Giant Circulating Cancer-Associated Macrophage-Like Cells Are Associated With Disease Recurrence and Survival in Non–Small-Cell Lung Cancer Treated With Chemoradiation and Atezolizumab

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Abstract

Development of distant metastatic disease is common in patients with locally advanced non–small-cell lung cancer, leading to high rates of cancer-related mortality. Monitoring for early signs of disease recurrence from peripheral blood markers is an attractive avenue toward personalizing cancer care. We identified a novel macrophage-like circulating cell whose size appears to associate with poorer survival and the development of metastatic disease shortly after completion of definitive treatment.

Background: Cancer-associated macrophage-like cells (CAMLs) are a potential peripheral blood biomarker for disease progression. This study used data from a phase 2 clinical trial to evaluate prognostic utility of CAMLs for locally advanced non–small-cell lung cancer treated with definitive chemoradiotherapy (CRT) and atezolizumab (DETERRED; ClinicalTrials.gov NCT02525757). **Patients and Methods:** Sample collection occurred at baseline (T0), during CRT (T1), at end of CRT (T2), and at first follow-up (T3). CAMLs were captured and quantified by the CellSieve system using multiplex immunostaining. Giant CAMLs were defined as characteristic CAMLs $\geq$ 50 μm. Kaplan-Meier methodology estimated progression-free survival, distant failure-free survival, relapse-free survival, and overall survival at 30 months. **Results:** Thirty-nine patients were evaluated between December 2015 and March 2018. Median follow-up was 27 months. Most disease was stage III (85%) and comprised squamous-cell carcinoma (38%) or adenocarcinoma (59%). In total, 267 blood samples were analyzed. Giant CAMLs were identified in 57%, 60%, 64%, and 63% of patients at T0, T1, T2, and T3, respectively. Patients with giant CAMLs at T3, occurring at a median of 30 days after completion of CRT, had significantly worse distant failure-free survival (hazard ratio [HR] 4.9, $P = .015$), progression-free survival (HR 2.5, $P = .025$), recurrence-free survival (HR 2.4, $P = .036$), and overall survival (HR 3.5, $P = .034$) compared to patients with small or no CAMLs. **Conclusions:** Presence of giant CAMLs after CRT completion was associated with development of metastatic disease and poorer survival despite the use of maintenance immunotherapy. Monitoring CAMLs may help risk-stratify patients for adaptive treatment strategies.

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Introduction

Early detection of recurrent cancer is essential to improve patient outcomes; emerging approaches continue to be developed in order to identify relapsed disease in a sensitive and accurate manner. To this extent, assessment of various tumor-associated cells in the peripheral blood using so-called liquid biopsies is a promising noninvasive diagnostic method to evaluate treatment response, recurrence risk, and prognosis. Identifying patients at risk for disease recurrence or death via simple, noninvasive techniques would allow for better treatment personalization and likely improved outcomes. Circulating tumor cells (CTCs) may provide such prognostic information in a variety of neoplasms,[1] but they are uncommon in nonmetastatic settings,[2] thus limiting their sensitivity as a biomarker for earlier-stage cancers.

Cancer-associated macrophage-like cells (CAMLs) are circulating multinucleated myeloid cells that exhibit expression of CD45/CD14/CD11c.[3] CAMLs occur in a wide variety of malignancies and can be detected in over 90% of such patients, including early and late stages of cancers; they are infrequently detected in benign neoplasms, and are not found in healthy volunteers.[3-6] CAMLs appear to have prognostic value for several neoplasms on the basis of the number and size of CAMLs, using a cut point of >5 CAMLs per 7.5 mL of peripheral blood, and ≥ 50 μm, respectively.[6-9] Compared to CAML number, CAML size 50 μm or more appeared to be associated with larger differences in progression-free survival (PFS) and overall survival (OS).[9] This study evaluated nearly 300 patients with a variety of solid malignancies, including breast, lung, prostate, pancreas, and kidney, suggesting wide application. It is speculated that these circulating cells could represent the inflammatory tumor microenvironment, but whether these cells would be associated with treatment response to immunotherapy is not known. This is particularly important in patients with unresectable locally advanced non−small-cell lung cancer (NSCLC), where the addition of consolidative immunotherapy is now the standard of care.[10,11]

Using samples collected from unresectable locally advanced NSCLC patients enrolled onto a prospective study treated with definitive chemoradiotherapy (CRT) and immunotherapy, we aimed to evaluate the prognostic utility of CAMLs for these patients. We hypothesized that giant CAMLs may be prognostic of higher risk of disease recurrence after CRT even with the addition of immunotherapy.

Patients and Methods

Patient Population

This study consisted of patients with unresectable locally advanced NSCLC enrolled onto a phase 2 prospective clinical trial, which combined CRT and atezolizumab followed by maintenance carboplatin/paclitaxel and atezolizumab (PD-L1 blockadE To Evaluate the safety of Lung CanceR therapy using Carboplatin, Paclitaxel, and Radiation combinEd with MPDL3280A, DETERRED; ClinicalTrials.gov NCT02525757).[12] Briefly, the trial was designed in two parts. Part 1 consisted of 10 patients who received standard-course CRT (60-66 Gy in 30-33 fractions) with once-weekly paclitaxel and carboplatin. As long as no progression was noted, patients received consolidative carboplatin and paclitaxel along with intravenous atezolizumab (1200 mg) every 3 weeks for 2 cycles, followed by maintenance atezolizumab for up to 1 year, or until disease progression, death, or toxicity led to drug discontinuation. In part 2, once it was determined that atezolizumab could be safely administered with consolidative chemotherapy, 30 patients received atezolizumab with CRT followed by the same consolidative and maintenance regimen from part 1. Thirty-nine of the 40 patients enrolled onto this trial were included for this biomarker collection study, 9 from part 1 and 30 from part 2. Anonymized peripheral blood samples from these patients were collected after obtaining written informed consent and according to the local institutional review board approval. Blood samples were processed on site. Two 7.5 mL tubes of blood per patient were collected at each interval, including baseline pretreatment (T0), during CRT (T1) and after CRT (T2), and at the first follow-up (T3). All patients who had blood samples collected were included in the analysis at every time point.

Patient disease was assessed by imaging and clinical evaluation at 3- or 4-month intervals for the first 1 to 2 years, then every 6 months thereafter. Radiographic interpretation of disease recurrence was performed according to institutional protocol.

Cellular Analysis

Blood samples (7.5 mL) were collected in CellSave preservative tubes and processed via the CellSieve Microfiltration Assay (Creatv MicroTech), using a low-pressure vacuum system. The CellSieve Microfiltration Assay isolates circulating cells based on size exclusion (>7 μm). CAMLs were identified[3] based on morphologic features and the phenotypic expression of CD45, EpCAM, cytokeratins 8, 18, 19, and 4′,6-diamidino-2-phenylindole (DAPI). CAMLs were morphologically identified using the following criteria: a single cell with an enlarged nucleus ($\geq$14 μm in diameter) or separated polymorphic nuclei contained within the cell. CAML size was measured by the length of the cytokeratin cytoplasmic signal (24-300 μm). An Olympus BX54WI fluorescent microscope with Carl Zeiss AxioCam and Zen2011 Blue (Carl Zeiss) was used for all imaging. Quantification was performed with the observer unaware of clinical information. All CAMLs in a given sample were analyzed for size, and any CAML ≥ 50 μm per time point was counted as a giant CAML. Patients with CAMLs < 50 μm or without identifiable CAMLs were counted as having none or small CAMLs.

