# Low Calretinin Expression and High Neutrophil-To-Lymphocyte Ratio Are Poor Prognostic Factors in Patients with Malignant Mesothelioma Undergoing Extrapleural Pneumonectomy

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**Introduction:** Survival after extrapleural pneumonectomy (EPP) is variable in patients with malignant pleural mesothelioma (MPM), and there are no validated prognostic factors that could be used preoperatively. We investigated the calretinin and D2-40 expression and the neutrophil-to-lymphocyte ratio (NLR), an index of systemic inflammation as potential preoperative prognostic factors.

**Methods:** Consecutive patients who underwent EPP were included in this retrospective study. Potential prognostic factors such as age, gender, histological subtype, baseline laboratory parameters including NLR, and immunohistochemical staining for calretinin and D2-40 were evaluated. Overall survival (OS) from the date of surgery was determined by the Kaplan-Meier method. The prognostic value of the variables was examined using Cox regression, and significant factors ($p < 0.05$) were entered into a multivariate model to determine their independent effect.

**Results:** A total of 85 patients were included: median age 58 years; 80% men; 77% epithelial and 23% biphasic MPM. The median OS was 19.7 months. The following variables were predictive of longer OS: female gender ($p = 0.02$), epithelial subtype ($p = 0.04$), low NLR ($p < 0.01$), and high calretinin score ($p < 0.001$). In a multivariate analysis, only NLR ≥3 (hazard ratio 1.79; 95% confidence interval: 1.04–3.07; $p = 0.04$) and calretinin score ≤33 versus more than 67% (hazard ratio 4.72; 95% confidence interval: 1.97–11.32; $p < 0.01$) remained independent predictors. The addition of calretinin score increased the explained variation by 10.1%.

**Conclusions:** Both low calretinin expression and high NLR were independently associated with poor prognosis in patients with MPM undergoing EPP, and the calretinin score seemed to improve the accuracy of the prognostic model.

**Key Words:** Malignant pleural mesothelioma, Calretinin, Neutrophil-to-lymphocyte ratio, Prognosis.

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Malignant pleural mesothelioma (MPM) is an aggressive and difficult-to-treat tumor. It is caused by the carcinogenic effect of asbestos on the mesothelium surrounding the serosal cavities in the thorax. Because of the long latency period between asbestos exposure and the occurrence of MPM, the incidence of the disease continues to increase even in countries where a ban on the use of asbestos is in place.[1] When the ongoing heavy use in some developing countries is considered, the disease is expected to remain a growing global problem for many decades to come.[1]

The standard of care for patients with MPM has yet to be established.[2,3] Although therapy has improved incrementally over the past 20 years, the disease remains almost invariably fatal with a median survival ranging from 7 to 24 months.[4,5] Extrapleural pneumonectomy (EPP) has been performed increasingly over the past two decades as an option for some patients with MPM. Some patients survive long periods after surgery, but in others the disease course does not seem to be affected by a radical (surgical) approach. To improve outcomes, many institutions now favor trimodality therapy combining EPP with chemotherapy and/or radiotherapy.[6–11]

The significant morbidity and mortality associated with EPP require careful selection of patients.[2,3,12,13] To qualify for the procedure, good performance status, adequate organ function, and a potentially resectable tumor are the most frequent

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criteria that must be met. Histological subtype and extrapleural nodal status have been consistently identified as independent prognostic factors in determining survival in patients undergoing trimodality treatment.[14–16] Despite these prognostic indicators, the preselection of patients coupled with an absence of randomized controlled trials make it difficult to assess the benefits of EPP. Besides histological subtype, there remains a lack of accurate prognostic factors to identify the patients more likely to benefit from EPP before the surgery is performed.

Immunohistochemical (IHC) assessment in the diagnosis of MPM is well established. A panel of positive and negative IHC markers for MPM is needed for the diagnosis as recommended by the International Mesothelioma Panel.[17] Two of the mesothelial-related markers commonly used in the diagnostic setting include calretinin and D2-40,[17,18] but their prognostic role is yet to be established.

