

# Hallmark Circulating Tumor-Associated Cell Clusters Signify 230 Times Higher One-Year Cancer Risk

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## ABSTRACT

We have previously shown that circulating ensembles of tumor-associated cells (C-ETACs) are a systemic hallmark of cancer based on analysis of blood samples from 16,134 individuals including 10,625 asymptomatic individuals and 5,509 diagnosed cases of cancer. C-ETACs were ubiquitously (90%) detected across all cancer types and were rare (3.6%) among the asymptomatic population. Consequently, we hypothesized that asymptomatic individuals with detectable C-ETACs would have a definitively elevated risk of developing cancer as compared with individuals without C-ETACs. In the present manuscript we present 1-year follow-up data of the asymptomatic cohort which shows that C-ETAC positive individuals have a 230-fold ( $P < 0.00001$ ) higher 1-year cancer risk as compared with individuals where C-ETACs were undetectable. Simultaneously,

we also expanded the study to include 4,419 symptomatic individuals, suspected of cancer, prior to undergoing an invasive biopsy for diagnosis. C-ETACs were detected in 4,101 (92.8%) of these 4,419 cases where cancer was eventually confirmed. We conclude that detection of C-ETACs can identify patients at risk of cancer and can be reliably used to stratify asymptomatic individuals with an elevated 1-year risk of cancer.

**Prevention Relevance:** The study evaluated a blood test that can determine if healthy ('asymptomatic') individuals without a history of cancer have an increased risk of developing cancer within the next one year. This test can significantly minimize radiological or invasive screening in the majority individuals who do not have any increased risk.

## Introduction

The WHO states that early detection of cancer greatly increases the chances of successful treatment (<https://www.who.int/cancer/prevention/diagnosis-screening/en/>). Mammography, low-dose computed tomography (LDCT) and colonoscopy are some methods presently in vogue, albeit with nagging reservations: the procedures pose several challenges including invasive nature of tests (1), discomfort (1), and radiation risks (2, 3) besides resource heavy settings. Population-based blood-based screening methods (mostly using cfDNA as the primary analyte) aim to definitively identify any individual with indication of malignant activity with the objective to intervene at the earliest stage and attempt curative procedures (4). Many of these tests are too sensitive and less specific, leading to false positive cases as they

may suffer from "source uncertainty" which is associated with circulating nucleic acid fragments: circulating mutant fragments of DNA can emanate from diverse sources that may not necessarily represent viable malignancy, for example alterations captured in cfDNA due to clonal hematopoietic mutations of indeterminate potential (5). Also, the test may turn out to be positive too early, making radiologic or clinical verification almost impossible, thereby causing overdiagnosis and anxiety (6, 7). For these reasons, blood-based tests have not yet gained wider acceptance or adoption.

An alternative to the "definitive positive selection" approach would be to risk-stratify asymptomatic individuals according to a "1 year" risk by periodic testing. The process would be to identify those individuals who are "biomarker positive" and risk stratify them as "average/higher risk" cohort for the purpose of follow-up monitoring with surveillance programs, whereas in those individuals where no hallmark bio-marker is detected could be classified as "low risk."

We have previously shown that circulating ensembles of tumor-associated cells (C-ETAC) are heterotypic clusters comprising tumor cells, immune cells, and fibroblasts, and are a systemic hallmark of cancer (8). The presence of C-ETACs either singly or in clusters offers a definitive head-start for risk-stratification since C-ETACs by their very nature are causatively linked to malignant activity. We show herein that this approach yields negative risk stratification benefit for identifying individuals who can be excluded from routine screening unless warranted by other clinical considerations. This is the first large-scale study where samples from suspected patients with cancer were obtained

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**Note:** Supplementary data for this article are available at Cancer Prevention Research Online (<http://cancerprevres.aacrjournals.org/>).

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before any biopsy. In other contemporary studies, samples appear to have been obtained from patients with cancer where there had already been a diagnosis based on a biopsy/surgery (9). This is relevant since breach of the basement membrane of the tumor would inevitably lead to release of tumor material in the blood, which may result in false higher sensitivity, especially in early-stage cancers. A real-world screening test would have to detect latent malignancy in asymptomatic individuals who would have not undergone any prior invasive procedure.

