IJC
International Journal of Cancer

Circulating ensembles of tumor-associated cells: A redoubtable new systemic hallmark of cancer

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Circulating ensembles of tumor-associated cells (C-ETACs) which comprise tumor emboli, immune cells and fibroblasts pose well-recognized risks of thrombosis and aggressive metastasis. However, the detection, prevalence and characterization of C-ETACs have been impaired due to methodological difficulties. Our findings show extensive pan-cancer prevalence of C-ETACs on a hitherto unreported scale in cancer patients and virtual undetectability in asymptomatic individuals. Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples of 16,134 subjects including 5,509 patients with epithelial malignancies in various organs and 10,625 asymptomatic individuals with age related higher cancer risk. PBMCs were treated with stabilizing reagents to protect and harvest apoptosis-resistant C-ETACs, which are defined as cell clusters comprising at least three EpCAM$^+$ and CK$^+$ cells irrespective of leucocyte common antigen (CD45) status. All asymptomatic individuals underwent screening investigations for malignancy including PAP smear, mammography, low-dose computed tomography, evaluation of cancer antigen 125, cancer antigen 19-9, alpha fetoprotein, carcinoembryonic antigen, prostate specific antigen (PSA) levels and clinical examination to identify healthy individuals with no indication of cancer. C-ETACs were detected in 4,944 (89.8%, 95% CI: 89.0–90.7%) out of 5,509 cases of cancer. C-ETACs were detected in 255 (3%, 95% CI: 2.7–3.4%) of the 8,493 individuals with no abnormal findings in screening. C-ETACs were detected in 137 (6.4%, 95% CI: 5.4–7.4%) of the 2,132 asymptomatic individuals with abnormal results in one or more screening tests. Our study shows that heterotypic C-ETACs are ubiquitous in epithelial cancers irrespective of radiological, metastatic or therapy status. C-ETACs thus qualify to be a systemic hallmark of cancer.

Introduction

The focus of prior research efforts in regard to the release of viable cells from the tumor has been to capture and characterize single cells rather than clusters. However, there is growing evidence that has led researchers to hypothesize that in addition to (or rather than) circulating tumor cells (CTCs), metastasis is facilitated more

Additional Supporting Information may be found in the online version of this article.

Key words: circulating tumor cells, circulating tumor-associated cells, circulating tumor emboli, circulating tumor cell clusters, cancer related thrombosis, circulating metastatic disease

Abbreviations: AFP: alpha fetoprotein; CA125: cancer antigen 125; CA19-9: cancer antigen 19-9; CAP: College of American Pathologists; CD45: leucocyte common antigen; CEA: carcinoembryonic antigen; C-ETACs: circulating ensembles of tumor-associated cells; CMD: circulating metastatic disease; CSC: cancer stem cells; CTCs: circulating tumor cells; EpCAM: epithelial cell adhesion molecule; HPE: histopathological evaluation; IHC: immunohistochemistry; LDCT: low-dose computed tomography; MRD: minimum residual disease; NED: no evidence of disease; pan-CK: pan-cytokeratins; PBMCs: peripheral blood mononuclear cells; PSA: prostate-specific antigen; SoC: standard of care; TAL: tumor-associated T-lymphocytes; TAM: tumor-associated macrophages; VTE: venous thromboembolism

Grant sponsor: Datar Cancer Genetics Limited

DOI: 10.1002/ijc.32815
History: Received 30 Aug 2019; Accepted 25 Nov 2019; Online 16 Dec 2019

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What's new?

Circulating Ensembles of Tumor Associated Cells (C-ETACs) comprised of tumor emboli, immune cells, and fibroblasts pose well-recognized risks of thrombosis and aggressive metastasis. However, the detection and characterization of C-ETACs have been impaired by methodological difficulties. Here, the authors have developed a label-free non-mechanical process that permits enrichment of viable apoptosis-resistant C-ETACs from peripheral blood. They show that heterotypic C-ETACs are not merely incidental findings in cancer but rather a systemic manifestation of malignancy. C-ETACs are present in a significant proportion of all solid organ malignancies and are rare in asymptomatic individuals. Monitoring of C-ETACs could help inform cancer management.

aggressively by dissemination of cell clusters containing CTCs.[1,2] Though there have been prior attempts at identification and characterization of CTC clusters, these efforts have employed methods and devices primarily designed for isolation of single CTCs, such as epitope (epithelial cell adhesion molecule [EpCAM]) capture or microfluidic devices.[3–5] There appear to be no reports on definitive methods for harvesting tumor derived emboli or CTC clusters. We hypothesized that prior attempts may have been suboptimal in recovering intact viable clusters due to methodological limitations, and may have inadvertently underrepresented the prevalence of CTC aggregates.[2] We have developed a label free nonmechanical process that permits enrichment of viable apoptosis resistant circulating tumor-associated cells (C-TACs) and their assemblages (circulating ensembles of tumor-associated cells [C-ETACs]) from peripheral blood. This process detects and yields C-ETACs for qualitative and quantitative analysis. Samples from a large cohort of cancer patients (n = 5,509) as well as asymptomatic individuals (n = 10,625) were processed to identify and harvest C-ETACs. We show that heterotypic C-ETACs comprising tumor cells and diverse immune cells are commonly detected in patients with epithelial solid organ malignancies at higher prevalence rates than previously thought and are virtually undetectable in the asymptomatic population. Our study findings qualify C-ETACs as a systemic hallmark of cancer with potential implications in cancer detection and management.

Methods

Study design

We present data from two separate prospective observational studies. The first observational study is titled, "Realtime Enrichment Screen for Outright detection of Latent Undiagnosed malignant Tumors in asymptomatic individuals Efficiently—RESOLUTE" (WHO ICTRP ID CTRI/2019/01/017219). The second observational study is titled "Tissue biopsy Replacement with Unique Evaluation of circulating bio-markers for morphological evaluation and clinically relevant molecular typing of malignancies from BLOOD sample—TrueBlood" (WHO ICTRP ID CTRI/2019/03/017918). Both studies have been approved by the respective Institutional Ethics Committees of participating centers. Evaluation of participant samples was carried out at a facility which offers College of American Pathologists (CAP) accredited services.

Study participants and samples

The present study screened 16,134 individuals including 5,509 cancer patients (TrueBlood) and 10,625 asymptomatic individuals (RESOLUTE). The TrueBlood Study recruited adult (≥18) male and female patients with confirmed diagnosis of solid organ cancers irrespective of stage, grade or therapy status (>21 days since most recent systemic therapy or radiology for pretreated patients). Details of the True Blood study are available at http://apps.who.int/trialsearch/Trial2.aspx?TrialID=CTRI/2019/03/017918. The RESOLUTE Study recruited adult males (49–75 years) and females (40–75 years) with no known diagnosis or clinical suspicion of cancer. Details of the RESOLUTE study are available at http://apps.who.int/trialsearch/Trial2.aspx?TrialID=CTRI/2019/01/017219. All screened individuals were counseled regarding the study objectives and procedures and those who provided written informed consent were enrolled. Venous blood was collected in EDTA containers from all recruited participants. Cancer patients in the TruBlood Study did not undergo any further evaluations and their most recent clinical records including histopathology, treatment summary and radiological evaluations were referred for disease status. All asymptomatic individuals in the RESOLUTE study underwent prescribed gender-relevant cancer screening procedures including mammography, low-dose computed tomography (LDCT) scan and PAP smear, as well as evaluation of cancer antigen 125 (CA125), cancer antigen 19-9 (CA19-9), carcinoembryonic antigen (CEA), alpha fetoprotein (AFP) and prostate-specific antigen (PSA) levels. Asymptomatic individuals with abnormal findings in any of the screening procedures (e.g., elevated CA marker or suspicious findings on imaging) were identified and considered as "at risk" population, while those with normal findings were considered as "healthy" population in all further evaluations. Demographic and clinical stratification details of cancer patients and asymptomatic individuals are provided in Supporting Information Tables S1 and S2, respectively.

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, Waltham, MA) and finally resuspended in buffer as per manufacturer's instructions. Resuspended PBMCs were divided into several aliquots, which were transferred into multiwell plates and treated with epigenetically activated media for up to 100 hr at 37°C under hypoxic (5% O_2) conditions. The

epigenetically activated media comprises of DMEM (Thermo Fisher) containing FBS (Thermo Fisher) which is enriched with Tumor Necrosis Factor Receptors (TNFR), Nuclear Factor kappa B (NF-kB) and the Janus kinase/signal transducer and activator of transcription (Jak/Stat) pathway related transcripts and factors. Additional cell growth factors (CGF) such as F12 nutrient mixture (Thermo Fisher), epidermal growth factor (EGF, Thermo Fisher), fibroblast growth factor (FGF, Thermo Fisher) and N-2 supplements (Thermo Fisher) are also blended. Since epithelial cells and hematolymphoid cells have significantly different apoptotic pathways, the media provokes differential apoptosis in cells of these lineages. This approach selectively kills hematolymphoid cells with proficient apoptotic mechanisms in response to intense progrowth stimuli. The cells which survive are "apoptosis resistant" and are therefore direct tumor cells or those who are recruited by the tumors such as, but not limited to, tumor-associated macrophages (TAMs) and tumor-associated fibroblasts (TAFs). The procedure being label-free and singularly premised upon the exploitation of apoptosis proficient/resistant characteristic of normal *versus* tumor cells affords the benefit of harvesting clusters without dependence on antigen epitopes or the mechanical hobbling or stresses typical of microfluidic devices. Processed samples were thereafter observed by phase contrast microscopy on the fifth day and cell clusters if any were harvested by aspiration for further characterization. Harvested clusters were immunostained with fluorophore conjugated antibodies against EpCAM (phycoerythrin [PE]), pan-cytokeratins (pan-CK; fluorescein isothiocyanate [FITC]) and leucocyte common antigen (CD45; CY5), and finally stained with the nucleic acid dye (4,6-diaminodino-2-phenylindole [DAPI]). Fluorescence imaging was performed on Cell Insight CX7 High-Content Screening Platform (ThermoFisher Scientific). For the purpose of our study, C-ETACs were defined as clusters of at least three cells that were positive by immunostaining for EpCAM and pan-CK, irrespective of CD45 status. The C-ETAC enrichment media formulation and isolation protocol is the subject matter of Patent applications (United States Patent Office Provisional Application Numbers 62849840 and 62796098).