Recently, we identified the neutrophil-to-lymphocyte ratio (NLR) as an independent prognostic factor for patients with MPM undergoing systemic therapy.[19] As an easily reproducible and inexpensive marker, NLR, a measure of systemic inflammation, has the potential to aid in the management of patients with MPM.

In this study, we have retrospectively assessed the prognostic values of calretinin and D2-40 IHC expression and NLR in predicting outcome following EPP. We also explored the relationship between pathological stage and the systemic inflammatory status indicated by NLR.

## PATIENTS AND METHODS

Consecutive patients with MPM who underwent EPP at Royal Prince Alfred and Strathfield Private Hospitals, Sydney, Australia, from 1994 to November 2009 were included in the study. The patient selection for EPP was as described previously.[14] The demographic and treatment details of the patients were kept in a prospectively collected database. This study was approved by the Human Research Ethics Committees at the Sydney South West Area Health Service—Concord Repatriation General Hospital Zone.

The histological diagnosis of MPM was confirmed by two pathologists. Diagnostic IHC evaluation included a standard panel of antibodies (calretinin, BG-8, and CD15), as described previously,[18] and D2-40. The histological subtypes were assigned in accordance to the World Health Organization criteria and recommendations.[20] Pathological stage was determined according to the American Joint Committee on Cancer Staging System.[21]

Full blood counts were collected the day before EPP as part of the preoperative assessment. Hemoglobin (Hb), platelet count, white cell count (WCC), and its differential counts were recorded. According to previous publications, Hb difference defined as the difference relative to 160 g/liter in men and 140 g/liter in women was dichotomized to $<10$ g/liter versus $\geq 10$ g/liter[22]; platelet count into $\leq 400 \times 10^9$/liter versus $>400 \times 10^9$/liter,[23] and WCC into $\leq 8.3 \times 10^9$/liter versus $>8.3 \times 10^9$/liter.[24] The NLR was defined as the absolute neutrophil count divided by the absolute lymphocyte count.

Archival formalin-fixed, paraffin-embedded tissues from the EPP procedure were retrieved. Serial 4-$\mu$m-thick paraffin sections of the tumor underwent IHC labeling for calretinin and D2-40. Primary rabbit anticalretinin antibody (dilution 1:2000; Invitrogen, Camarillo, CA) and primary anti-D2-40 monoclonal antibody (dilution 1:100; Signet) were applied to incubate the sections overnight at 4°C after citric acid retrieval (1:10 dilution of 0.1 M citrate buffer, pH 6.0) in a microwave. All primary antibodies were diluted in 10% normal goat serum. The reaction with calretinin was developed with the Novocastra Polymer System (Leica Biosystems Newcastle Ltd, Newcastle Upon Tyne, UK), using the Liquid DAB and Substrate Chromogen System (Dako North America Inc, Carpinteria, CA), whereas the reaction with D2-40 was developed with the EnVision + Dual Link System (Dako), using the DAB Substrate Kit (Cell Marque, Rocklin, CA). For the quantitative evaluation of IHC scores of calretinin and D2-40, the percentage of cells labeled by the antibodies was recorded, irrespective of the intensity, resulting in a percentage score that ranged from 0 to 100%. All sections were assessed by two independent examiners in a blinded fashion. If there were scoring discrepancies of more than 10%, sections were reviewed jointly and a consensus reached.

### Statistical Analysis

Overall survival (OS) was calculated from the date of EPP and the date of death or last follow-up. Patients were censored at last follow-up if still alive or lost to follow-up. The prognostic value of the variables was examined using univariate Cox regression with either continuous (age, NLR, calretinin, and D2-40 scores) or categorical (gender, histological subtype, Hb difference, platelet count, and WCC) variables. NLR was log transformed. Variables with $p$ value less than 0.05 were considered statistically significant and Kaplan-Meier curves examined. Individually significant variables were entered into a multivariate model together with the established risk factors: age, gender, and histological subtype. The relationship between pathological stage and NLR was assessed using Spearman's correlation. These analyses were performed using SPSS for Windows version 17.0.

The additive discriminatory accuracy of calretinin and NLR, on top of the standard age/gender/subtype variables, was investigated using the method of Schemper and Henderson,[25] implemented in the R package surev.[26] Briefly, this was assessed by calculating the mean absolute difference between observed survival outcome and the model predictions. Explained variation was also computed and represents a measure equivalent to $R^2$ in linear regression. Standard errors were obtained by bootstrapping 200 samples.