## Materials and Methods

### Study design

The RESOLUTE and TRUBLOOD trials (CTRI Registration Nos. CTRI/2019/01/017219 and CTRI/2019/03/017918, respectively) are complimentary prospective observational studies for establishing the viability of circulating tumor cells (CTCs) and their clusters (C-ETACs) for screening, diagnostic, and prognostic purposes. Both studies have been previously reviewed by the Ethics Committees of the Study Sponsor (Datar Cancer Genetics, DCG) as well as the participating institutions. Both trials were conducted in accordance with existing regulatory and ethical guidelines such as the Declaration of Helsinki. Details of both studies may be obtained from WHO ICTRP.

### Study population

The RESOLUTE study recruited asymptomatic adults (males and females) with only age-associated elevated risk of cancer and no prior diagnosis of cancer. Study participants underwent protocol screening investigations for cancer including LDCT, mammography, Pap Smear as well as evaluation of serum cancer antigens (CA125, PSA, CA19-9, AFP, and CEA). The TrueBlood Study recruited adults (males and females) with symptoms suspected of cancer and those with prior confirmed diagnosis of solid organ cancers. For this study, all solid organ cancers are considered except hematolymphoid and CNS malignancies. Eligible volunteers for both studies were counselled regarding the respective study objectives, procedures, and sample requirements. Thereafter willing volunteers provided informed written and signed informed consent.

### Samples

A total of 15 mL blood samples were collected from participants in both studies. In case of the asymptomatic individuals, blood was collected prior to undergoing the screening investigations. In case of patients diagnosed with or suspected of cancer, the blood was collected prior to a biopsy, any other invasive procedure or a radiologic scan. In case of patients diagnosed with or suspected of cancer, all biopsies, other invasive procedures, and radiologic imaging scans were as part of routine diagnostic work-up and not as part of the Study. Blood samples from all study participants were processed at the CLIA, CAP, and NABL-ILAC accredited laboratory of the Study Sponsor.

### Enrichment and harvesting of C-ETACs

Peripheral blood mononuclear cells (PBMCs) were obtained from 15 mL whole blood using RBC lysis buffer (Thermo Fisher Scientific) as per manufacturer's instructions and aliquots were transferred into multiwell plates for treatment with epigenetically activating media as described previously (8). Processed samples were observed by phase contrast microscopy on the fifth day. Viable apoptosis-resistant (malignant) tumorigenic cells and their clusters were harvested by aspiration for further processing. Harvested cells clusters were gently transferred to coated glass slides for identification of C-ETACs by immunostaining. C-ETACs were defined as clusters of  $\geq 3$  cells with characteristic immunostaining pattern as per cancer type, including epithelial carcinoma (EPCAM+, panCK+, CD45 $\pm$ ), sarcoma (SMA+, Desmin+, CD45 $\pm$ ), or neuroendocrine tumor (Synaptophysin+, Chromogranin+, CD45 $\pm$ ). Immunocytochemistry (ICC) procedure for immunostaining of C-ETACs is provided below.

### ICC workflow

C-ETACs were fixed on slides with 4% paraformaldehyde (pH 6.9, 20 minutes). Cell permeabilization was achieved with 0.3% Triton-X 100 (15 minutes), followed by blocking with 3% BSA (30 minutes). Cells were immunostained with primary antibodies (60 minutes), washed with PBS (pH 7.4), incubated with secondary antibodies (60 minutes), washed with PBS, and then incubated with 4',6-diamidino-2-phenylindole dihydrochloride in dark (15 minutes). All incubations were at ambient temperature (20°C–25°C). Positive and negative cell line controls were also processed with each batch of samples (Supplementary Table S1). All cell lines were procured within the last 3 years. All cell lines were mycoplasma-free.

### Detection of C-ETACs

ICC slides were scanned by Cell Insight CX7 High-Content Screening Platform (Thermo Fisher Scientific). Scanned slides were reviewed using the colony detecting assay of the Cellinsight Software (Thermo Fisher Scientific) to detect C-ETACs using a surface area threshold of ( $\geq$ )120  $\mu\text{m}^2$ .