Statistical Analysis

Assessments of survival end points were conducted by the Kaplan-Meier method or Cox regression analysis. PFS was defined as the time from enrollment to the date of tumor recurrence (any location) or death, whichever occurred first. Locoregional failure-free survival (LRFFS) was defined as the time between CRT completion and development of local or regional disease recurrence. Regional recurrence included nodal relapses in the ipsilateral hilar, mediastinal, or supraclavicular lymph node basins. Distant failure-free survival (DFFS) referred to the time between CRT completion and the radiologic detection of distant (nonlocal/regional) metastatic disease. Relapse-free survival (RFS) was the time from completion of CRT and development of any disease recurrence, locoregional and/or distant. OS was the time between CRT

completion and death from any cause. All outcomes were censored at 30 months.

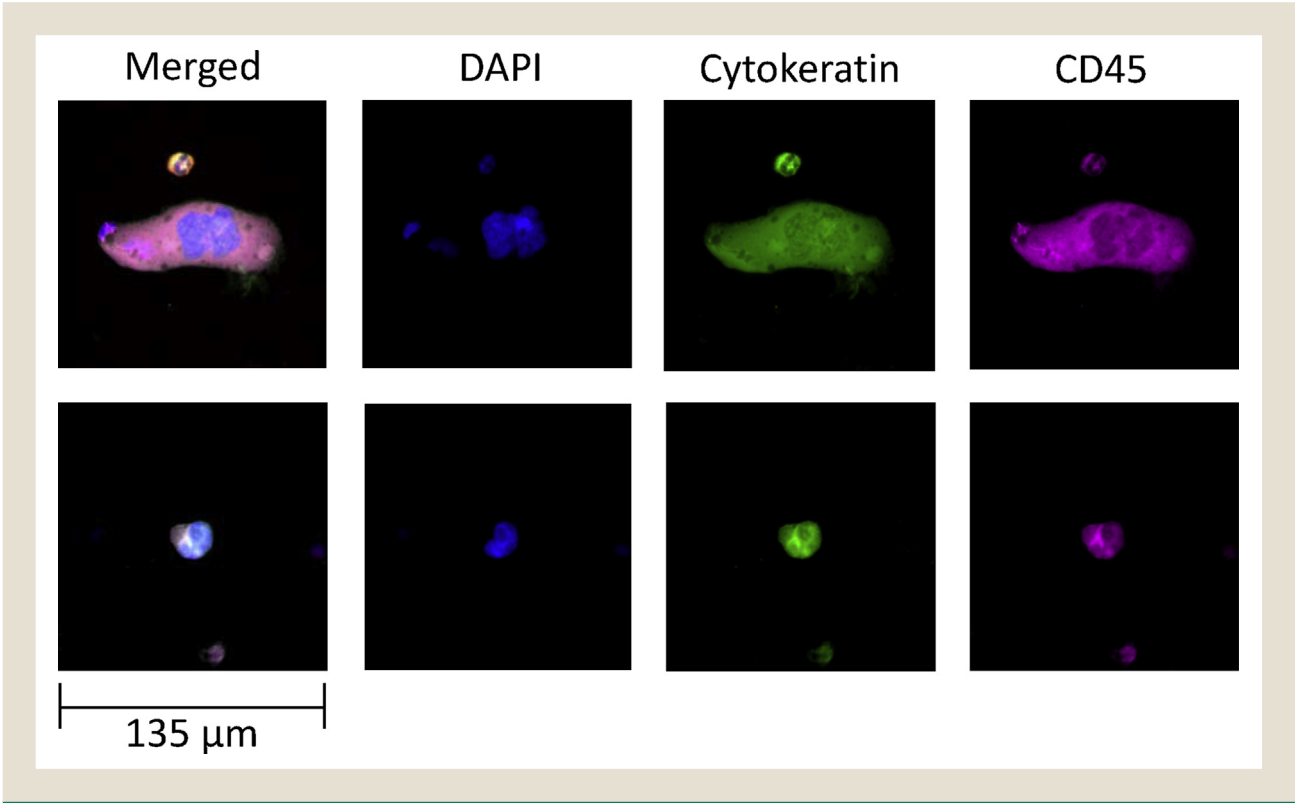
Results

The population of this study consisted of patients with unresectable locally advanced NSCLC (n = 39) treated with concurrent CRT with (n = 30) or without (n = 9) atezolizumab, followed by maintenance chemotherapy and atezolizumab treated on a phase 2 single-arm protocol. The median follow-up was 27 months (range, 1.5-43 months). A total of 267 blood samples were analyzed. Immediately before treatment (T0), 69 samples were obtained while 65 samples were obtained during CRT (T1), 64 at the end of CRT (T2), and 67 at the first follow-up (T3). The first follow-up occurred at a median of 30 days after completion of CRT (range, 26-89 days). A characteristic example of patients with small and giant CAMLs, respectively, is presented in Figure 1. Table 1 displays the clinical characteristics of this patient population, while Figure 2 and Supplementary Table 1 in the online version show the blood sample numbers and patient numbers at each time point. Of note, the median age was 66 years (range, 50-83 years), most patients had stage III disease (85%), and most had had squamous-cell carcinoma (38%) or adenocarcinoma (59%).

The number of patients and samples was well balanced across the 4 time points. CAMLs were identified in 76% of samples overall (202/267): 74% at T0 (51/69, range: 0-23), 74% at T1 (48/65, range: 0-14), 75% at T2 (48/64, range: 0-23), and 80% at T3 (56/70, range: 0-16). Because most patients provided two samples per time point, 91% (32/35) of patients had CAMLs identified at T0, 91% (30/33) at T1, 88% (29/33) at T2, and 100% (35/35) at T3 (Figure 2; Supplementary Table 1 in the online version). CAMLs ≥ 50 μm were identified in 42% of samples overall (111/267, range: 50-239 μm): 41% at T0 (28/69, range: 52-194 μm), 42% at T1 (27/65, range: 50-176 μm), 41% at T2 (26/64, range: 50-239 μm), and 43% at T3 (30/70, range: 50-222 μm). There were no significant differences in the number of patients with giant CAMLs identified between time points: T0 (n = 20; 57%), T1 (n = 22, 67%), T2 (n = 21; 64%), and T3 (n = 22; 63%). Not all patients had samples obtained at each time point; although 39 patients in total were assessed at all time points, 35 patients provided samples at T0, 33 at T1, 33 at T2, and 35 at T3 (Figure 2; Supplementary Table 1 in the online version). There was no statistical difference in the number of CAMLs identified between the 4 time points (there were 6, 5, 6, and 7 patients with ≥ 6 CAMLs at T0, T1, T2, and T3, respectively).

