

Circulating Cancer-Associated Macrophage-Like Cells Differentiate Malignant Breast Cancer and Benign Breast Conditions

Daniel L. Adams¹, Diane K. Adams², R. Katherine Alpaugh³, Massimo Cristofanilli⁴, Stuart S. Martin⁵, Saranya Chumsri^{5,6}, Cha-Mei Tang⁷, and Jeffrey R. Marks⁸

Abstract

Background: Blood-based testing can be used as a noninvasive method to recover and analyze circulating tumor-derived cells for clinical use. Circulating cancer-associated macrophage-like cells (CAML) are specialized myeloid cells found in peripheral blood and associated with the presence of solid malignancies. We measured CAMLs prospectively in peripheral blood to ascertain their prevalence, specificity, and sensitivity in relation to breast disease status at clinical presentation.

Methods: We report on two related but separate studies: 1) CellSieve microfilters were used to isolate CAMLs from blood samples of patients with known malignant disease ($n = 41$). Prevalence and specificity was compared against healthy volunteers ($n = 16$). 2) A follow-up double-blind pilot study was conducted on women ($n = 41$) undergoing core-needle biopsy to diagnose suspicious breast masses.

Results: CAMLs were found in 93% of known malignant patients ($n = 38/41$), averaging 19.4 cells per sample, but none in the healthy controls. In subjects undergoing core biopsy for initial diagnosis, CAMLs were found in 88% of subjects with invasive carcinoma ($n = 15/17$) and 26% with benign breast conditions ($n = 5/19$).

Conclusion: These preliminary pilot studies suggest that the presence of CAMLs may differentiate patients with malignant disease, benign breast conditions, and healthy individuals.

Impact: We supply evidence that this previously unidentified circulating stromal cell may have utility as a screening tool to detect breast cancer in various malignancies, irrespective of disease stage. *Cancer Epidemiol Biomarkers Prev*; 25(7): 1–6. ©2016 AACR.

Introduction

Blood-based biopsies, that is, "liquid" biopsies, using the peripheral blood of cancer patients have identified various tumor-associated circulating cells, including circulating tumor/epithelial cells (CTC) and cancer-associated macrophage-like cells (CAML; refs. 1–3). CTCs are malignant cells derived from solid tumors and may enter the circulatory system after dissociating from their original tumor site (4). CAMLs are clearly distinct from CTCs as they have properties of phagocytic myeloid cells and may be derived from an immunologic response to the tumor (5). In both cases, these cells should be an indication of the presence of cancer and the extent

of disease burden, particularly metastatic disease (6–8). However, CTCs are rarely found in nonmetastatic disease and do not provide the sensitivity needed for early detection although their presence in early-stage disease may be prognostic (9). In contrast, CAMLs appear to be more prevalent in all cancer patients, regardless of stage, but their underlying biology and clinical utility are still undetermined (5).

CAMLs are specialized myeloid cells (CD14⁺) that are found transiting the circulation of patients in all stages of cancer and in a variety of malignancies (5). On the basis of our preliminary studies, these cells are responsive to cancer treatment and are linked with poor prognosis in pancreatic, prostate, and breast cancer (5, 10, 11). Initially observed many years ago in both circulation and in tumor masses (12–16), the clinical value of these cells for detecting, staging, or monitoring malignancies has not been tested. We and others have previously reported that size-based filtration can rapidly and efficiently isolate multiple varieties of circulating tumor-associated cells (5, 17), including CAMLs and CTCs, from peripheral blood. It is therefore possible to study both cell types in conjunction with and in relation to malignant or benign conditions.

Our approach employs CellSieve microfilters that are lithographically fabricated membranes with high porosity, precise pore dimensions, and regular pore distribution to isolate peripheral blood cells larger than 7 μm . In this article, we describe two separate but related prospective studies using our CAML isolation and analysis procedures in the context of breast cancer detection. Results from these studies suggest that this circulating stromal cell occurs as an early event in the progression of the disease and may

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Note: Supplementary data for this article are available at Cancer Epidemiology, Biomarkers & Prevention Online (<http://cebp.aacrjournals.org/>).

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have utility as a noninvasive tool to differentiate breast cancer from benign breast conditions.