## RESULTS

### Patient Cohort

Eighty-five patients underwent EPP over the study period, and archival tissue from 80 patients was available for further study. The baseline characteristics of this cohort and the epithelial and biphasic subgroups are summarized in Table 1. Briefly, for the entire cohort: median age 58 years;

**TABLE 1.** Baseline Characteristics of the Extrapleural Pneumonectomy Cohort

| Variables | Total Cohort ($n$ = 85) | Epithelial Subgroup ($n$ = 65) | Biphasic Subgroup ($n$ = 20) |
|---|---|---|---|
| Age (yr) | | | |
| Median (range) | 58 (22–74) | 58 (22–70) | 60.5 (43–74) |
| Gender | | | |
| Male | 68 | 49 | 19 |
| Female | 17 | 16 | 1 |
| Laterality | | | |
| Right | 49 | 40 | 9 |
| Left | 36 | 25 | 11 |
| Hb difference (g/liter) | | | |
| <10 | 21 | 18 | 3 |
| ≥10 | 63 | 46 | 17 |
| Missing | 1 | 1 | 0 |
| Baseline platelet counts ($\times 10^9$/liter) | | | |
| ≤400 | 63 | 48 | 15 |
| >400 | 20 | 15 | 5 |
| Missing | 2 | 2 | 0 |
| Baseline white cell count ($\times 10^9$/liter) | | | |
| <8.3 | 48 | 37 | 11 |
| ≥8.3 | 36 | 27 | 9 |
| Missing | 1 | 1 | 0 |
| Pathological stage | | | |
| Complete response | 2 | 1 | 1 |
| I | 5 | 4 | 1 |
| II | 18 | 15 | 3 |
| III | 54 | 43 | 11 |
| IV | 6 | 2 | 4 |
| Neutrophil-to-lymphocyte ratio | | | |
| Median (range) | 3 (1–13.9) | 2.9 (1.2–7.9) | 3.4 (1–13.9) |
| <3 | 40 | 33 | 7 |
| ≥3 | 44 | 31 | 13 |
| Missing | 1 | 1 | 0 |
| Calretinin score (%)[a] | | | |
| Median (range) | 57.5 (0–100) | 60 (0–100) | 30 (0–75) |
| ≤33% | 22 | 11 | 11 |
| 34–67% | 29 | 23 | 6 |
| >67% | 29 | 27 | 2 |
| Missing | 5 | 4 | 1 |
| D2-40 score (%)[a] | | | |
| Median (range) | 50 (2–95) | 58 (3–95) | 9 (2–53) |
| Overall survival (mo)[a] | | | |
| Median (95% CI) | 19.7 (13.8–25.6) | 23.2 (12.8–33.5) | 12.2 (1.1–23.2) |

[a] Statistically different between epithelial and biphasic MPM (defined as $p < 0.05$ in Kaplan-Meier method for survival and independent $t$ test for calretinin and D2-40).
Hb, hemoglobin; CI, confidence interval; MPM, malignant pleural mesothelioma.

80% men; 77% epithelial MPM, and 23% biphasic MPM; and median OS 19.7 months (95% confidence interval: 13.8–25.6 months). Nineteen patients underwent neoadjuvant chemotherapy: 8 with carboplatin and pemetrexed, 10 with cisplatin and pemetrexed, and 1 with carboplatin and vinorelbine. Two of them attained complete response with no tumor being demonstrable in the surgically excised tissue.

There was a wide range of calretinin and D2-40 expression. For calretinin, the median score was 57.5% (range: 0–100%) with a median score of 60% for epithelial and 30% for biphasic subtype. For D2-40, the median score was 50% (range 2–95%) with a median score of 58% for epithelial and 9% for biphasic subtype.

## Univariate and Multivariate Analysis for Prognostic Factors

In the univariate analysis, gender and histological subtype, NLR, calretinin, and D2-40 score were all significant prognostic factors. Including these significant variables with age in a multivariate Cox regression model, NLR, and calretinin score remained independent prognostic factors (Table 2). After converting NLR and calretinin score into categorical variables (NLR categorized into <3 and ≥3 and calretinin score into <33%, 34–67%, and >67%), they remained significant in the multivariate model along with age, gender, and histological subtype (Table 3).