## Results

### Study participants

In our previously published data, we reported findings based on 16,134 study participants including 10,625 asymptomatic individuals and 5,509 patients with cancer. We subsequently enrolled an additional 4,743 eligible and consenting individuals suspected of solid organ cancer who had been advised an invasive biopsy into the TrueBlood Study; in these individuals blood samples were collected prior to an invasive biopsy. The additional patients were enrolled to obtain a numerically significant population to evaluate the extent of C-ETACs in symptomatic individuals presenting for a diagnostic biopsy/FNAC and have not undergone any prior invasive procedures. Among these 4,743 individuals, 4,419 (Supplementary Table S2) were subsequently diagnosed with cancer (2,129 nonmetastatic and 2,290 metastatic) and 324 (Supplementary Table S3) with a benign condition.

**Detection of C-ETACs**

In the prior report (8), we had indicated that C-ETACs were detected in 392 individuals (3.69%) of the entire asymptomatic cohort of 10,625 individuals, based on direct ocular assessment of samples (immunostained slides) by the operator. In this study, the data were re-examined using colony detecting assay of the Cellinsight Software (Thermo Fisher Scientific). During re-analysis, an additional 78 samples were identified as C-ETAC positive, leading to a cumulative detection in 470 (4.42%) individuals. Similarly, prior assessment of immunostained slides had indicated 4,944 C-ETAC positive samples (89.7%) in the cohort of 5,509 cancer cases. Re-evaluation of these samples with the colony detecting assay with same detection thresholds indicated C-ETAC positivity in an additional 179 samples leading to a cumulative detection in 5,123 (93.0%) patients. In the additional cohort of 4,419 cases eventually confirmed with cancer, C-ETACs were detected in 4,101 (92.8%) cases, including 1,980/2,129 (93.0%) nonmetastatic cases and 2,121/2,290 (92.6%) of the metastatic cases. C-ETACs were also detected in 8 of 324 (2.47%) cases of benign tumors.

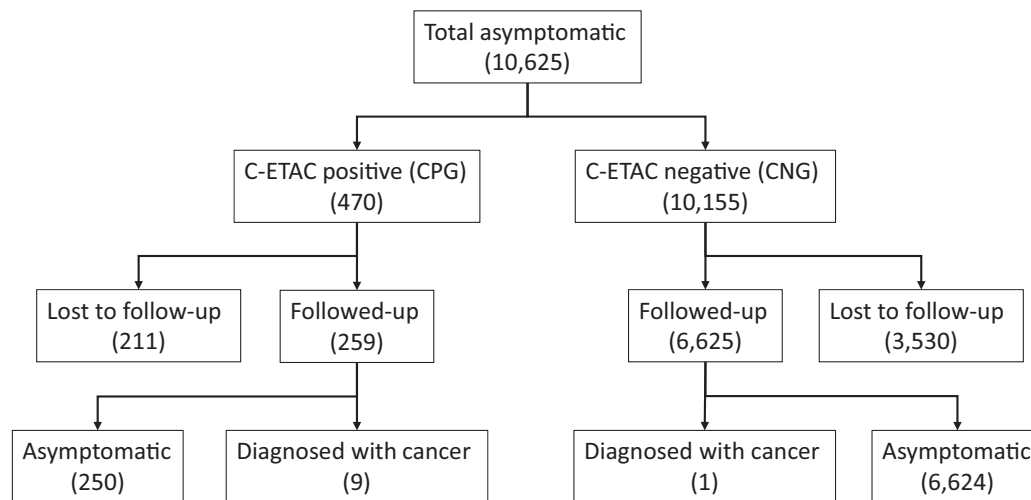
**Follow-up of asymptomatic individuals**

Between 14 February 2019 and 30 June 2019, 10,625 asymptomatic individuals were enrolled into the RESOLUTE study. Demographic details of this population have been published previously (8). Among this cohort, 10,155 individuals were determined to be C-ETAC negative whereas 470 were determined to be C-ETAC positive. Study participants were blinded to status of C-ETACs in their blood samples at all times. All study participants were followed up telephonically between 10 May 2020 and 27 May 2020 (Median duration of 379 days between recruitment and follow-up) with a brief questionnaire