Immunostaining for identification and characterization of C-ETACs

Harvested cell clusters were used for preparation of cytospin slides by using standard procedures. One slide was used for identification of C-ETACs by immunofluorescent staining using anti-EpCAM, anti-panCK and anti-CD45 antibodies, as well as DAPI to confirm intact (nucleated) cells. Additional slides were used for immunostaining with markers such as CD44 (cancer stem cells [CSCs]) and CD8 (tumor-associated leucocytes). In a set of samples from known cases of breast, lung, prostate, cervix and gastric cancers, general, organ-specific and nonorgan specific markers were evaluated by immunostaining of C-ETACs. All slides were scanned using a multiwavelength fluorescent scanner (CellInsight, Thermofisher). A sample was treated as positive if at least one C-ETAC was detected in 1 ml PBMC equivalent of peripheral blood. All primary and secondary antibodies used in immunostaining, their manufacturers as well as cell lines used as positive controls for each antibody are listed in Supporting Information Table S3. The immunostaining workflow is provided in Supporting Information Table S6. All antibodies were used at manufacturer recommended dilutions with dilutions being prepared in manufacturer provided or recommended dilution buffers. All human cell lines were procured within the last 3 years. All experiments were performed with mycoplasma-free cells.

Tumorigenic origin of C-ETACs

In order to establish a direct causative link with the existence of a tumor, we obtained samples from a subcohort of 223 cancer patients prior to undergoing surgical resection of the tumor as well as 8 hr after the surgical procedure. C-ETACs were harvested and enumerated to discern their presurgery and postsurgery numbers. Details of this subcohort are provided in Supporting Information Table S4.

C-ETACs and radiological status

Another subcohort of the study population included 589 patients who had previously (>21 days ago) received treatments for cancer and where recent radiological evaluation indicated no evidence of disease (NED). Details of this subcohort are provided in Supporting Information Table S5. Samples from this subcohort were compared to those of patients with radiological evidence of disease to determine differences in prevalence of C-ETACs.

Data availability

Data may be made available from the authors upon reasonable request.

Results

Study cohort

The present study included 5,509 patients with a confirmed diagnosis of cancer (Supporting Information Table S1) with a median age of 55 years including 2,482 (45.1%) males and 3,027 (54.9%) females. Then, 4,920 patients had radiological evidence of active cancer at the time of blood sampling (irrespective of prior treatment status) and 589 had no radiological evidence of disease post prior treatment(s). Then, 3,098 patients (56.2%) had metastatic disease and 1,138 (20.7%) cases were nonmetastatic; metastatic status was unavailable in 1,273 (23.1%) cases. Then, 3,413 patients (62.0%) had received prior treatment whereas 1,828 (33.2%) were treatment naïve. Therapy status of 268 patients (4.8%) was unknown. The asymptomatic cohort (Supporting Information Table S2) screened 10,625 individuals with a median age of 54 years including 3,898 (36.7%) males and 6,727 (63.3%) females, of whom 3,475 were postmenopausal. Among these 10,625 individuals, 2,132 (898 males +1,234 females) had either significant findings in LDCT,

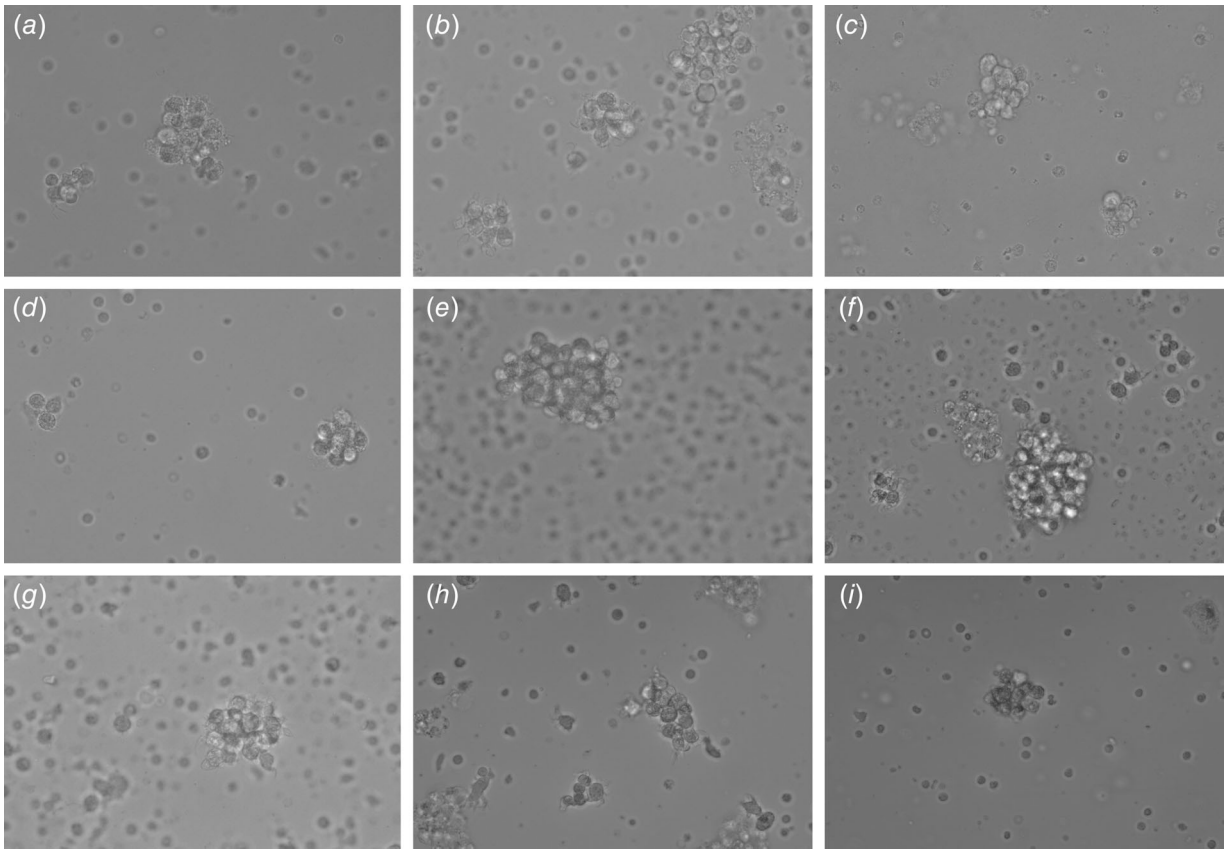

Figure 1. Cell assemblages on Day 5. Viable intact cell assemblages (white arrow) were imaged under a phase contrast microscope at 40× magnification. Samples from various cancer types are depicted (*a*) breast, (*b*) lung, (*c*) prostate, (*d*) stomach, (*e*) gallbladder, (*f*) kidney, (*g*) bladder, (*h*) buccal mucosa and (*i*) pancreas. Field width is ~160 μm.

Mammography or PAP smear or elevated level(s) of CA125, CA19-9, CEA, AFP or PSA and were hence considered as "at risk" population. The remaining 8,493 individuals were considered as healthy population and consisted of 3,000 (35.3%) males and 5,493 (64.7% females) with a median age of 53 years (range: 40–75 years).