The presence of giant CAMLs compared to no or small CAMLs was associated with significantly worse 30-month outcomes only from samples obtained at the time of first follow-up (T3). No events occurred beyond 30 months for all measured outcomes. Patients with giant CAMLs at the first follow-up were significantly more likely to develop distant metastases (30 month DFFS; median: not

Figure 1 Identification of Giant and Small CAML Top Row Shows Giant CAML (≥50 μm) and Bottom Row Small CAML (<50 μm). Blue Indicates DAPI; Green, Cytokeratin; and Purple, CD45. Each Box is 135 μm Square



Abbreviations: CAML = cancer-associated macrophage-like cell; DAPI = 4′,6-diamidino-2-phenylindole.

Table 1 Patient Characteristics

Characteristic	Value
Age at diagnosis (years), median (range)	66 (50-83)
Sex	
Male	26 (66)
Female	13 (33)
ECOG performance status	
0	16 (41)
1	23 (59)
2-5	0
Race/ethnicity	
Black/African American	4 (10)
White	35 (90)
Smoking history	
Yes	35 (90)
No	4 (10)
Histology	
Adenocarcinoma	23 (59)
Squamous-cell carcinoma	15 (38)
NSCLC-NOS	1 (3)
Stage (AJCC 7th ed.)	
I	0
II	6 (15)
III	33 (85)
IV	0
Radiation modality	
IMRT/VMAT	31 (79)
Proton	8 (21)
Radiation prescription dose	
<60 Gy	0
60-66 Gy	38 (97)
>66 Gy	1 (3)
Total no. of atezolizumab cycles	
1-5	9 (23)
6-10	12 (31)
11-19	17 (44)

Data are presented as n (%) unless otherwise indicated.
Abbreviations: AJCC = American Joint Committee on Cancer; ECOG = Eastern Cooperative Group; IMRT = intensity modulated radiation therapy; NSCLC-NOS = non–small-cell lung cancer not otherwise specified; VMAT = volumetric modulated arc therapy.

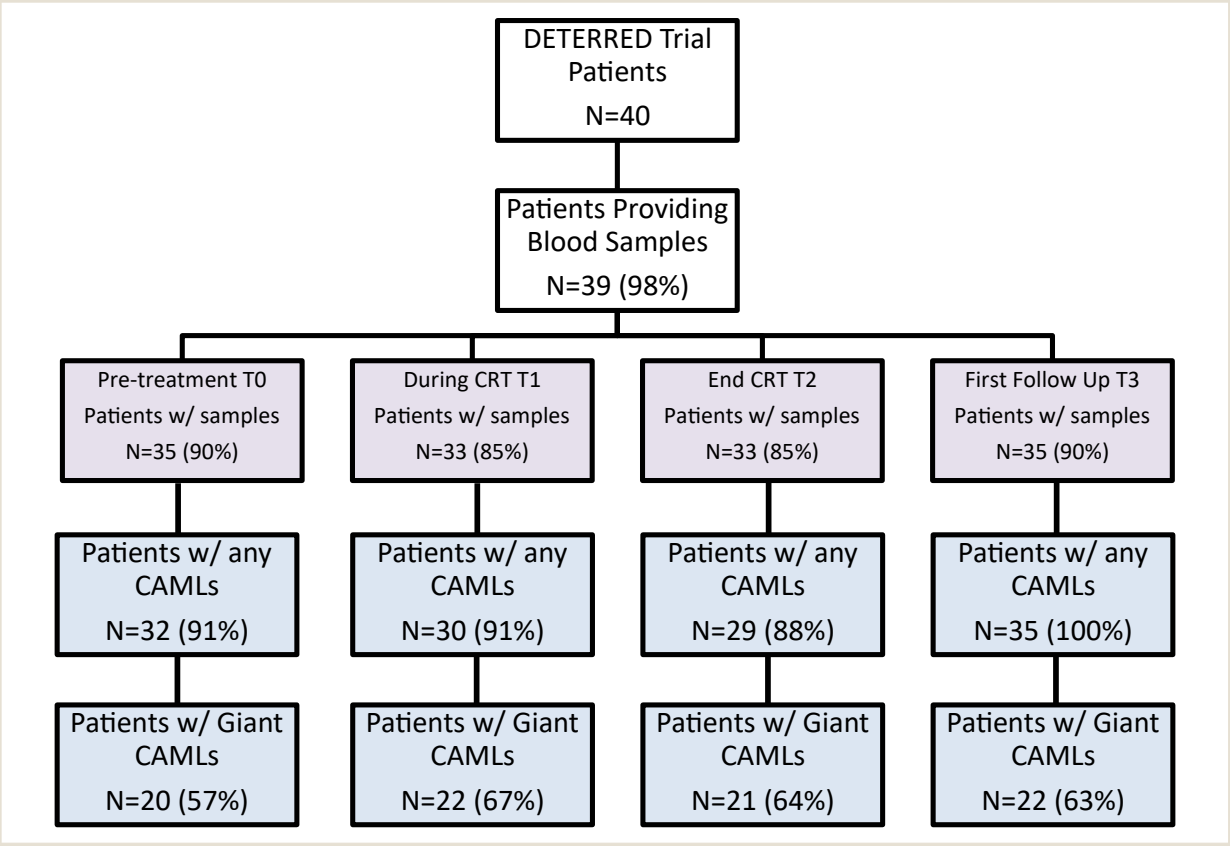
reached vs. 25 months; hazard ratio [HR] 4.9; 95% CI 1.7-13.9, $P = .015$; Figure 3) and also developed more frequent disease relapses overall (30-month RFS: median: not reached vs. 8 months; HR 2.4; 95% CI 1.0-5.6, $P = .036$; Figure 3). We identified worse 30-month PFS in patients with giant CAMLs at T3 (PFS; median: not reached vs. 8 months; HR 2.5; 95% CI 1.1-5.8, $P = .025$; Figure 3), and worse OS (median: not reached vs. 25 months; HR 3.5; 95% CI 1.3-9.6, $P = .034$; Figure 3). Giant CAMLs were identified in 22 (63%) of 35 patients at the first follow-up. Patients with giant CAMLs identified at baseline before initiation of definitive CRT (T0) or during CRT (T1) or at the end of CRT (T2) were not found to have worse DFFS ($P = .286$ for T0, $P = .569$ for T1, $P = .190$ for T2), PFS ($P = .768$ for T0, $P = .846$ for T1, $P = .285$ for T2), RFS ($P = .531$ for T0, $P = .730$ for T1, $P = .218$ for T2), or OS ($P = .861$ for T0, $P = .978$ for T1, $P = .515$ for T2), respectively (Supplementary Figure 1 and Supplementary Table 2 in the online version). There was no statistically significant difference in LRFFS at all time points, including T3 (Supplementary Figure 2 in the online version). Although there were numerically more locoregional failures in patients with CAMLs < 50 μm (5 of 13%; 38%) compared to patients with giant CAMLs (4 of 22%; 18%) at T3, this difference was not statistically significant ($P = .372$) (Supplementary Figure 2, Supplementary Table 3 in the online version). Supplementary Table 3 in the online version lists the location of first recurrence according to CAML size at T3. To further evaluate CAML size as a biomarker of disease progression and survival, we performed time-dependent Cox regression analysis using CAML size as a continuous variable. This did not result in statistical differences in any of the outcome measures (LRFFS, DFFS, RFS, PFS, and OS) at any of the time points. Using a cutoff of 40 μm resulted in similar statistically significant outcomes at T3 compared to a 50 μm cutoff; however, the number of patients with CAMLs < 40 μm at each time point was small (12, 6, 6, and 6 at T0, T1, T2, and T3, respectively). Using a cutoff of 60 μm demonstrated a significant difference in DFFS at T3 ($P = .04$), but not any other outcome measure. Finally, cutoffs of 70, 80, 90, or 100 μm did not demonstrate any differences in outcomes at any of the time points.