(Supplementary Table S4) asking about detection of cancer. Consequently, out of the 10,155 individuals in the C-ETAC Negative Group (CNG), 6,625 (61.3%) could be contacted whereas 3,530 individuals (38.7%) were either lost to follow-up or withdrew consent for further follow-up. Among these 6,625 individuals, 6,624 (99.984%) stated that there was no diagnosis of cancer whereas one individual (0.015%) was diagnosed with breast cancer. Among the 470 individuals in the C-ETAC Positive Group (CPG), 259 (55.10%) could be contacted whereas 211 (44.9%) were either lost to follow-up or withdrew consent for further follow-up. Among these 259 individuals, cancer was detected in nine cases (3.47%) of whom four had breast cancer, two refused to disclose the cancer type and 1 each had ovarian, esophageal, and colon cancer. One individual detected with breast cancer had BIRAD 5 status at the time of enrolment. Stage and grade of the cancer cases was not ascertainable. A summary of the follow-up findings is depicted in Fig. 1. Thus, the detection rates of cancer were 0.015% in the CNG and 3.47% in the CPG, indicating 230-fold ( $P < 0.00001$ ) increase in 1-year cancer risk associated with detection of C-ETACs. If the lost to follow-up participants are included in the overall computation by accounting for the average age standardized cancer incidence rate of 0.089%, the detection rates would be 2.13% in the CPG and 0.04% in the CNG respectively, yielding a 54-fold ( $P < 0.00001$ ) 1 year elevated cancer risk in the CPG.

**Discussion**

Because C-ETACs are directly derived from a tumor mass, they are a direct evidence of malignancy and can be conveniently construed as a microbiopsy. We had previously demonstrated that C-ETACs are ubiquitous in various solid organ



**Figure 1.**

Summary of follow-up findings in the cohort of 10,625 asymptomatic individuals. All study participants were contacted approx. One year after initial enrollment to determine the proportion of individuals with diagnosis of cancers. Follow-up was possible in 6,884 cases whereas patients were lost to follow-up (or withdrew consent) in 4,141 cases. Higher detection rates of cancer were observed in the C-ETAC positive group as compared with the C-ETAC negative group (3.47% vs. 0.015%,  $P < 0.00001$ ) indicating 230-fold higher 1-year cancer risk associated with detection of C-ETACs.

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cancers and are rare among asymptomatic individuals; 89.8% of 5,509 patients with cancer were positive for C-ETACs as opposed to 3.6% of 10,625 asymptomatic individuals. We hence hypothesized that detection of C-ETACs in asymptomatic individuals may be indicative of a latent/undiagnosed malignancy and precede a future diagnosis of cancer. On the basis of this premise, we risk-stratified the 10,625 asymptomatic individuals as elevated or baseline risk of malignancy based on detection of C-ETACs in blood samples. On the basis of the recommendations of the United States Preventive Screening Task Force (USPSTF; <https://www.uspreventiveservicestaskforce.org>) existing at the time of initial enrolment, the study population included adult females above the age of 45 and adult males above the age of 50 who are generally considered at an elevated age associated risk of most cancers. The 1-year follow-up of these individuals from the largest study of viable C-ETACs was intended to assess if their detection has a higher consequential risk of manifest malignancy in a finite period for individuals who were C-ETAC positive in the first instance. The study findings demonstrate a definitive risk for C-ETAC positive individuals to be detected with cancer within 1 year. No significant differences were observed between age-wise subgroups. The authors are mindful that, given the nature and biology of cancer, it is impossible to predict the radiological or symptomatic manifestation of the disease in C-ETAC positive cases. This is especially so since circulating tumor cells have been previously shown to be detectable several years before the disease becomes apparent symptomatically or on imaging (10). Correspondingly, the study also evaluated whether individuals with no detectable C-ETACs would have a meaningful reassurance of being free from the risk of cancer for a length of time.

Considering that C-ETACs are extremely unlikely to be influenced by ethnicity, the present strategy offers a viable approach to stratification-based screening of populations irrespective of demographic subtypes. The high sensitivity and specificity of C-ETAC detection-based approach can facilitate accurate triaging of at-risk populations. Additional prospective studies will help us understand if the test could be considered for all asymptomatic individuals above the age of 45 (females) and 50 (males) with no prior diagnosis of cancer. The risk stratification can be used to identify individuals who have negative C-ETAC status and can be excluded from current screening modalities if there are no other clinical reasons warranting such investigations. In this study, ~96% of the asymptomatic population were deemed at a lower risk due to absence of C-ETACs. Relief from conventional screening in this sizeable proportion of individuals translates to an appreciable reduction in logistical, operational, and financial burden on the present cancer screening infrastructure which is reliant on resource intensive methods. Additional prospective studies will help us understand if the savings can readily absorb any additional follow-up costs in the ~4% “at risk” population.