Materials and Methods

Anonymized peripheral blood samples ($n = 41$) were supplied through collaborative agreements with Fox Chase Cancer Center (FCCC) and University of Maryland Baltimore (UMB) with written informed consent and according to the local Institutional Review Board (IRB) approval at each institution. The 41 whole peripheral blood samples were drawn from women who were actively undergoing treatment for previously confirmed stage II, III, or IV breast cancer at either FCCC or UMB between 2011 and 2013. The study group characteristics can be found in Supplementary Table S1. All blood samples were drawn into CellSave preservative tubes (~9 mL, Janssen Diagnostics) and shipped to Creatv MicroTech (Creatv) for processing. Results and patient identification from institutions were not shared or communicated until completion of study.

Anonymized healthy women volunteers donated blood samples ($n = 16$) with written informed consent and IRB approval by Western Institutional Review Board. Donor samples were procured on a voluntarily basis at a blood collection center with no selection process, outside standard exclusion criteria. As such, all samples were considered to be from healthy individuals and were drawn into CellSave preservative tubes and shipped to Creatv for processing. The 41 cancers and 16 healthy controls constitute Study A in this report (Fig. 1A).

Study B was a double-blind prospective study of peripheral blood samples from 41 subjects undergoing tissue diagnosis by core-needle biopsy, performed as a collaboration between Duke University Medical Center and Creatv (Fig. 1B). Blood was procured from subjects undergoing core biopsy, either immediately before or immediately after core biopsy (see Table 1), after referral from either an abnormal screening mammogram or clinical exam

and were shipped overnight to Creatv for processing. Investigators at Duke University, Creatv, and the subjects themselves were unaware of the pathologic diagnosis at the time of the blood draw and CAML analysis. Follow-up information derived from pathology reports on the core biopsy and subsequent surgeries (if performed) were collected. This information was only shared with investigators at Creatv after CAML analysis was performed and returned to Duke.

CellSieve microfilters were used to isolate CAMLs and CTCs from 7.5 mL of whole peripheral blood, using 7- μ m pores to separate CAMLs from white blood cells based on size as described previously (18–20). CAMLs collected for Study A were fixed, permeabilized, and stained with 4',6-diamidino-2-phenylindole (DAPI) and an antibody cocktail against cytokeratins 8, 18, and 19, EpCAM, and CD45. CAMLs collected for Study B were fixed, permeabilized, and stained with DAPI and an antibody cocktail against cytokeratin 8, 18, and 19, CD14, and CD45. CAMLs were defined as enlarged (≥ 30 μ m in diameter), multinuclear cells with diffuse cytoplasmic cytokeratin and either CD14⁺ or CD45⁺. As described previously, pathologically definable CTCs were defined as filamentous cytokeratin-positive cells, with pleomorphic nuclei that were negative for CD45 and CD14 (Fig. 2; ref. 20). Epithelial–mesenchymal transition CTCs (EMT-CTCs) were defined as diffuse/nonfilamentous cytokeratin-positive cells with pleomorphic nuclei that were negative for CD45 and CD14 (20).

Results

Prevalence of CAMLs in advanced-stage breast cancer compared with healthy controls (Study A)

Our initial studies on several types of carcinomas indicated that the number of CAMLs may be related to stage of disease and response to therapy (5). To further explore this relationship specifically in breast cancer, we analyzed blood from 41 patients

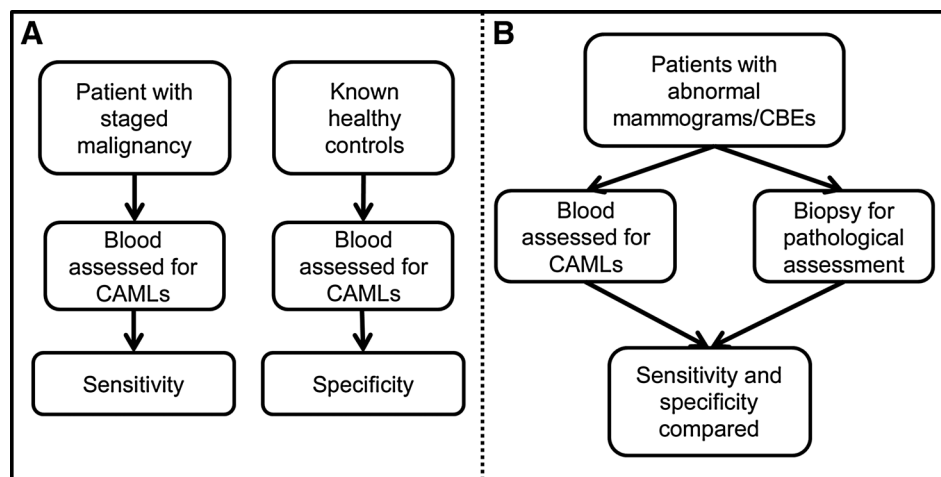


Figure 1.