We evaluated change in CAML size over time with each line representing an individual patient (Figure 4). Assessing 35 patients with either giant CAMLs (n = 22) or small CAMLs (n = 13) from the first follow-up time point who had at least one blood sample taken at an earlier time point, patients with giant CAMLs at T3 tended to have giant CAMLs throughout treatment. Fifty-five percent of these patients developed metastatic disease (12 of 22; Figure 4, red lines). Patients with small CAMLs at T3 tended to have small CAMLs throughout treatment, and 85% of these patients did not develop distant metastatic disease (11 of 13; Figure 4, blue line).

Combining all CAML data at T0-2 from patients who had giant CAMLs at T3 and comparing it to patients with small CAMLs at T3 revealed a significant difference in CAML size between the populations (Figure 5). The median CAML size at T0-2 for patients with giant CAMLs at T3 was 76 μm and 48 μm for patients with small CAMLs at T3. Mean values for the same groups were 84.4 μm and 51.1 μm, and this difference was significant (unpaired *t* test, $P = .0001$). Univariate Cox regression revealed that the presence of giant CAMLs was a predictor for poorer DFFS, RFS, PFS, and OS at the first follow-up time point (T3) compared to age, Eastern Cooperative Oncology Group performance status, radiation dose, smoking pack-years, and tumor histology (Supplementary Table 4 in the online version). OS did correlate with smoking pack-years, but not other variables, as patients with higher documented pack-year smoking history experienced poorer survival (Supplementary Table 4 in the online version) in the online versionz. Multivariate Cox regression analysis was not possible because of the low number of events.

There was no association with disease or survival outcomes when evaluating primary tumor programmed death ligand 1 (PD-L1)

Figure 2 Flowchart Indicating Identification of CAMLs at 4 Time Points Shown are Number of Patients Providing Samples, Number of Patients With CAMLs Identified, and Number of Patients With Giant CAMLs for Samples Obtained at Each Time Point. T0 Indicates Baseline; T1, During CRT; T2, End of CRT; and T3, First Follow-up)



Abbreviations: CAML = cancer-associated macrophage-like cell; CRT = chemoradiotherapy.

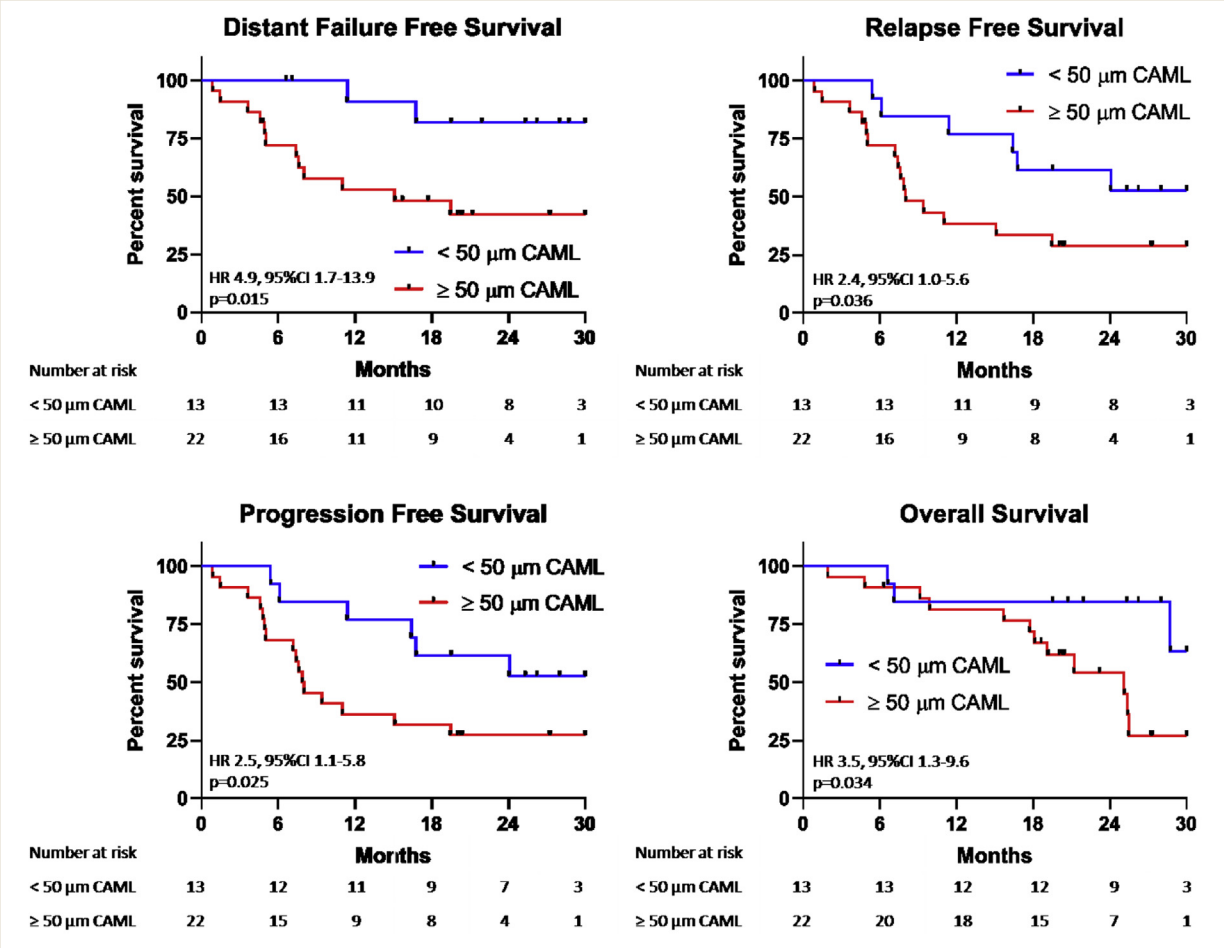
expression status. Stratification of patients on the basis of PD-L1 immunohistochemical expression of ≥ 1% or ≥ 50% did not reveal any statistically significant association with OS or DFFS (Supplementary Figure 3 in the online version). Additionally, there was no clear association with PD-L1 expression and presence of giant CAMLs at any time point, although PD-L1 expression 50% or more appeared to correlate with presence of small CAMLs only from the baseline, pretreatment samples (Supplementary Table 5 in the online version).