## Kaplan-Meier Curves

Figure 1*A* shows the Kaplan-Meier curve for NLR: median OS of 26.7 months for NLR less than 3 and 12.6 months for NLR ≥3 ($p = 0.006$). Figure 1*B* shows the Kaplan-Meier curve for calretinin score: median OS of 35.8 months for calretinin score more than 67%; 14.5 months for calretinin score of 34 to 67%; and 6.9 months for calretinin score ≤33% ($p < 0.001$).

With the aim of developing a more accurate prognostic tool, we then combined NLR and calretinin scores: category 1 = NLR less than 3 and calretinin more than 67%; category 2 = NLR less than 3 and calretinin ≤67%; or NLR more than 3 and calretinin more than 33%; category 3 = NLR ≥3 and calretinin ≤33%. Figure 1*C* demonstrates the Kaplan-Meier curve for this composite score and shows that the median OS was 48.7 versus 15.9 versus 6.4 months for category 1, 2, and 3, respectively.

## Discriminative Accuracy

The predicted inaccuracy for a model without predictors (independent variables) is 0.328 for this dataset, representing the maximum level of inaccuracy to predict OS. Using NLR in addition to the standard variables had only a minor impact on the predictive inaccuracy (reduced from 0.309 to 0.300). Modest improvement was seen when calretinin was incorporated into the model, with a slight improvement again seen for the composite score. This final model also had the highest explained variation (Table 4).

## Relationship Between Stage and NLR

The mean NLR for stage I disease was 2.4 (standard deviation [SD]: 0.7); 2.9 (SD: 1.5) for stage II; 3.7 (SD: 2.1)

**TABLE 2.** Univariate and Multivariate Analysis for Prognostic Factors

| | Univariate Analysis | | | Multivariate Analysis | | |
|---|---|---|---|---|---|---|
| | **HR** | **95% CI** | ***p*** | **HR** | **95% CI** | ***p*** |
| Age (yr)[a] | 1.14 | 0.89–1.48 | 0.30 | 0.88 | 0.63–1.23 | 0.44 |
| Gender | | | | | | |
| Male | | | 1 (reference) | | | |
| Female | 0.44 | 0.21–0.90 | 0.02 | 0.47 | 0.20–1.10 | 0.82 |
| Histological subtype | | | | | | |
| Epithelial | | | 1 (reference) | | | |
| Biphasic | 1.80 | 1.04–3.11 | 0.04 | 0.84 | 0.41–1.71 | 0.62 |
| Haemoglobin difference | | | | | | |
| <10 | | | 1 (reference) | | | |
| ≥10 | 1.64 | 0.91–2.97 | 0.10 | | | |
| White cell count | | | | | | |
| <8.3 | | | 1 (reference) | | | |
| ≥8.3 | 1.49 | 0.91–2.44 | 0.11 | | | |
| Platelet count | | | | | | |
| ≤400 | | | 1 (reference) | | | |
| >400 | 1.27 | 0.72–2.24 | 0.42 | | | |
| NLR log[b] | 4.64 | 1.66–12.95 | <0.01 | 3.24 | 1.06–10.71 | 0.04 |
| Calretinin score (%)[a] | 0.83 | 0.75–0.92 | <0.001 | 0.86 | 0.76–0.98 | 0.03 |
| D2-40 score (%)[a] | 0.89 | 0.81–0.98 | 0.02 | 0.95 | 0.85–1.06 | 0.35 |

[a] Increment of 10 units.
[b] Increment of 1 unit.
HR, hazard ratio; CI, confidence interval; NLR, neutrophil-to-lymphocyte ratio.