Overview of sensitivity and specificity determination based on our two studies, either Study A: populations of women with diagnosed breast cancer or Study B: populations of women with positive clinical breast exam (CBE)/mammography but before diagnosis. A, patients with invasive breast malignancy were used to determine sensitivity of an assay based on CAML presence. Healthy controls were used to determine the specificity. B, the assay was then tested using patients with positive breast mammography, or positive CBEs, for CAML presence by standard blood draw at the time core biopsy was taken. Standard pathologic assessment was used to determine invasive malignant disease, benign condition, or noninvasive disease/stage 0. Patient information was then unblinded and sensitivity/specificity of CAMLs in relation to pathologic assessment was compared.

Table 1. Subject characteristics from Study B: patients with abnormal mammogram, or CBE

	Benign (<i>n</i> = 19)	ADH+DCIS (<i>n</i> = 5)	Invasive (<i>n</i> = 17)
Age (mean)	55.2	58.4	57.1
Race, <i>n</i>			
White	14	5	16
Black	5	0	0
Asian	0	0	1
Body mass index (mean)	28.7	26.0	31.8
Prior invasive cancer (%)	0	0	6% ^a
Detected by screening mammography	58% ^b	80%	35% ^c
Detected by self-exam	37%	20%	53%
Drawn before biopsy (%)	32%	0%	29%

^aOne of the invasive breast cancer subjects had an early invasive breast cancer diagnosed >20 years before the current encounter.

^bOne of the subjects with a benign breast condition was referred for biopsy from an incidental finding from a CT exam.

^cTwo of the subjects with invasive cancer were referred on the basis of findings from CT and PET exams.

with known invasive breast cancer who were undergoing treatment for the disease. CAMLs were found in 93% of blood samples (Fig. 3A). Specifically, CAMLs averaged 19.4 per 7.5 mL blood sample (2.6 CAML/mL), median 8.0 per sample, low of 0 per sample, and a high of 105 per sample (Fig. 3B; Supplementary Table S1). We compared these findings to a group of healthy women of comparable age with no known malignant conditions. No CAMLs were detected in any of the 16 peripheral blood samples from these control subjects (Fig. 3). Therefore, using a threshold of 1 CAML as a positive finding, the results from Study A are summarized by an ROC curve with an AUC of 0.96 (95% CI, 0.91–1.0; Fig. 3C) with sensitivity of 93% (95% CI, 80–99), specificity of 100% (95% CI, 79–100), positive predictive value (PPV): 100% (95% CI, 91–100), and negative predictive value (NPV): 84% (95% CI, 60–97).

Detection of CAMLs in women undergoing breast cancer diagnosis (Study B)

To determine the utility of CAMLs for detection and discrimination of cancer from benign conditions, we performed a PRoBE (21) designed prospective study by recruiting women undergoing image-guided biopsies for breast cancer diagnosis. Principles of PRoBE design are a prospective study that mirrors the population of subjects for which the test is designed followed by a blinded evaluation. In this case, all parties were blinded to the case-control status including the subjects who were having a tissue