Discussion

This study focused on patients enrolled onto a recently published prospective phase 2 trial evaluating addition of concurrent and adjuvant atezolizumab to standard-of-care CRT for patients with unresectable locally advanced NSCLC.[12] We collected blood samples from 39 of 40 patients enrolled onto this trial and evaluated the presence of a recently described circulating stromal cell that we call cancer-associated macrophage-like cell, or CAML. Our previous experience with this cell type from a multi-institutional prospective 2-year study of 293 cancer patients with 6 primary solid tumor types and identified both CAML number per sample and CAML size to be prognostic, with CAML size ≥ 50 μm exhibiting a larger hazard ratio for PFS.[9] However, none of those patients was managed with immunotherapy. We therefore sought to determine whether CAML size remains an independent prognostic factor in patients with locally advanced NSCLC treated with definitive CRT and immunotherapy.

We prospectively collected blood samples before CRT (T0), during CRT (T1), at the end of CRT (T2), and at the time of first follow-up (T3), then analyzed those samples for CAML size. Overall, 267 blood samples were analyzed, and CAMLs were identified in 75% of samples. Samples collected, number of patients, number of CAMLs identified, and the number of patients with giant CAMLs were well balanced between the 4 time points. Patients found to have at least one CAML ≥ 50 μm at the time of the first follow-up after completing definitive CRT had significantly more metastatic recurrences compared to those who had small CAMLs or no CAMLs identified at the same time point. This finding is independent of the fact that the incidence of giant CAMLs was not different between time points. Because the primary mode of failure for patients with locally advanced NSCLC is distant, this is a highly relevant finding for this population that may be utilized in future clinical trials to help direct treatment strategies. Additionally, we identified that patients with giant CAMLs had

Figure 3 Survival According to CAML Size Distant Failure–free Survival, Relapse-free Survival, Progression-free Survival, and Overall Survival Based on CAML Size From Samples Collected at Time of First Follow-Up (T3 Time Point) after Completion of Definitive CRT



Abbreviations: CAML = cancer-associated macrophage-like cell; CRT = chemoradiotherapy.

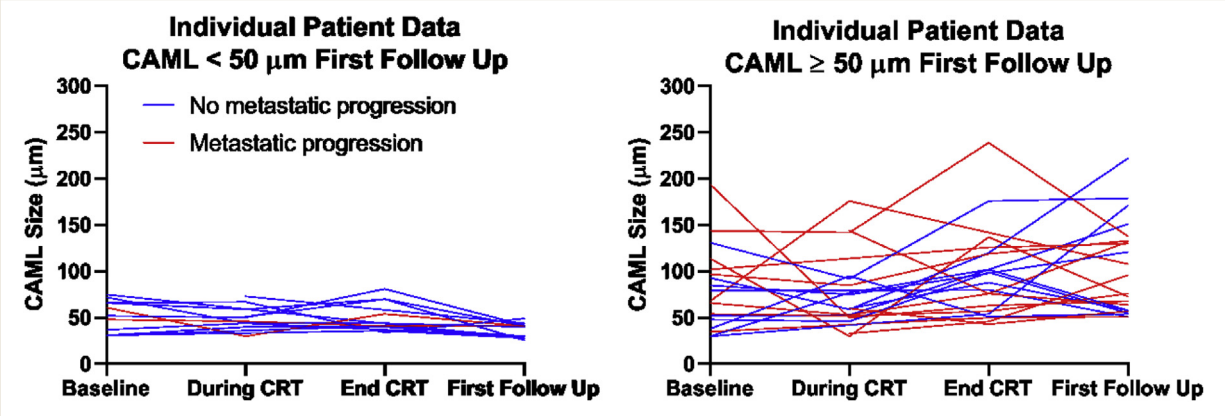
worse PFS and RFS on the basis of samples obtained at the first follow-up time point, as well as worse OS. Metastatic recurrences translated to cancer-specific mortality despite the availability of first-line salvage therapies.

Our data reveal that patients with giant CAMLs at the first follow-up were significantly more likely to have giant CAMLs at the earlier time points (pretreatment baseline, during CRT, and at the end of CRT), although there was no association with outcomes or prognosis at those time points. One possibility is that local treatment with radiation modulates CAMLs and affects their size, which occurs after completion of definitive therapy and therefore becomes evident during follow-up. A second possibility is that giant CAMLs are indeed prognostic at any time point, but a larger data set would be needed to explore this further.

A prior report suggests that CAMLs are a subset of disseminated tumor-associated macrophages and therefore it is possible that CRT mobilizes these cells into the circulation.[3] It will be important to continue obtaining blood samples from patients throughout follow-up, particularly in the era of consolidative immunotherapy, to determine whether any trends exist in CAML size that point to treatment response or disease prognosis. We previously evaluated the expression of PD-L1 on CAMLs and CTCs from patients receiving radiation or CRT therapy for stage I-IV lung cancer and found that PD-L1 was induced in 49% of patients assessed, suggesting that inducible PD-L1 could be predictive of immunotherapy response.[5,13] Future studies will determine whether PD-L1 expression or induction during definitive therapy are predictive for patients with locally advanced NSCLC, with or without immunotherapy.

The function of CAMLs has yet to be fully elucidated, as this novel biomarker is being explored in a few lung cancer trials (eg, NCT03992183, NCT03923777). Nevertheless, our report points to CAMLs as an intriguing biomarker. CAMLs were previously evaluated in a combination of other disease sites[6,9] (eg, neoplasms of the breast, esophagus, prostate, pancreas, lung, and kidney), where it was identified that CAML size (50 µm cutoff) was a better prognostic marker than CAML number. As such, we performed an exploratory analysis of various CAML sizes (data not shown)

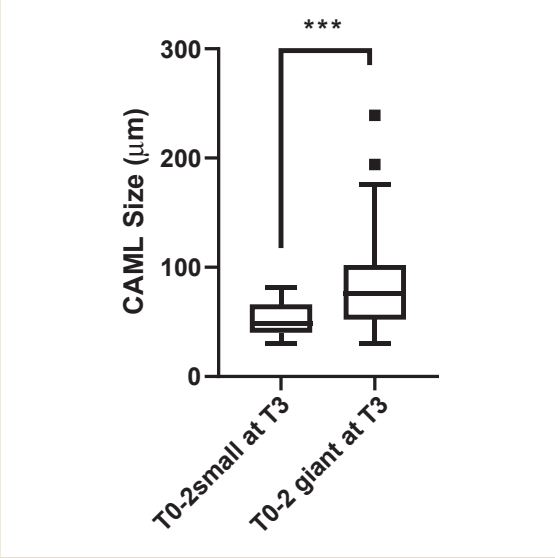
Figure 4 Disease Progression of Patients With CAML Size < 50 μm (Left) and CAML Size ≥ 50 μm (Right) at Time Point T3 Individual Patient CAML Size (Largest in Sample) Plotted Across all Time Points (T0-T3). Blue Lines Represent Patients Without Metastatic Progression; Red Lines, Patients With Metastatic Progression. T0 Indicates Baseline; T1, During CRT; T2, End of CRT; and T3, First Follow-up



Abbreviations: CAML = cancer-associated macrophage-like cell; CRT = chemoradiotherapy.