**TABLE 3.** Multivariate Analysis After Categorizing Variables

| | **HR** | **95% CI** | ***p*** |
|---|---|---|---|
| Age[a] | 0.81 | 0.57–1.16 | 0.25 |
| Gender | | | |
| Male | | 1 (reference) | |
| Female | 0.43 | 0.18–1.03 | 0.06 |
| Histological subtype | | | |
| Epithelial | | 1 (reference) | |
| Biphasic | 0.77 | 0.38–1.56 | 0.47 |
| NLR | | | |
| <3 | | 1 (reference) | |
| ≥3 | 1.79 | 1.04–3.07 | 0.04 |
| Calretinin | | | |
| ≤33% | 4.72 | 1.97–11.32 | <0.01 |
| 34–67% | 1.57 | 0.83–2.97 | 0.17 |
| >67% | | 1 (reference) | |

[a] Increment of 10 yr.
HR, hazard ratio; CI, confidence interval; NLR, neutrophil-to-lymphocyte ratio.

for stage III; and 3.1 (SD: 0.9) for stage IV. The Spearman's correlation between NLR and pathological stage was 0.25 ($p = 0.03$).

## DISCUSSION

The effectiveness of EPP in extending the survival of patients with MPM remains controversial.[27] Several criteria are used to select patients for this procedure, but even in this preselected patient population, survival after EPP is highly variable. Currently, there is no method to identify the subpopulation of patients most likely to benefit from the radical approach.

Unlike other solid tumors where tumor grade is routinely reported and often provides important prognostic information, there is currently no consensus as to how best to assign tumor grade in MPM.[28] It is not routinely reported in clinical practice or clinical trials. Renewed interest in tumor grade in MPM has followed a large population-based study using the Surveillance, Epidemiology, and End Results database, which demonstrated tumor grade to be a significant prognostic factor.[29] However, because of the retrospective nature of the study, and the absence of pathology review, the definition of the tumor grade was criticized, and it is worth noting that 90% of the patients in the study did not have an assigned grade.[29] Cunto-Amesty et al.[30] presented a pilot study of reporting tumor grade using a morphologicallybased grading system, examining the cellular and architectural morphology, necrosis, and mitotic rates. They found that well-differentiated tumors trended toward having longer survival than those with poorly differentiated tumors in 20 cases of stage II patients with MPM who were treated with EPP.[30] Similarly, Takeshima et al.[31] demonstrated the prognostic role of the tumor grade in 53 patients with epithelial MPM and found the expression of calretinin (scored from 0 to 3+) to be highly correlated with tumor differentiation, i.e., higher calretinin scores in the more differentiated tumors. As such, the calretinin scores may reflect the grading of the MPM tumors.

In this study, we used a retrospective series of 85 patients with MPM who underwent EPP to identify potential factors that could be used to stratify patients before the