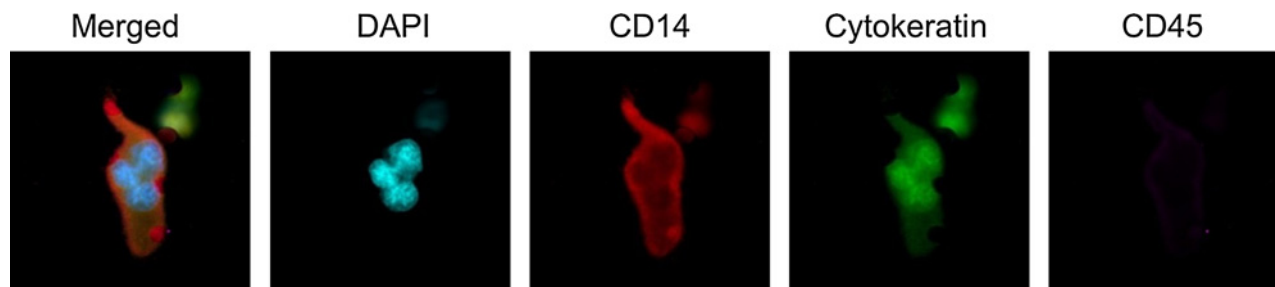
sampling procedure for the purposes of diagnosis. From 41 total subjects recruited over the course of a 6-month period (October 2014–April 2015), CAMLs were detected in 15 of 17 subjects newly diagnosed with invasive breast cancer (88% sensitivity; Fig. 4A; Supplementary Table S2). In general, fewer CAMLs were detected in the subjects undergoing diagnosis compared with those under treatment (Study A). From a total of 19 patients diagnosed with benign breast conditions, CAMLs were detected in 5 (26%) subjects. In addition, 5 of 5 subjects who were diagnosed with high-risk noninvasive conditions [ductal carcinoma in situ (DCIS), stage 0, and atypical ductal hyperplasia] had detectable CAMLs (Fig. 4B; Table 1). We saw no statistical difference in CAML size, morphology, or staining pattern between true positives and false positives. Comparing subjects with benign conditions (*n* = 19, excluding the high-risk noninvasive lesions) to those with invasive carcinoma (*n* = 17) results in an ROC curve with an AUC of 0.78 (95% CI, 0.63–0.92), using a threshold of 1 CAML as a positive finding (Fig. 4C). This threshold returns a sensitivity of 88% (95% CI, 64–99), specificity of 74% (95% CI, 49–91), PPV of 75% (95% CI, 51–91), and a NPV of 88% (95% CI, 62–99; Supplementary Table S3).

Aggregating the results from both studies shows that all breast cancer subtypes based on ER, PR, and HER2 status produce detectable levels of CAMLs and that there is not a pronounced effect of tumor stage or nodal status on the presence of these cells.

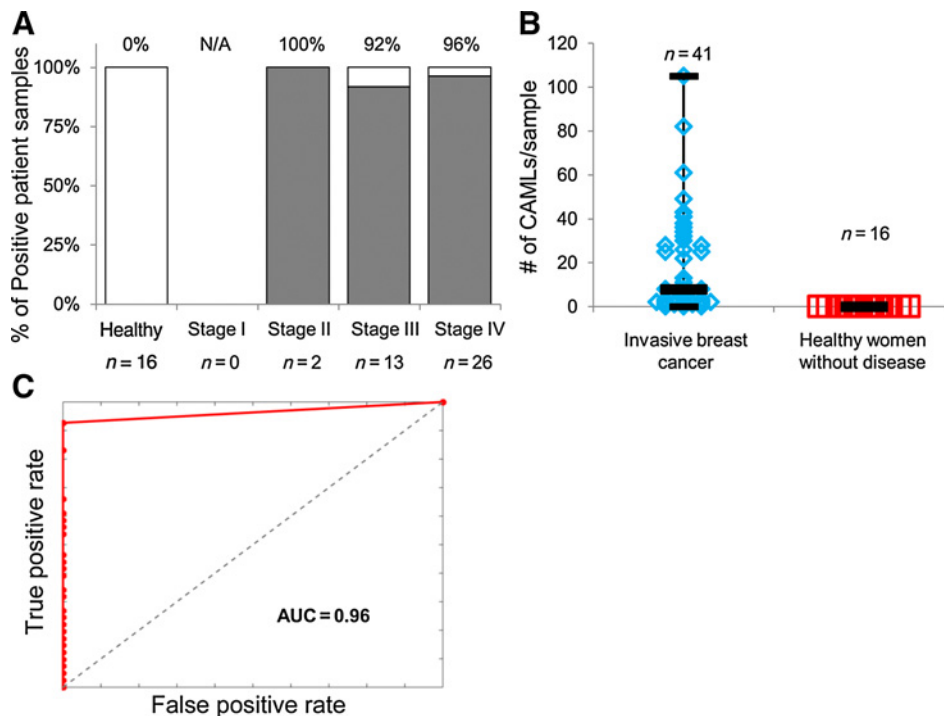
In comparison, for Study A none of the healthy control samples had any type of detectable CTCs, as defined previously (20), but 76% of patients with known breast carcinomas had at least 1 pathologically definable CTC and 54% had at least 1 EMT-like CTC. In Study B, none of the samples had pathologically definable CTCs, whereas EMT-like CTCs were found in 18% (*n* = 3/17) of invasive carcinomas and in 37% (*n* = 7/19) of patients with benign conditions (Supplementary Fig. S1). The finding that no pathologically definable CTC was found in any patient sample with early disease, whereas EMT-like CTCs were found in 18% of patients, is at par with previous studies comparing CTCs in early-stage disease. A variety of studies have shown that CTC isolation techniques may identify CTCs in approximately 20% of early-stage disease (9, 22) while having a false positive rate of 7.5%–19%, compared to individuals with benign conditions (23, 24).