(40-100 μm, intervals of 10 μm), which showed that a cutoff of 40 μm exhibited worse DFFS at T2 and worse DFFS, RFS, and PFS at T3 (with a nonsignificant trend for OS at T3). A 60 μm threshold produced significant DFFS differences at T3, but not other parameters. Moreover, performing Cox regression using CAML size as a continuous variable did not produce statistically significant differences, indicating that the prognostic value of CAML size may be more deterministic than stochastic (owing to a threshold). We also evaluated CAML number and found that patients with 6 or more CAMLs at T3 did not have worse outcomes compared to patients with 5 or fewer CAMLs (OS $P = .96$; PFS $P = .46$; DFFS $P = .21$), although the number of patients with 6 or more CAMLs at each time point was small (6, 5, 6, and 7, respectively). CAML number cutoffs of 3, 4, or 5 CAMLs produced similar nonsignificant findings at each of the time points. These data reinforce the hypothesis that CAML size 50 μm or more is associated with disease progression and survival.

Figure 5 Overall CAML Size Overall CAML Size From all Patients From Time Points T0 to T2 Based on CAML Size < 50 μm or ≥ 50 μm at Time Point T3. T0 Indicates Baseline; T1, During CRT; T2, End of CRT; and T3, First Follow-up (***$P < 0.001$)



Abbreviation: CAML = cancer-associated macrophage-like cell.

One benefit of CAMLs compared to other circulating tumor–associated cells such as CTCs is the frequency of identification is very high. Overall, every patient from our cohort had at least one CAML identified when considering all time points (T0 to T3), while at any single time point the likelihood of identifying a CAML was between 88% and 100%. Comparatively, CTCs are identified in approximately 10% to 50% of patients with metastatic disease, and lower rates of detection in patients with locoregional disease. This suggests that CAMLs are better suited for assessing prognosis and possibly prediction of disease response resulting from higher frequency of detection in patients with localized disease; however, further studies are necessary to evaluate the predictive and prognostic power of this biomarker.

The results of this study are overall in keeping with the few available data on this topic; however, it currently remains unclear whether CAMLs represent a cause or effect of poor prognosis in these patients. Because CAMLs interact with CTCs,[3] which in turn are linked with metastatic potential and prognosis of NSCLC,[14] it is possible that CAMLs may reflect a higher propensity for systemic disease burden. However, it is also possible that CAMLs may drive tumor invasion and metastasis, since macrophages are known to play a role in these events.[15] Our study focused on CAML size based on prior data.[9] It is possible that CAML size is a surrogate for

attenuated antitumor immune response and/or a surrogate for an immunosuppressive tumor microenvironment. More CAMLs were identified at a median of 30 days after completing definitive therapy, suggesting that CRT is able to mobilize these cells into the circulation. It is not clear whether concurrent immunotherapy influenced the mobilization of CAMLs during treatment with concurrent CRT and checkpoint inhibition; however, because checkpoint inhibition largely acts on T cells and not macrophages, it is possible that immunotherapy did not play a significant role in this observation. Macrophages (largely M2 subtypes) exert tumor-stimulating effects, which may limit the effectiveness of immunotherapy and contribute to the relatively low response rates to these agents. Macrophage-specific immunotherapies are a focus of some ongoing investigations, and could influence the prognostic value of CAMLs following administration of those compounds.[16,17] Identification of polyploid giant cells in the tumor microenvironment has been previously described in different solid tumor types, suggesting that these cells may be mobilized into the circulation; however, detailed characterization is still lacking and the subject of ongoing investigation.[18,19]

Interestingly, there was no association with tumor baseline PD-L1 expression and OS or DFFS, and there was also no association with PD-L1 expression and presence of giant CAMLs at any time point. This suggests that CAMLs are not greatly modulated by the addition of immunotherapy during CRT; although the possibility exists that CAMLs may be modulated during maintenance immunotherapy, exploring this would require serial blood samples obtained over time throughout maintenance treatment and follow-up. Although tumor PD-L1 expression may not be associated with disease outcomes or CAML size, we have previously shown that PD-L1 expression is induced on circulating stromal cells during definitive CRT.[5] We also showed that induction of PD-L1 expression on circulating stromal cells including CTCs and CAMLs after treatment may also be associated with disease outcomes, while baseline circulating cell PD-L1 expression was not.[13] Dynamic tracking of PD-L1 may therefore serve as another circulating biomarker of disease outcomes and immunotherapy effectiveness, along with CAML size; however, further research is necessary to verify these preliminary findings.

There are several strengths of this work, including the standardized assessment of CAML detection and the prospective population from which these data were collected. However, there are several limitations of our study. First, the sample sizes and follow-up time require further verification by larger and longer-term data. Second, it is possible that different tumor mutations and/or polymorphisms (not accounted for herein) may be associated with a differential rate of CAML development, along with other molecular factors that could not be evaluated in this study. Third, it may also be important to consider our a priori cutoff of 50 μm was based on prior empirical studies that also utilized a priori thresholds.[8] Last, we cannot comment on whether changes in CAML size over time are related to enlargement of existing CAMLs or increased production of giant CAMLs from the tumor stroma through an adaptive process in the microenvironment during the course of therapy. Overall, our results lend credence to the importance of noninvasive peripheral blood–based biomarkers to evaluate disease recurrence and prognosis, and help personalize cancer care.

Conclusion

This study of patients enrolled onto on a prospective phase 2 clinical trial provides support to the notion that giant CAMLs are associated with outcomes after definitive CRT for locally advanced NSCLC treated in the consolidative immunotherapy era. Although these data are intriguing, our results require continued prospective validation to further validate CAMLs as a prognostic biomarker.

Clinical Practice Points

- Early assessment of disease prognosis in locally advanced NSCLC patients is needed to identify those at high risk for disease recurrence. Noninvasive so-called liquid biopsy methods are well suited for this task because they pose a low risk to the patient and can be monitored serially over time.
- We identified a novel circulating cell in the peripheral blood whose size is associated with disease recurrence and poorer survival in locally advanced NSCLC patients from samples obtained shortly after completion of definitive CRT.
- These cells, which are myeloid derived and appear macrophage-like (CAML), were identified in 76% of samples using microfiltration, immunostaining, and microscopy.
- Patients harboring at least one CAML 50 μm or larger after completing CRT were significantly more likely to develop distant metastases and experience worse survival.
- Our simple method permits assessment of an early biomarker that identifies patients at high risk for disease recurrence, which may be used to guide future clinical trials.

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Disclosure

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Supplementary Data

Supplementary tables and figures accompanying this article can be found in the online version https://doi.org/10.1016/j.cllc.2020.06.016.