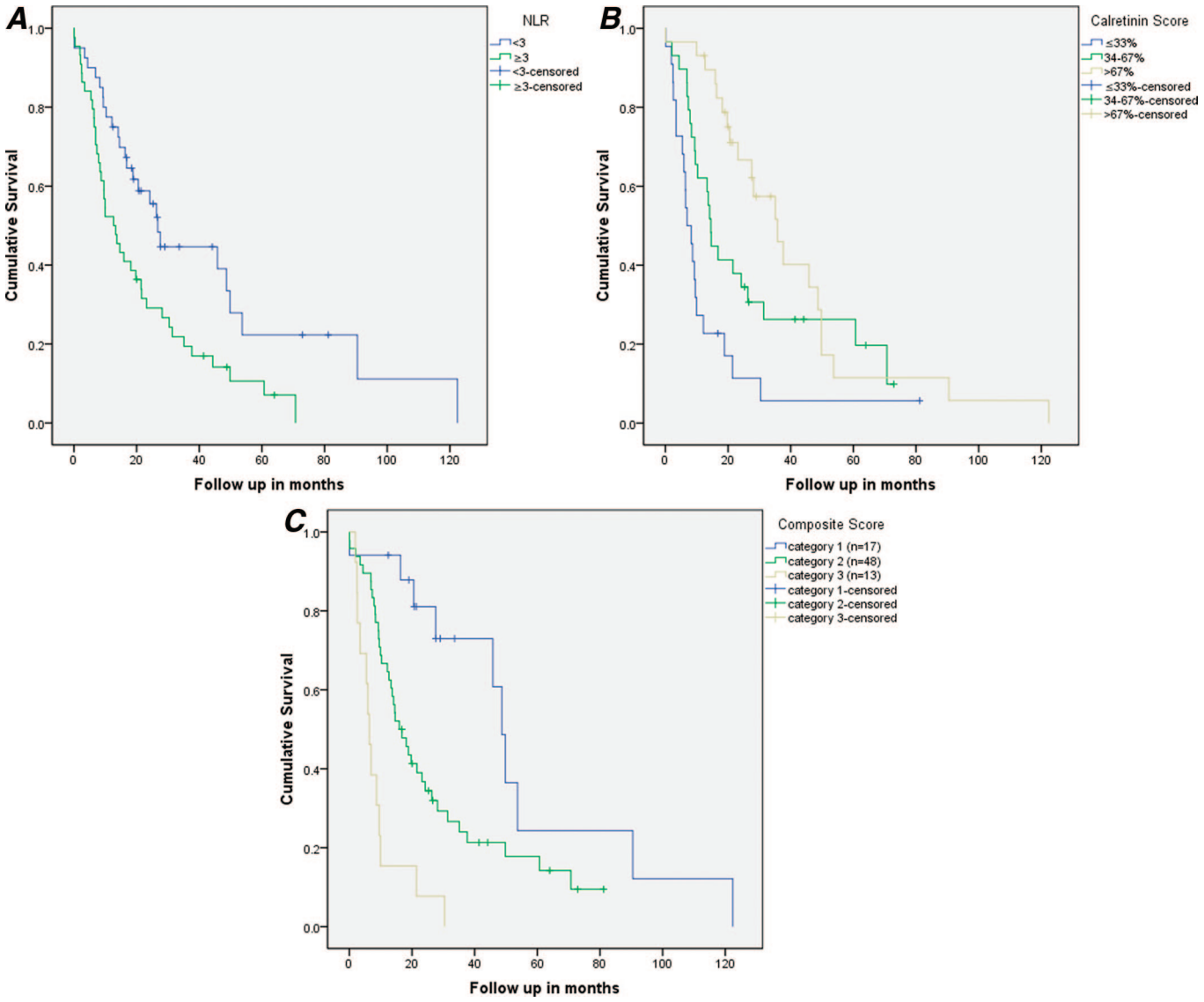


**FIGURE 1.** *A,* Kaplan-Meier curve for overall survival stratified by neutrophil-to-lymphocyte ratio (NLR). Median overall survival was 26.7 months versus 12.6 months for NLR <3 and ≥3, respectively. *B,* Kaplan-Meier Curve for overall survival stratified by calretinin. Median overall survival was 35.8 versus 14.5 versus 6.9 months for calretinin scores more than 67%, 34 to 67%, and ≤33%, respectively. *C,* Kaplan-Meier curve for overall survival stratified by the composite score. Median overall survival was 48.7 months versus 15.9 months versus 6.4 months for categories 1, 2, and 3 respectively. Category 1 = NLR less than 3 and calretinin more than 67%; category 2 = NLR less than 3 and calretinin ≥67% or NLR more than 3 and calretinin more than 33%; and category 3 = NLR ≥3 and calretinin ≤33%.

radical surgery. We found that the extent of the calretinin labeling was associated with OS, with more extensive calretinin labeling linked to longer OS. Our results further suggest that the tumor differentiation indicated by calretinin labeling in patients with resectable MPM provides important information. To the best of our knowledge, this is the first report to implicate calretinin as a prognostic factor. Pathological staging was deliberately not accounted for in the multivariate model as it often cannot be accurately assessed preoperatively and as a result is not available for consideration. Even taking pathological staging into account (data not shown), calretinin score remained independently associated with prognosis.

In this study, calretinin score was assessed by the extent of labeling (i.e., the percentage of labeled cells) regardless of the intensity. There were multiple reasons for not considering intensity in the score: assessment of intensity can be subjective and several factors can alter the outcome such as uneven thickness of the section created by differing sharpness of the blade of the microtome; inconsistencies of manual incubation times between batches of the experiments; and degree of background staining. Our proposed score is easy to adopt in the daily clinical practice of a diagnostic pathologist, particularly when calretinin is routinely applied in the diagnosis of MPM.