C-ETACs are heterotypic

Figure 1 shows representative phase contrast microscope images of cell assemblages from various cancers as observed on the fifth day. Figures 2*a*–2*i* show representative images of clusters (3–50 cells) staining positively for EpCAM, CK or CD45. C-ETACs included cells that were negative for EpCAM and CK but positive

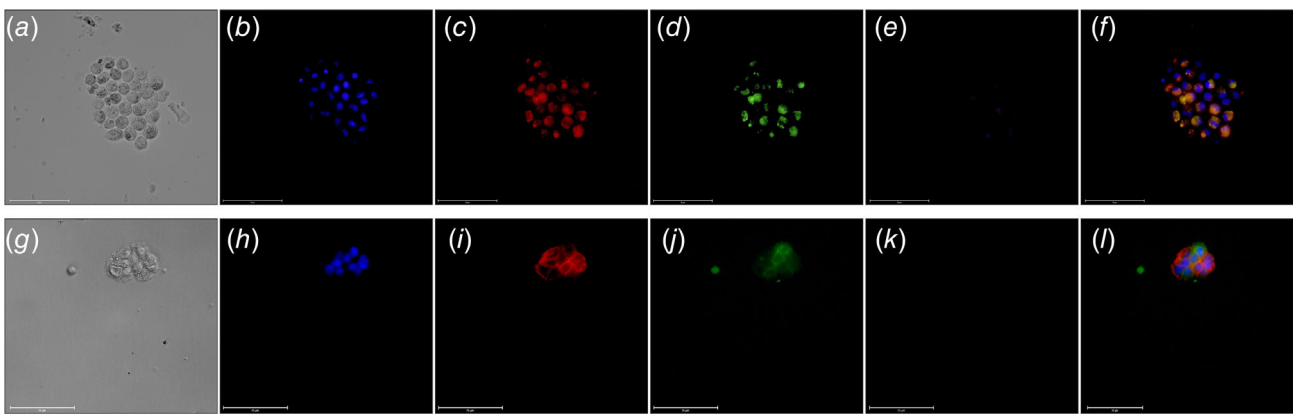

Figure 2. Immunostaining of C-ETACs. Cytospin smears prepared from cell-assemblages obtained on Day 5 from a case of Ca lung (*a*–*f*) and Ca endometrium (*g*–*l*) were stained with DAPI, anti-EpCAM, anti-CK and anti-CD45. (*a*, *g*) Bright field; (*b*, *h*) DAPI; (*c*, *i*) EpCAM; (*d*, *j*) panCK; (*e k*) CD45; (*f*, *l*) composite overlay (without bright field).

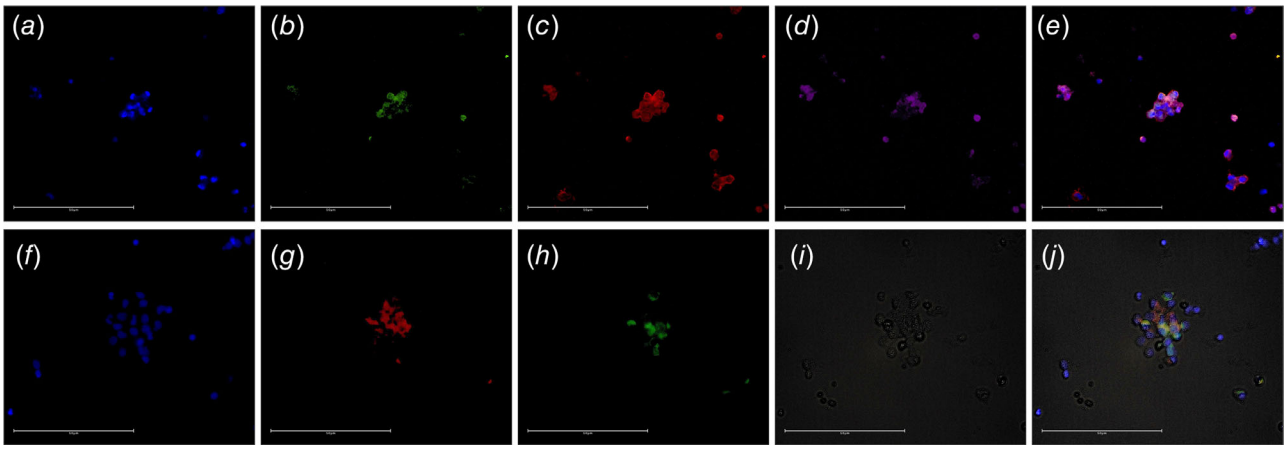

Figure 3. C-ETACs are heterotypic. Cytospin smears of confirmed C-ETAC samples were immunostained for CD44 in a known case of Ca buccal mucosa (*a–e*) and CD8a in a case of Ca Breast (*f–j*). C-ETACs in *a–d* were stained for DAPI, panCK, CD44 and CD45, respectively, while *e* is the composite overlay. C-ETACs in *f–h* were stained for DAPI, EpCAM and CD8a, while *i* is the bright field image and *j* is the composite overlay.

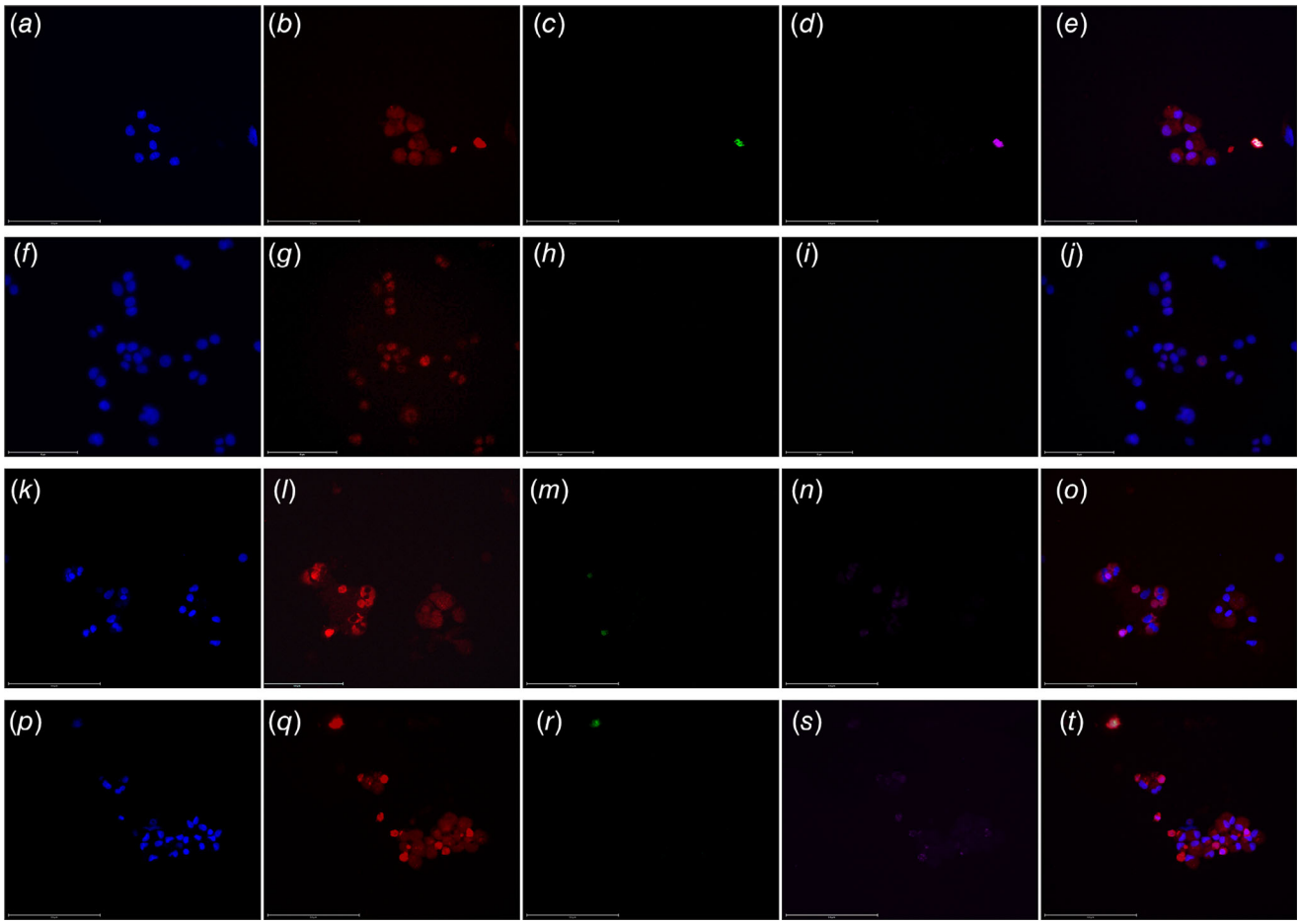

Figure 4. Organ specificity of C-ETACs. Cytospin smears of confirmed C-ETAC samples were immunostained for organ-specific and organ nonspecific markers in a case of Ca Breast (*a–e*), Ca Colon (*f–j*), Ca Ovary (*k–o*) and Ca Prostate (*p–t*). C-ETACs from Ca Breast were stained for DAPI, specific marker GCDFP15 (unconjugated primary and PE-conjugated secondary), negative marker CDX-2 (FITC) and CD45 (Cy5.5). C-ETACs from Ca Colon were stained for DAPI, specific marker CDX-2 (unconjugated primary and PE-conjugated secondary), negative marker GCDFP-15 (FITC) and CD45 (Cy5.5). C-ETACs from Ca Ovary were stained for DAPI, specific marker CA125 (unconjugated primary and PE-conjugated secondary), negative marker GFAP (FITC) and CD45 (Cy5.5). C-ETACs from Ca Prostate were stained for DAPI, AMACR (unconjugated primary and PE-conjugated secondary), negative marker GFAP (FITC) and CD45 (Cy5.5).