Discussion

There has been a great deal of interest in using blood-based biopsies, or so-called "liquid biopsies" to diagnose, categorize,

**Figure 2.**

Appearance of CAMLs isolated on filters from cancer patients. Single approximately 100- μ m CAML from a breast cancer patient with a polymorphonuclear DAPI-stained structure (blue), intense CD14 (red) staining, some cytokeratin staining (green), and weak CD45 (purple). Box size = 100 μ m.

**Figure 3.**

Study A: presence, sensitivity, and specificity of CAMLs in patients with known invasive breast cancer versus healthy control volunteers. A, CAMLs were found in 38 of 41 patients with known invasive breast cancer, but in none of the 16 healthy controls. B, the number of CAMLs ranged 0–105 per 7.5-mL blood sample in patients with cancer. C, ROC curve shows a clear stratification between a healthy control population from a malignant population, AUC = 0.96. Sensitivity: 93% (95% CI, 80–99); specificity: 100% (95% CI, 79–100); PPV: 100% (95% CI, 91–100); NPV: 84% (95% CI, 60–97).

and monitor disease status in clinical oncology settings (3). One approach for isolating cancer-associated cells from the peripheral blood is microfiltration which takes advantage of the typically larger size of these cells compared with the vast majority of the mononuclear cells found in circulation. Microfiltration has been shown to capture a variety of circulating tumor-derived cells, that is, CTCs and CAMLs, without relying on specific cell surface markers for antibody capture. CAMLs have properties that are consistent with disseminated tumor-associated macrophages containing phagocytosed elements of the primary tumor and as such may be specific and sensitive for the presence of cancer. We have identified these cells in multiple cancer types and at all stages of disease (5).

In this article, we describe two related studies to determine the prevalence and specificity of CAMLs for detecting breast cancer in independent populations of women undergoing diagnosis or treatment for the disease. Laboratory, evaluation methods, and criteria used to identify and enumerate CAMLs were nearly identical for both studies. In the first study, women with already diagnosed advanced stage breast cancer were compared with healthy age-matched controls. Nearly all of the cases ($n = 38/41$) and none of the controls ($n = 0/16$) had detectable CAMLs yielding an AUC of 0.96. This degree of specificity and sensitivity support our prior work that the presence of CAMLs may be useful for disease monitoring (5, 10, 11). Furthermore, the high fraction of positive cases, even in stage II cancers, shows the potential utility over current cell-based liquid biopsy methods which are limited by cell surface antigen expression required for antibody enrichment (20).

The second study was designed to test whether the presence of CAMLs could be useful in the diagnostic setting. There are a large number of breast biopsies performed each year in the United States after referral from imaging, clinical exam, or self-exam. The majority of these biopsies return benign findings resulting

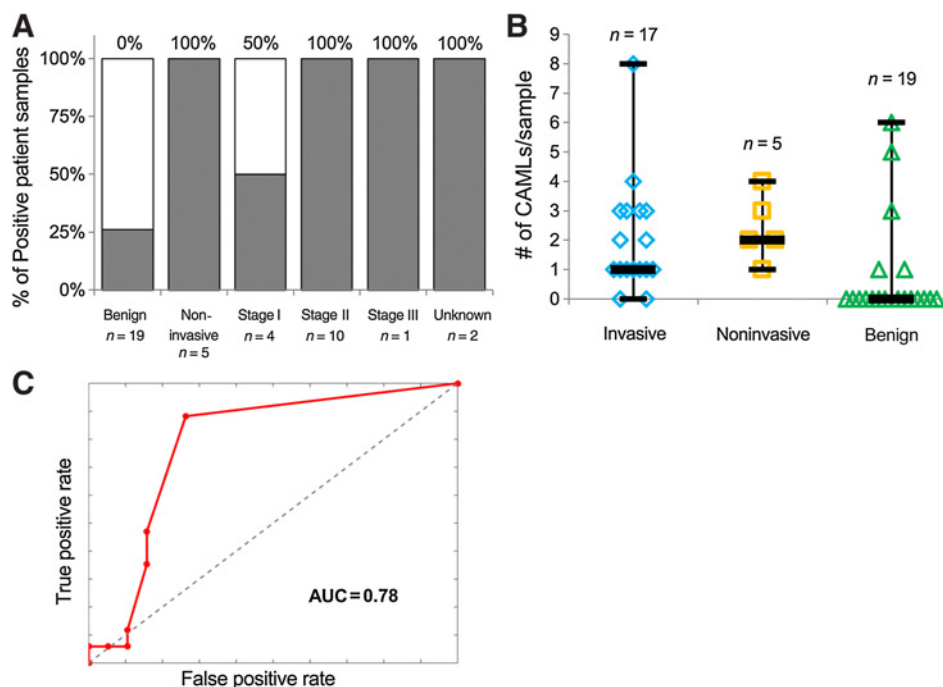
in no useful clinical recommendations other than continued surveillance. A cohort of women undergoing image-guided tissue diagnosis was enrolled in the study and blood was drawn either immediately before or immediately after the core biopsy procedure. Pathology and staging information were obtained in the weeks after enrollment and remained blinded to the scientists performing the CAML assay. This rigorous study design with an abundance of early-stage newly diagnosed cancers still produced a meaningful but less dramatic separation between invasive cancers and benign conditions (AUC = 0.78). Interestingly, all 5 subjects with noninvasive breast disease (carcinoma *in situ* and atypical hyperplasia) had detectable CAMLs, suggesting that these high-risk (25–27) conditions may cause an influx of CAMLs into circulation. As this was a prospective study, we have only a short interval of follow-up and none of these subjects have been diagnosed with invasive cancer within this limited time period.

