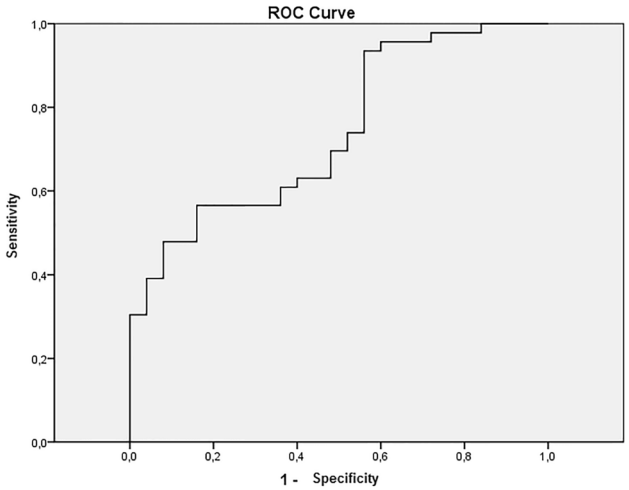

Fig. 2 ROC curve of the PF/PB ratio of the CD56 dim CD16− NK subpopulation to differentiate malignant and paramalignant pleural effusions from benign ones

benign cases, we still observed the same statistically significant differences as in the analysis done per groups. The PF/PB ratio of the CD56 bright CD16− NK subpopulation had an AUC of 0.757 to discriminate a BPE from a malignant–paramalignant one (95% CI 0.602–0.912; $p=0.003$). If the cut-off point was 7.38%, then sensitivity would be 81% and specificity would be 62% (Fig. 1). The PF/PB ratio of the CD56 dim CD16− NK subpopulation was determined to differentiate a malignant–paramalignant pleural effusion from a benign one, where the AUC was 0.741 (95% CI 0.624–0.858; $p=0.001$). If the cut-off point was 1.20%, sensitivity would be 94% and specificity would be 44% (Fig. 2).

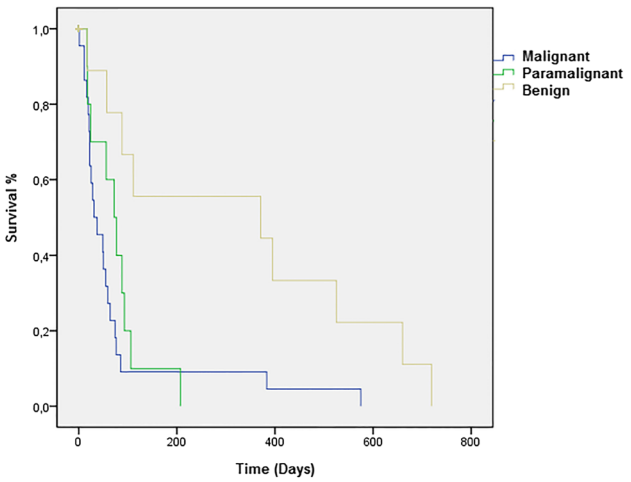

Fig. 3 Kaplan–Meier survival curve

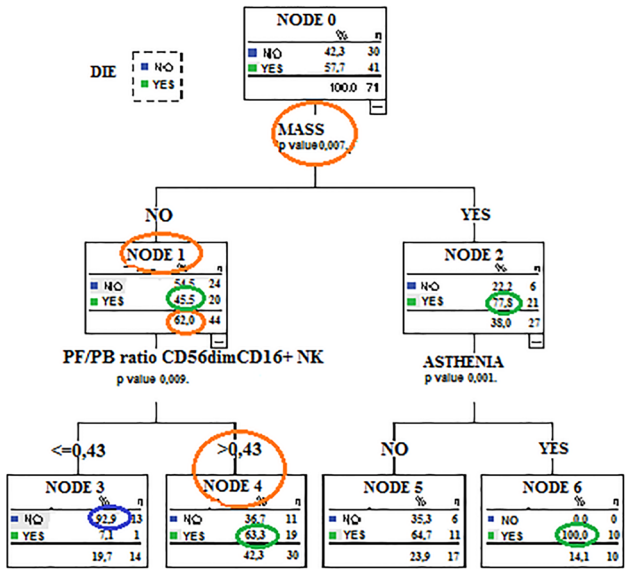

Fig. 4 Decision tree for MPE prognosis

Table 4 Crude and adjusted Cox's regression model according to the risk of patients dying versus patients surviving

	HR (95% CI)	*p* Value
Crude Cox's regression model		
PF/PB ratio CD16+ NK	5.025 (1.52–16.57)	0.008**
PF/PB ratio CD57+ NK	2.936 (1.04–8.28)	0.042*
Adjusted Cox's regression model		
Lung mass	2.47 (1.07–5.71)	0.035*
PF/PB ratio CD16+ NK	6.1 (1.76–21.1)	0.004**
Malignant	3.35 (1.22–9.17)	0.019*
Paramalignant	4.74 (1.21–18.55)	0.025*

HR hazard ratio, *CI* confidence interval, *NK* natural killer
*$p<0.05$;**$p<0.01$

Table 5 Classification according to death from pleural effusion of unknown aetiology (CHAID)

Observed	Prognosis		
	Survived	Died	Correct percentage
Survived	13	17	43.3%
Died	1	40	97.6%
Overall percentage	19.7%	80.3%	74.6%
Cross-validation			0.059

Growth method: CHAID

The Prognostic Value of the NK and NK Subpopulations in MPE Patients

The follow-up of the whole population lasted 640.4 ± 242 days, during which 57.7% died. Of these, 53.7% were MPEs and 24.4% were PPEs, with a median survival of 31 days and 72 days, respectively. A statistically significant relation ($p=0.005$) was observed between the malignant, paramalignant or BPE and survival time (Fig. 3). The most frequent cause of death was attributed to a primary tumour (95.5% in MPE patients and 100% in PPE patients). According to the crude Cox's regression analysis, the PF/PB ratio of the CD16+ NK and CD57+ NK subpopulations obtained a higher HR (Table 4). In the adjusted Cox's regression analysis, the PF/PB ratio of the CD16+ NK subpopulation obtained a higher HR of 6.1 (1.76–21.1) ($p=0.004$) (Table 4).