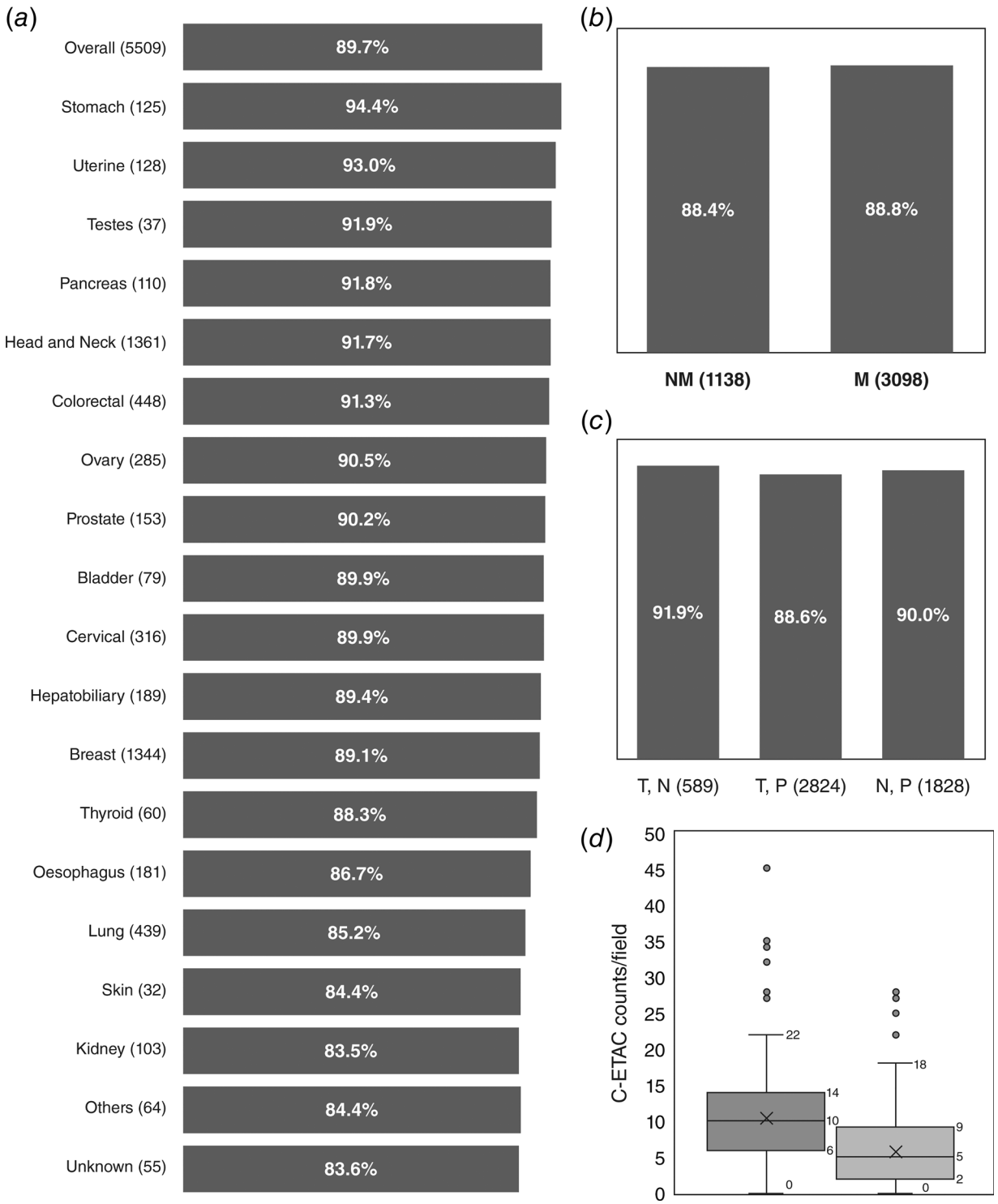

Figure 5. Ubiquity of C-ETACs. (*a*) C-ETACs were evaluated in 5,509 previously diagnosed cases of cancers. Dark bars represent percentage of total samples in each cancer type (and overall) where C-ETACs could be detected. (*b*) C-ETACs were detected with comparable frequency in metastatic (M) as well as nonmetastatic (NM) cancer samples (UA: metastatic status unavailable). (*c*) C-ETACs detection was irrespective of treatment and radiological status. T, N: treated with presently no radiological evidence of disease; T, P: treated with radiologically evident disease; N, P: therapy naïve with radiologically evident disease. (*d*) C-ETAC counts in presurgery (dark bar) and postsurgery (light bar) sample.

for CD8a (tumor-associated leucocytes; Figs. 3*a*–3*e*) as well as cells that stained positively for CD44 (CSCs, Figs. 3*f*–3*j*). The varied immunomorphology indicated that these C-ETACs are not merely aggregates of CTCs but represent a snapshot of heterotypic multicellular associations. C-ETACs were immunostained with organ of origin specific markers in samples from Ca Breast (Figs. 4*a*–4*e*), Ca Lung (Figs. 4*f*–4*j*), Ca Ovary (Figs. 4*k*–4*o*) and Ca Prostate (Figs. 4*p*–4*t*) indicating that the C-ETAC can be used to identify the organ of origin. All tested samples were found to be concordant for the organ-specific markers tested and had undetectable reactivity with nonspecific markers.

C-ETACs are ubiquitous in epithelial malignancies

Blood samples from 5,509 cancer patients were processed for stabilization and isolation of C-ETACs. Viable C-ETACs were discernible in 4,944 cases (89.7%) across all epithelial solid organ malignancies. Figure 5*a* depicts the cancer-wise proportion of samples where C-ETACs were detectable. Histopathological evaluation (HPE) and tumor grade data was available for a subset of samples; however, no significant differences were observed based on differences in HPE subtype or tumor grade (data not shown). C-ETACs were detected in 1,006 (88.4%) patients out of 1,138 with local disease and 2,750 (88.8%) patients of 3,098 with metastatic disease (Fig. 5*b*). C-ETACs were detected in 1,645 (90.0%) of 1,828 recently diagnosed (radiologically evident) therapy naïve patients as well as in 3,062 (89.7%) of 3,413 pretreated patients irrespective of present radiological status. C-ETACs were also detectable in 4,403 (89.5%) of 4,920 patients with radiologically evident disease, irrespective of treatment status. A subset (n = 589) of the pretreated cancer population included patients with NED in the most recent radiological scan; C-ETACs were detected in 541 (91.9%) of these patients (Fig. 5*c*). In another subcohort of 223 cancer patients (Supporting Information Table S4) who underwent surgical resection of tumor, C-ETACs were enumerated in samples collected prior to surgery and 8 hr postsurgery. It was observed that while presurgery samples had a median density of 10 C-ETACs/field, postsurgery samples had a median density of 5 C-ETACs/field (Fig. 5*d*). In postsurgery samples, majority (77%) of samples showed decrease in C-ETACs, while 15% of samples showed increased C-ETACs and 8% of samples showed no change.

C-ETACs in asymptomatic population

Among the 10,625 asymptomatic individuals, 8,493 had no abnormal findings in screening for cancer and were deemed as healthy population. C-ETACs were detected in 255 (3.0%) of these 8,493 individuals' samples. Among the 2,132 individuals with deranged findings on any of the screening investigations, C-ETACs were detected in 137 (6.4%) cases. The occurrence of C-ETACs in patients with normal (negative) and abnormal (elevated/significant) findings in various screening investigations are provided in Supporting Information Table S7. In males, higher probability of C-ETACs detection was associated with abnormal findings in CA-19-9 (10.3%) and total PSA (8.9%). In females, higher probability of C-ETACs detection was associated with abnormal findings in CEA (8.5%) and PAP smear (10.3%). Among the 487 (out of 10,625) asymptomatic individuals with a known family history (first-/second-degree blood relatives) of cancer, C-ETACs were detected in 14 (2.9%) of these cases which was comparable to the 3% detection rate in asymptomatic individuals with no aberrant findings. Among the 985 (out of 10,625) asymptomatic individuals who reported habits such as tobacco addiction as well as individuals with risk of exposure to carcinogens due to occupational hazard, C-ETACs were detected in 47 (4.8%) cases. At the time of submission of this article, none of the 392 individuals where C-ETACs were detected had presented with clinical or radiological manifestations of cancer. However, they have been advised follow up to identify any early clinical presentation.

Discussion

The onset of any sustained neoplastic expansion that disturbs the cellular equilibrium in the human body is a major disruptive event with possibly fatal consequences. Such uncontrolled cell growth coupled with resistance to apoptosis is part of a cascade of survival and proliferative events that form the cellular and molecular hallmarks of malignancy.[6,7] What remains largely unknown is the existence of systemic hallmarks of cancer, that is, extracellular features or events that are ubiquitous to cancers and actively involved in oncological processes. Although the significance of CTCs in cancer has been extensively studied,[8] the prevalence of CTC assemblages has been largely underestimated[2] and not been studied from the perspective of being definitive attributes of malignant neoplasia. Here, we present evidence which indicates that C-ETACs qualify as a systemic hallmark of cancer.