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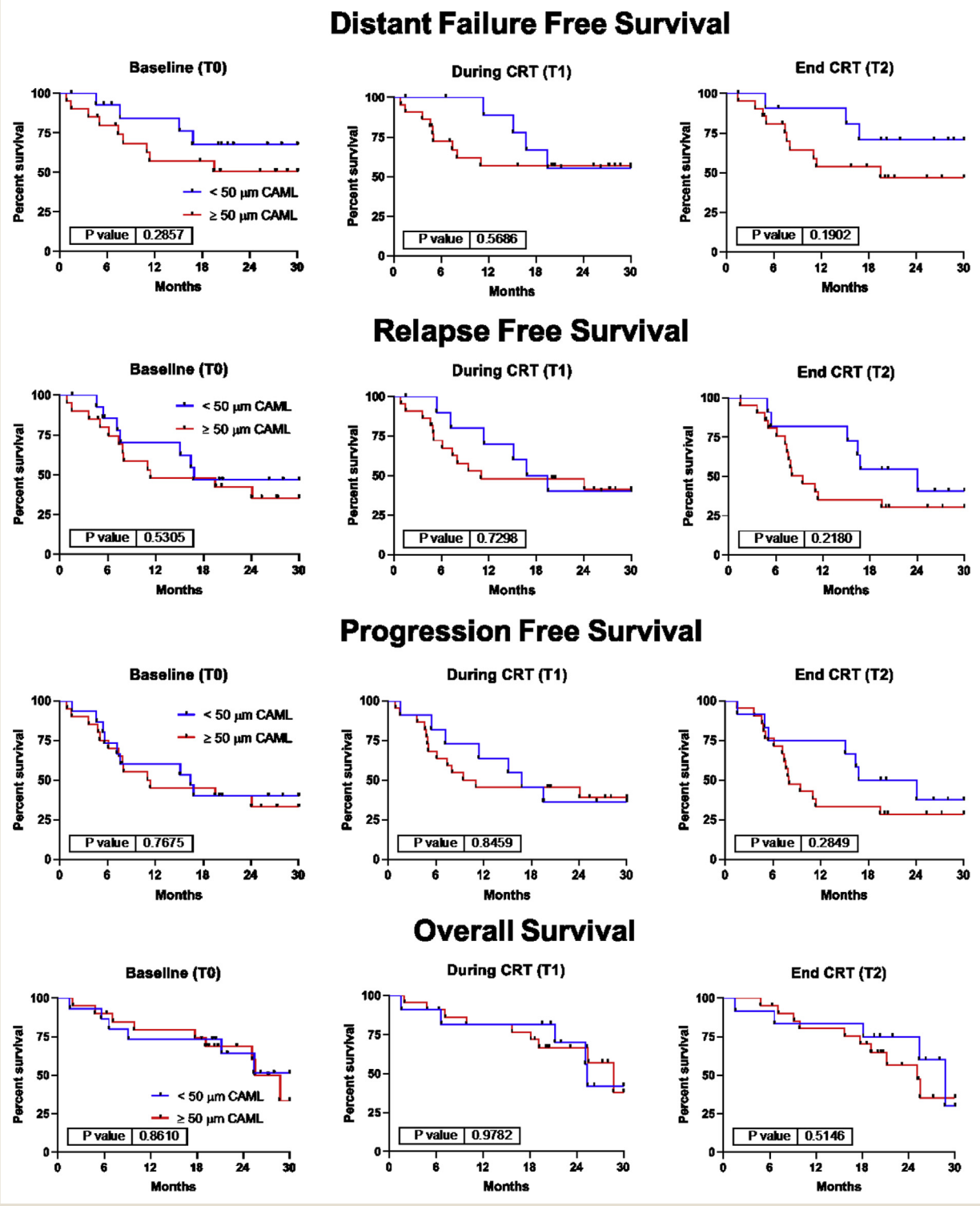
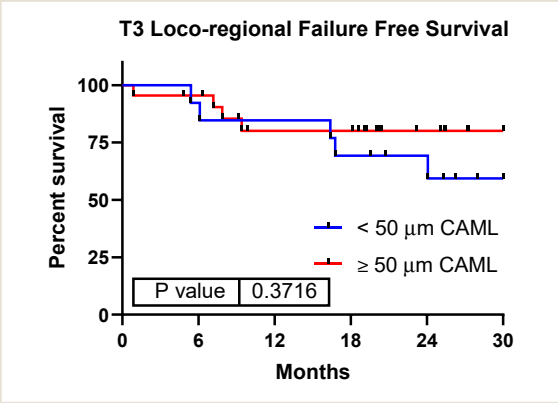
Supplemental Figure 1 DFFS, RFS, PFS, and OS Based on CAML Size < 50 μm or ≥ 50 From Time Points T0, T1, and T2 T0 Indicates Baseline; T1, During CRT; T2, End of CRT; and T3, First Follow-up



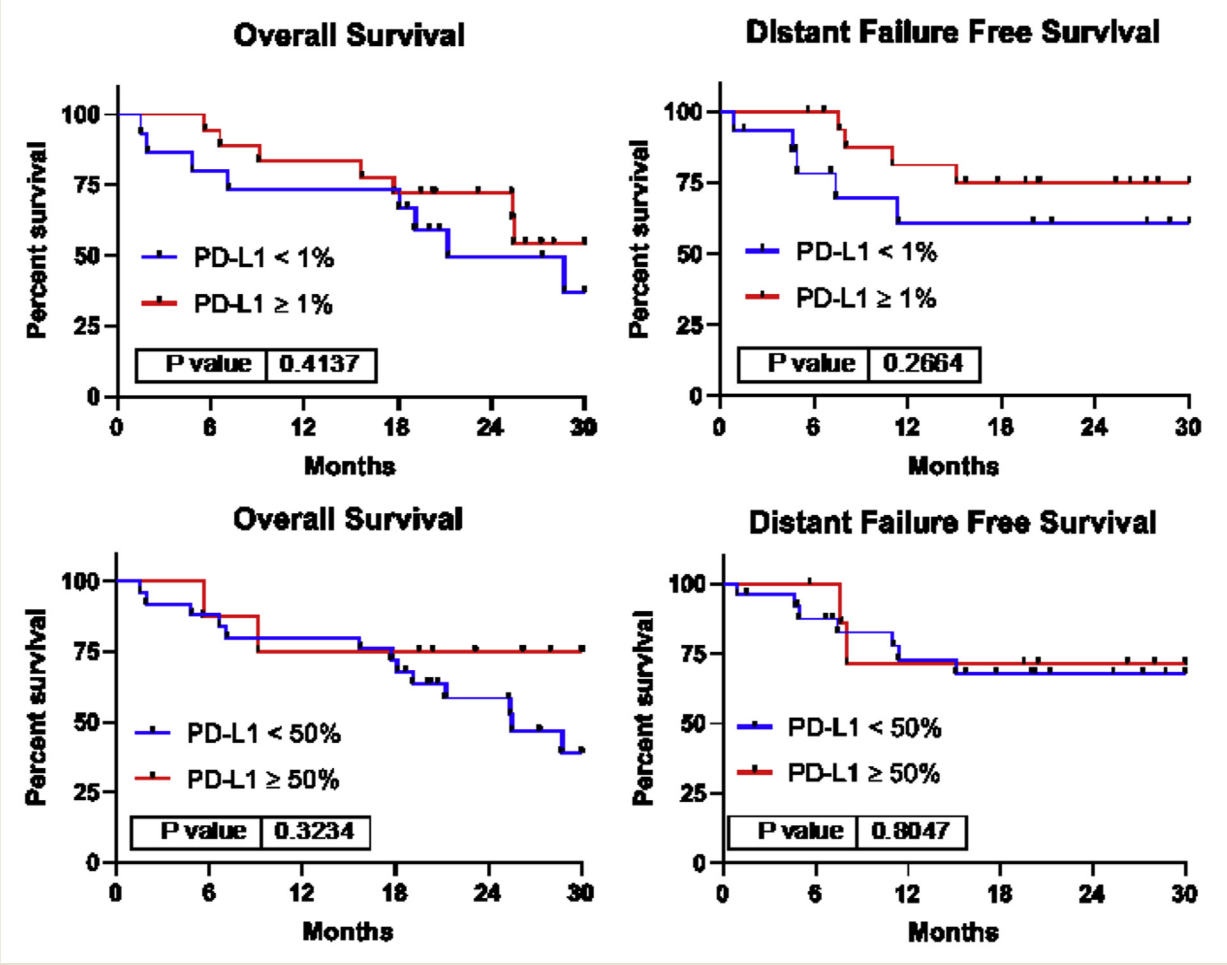
Abbreviations: CAML = cancer-associated macrophage-like cell; CRT = chemoradiotherapy; DFFS = distant failure-free survival; OS = overall Survival; PFS = progression-free survival; RFS = progression-free survival.