Decision Tree for the Prognosis of Pleural Effusion of Unknown Aetiology

The dependent variable of this tree diagram (Fig. 4) was death, while its independent variables were: lung mass in the CT, asthenia and the PF/PB ratio of the CD56 dim CD16+ NK subpopulation. However, the presence of lung mass was the strongest predictor of death during the study period. According to node 2, 77.8% of the patients with lung mass died, and this likelihood rose to 100% if they also had asthenia (node 6). According to node 3, however, we observed that 92.9% of those who did not die had a PF/PB ratio of the CD56 dim CD16+ NK subpopulation ≤ 0.43. The model correctly classified 74.6% of the patients in general (Table 5).

Discussion

The NK subpopulations with cytotoxic function should reflect increased cytolytic activity in MPEs given the presence of neoplastic cells in PF. In this study, we evaluated if these NK subpopulations analysed in PF could be a new diagnostic and prognostic indicator for MPE. We found that even though isolated PF values were not useful, when they were related with PB (PF/PB ratio), they provided relevant information about the diagnosis, and particularly about the prognosis, of MPE. Thus, the PF/PB ratio of the CD56 bright CD16− and CD56 dim CD16− NK subpopulations was higher in MPEs and PPEs than in benign cases. As far as we know, almost all the studies conducted to date present absolute values or percentages for NK in PF, but not in relation to PB [3, 16, 17]. Only Atanackovic et al. [18] have used the PF/PB ratio of NK (CD3− CD56+) to differentiate MPEs from benign ones. These authors reported a lower ratio in MPE patients. In our work, this ratio showed no inter-group differences, but it is difficult to compare both results because different methodologies were used. Our results agree with previously published works about the distribution of NK subpopulations in PF and PB [16, 17, 19, 20].

For the prognosis value of the NK cells in MPEs, we demonstrated that the high CD57+ NK values, mainly for CD16+ NK, would have a poor prognosis in MPE patients. This was expected because both subpopulations displayed strong cytotoxic activity. Despite CD16+ expression having been reported as a good prognostic factor with certain tumours [21, 22], only one article [23] has described the CD16+ NK subpopulation in MPE, and observed that its presence in PF was associated with worse survival, which was also the case in our series. No data are currently available about the prognostic value of CD57+ NK in PF in MPE patients.

According to the decision tree created to approach MPE prognosis, the presence of lung mass by CT imaging was the strongest prognostic predictor of death during the study period, with a 100% probability of death in those patients with lung mass and asthenia. The probability of survival was 92.9% for those with no lung mass and a PF/PB ratio of the CD56 dim CD16+ NK subpopulation (cytotoxic) ≤ 0.43, which is the cut-off point established by CHAID. Thus , the fact that fewer cytotoxic NK cells are present in PF indicates a lower tumour burden in the pleural cavity and better survival.

The strong point of our work is that it includes a new indicator that has not been previously studied in MPE. As we indicate herein, the PF/PB ratio of the cytotoxic NK subpopulation increases the risk of death in MPE. Currently, we do not exactly know the factors that predict a poor prognosis in some patients with MPE upon diagnosis, especially in those with MPE of unknown primary tumour. So, determining the PF/PB ratio of the cytotoxic NK subpopulation could help to make therapeutic management decisions, such as performing pleurodesis or tunnelled indwelling pleural catheter placement. However, to draw these conclusions, more studies should be conducted to validate this new prognostic marker.

The main limitation of this study is its sample size as it would have been more conclusive if a larger number of pleural effusions had been included. Likewise, we were unable to differentiate between malignant and paramalignant effusions with the number of patients included in both groups. Another study limitation is the inexistent, scarce or methodologically different literature published to date on the topic, which makes comparing our results very hard.

In conclusion, according to our data, the PF/PB ratio of the CD56 bright CD16− NK and CD56 dim CD16− NK subpopulations could help to distinguish patients with MPE and PPE from patients with BPE. Regarding prognostics, the PF/PB ratio of the cytotoxic CD16+ NK subpopulation increases the risk of death by 6.1-fold in MPE patients. Therefore, its determination would help to estimate the prognosis of these patients, and consequently, their management.

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Author Contributions SHL designed and conducted the research, collected, analysed and interpreted data, and wrote the manuscript. EF-F designed and conducted the research, interpreted the data, wrote the manuscript and critically revised the article. GJS critically revised the article. JMB analysed and interpreted the data. RAL analysed and interpreted the data. APM provided technical assistance. MMS-V analysed and interpreted the data, performed the statistical analysis and critically revised the article.

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Compliance with Ethical Standards

Conflict of interest We wish to confirm that there are no known conflicts of interest associated with this publication, and there has been no financial support for this work that could have influenced its outcome.

Ethical Approval We further confirm that any aspect of the work covered herein that has involved human patients has been conducted with the ethical approval of all the relevant bodies, and that such approvals are acknowledged in the manuscript.

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