Mechanistically, an important feature of the disorganized process of uncontrolled proliferation of cells in solid organ tumors is the outflow of loosely attached epithelial cells and their emboli into the vasculature.[1,9,10] Normal parenchymal cells which have torn away from their cellular scaffolds due to either injury or infections but are not part of the malignant or premalignant population succumb to anoikis.[11,12] However, cells that have acquired apoptosis-resistant phenotypes adapt to the hematolymphoid habitat and survive for extended periods of time or remain senescent in safe niches.[13,14] Recent studies have shown that the lineage and ensemble of such cells is quite diverse including tumor-associated macrophages (TAM), tumor-associated lymphocytes, CSCs and TAM-cancer cell hybrids,[15–18] and that they perhaps obtain immune privilege using multiple camouflages and even get layered protection from treatment agents[19] as well as any other extrinsic antitumor factors. Indeed, the active recruitment and reprogramming of normal cells, including immune cells, by tumor cells has been previously described[20] as one of the means by which tumor cells subvert immune machinery to achieve tumor survival and proliferation. This agrees with our own observations of CD8a positive cells in the C-ETACs. Owing to the

selective cytotoxicity of our process, all immune cells (being nontumorigenic) are eliminated. Accordingly, neither single immune cells nor homotypic clusters of immune cells were detectable and the only detectable immune cells (CD8a+) were found in C-ETACs. We speculate that the CD8a+ cells may have undergone some reprogramming after recruitment by the tumor-associated cells.

C-ETACs are a further potent danger because it has been shown that they have a very high metastatic potential[1,9,10,21] besides posing the imminent threat of thromboembolic complications.[22,23] Though C-ETACs have received due attention in recent years, their composite detection, harvest and culture has remained difficult and sporadic, with only few anecdotal successes.[3] The limited successes of prior efforts may be attributable to the processes relying on devices and methods originally designed for detection/capture of single CTC. Microfluidic devices[4,5] used for single cell capture may be associated with shear forces which could lead to destruction of cells or disruption of cell clusters and result in lower detection rates.[4] Label (e.g., EpCAM) based detection/isolation methods have been used extensively; the FDA-approved CellSearch[24] is a more contemporary example where CTCs are defined as EpCAM+, CK+ and CD45−. However, EpCAM-based approaches are not suitable for identification of CTCs that have undergone epithelial to mesenchymal transition.[25] EpCAM-based approaches also have limited efficacy in isolation of heterotypic C-ETACs for the same reason: EpCAM+ cells in viable C-ETACs can be obscured from detection due to sequestration with a plethora of cells such as post-EMT CTCs, tumor-associated T-lymphocytes (TAL), TAM and CSCs.[15–18] The C-ETAC isolation process used in our approach is neither microfluidic nor epitope-based and is hence unaffected by the limitations of the respective approaches. *In vitro* processing of viable cells may introduce artifacts due to inherent complexities in tumor biology as well as interactions with media or reagents. Such artifacts may include passive cell aggregation due to metabolic intermediates,[26] as well as active chemotaxis and cellular-adherence in viable cells induced by media or reagents. Supporting Information Video S1 is a time lapse video (Day 0–Day 5) of a representative sample showing persistence of existing clusters (stabilization), elimination of most single cells and absence of new cluster formation. The *in vitro* C-ETAC isolation process was also used with cell lines (SiHa Cervical Cancer, SKBR3 Breast Cancer) and TDCs from freshly biopsied tumor (Liver, Ovarian) tissue and PBMC samples from healthy individuals and no cell assemblages were observed in any of these samples (Supporting Information Fig. S1) indicating the fidelity of the process.

We evaluated the prevalence of C-ETACs across a range of cancer types in 5,509 samples. Prior investigations[2,27–32] reported significant variations in detection of cell clusters ranging between 14.5% ($n = 55$) to 100% ($n = 7$) in lung cancers, 17.4% ($n = 115$) to 61.9% ($n = 21$) in breast cancers, 50% ($n = 8$) to 68.8% ($n = 32$), 4.5% ($n = 44$) in hepatocellular carcinoma, 33.3% ($n = 42$) in renal cell carcinoma, 2.8% ($n = 36$) to 80% ($n = 10$) in prostate cancers and 22% ($n = 18$) to 96.2% ($n = 53$) in pancreatic cancers. In contrast, we report a pan-cancer (epithelial malignancies) C-ETAC prevalence of 89.7%.

It may be intuitive to expect a higher incidence of CTCs and tumor emboli in metastatic cancers. However, C-ETACs were detected at comparable frequencies in metastatic as well as nonmetastatic patients in our study. Though nonmetastatic solid organ cancers may be viable for surgical resection with curative intent, presence of C-ETACs in patients with nonmetastatic disease emphasizes the need for proactive disease surveillance postsurgery. Some reports have suggested that tumor cell clusters may increase after surgical resection.[33,34] In the subcohort of patients with paired presurgery and postsurgery samples, a decreasing trend of C-ETACs was observed postsurgery. These observations also reaffirm the tumorigenic origin of C-ETACs.

No significant differences were observed between detection rates of C-ETACs in therapy naïve and pretreated individuals. In a subset of the study cohort where patients had received prior treatment and recent radiological scan indicated NED, C-ETACs were detected in 91.9% of these patients. The findings suggest that C-ETACs tend to remain in circulation for extended periods even though the disease is radiologically undetectable posttherapy, and may also be indicative of potential predisposition toward recurrence or metastasis, subject to availability of supportive niches. Although it is well accepted that absence of radiological evidence does not infer absence of malignancy, NED is often a significant yardstick for critical treatment-related decisions including drug and dose modifications or a shift to metronomic regimens. Akin to the concept of minimum residual disease[35] (MRD) in hematological malignancies, viable remnant CTCs in radiologically undetectable cancers are linked to risks of recurrence due to drug resistant clonal subtypes as well as resurgent populations in light of therapy inadequacy. Hence, we propose the term circulating metastatic disease (CMD) in solid organ malignancies, which can be accurately determined by evaluation of C-ETACs to better guide disease management especially treatment related decisions.

Treatment decisions in standard of care (SoC) are based on organ of origin and often use information on antigen markers determined by immunohistochemistry (IHC) analysis on tumor tissue after biopsy. Prior efforts[36,37] at determination of organ-specificity and replication of IHC markers using CTCs favor the development of these noninvasive assays. Accordingly, we evaluated C-ETACs from various cancer types and observed that they reported organ-specificity with high fidelity, with little or no interference from other organ-specific markers. Based on these findings, we have initiated a larger study on utility of C-ETACs for diagnosis and treatment decisions in cancers. The study data will be published separately.

C-ETACs were detected in 3% of the 8,493 healthy individuals as well as 6.4% of the 2,132 asymptomatic individuals with aberrant findings on screening investigations. The C-ETAC detection rates among the screened negative (healthy) as well as at risk populations have to be viewed primarily in the context of age-

associated higher risk of cancer. Elevated levels of CA19-9 and PSA appeared to be most highly associated with increased incidences of C-ETAC detection among males whereas the findings of LDCT (higher lung-RADS) inversely correlated with C-ETACs presence. Similarly, elevated levels of CEA as well as suspicious findings in PAP smear appeared to be most highly associated with increased incidences of C-ETAC detection among females while lung-RADS appeared to have lowest association. We did not investigate association of C-ETACs with quantitative differences in any of the screening investigations since it was beyond the scope of the present study. Individuals with risks of carcinogen exposure due to tobacco addiction as well as occupational hazards appeared to be at a higher risk of C-ETAC positivity as compared to the asymptomatic population. Surprisingly, individuals with a known family history (first-/second-degree blood relatives) of cancer did not appear to be at a higher risk of C-ETAC positivity. The extremely high incidence of C-ETACs in the cancer cohort indicates that C-ETACs represent the biological prevalence of malignancy, irrespective of clinical or radiological status. However, the probability of a future clinical presentation of cancer in these 392 asymptomatic individuals (with C-ETAC positivity) cannot be presently predicted, nor can the "clinical false-positive" fraction, that is, those individuals among the 392 in whom cancer will not manifest clinically in their lifetimes. Hypothesizing that the cancer will not clinically manifest in any of the 392 individuals among the total 10,625 yields a hypothetical-maximum false-positive rate of 3.7% which is yet significantly and unambiguously lower than the false positives observed for LDCT (12.9–25.9%),[38] mammography (7–12% at first mammogram[39] and 50–60% after 10 yearly mammograms[40]) and CA markers (e.g., 66% for PSA,[41] 29% for CA-125,[42] 10–60% for CA19-9[43]) which are routinely used in early detection screening. Radiological scans such as LDCT and mammography not only have high false positive rates, but are also nonconfirmatory, that is, necessitate an invasive biopsy for histopathological confirmation of suspected malignancy, as well as being associated with radiation exposure risks.[44,45] Though PAP smears offer direct evidence of malignancy, false-negative findings due to suboptimal samples are not uncommon.[46] Coupled with the high specificity for cancers as well as the noninvasiveness of the procedure, C-ETACs appear to be a superior analyte for detection of malignancy in asymptomatic individuals. C-ETAC based cancer screening of populations is also expected to significantly reduce instances of confirmatory biopsies as well as radiological scans, both of which may be unnecessarily necessitated in suspected cases due to false positives.

The scope of the present study extended to establish the ubiquity of C-ETACs in epithelial malignancies and rarity in asymptomatic populations, which has been demonstrated. Enumeration of C-ETACs is presently a cumbersome and laborious manual process and hence has been attempted only in a single subcohort. Further development and refinements of methods will enable quantitative correlation of C-ETACs with treatment status, radiological findings and extent of disease. These findings will be published at fruition. Though the present study was based on a South Asian population, we do not anticipate variations based on ethnicity or geographical location. We conclude that C-ETACs are not merely incidental findings in malignancy but rather its systemic manifestation, the monitoring of which would better inform cancer management. Ubiquitous C-ETACs qualify as a systemic hallmark of cancer and their presence in an individual's blood is the colloquial "smoking gun"—the absolute and direct evidence of viable neoplastic disease.