 

**TABLE 4.** Explained Variation and Discriminative Accuracy for Overall Survival

| Model | Predicted Inaccuracy, SE | Explained Variation, SE (%) |
|---|---|---|
| No predictors | 0.328 | |
| Age + gender + subtype (base model) | 0.309 ± 0.020 | 5.8 ± 4.9 |
| Base model + NLR | 0.300 ± 0.021 | 8.5 ± 5.0 |
| Base model + calretinin | 0.276 ± 0.023 | 15.9 ± 6.1 |
| Base model + NLR/calretinin composite | 0.273 ± 0.022 | 16.8 ± 5.7 |

SE, standard error; NLR, neutrophil-to-lymphocyte ratio.

From the literature, it is clear that even a significant prognostic factor with a large hazard ratio may not contribute to a meaningful improvement in the discriminative accuracy.[25] We found when calretinin score was considered in addition to the traditional prognostic factors of age, gender, and histological subtype, the explained variation in survival was increased by 10.1%. As such, it seemed that calretinin score has additional prognostic value for OS in patients with MPM undergoing EPP. Given calretinin is routinely used in the diagnosis of MPM and the proposed scoring method is simple, we believe that there is value in using calretinin score in clinical practice.

NLR has been implicated as a prognostic factor in several tumor types, including MPM.[19] It is an inflammation-based score, and an elevated NLR is thought to reflect an exaggerated systemic inflammatory response. In our previous report, an elevated NLR (defined as $\geq 5$) predicted shorter survival in patients with unresectable MPM treated with systemic therapy.[19] In this study on resectable patients treated with EPP, preoperative NLR was also independently associated with prognosis; after adjusting for known prognostic factors such as histological subtype, age, and gender, patients with NLR less than 3 had longer OS than those with NLR $\geq 3$ ($p = 0.04$). The cutoff of 3 for NLR was chosen in this study as it is the median value for this series, and only 13% of patients had a NLR of more than 5. However, when considered in the prognostic model, neither NLR alone nor the combination of calretinin score and NLR was significantly superior to calretinin score alone in discriminative accuracy.

Despite the lack of NLR to increase the discriminative accuracy, systemic inflammation remains an important factor to consider in patients with resectable MPM. The hypothesis that systemic inflammation becomes more exaggerated as the disease progresses is supported by the fact that the median NLR in this EPP cohort was 3, whereas the median NLR in the patients with unresectable MPM treated with systemic therapy in our previous series was 5.[19] This is also reflected in the positive correlation between increasing NLR and progressive stage in our study.

This study has its limitations. Despite the promising results on the prognostic role of the calretinin score in the patients undergoing EPP, this study is retrospective. As such it suffers from the inherent problems associated with retrospective analysis. Although the histological subtype was taken into account when performing the multivariate analysis, there was an absence of the sarcomatoid subtype of MPM in our series. This highlights the other limitation of the study as our study focused on a specific subset of patients with MPM who were eligible for EPP, which renders the results less generalizable to the whole MPM population. Nevertheless, the study cohort is representative of the typical patient group that is suitable for EPP. Our results need validation in an independent series of patients, preferably in a prospective fashion. This should be done in all patients presenting with MPM to confirm the prognostic value of calretinin in all patients with MPM.

Furthermore, even though calretinin score and NLR were significant independent factors, together with the traditional factors of histological subtype, age, and gender, they only explained 16.8% of the variation in this cohort's survival. This is a potent reminder that further advance in our knowledge of the biology of MPM is urgently required to explain major survival differences.

In conclusion, this study has addressed the need for preoperative factors that can better predict outcome of patients with MPM undergoing EPP. Extent of calretinin labeling seems to be a useful factor to consider preoperatively. With a median survival of 6.9 months for patients with a calretinin score of less than 33%, one may argue that surgery does not seem to benefit such patients and this may reflect the more aggressive biology of a tumor that is not altered by the treatment. Given that calretinin is routinely used for the pathological diagnosis of MPM, we believe that it is relatively easy to prospectively confirm the prognostic value of this marker, in particular, in an adequately sized diagnostic biopsy in advance of EPP.

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