While our study in women undergoing diagnosis contains appropriately matched cases and controls, we identify one caveat to the results. It is formally possible that the core biopsy itself produced a transient increase in CAML-type cells. To account for this possibility, we compared the timing of blood draw in relation to the biopsy. In Study B, approximately 70% of the subjects had their blood drawn immediately after the biopsy procedure with the remaining 30% drawn immediately before the biopsy (Supplementary Fig. S2). In comparing the dataset, we did not observe a consistent trend in presence or number of CAMLs and the timing of the sample draw in relation to biopsy.

It has been argued that cancer screening assays should meet a minimum sensitivity and a minimum specificity, though usually screening assays will sacrifice specificity for sensitivity, or vice versa. Many would agree that >90% sensitivity and >90% specificity is optimal if cancer screening platforms are to be clinically useful. These data suggest that the presence

Figure 4.

Study B: Presence, sensitivity, and specificity of CAMLs in patients with positive mammograms/CBEs compared with standard pathologic assessment. A, CAMLs were found in all patients with noninvasive disease/stage 0, in 88% of patients with invasive disease, and 26% of patients with benign conditions. B, CAMLs were found in a range of 0–8 cells per 7.5-mL sample. C, ROC curves comparing invasive breast cancer ($n = 17$) to benign conditions ($n = 19$) showed an AUC = 0.78. Sensitivity: 88% (95% CI, 64–99); specificity: 74% (95% CI, 49–91); PPV: 75% (95% CI, 51–91); NPV: 88% (95% CI, 62–99).



of CAML cells as a tool for cancer screening may meet the desired sensitivity (93%) and specificity (100%), when persons with breast carcinomas are compared against a healthy population. However, comparing patients with carcinoma versus persons with benign conditions, whereas the sensitivity remains high (88%), the specificity drops to 74%. This was tested in patients where the current screening tools were unable to differentiate carcinomas and benign conditions, that is, patients with a positive mammography exam, or breast exam. In this context, this study appears promising and dictates the need for additional biologic and clinical studies to expand our understanding of CAMLs for its potential utility as a screening tool.

These studies, although preliminary and on relatively small sets of subjects, indicate that the use of CAMLs as a blood-based biomarker of malignant disease shows promise. The high frequency and high specificity of CAMLs suggest a possible use as a biomarker for early detection of solid tumors including breast cancer. In addition, further proteomic and genomic characterization of CAMLs may allow for more discrete identification of cancer type during initial disease screening and a more accurate differentiation of benign diseases. An expanded study cohort should include (i) a larger population of patients, (ii) more patient subsets with and without malignant disease, (iii) the inclusion of women with other conditions, and (iv) reading samples at multiple site to better gauge the interassay and interobserver variability. Although this pilot study appears promising, the additional studies described will be necessary to fully assess the clinical utility of this cellular biomarker.

Disclosure of Potential Conflicts of Interest

D.L. Adams is a senior research scientist at and has ownership interest (including patents) in Creatv MicroTech, Inc. C.-M. Tang has ownership interest (including patents) in Creatv MicroTech, Inc. No potential conflicts of interest were disclosed by the other authors.

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