Acknowledgements

The authors acknowledge all the patients and asymptomatic individuals who consented to participate in the study and provide blood samples. Samples from asymptomatic individuals were obtained from Medall Healthcare Pvt. Ltd. (multiple pan-India locations) while cancer patients' samples were obtained from HCG Manavata Cancer Centre (Nasik, India), NueClear Healthcare (Mumbai, India), Chandak Cancer Hospital (Jalgaon, India) and HCG Cancer Centre (Bengaluru, India). Contributions of Ms Swati Deshpande, Mr Pankaj Porje, Mr Milind Agnihotri, Dr Jitendra Karlekar, Dr Shalom Syed, Ms Rukhsar Patel, Ms Ashwini Pawar, Mr Ashish Rojekar, Ms Rimple Shah, Ms Disha Mathew, Ms Shweta Shinde and Ms Kanchan Tidke in managing various aspects of the study are acknowledged. The entire study was self-funded by Datar Cancer Genetics Limited and no external funding was received for our study.

Conflict of interest

D.A., D.P., V.D., C.S., R.P., P.F., P.F., N.S., P.D., S.A., S.P., S.P., A.A., S.P., A.A., R.C., M.A. and A.S. are in full time employment of the Study Sponsor. R.D. is the Founder and CMD of the Study Sponsor. T.C., S.L., R.P. and A.R. have no competing interests.

References

1. Giuliano M, Shaikh A, Lo HC, et al. Perspective on circulating tumor cell clusters: why it takes a village to metastasize. *Cancer Res* 2018;78:845–52.
2. Hong Y, Fang F, Zhang Q. Circulating tumor cell clusters: what we know and what we expect (review). *Int J Oncol* 2016;49:2206–16.
3. Au SH, Storey BD, Moore JC, et al. Clusters of circulating tumor cells traverse capillary-sized vessels. *Proc Natl Acad Sci USA* 2016;113:4947–52.
4. Au SH, Edd J, Stoddard AE, et al. Microfluidic isolation of circulating tumor cell clusters by size and asymmetry. *Sci Rep* 2017;7:2433.
5. Sarioglu AF, Aceto N, Kojic N, et al. A microfluidic device for label-free, physical capture of circulating tumor cell clusters. *Nat Methods* 2015;12:685–91.
6. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;100:57–70.
7. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011;144:646–74.
8. Plaks V, Koopman CD, Werb Z. Cancer. Circulating tumor cells. *Science* 2013;341:1186–8.
9. Dasgupta A, Lim AR, Ghajar CM. Circulating and disseminated tumor cells: harbingers or initiators of metastasis? *Mol Oncol* 2017;11:40–61.
10. Aceto N, Toner M, Maheswaran S, et al. En route to metastasis: circulating tumor cell clusters and epithelial-to-mesenchymal transition. *Trends Cancer* 2015;1:44–52.
11. Frisch SM, Screaton RA. Anoikis mechanisms. *Curr Opin Cell Biol* 2001;13:555–62.
12. Taddei ML, Giannoni E, Fiaschi T, et al. Anoikis: an emerging hallmark in health and diseases. *J Pathol* 2012;226:380–93.
13. Cao Z, Livas T, Kyprianou N. Anoikis and EMT: lethal "liaisons" during cancer progression. *Crit Rev Oncog* 2016;21:155–68.

14. Paoli P, Giannoni E, Chiarugi P. Anoikis molecular pathways and its role in cancer progression. *Biochim Biophys Acta* 2013;1833:3481–98.
15. Song W, Mazzieri R, Yang T, et al. Translational significance for tumor metastasis of tumor-associated macrophages and epithelial-mesenchymal transition. *Front Immunol* 2017;8:1106.
16. Hamilton G, Rath B. Circulating tumor cell interactions with macrophages: implications for biology and treatment. *Transl Lung Cancer Res* 2017;6:418–30.
17. Ding J, Jin W, Chen C, et al. Tumor associated macrophage × cancer cell hybrids may acquire cancer stem cell properties in breast cancer. *PLoS One* 2012;7:e41942.
18. Agnoletto C, Corrà F, Minotti L, et al. Heterogeneity in circulating tumor cells: the relevance of the stem-cell subset. *Cancers (Basel)* 2019;11:483.
19. Liu Q, Liao Q, Zhao Y. Myeloid-derived suppressor cells (MDSC) facilitate distant metastasis of malignancies by shielding circulating tumor cells (CTC) from immune surveillance. *Med Hypotheses* 2016;87:34–9.
20. Hanahan D, Coussens LM. Accessories to the crime: functions of cells recruited to the tumor microenvironment. *Cancer Cell* 2012;21:309–22.
21. Divella R, Daniele A, Abbate I, et al. The presence of clustered circulating tumor cells (CTCs) and circulating cytokines define an aggressive phenotype in metastatic colorectal cancer. *Cancer Causes Control* 2014;25:1531–41.
22. Ünlü B, Versteeg HH. Cancer-associated thrombosis: the search for the holy grail continues. *Res Pract Thromb Haemost* 2018;2:622–9.
23. Tormoen GW, Haley KM, Levine RL, et al. Do circulating tumor cells play a role in coagulation and thrombosis? *Front Oncol* 2012;2:115.
24. Cellsearch. Available from https://www.cellsearchctc.com/. Accessed July 18, 2019.
25. Gorges TM, Tinhofer I, Drosch M, et al. Circulating tumour cells escape from EpCAM-based detection due to epithelial-to-mesenchymal transition. *BMC Cancer* 2012;12:178.
26. Sigma-Aldrich. Available from https://www.sigmaaldrich.com/technical-documents/articles/biology/cell-culture-troubleshooting-cell-clumping.html. Accessed July 18, 2019.
27. Molnar B, Ladanyi A, Tanko L, et al. Circulating tumor cell clusters in the peripheral blood of colorectal cancer patients. *Clin Cancer Res* 2001;7:4080–5.
28. Mu Z, Wang C, Ye Z, et al. Prospective assessment of the prognostic value of circulating tumor cells and their clusters in patients with advanced-stage breast cancer. *Breast Cancer Res Treat* 2015;154:563–71.
29. Wendel M, Bazhenova L, Boshuizen R, et al. Fluid biopsy for circulating tumor cell identification in patients with early-and late-stage non-small cell lung cancer: a glimpse into lung cancer biology. *Phys Biol* 2012;9:016005.
30. Kats-Ugurlu G, Roodink I, de Weijert M, et al. Circulating tumour tissue fragments in patients with pulmonary metastasis of clear cell renal cell carcinoma. *J Pathol* 2009;219:287–93.
31. Loh J, Jovanovic L, Lehman M, et al. Circulating tumor cell detection in high-risk non-metastatic prostate cancer. *J Cancer Res Clin Oncol* 2014;140:2157–62.
32. Chang MC, Chang YT, Chen JY, et al. Clinical significance of circulating tumor microemboli as a prognostic marker in patients with pancreatic ductal adenocarcinoma. *Clin Chem* 2016;62:505–13.
33. Martin OA, Anderson RL, Narayan K, et al. Does the mobilization of circulating tumour cells during cancer therapy cause metastasis? *Nat Rev Clin Oncol* 2017;14:32–44.
34. Katharina P. Tumor cell seeding during surgery-possible contribution to metastasis formations. *Cancers (Basel)* 2011;3:2540–53.
35. Luskin MR, Murakami MA, Manalis SR, et al. Targeting minimal residual disease: a path to cure? *Nat Rev Cancer* 2018;18:255–63.
36. Kang YT, Kim YJ, Lee TH, et al. Cytopathological study of the circulating tumor cells filtered from the cancer patients' blood using hydrogel-based cell block formation. *Sci Rep* 2018;8:15218.
37. Cummings J, Sloane R, Morris K, et al. Optimisation of an immunohistochemistry method for the determination of androgen receptor expression levels in circulating tumour cells. *BMC Cancer* 2014;14:226.
38. Pinsky PF, Bellinger CR, Miller DP Jr. False-positive screens and lung cancer risk in the National Lung Screening Trial: implications for shared decision-making. *J Med Screen* 2018;25:110–2.
39. Nelson HD, Fu R, Cantor A, et al. Effectiveness of breast cancer screening: systematic review and meta-analysis to update the 2009 U.S. preventive services task force recommendation. *Ann Intern Med* 2016;164:244–55.
40. Hubbard RA, Kerlikowske K, Flowers CI, et al. Cumulative probability of false-positive recall or biopsy recommendation after 10 years of screening mammography: a cohort study. *Ann Intern Med* 2011;155:481–92.
41. Kilpeläinen TP, Tammela TL, Roobol M, et al. False-positive screening results in the European randomized study of screening for prostate cancer. *Eur J Cancer* 2011;47:2698–705.
42. Moss EL, Hollingworth J, Reynolds TM. The role of CA125 in clinical practice. *J Clin Pathol* 2005;58:308–12.
43. Ballehaninna UK, Chamberlain RS. The clinical utility of serum CA 19-9 in the diagnosis, prognosis and management of pancreatic adenocarcinoma: an evidence-based appraisal. *J Gastrointest Oncol* 2012;3:105–19.
44. Fabrikant MS, Wisnivesky JP, Marron T, et al. Benefits and challenges of lung cancer screening in older adults. *Clin Ther* 2018;40:526–34.
45. Heywang-Köbrunner SH, Hacker A, Sedlacek S. Advantages and disadvantages of mammography screening. *Breast Care (Basel)* 2011;6:199–207.
46. Lieu D. The Papanicolaou smear: its value and limitations. *J Fam Pract* 1996;42:391–9.