Supplemental Figure 2 Locoregional Failure–free Survival Based on CAML Size < 50 μm or ≥ 50 From Time Point T3 T0 Indicates Baseline; T1, During CRT; T2, End of CRT; and T3, First Follow-up



Abbreviations: CAML = cancer-associated macrophage-like cell; CRT = chemoradiotherapy.

Supplemental Figure 3 Overall Survival and Distant Failure-free Survival Based on PD-L1 Expression Using Cutoff of 1% or 50%



Abbreviation: PD-L1 = programmed death ligand 1.

Supplemental Table 1 Blood Sample and CAML Size Characteristics at Each Time Point

Characteristic	Value
Total patients	39
Total blood samples	267
T0 (baseline)	
Patients	35 (90)
Samples	69 (26)
Samples with CAMLs	51 (74)
CAMLs ≥50 μm	28 (41)
T1 (during CRT)	
Patients	33 (85)
Samples	65 (24)
Samples with CAMLs	48 (74)
CAMLs ≥50 μm	27 (42)
T2 (end of CRT)	
Patients	33 (85)
Samples	64 (24)
Samples with CAMLs	48 (75)
CAMLs ≥50 μm	26 (41)
T3 (first follow-up)	
Patients	35 (90)
Samples	70 (26)
Samples with CAMLs	56 (80)
CAMLs ≥50 μm	30 (43)

Data are presented as n (%) unless otherwise indicated.
Abbreviations: CAML = cancer-associated macrophage-like cell; CRT = chemoradiotherapy.

Supplemental Table 2 Univariate Cox Regression Analysis Based on CAML Size < 50 μm or ≥ 50 μm From Time Points T0, T1, T2, and T3

Survival	*P*
Overall survival	
CAML ≥50 μm at baseline	.861
CAML ≥50 μm during CRT	.978
CAML ≥50 μm at end of CRT	.515
CAML ≥50 μm at first follow-up	.034[a]
Distant failure-free survival	
CAML ≥50 μm at baseline	.286
CAML ≥50 μm during CRT	.569
CAML ≥50 μm at end of CRT	.190
CAML ≥50 μm at first follow-up	.015[a]
Relapse-free survival	
CAML ≥50 μm at baseline	.531
CAML ≥50 μm during CRT	.730
CAML ≥50 μm at end of CRT	.218
CAML ≥50 μm at first follow-up	.036[a]
Progression-free survival	
CAML ≥50 μm at baseline	.768
CAML ≥50 μm during CRT	.846
CAML ≥50 μm at end of CRT	.285
CAML ≥50 μm at first follow-up	.025[a]

T0 indicates baseline; T1, during CRT; T2, end of CRT; and T3, first follow-up
Abbreviations: CAML = cancer-associated macrophage-like cell; CRT = chemoradiotherapy.
[a]Statistically significant ($P \leq 0.05$).

Supplemental Table 3 Patient Recurrence by CAML Size at First Post-CRT Follow-up (Time Point T3)

Patient No. With Recurrence for:	Recurrence Pattern	Recurrence Location
CAMLs <50 μm at first follow-up		
1	Locoregional	Primary site
2	Locoregional	Primary site
3	Locoregional	Primary site
4	Locoregional	Primary site
5	Locoregional and distant	Primary site, lung, bone
6	Distant	Bone
CAMLs ≥50 μm at first follow-up		
1	Locoregional	Primary site
2	Locoregional	Primary site
3	Locoregional	Primary site
4	Locoregional and distant	Primary site, bone
5	Distant	Brain
6	Distant	Bone
7	Distant	Lung
8	Distant	Brain
9	Distant	Liver
10	Distant	Lung, peritoneum, liver, spleen
11	Distant	Bone
12	Distant	Adrenal, brain, lung
13	Distant	Brain
14	Distant	Flank
15	Distant	Liver

Abbreviations: CAML = cancer-associated macrophage-like cell; CRT = chemoradiotherapy.

Supplemental Table 4 Univariate Cox Regression Analysis at Time Point T3

Survival	*P*
Overall survival	
CAML ≥50 μm at first follow-up	.034[a]
Age	.166
Sex	.759
ECOG PS	.785
Radiation prescription dose	.677
Histology	.991
Smoking pack-years	.039[a]
Distant failure-free survival	
CAML ≥50 μm at first follow-up	.015[a]
Age	.650
Sex	.321
ECOG PS	.110
Radiation prescription dose	.908
Histology	.124
Smoking pack-years	.233
Relapse-free survival	
CAML ≥50 μm at first follow-up	.036[a]
Age	.709
Sex	.862
ECOG PS	.313
Radiation prescription dose	.939
Histology	.280
Smoking pack-years	.746
Progression-free survival	
CAML ≥ 50 μm at first follow-up	.025[a]
Age	.861
Sex	.875
ECOG PS	.380
Radiation prescription dose	.873
Histology	.496
Smoking pack-years	.462

Abbreviations: CAML = cancer-associated macrophage-like cell; ECOG PS = Eastern Cooperative Oncology Group performance status.
[a]Statistically significant.

Supplemental Table 5	Chi-Square Calculations Assessing Association Between PD-L1 Expression and CAML Size
Characteristic	***P***
PD-L1 expression cutoff $\geq$ 1%	
CAML <50 μm at T0	.534
CAML <50 μm at T1	.686
CAML <50 μm at T2	.247
CAML <50 μm at T3	.550
PD-L1 expression cutoff $\geq$ 50%	
CAML <50 μm at T0	.044[a]
CAML <50 μm at T1	.291
CAML <50 μm at T2	.201
CAML <50 μm at T3	.229

T0 indicates baseline; T1, during CRT; T2, end of CRT; and T3, first follow-up.
Abbreviations: CAML = cancer-associated macrophage-like cell; CRT = chemoradiotherapy; PD-L1 = programmed death ligand 1.
[a]Statistically significant.