A positive C-ETAC result narrows down the focus on the “higher/average risk” population and can reduce the burden on the cancer screening, detection, and diagnosis infrastructure.

Simultaneously, a negative C-ETAC result will lighten the anxiety of cancer. Our study shows that the detection of C-ETACs is largely unaffected by metastatic/nonmetastatic status of the disease. The findings of this study also reinforce the case for a pan-cancer screening test rather than separate investigations for different cancers, which are largely tied to anatomical features such as primary organ. The test when offered at a population level should not cost more than \$200, which compares favorably with other screening modalities such as LDCT, mammography, or colonoscopy with the added advantage of it being a blood test with no concerns about radiation or invasive procedures.

Accurate risk stratification can reduce the time to detection and treatment of cancers (11, 12). The detection of cancer in 9 individuals within 1 year among the C-ETAC positive asymptomatic cohort of 259 participants versus 1 individual in the C-ETAC negative asymptomatic cohort of 6,224 individuals shows that individuals in the CPG had a 230 times higher risk of developing cancer than those in the CNG ( $P < 0.00001$ ). This is a statistically significant basis for classification of high-risk and low-risk groups. We speculate that further follow-up of the higher risk (C-ETAC positive) population would establish the higher incidence of cancer. The present absolute risk must be viewed in the context of a 1-year follow-up, which indicates that the absolute risk is not insignificant. Moreover, the high sensitivity of 92.8% in the expanded real-world “pre-biopsy” cohort of 4,419 shows that C-ETACs are a reliable means of detecting cancer even at the stage of (symptomatic) presentation. This approach extends to cover cancers which cumulatively cause >85% deaths worldwide and facilitates early detection which can impact outcomes and the cost of treatments. Among the asymptomatic individuals (CPG or CNG) about whom information could not be gathered were 15 participants who died in the intervening period of enrolment to follow-up, due to reasons other than cancer. In conclusion, adoption of the C-ETAC-detection based cancer risk stratification is a viable strategy for screening of asymptomatic individuals above the age of 40 years, considering that there is evidence of shifting of age risks towards younger adults.

The results and conclusions from our work should be interpreted cautiously. This study was limited to ascertaining primarily the comparative detectability of C-ETACs between individuals presenting with symptoms of cancer (therapy naïve and before any invasive procedure) and those without symptoms of cancer. The secondary objective was to evaluate the manifestation of cancer as an annual risk to determine the feasibility of using the test for risk stratification. A major limitation of this work is that over 40% of patients were lost to follow-up. In addition, simply asking patients if they had a cancer diagnosis could be prone to error. Given the types of cancers that were identified in the 9 participants, specific attention to length time bias and lead time bias should be given in future prospective trials designed to investigate the value of this test as a stratification and/or early detection tool.

## Authors' Disclosures

D. Akolkar reports other from Datar Cancer Genetics (full time employment) outside the submitted work. D. Patil reports personal fees from Datar Cancer Genetics (professional fees for employment) during the conduct of the study; as well as personal fees from Datar Cancer Genetics (professional fees for employment) outside the submitted work. No disclosures were reported by the other authors.

## Authors' Contributions

**A. Ranade:** Conceptualization, data curation, formal analysis, investigation, visualization, project administration, writing-review and editing. **A. Bhatt:** Conceptualization, data curation, formal analysis, supervision, validation, investigation, visualization, project administration, writing-review and editing. **R. Page:** Conceptualization, data curation, formal analysis, validation, visualization, methodology, writing-original draft, writing-review and editing. **S. Limaye:** Conceptualization, data curation, formal analysis, supervision, visualization, writing-original draft, writing-review and editing. **T. Crook:** Conceptualization, formal analysis, supervision, visualization, writing-original draft, writing-review and editing. **D. Akolkar:** Conceptualization, resources, data curation, formal analysis, supervision, methodology, writing-original draft, project administration, writing-review and editing. **D. Patil:** Conceptualization, data curation, formal analysis, supervision,

validation, investigation, visualization, methodology, writing-original draft, project administration, writing-review and editing.

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