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.

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 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

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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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Cancer Prev Res 2020;XX:XX–XX

doi: 10.1158/1940-6207.CAPR-20-0322

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 ≥3 cells with characteristic immunostaining pattern as per cancer type, including epithelial carcinoma (EPCAM+, panCK+, CD45±), sarcoma (SMA+, Desmin+, CD45±), or neuroendocrine tumor (Synaptophysin+, Chromogranin+, CD45±). 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 (≥)120 μm^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

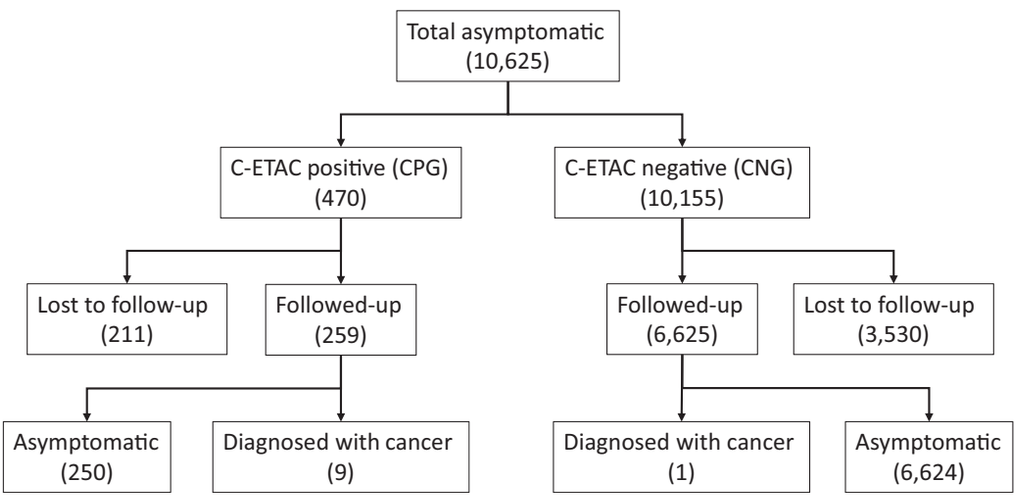

Figure 1.

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.

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/yet 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.

Disclosure of Potential Conflicts of Interest

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 potential conflicts of interest were disclosed 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.

Acknowledgments

The authors acknowledge all the patients and asymptomatic individuals who consented to participate in the study and provide blood samples. Samples from asymptomatic individuals were obtained from Medall Spark Diagnostics (multiple pan-India locations) whereas cancer patients' samples were obtained from HCG Manavata Cancer Centre (Nasik), NueClear Healthcare (Mumbai), Chandak Cancer Care (Jalgaon), and HCG Cancer Centre (Bengaluru). The authors acknowledge the contributions of all DCG staff and Scientists in managing various operational aspects of the study. The entire study was self-funded by Datar Cancer Genetics and no external funding was received for our study.

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Received June 19, 2020; revised July 22, 2020; accepted September 15, 2020; published first September 21, 2020.

References

1. Kavic SM, Basson MD. Management of complications of colonoscopy. In: Holzheimer RG, Mannick JA, editors. Surgical treatment: evidence-based and problem-oriented. Munich: Zuckschwerdt; 2001.
2. Fabrikant MS, Wisnivesky JP, Marron T, Taioli E, Veluswamy RR. Benefits and challenges of lung cancer screening in older adults. Clin Ther 2018;40:526–34.
3. Heywang-Köbrunner SH, Hacker A, Sedlacek S. Advantages and disadvantages of mammography screening. Breast Care 2011;6: 199–207.
4. Barbany G, Arthur C, Liedén A, Nordenskjöld M, Rosenquist R, Tesi B, et al. Cell-free tumour DNA testing for early detection of cancer - a potential future tool. J Intern Med 2019;286:118–36.
5. Steensma DP. Clinical consequences of clonal hematopoiesis of indeterminate potential. Blood Adv 2018;2:3404–10.
6. Elmore JG, Fletcher SW. Overdiagnosis in breast cancer screening: time to tackle an underappreciated harm. Ann Intern Med 2012;156: 536–7.
7. Kalager M, Wieszczy P, Lansdorp-Vogelaar I, Corley DA, Bretthauer M, Kaminski MF. Overdiagnosis in colorectal cancer screening: time to acknowledge a blind spot. Gastroenterology 2018;155:592–5.
8. Akolkar D, Patil D, Crook T, Limaye S, Page R, Datta V, et al. Circulating ensembles of tumor-associated cells: a redoubtable new systemic hallmark of cancer. Int J Cancer 2020;146:3485–94.
9. Liu M, Oxnard G, Klein E, Swanton C, Seidon MV; On behalf of the CCGA Consortium. Sensitive and specific multi-cancer detection and localization using methylation signatures in cell-free DNA. Ann Oncol 2020;31:745–59.
10. Ried K, Eng P, Sali A. Screening for circulating tumour cells allows early detection of cancer and monitoring of treatment effectiveness: an observational study. Asian Pac J Cancer Prev 2017;18:2275–85.
11. Gnanapragasam VJ, Lophatananon A, Wright KA, Muir KR, Gavin A, Greenberg DC. Improving clinical risk stratification at diagnosis in primary prostate cancer: a prognostic modelling study. PLoS Med 2016;13:e1002063.
12. Chou WC, Wang F, Cheng YF, Chen MF, Lu CH, Wang CH, et al. A simple risk stratification model that predicts 1-year postoperative mortality rate in patients with solid-organ cancer. Cancer Med 2015;4:1687–96.

Original Article

Evaluation of Circulating Tumor Cell Clusters for Pan-Cancer Noninvasive Diagnostic Triaging

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BACKGROUND: Histopathologic examination (HPE) of tumor tissue obtained by invasive biopsy is the standard for cancer diagnosis but is resource-intensive and has been associated with procedural risks. The authors demonstrate that immunocytochemistry (ICC) profiling of circulating ensembles of tumor-associated cells (C-ETACs) can noninvasively provide diagnostic guidance in solid organ cancers. **METHODS:** The clinical performance of this approach was tested on blood samples from 30,060 individuals, including 9416 individuals with known cancer; 6725 symptomatic individuals with suspected cancer; and 13,919 asymptomatic individuals with no prior diagnosis of cancer. C-ETACs were harvested from peripheral blood and profiled by ICC for organ-specific and subtype-specific markers relevant to the cancer type. ICC profiles were compared with HPE diagnoses to determine concordance. **RESULTS:** The presence of malignancy was confirmed by the detection of C-ETACs in 91.8% of the 9416 individuals with previously known cancer. Of the 6725 symptomatic individuals, 6025 were diagnosed with cancer, and 700 were diagnosed with benign conditions; C-ETACs were detected in 92.6% of samples from the 6025 individuals with cancer. In a subset of 3509 samples, ICC profiling of C-ETACs for organ-specific and subtype-specific markers was concordant with HPE findings in 93.1% of cases. C-ETACs were undetectable in 95% of samples from the 700 symptomatic individuals who had benign conditions and in 96.3% of samples from the 13,919 asymptomatic individuals. **CONCLUSIONS:** C-ETACs were ubiquitous (>90%) in various cancers and provided diagnostically relevant information in the majority (>90%) of cases. This is the first comprehensive report on the feasibility of ICC profiling of C-ETACs to provide pan-cancer diagnostic guidance with accuracy comparable to that of HPE. ***Cancer Cytopathol* 2021;129:226-238**.

KEY WORDS: circulating ensembles of tumor-associated cells (C-ETACs); circulating tumor cells (CTCs); diagnosis; diagnostic triaging; liquid biopsy; noninvasive; solid organ cancers.

INTRODUCTION

Cancers are traditionally diagnosed using histologic examination (HPE) of tumor tissue obtained by invasive biopsy to identify morphologic irregularities and nuclear features.[1] Tissue biopsies, which are usually image-guided,

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We acknowledge all patients and asymptomatic individuals who consented to participate in this study and provided blood samples. Samples from asymptomatic individuals were obtained from Medall Spark Diagnostics (multiple pan-India locations), and samples from patients with cancer were obtained from HCG Manavata Cancer Center (Nasik), NueClear Healthcare (Mumbai), Chandak Cancer Care (Jalgaon), and HCG Cancer Center (Bengaluru). We also acknowledge the contributions of all Datar Cancer Genetics staff and scientists in managing various operational aspects of the study.

Additional supporting information may be found in the online version of this article.

Received: August 3, 2020; **Revised:** August 29, 2020; **Accepted:** September 8, 2020

Published online September 30, 2020 in Wiley Online Library (wileyonlinelibrary.com)

DOI: 10.1002/cncy.22366, wileyonlinelibrary.com

are specialized, invasive procedures with significant morbidity and financial implications. In addition, they necessitate patient visits to a tertiary care center with specialized facilities. Apart from the logistical aspects, there are other factors that affect tumor tissue procurement, such as inaccessibility of the tumor, proximity of the tumor to vital organs or vasculature, patients' comorbidities, and even patients' reluctance because of procedural risks. Repeat biopsies often may be desirable, such as 1) if the prior tissue sample was insufficient[2] or poorly representative,[3] 2) to determine the status of therapeutically relevant biomarkers,[4] 3) to characterize recurrent lesions, or 4) to identify a new lesion as a second primary or metastasis.[5] However, repeat biopsies are associated with increased procedural risks.

Noninvasive alternatives for obtaining representative tumor samples or tumor-derived analytes can alleviate the challenges encountered with invasive procedures.[6] Circulating tumor cells[7] (CTCs) are malignant cells shed by tumors into the vasculature or lymphatics either as single cells or in clusters (eg, ≥2 cells). Because they are derived from the tumor mass itself, CTCs and their clusters are analytically equivalent to the tumor tissue. Harvesting sufficient, viable CTCs and their clusters from peripheral blood thus is comparable to obtaining a representative tissue biopsy with minimal stromal tissue or other nontumor content and may conveniently be described as *oligobiopsy* or *microbiopsy*.

Previous reports also have indicated that CTCs convey the status of diagnostic or theranostic antigens that are otherwise routinely evaluated in tumor tissue.[8] However, the application of CTCs in the clinical setting is currently confined to numerical evaluation for prognostication in a few metastatic cancers.[9-11] The clinical potential of CTC-based diagnosis has not been realized because current methods and devices to harvest CTCs and their clusters from peripheral blood principally rely on immunomagnetic enrichment or microfluidic separation, neither of which yields sufficient numbers for meaningful applications.[12-14] We previously described the ubiquity of circulating ensembles of tumor-associated cells (C-ETACs) in solid organ tumors; C-ETACs include CTCs, which are CD45-negative cells, as well as CD45-positive and CD8-positive cells, such as tumor-associated macrophages and tumor-associated leucocytes, in addition to cancer stem cells (CD44-positive).[15] We previously described a novel approach for the negative enrichment of C-ETACs (and CTCs) from peripheral blood samples based on the apoptosis resistance of malignant cells of tumorigenic origin.[15] We used this approach to achieve high detection and harvest rates of C-ETACs in a large cohort of patients who had prior diagnoses of various cancers and in symptomatic individuals who had results that were suspicious for cancer. In a subset analysis, C-ETACs were characterized by immunocytochemistry (ICC) profiling for organ-specific and subtype-specific (OSS) antigens, which are routinely evaluated in HPE and ICC, to determine the tissue of origin. Here, we describe the suitability of this approach for adoption in clinical practice because it noninvasively provides diagnostically relevant information not inferior to that obtained by HPE of tumor tissue.

MATERIALS AND METHODS

Study Design

The data presented in this report were derived from patient samples obtained during the course of 4 observational studies: the *TrueBlood study* (Tissue Biopsy Replacement With Unique Evaluation of Circulating Biomarkers for Morphological Evaluation and Clinically Relevant Molecular Typing of Malignancies From Blood Samples; Clinical Trials Registry-India [CTRI]/2019/03/017918); the *ProState study* (Utility of ProState, the Liquid Biopsy Platform, in Distinguishing Prostate Malignancies From Benign Prostatic Hyperplasia; CTRI/2019/02/017863), the *GlioLENS study* (Utility of Gliotrack, the Liquid Biopsy Platform for Gliomas, in Distinguishing Glioblastoma From Other Central Nervous System Lesions With Equivocal Findings on Neuroimaging; registered on the World Health Organization International Clinical Trials Registry Platform; CTRI/2019/02/017663), and the *RESOLUTE study* (Realtime Enrichment Screen for Outright Detection of Latent Undiagnosed Malignant Tumors in Asymptomatic Individuals Efficiently; CTRI/2019/01/017219). All studies were evaluated by the institutional review boards and approved by the ethics committees of the study sponsor (Datar Cancer Genetics) and of the respective participating institutions. All trials were conducted in accordance with existing ethical guidelines and regulations, such as those of the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use-Good Clinical Practice (ICH-GCP), as well as the Declaration of Helsinki.

The TrueBlood study enrolled adult men and women (aged ≥18 years) who had a histopathologically confirmed

diagnosis of a solid organ cancer irrespective of the extent of disease or therapy status. The ProState study enrolled adult men (aged ≥18 years) who had a confirmed diagnosis of either prostate cancer or benign prostate enlargement as well as individuals who had results that were suspicious for prostate cancer. The GlioLENS study enrolled adults (aged ≥18 years) who presented with radiologic intracranial space-occupying lesions that were suspicious for central nervous system (CNS) malignancies. The RESOLUTE study enrolled asymptomatic adult men and women who had an age-associated elevated risk of cancer. Details of all studies are available online by querying for the respective trial identification numbers (https://apps.who.int/trialsearch/, last accessed on 29-Aug-2020).

Study Participants and Samples

For the current study, we primarily evaluated 9416 patients who had prior diagnoses of various cancers and 6725 who had suspected cancers, among whom 6025 were subsequently diagnosed with cancer and 700 were diagnosed with benign or inflammatory conditions of various organs. Clinical details of these patients' cancers were determined from the most recent clinical reports. Finally, the study evaluated 13,919 asymptomatic individuals who had an age-associated elevated risk of cancer but had negative (normal) findings on screening investigations for cancer, including low-dose computed tomography, mammography, Papanicolaou smears, as well as serum antigens (cancer antigen 125 [CA125], prostate-specific antigen [PSA], carbohydrate antigen 19-9 [CA19-9], α-fetoprotein [AFP], and carcinoembryonic antigen [CEA]). Demographic data from study participants are provided in Table 1. Details of various observational trials from which our study cohort was populated are provided in Supporting Table 1. Details of cancer types and benign conditions are provided in Supporting Tables 2 and 3, respectively. All study participants were counselled regarding the aims and scope of each study, after which they provided signed, written informed consent. From 15 to 20 mL of peripheral blood was collected into ethylenediaminetetraacetic acid vacutainer tubes from all study participants. For the 6725 individuals with suspected cancer, blood was collected before undergoing a biopsy or any other invasive procedure. Blood samples were transported to the laboratory at between 2 °C and 8 °C within 48 hours. All samples were processed at the facility of the study sponsor, which is accredited by the College of American Pathologists under the Clinical Laboratory Improvement Amendments and by the International Organization for Standardization number 15189:2012 (National Accreditation Board for Testing and Calibration Laboratories-International Laboratory Accreditation Cooperation, NABL-ILAC).

TABLE 1. Patient Demographics[a]

Characteristic	Cancer	Benign	Asymptomatic
Sex			
Men	6773	434	5807
Women	8668	266	8112
Total	15,441	700	13,919
Age: Median (range), y	57 (18-102)	55 (18-90)	53 (40-75)
Therapy status			
Naive	6025	—	—
Treated	9416	—	—
Metastatic status			
Nonmetastatic	3947	—	—
Metastatic	9675	—	—
Unavailable	1819	—	—

[a]The study cohort included 9416 previously diagnosed and treated cases of cancer, 6025 recently diagnosed therapy-naive cases of cancer, 700 individuals with benign conditions, and 13,919 asymptomatic individuals.

Enrichment, Harvesting, and Detection of C-ETACs

Peripheral blood mononuclear cells were obtained from 15 mL of whole blood using RBC Lysis Buffer (Thermo Fisher Scientific USA) according to the manufacturer's instructions, and aliquots were transferred into multiwell plates for treatment with epigenetically activating media, as described previously.[15] 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 single cells and clusters were gently transferred to 96-well, imaging-compatible plates for the identification of C-ETACs and CTCs by ICC (see Immunocytochemistry Workflow, below). C-ETACs were defined as epithelial cell adhesion molecule (EpCAM)-positive, pan-cytokeratin (PanCK)-positive, and irrespective of CD45 status for all epithelial malignancies (carcinomas); as cell-surface vimentin -positive and smooth muscle actin -positive/S100-positive, irrespective of CD45 status for all sarcomas; and as glial fibrillary acidic protein (GFAP)-positive, S100-positive/Nestin-positive and CD45-negative for all glial CNS malignancies. To differentiate active C-ETACs from random/transient associations of cells, C-ETACs were defined as clusters of ≥3 cells that stained positive for the

TABLE 2. Organ-Specific and Subtype-Specific Antibodies[a]

Cancer Type	Marker 1	Marker 2	Marker 3	Marker 4
Bladder	Uroplakin-II	GATA3	CK20	CK7
Breast	GCDFP-15	GATA3	EMA	CK7
CNS	GFAP	S100	Nestin	Olig-2
Cervix	p63	p16	CEA	CK7
Colorectum	CDX2	MUC2	CK20	—
Gallbladder	CEA	Maspin	CK19	CK7
Head and neck	p63	HMWCK	CK5/CK6	—
Kidney	CA-IX	RCC	CD10	Pax-8
Liver	Glypican 3	Hep Par-1	AFP	Arginase
Lung	Napsin-A	TTF-1	p40	CK7
Esophagus	p63	CK5/CK6	MUC2	CK7
Ovary	CA125	WT-1	Pax-8	CK7
Pancreas	CA19.9	CK19	Maspin	CK7
Prostate	AMACR	PSMA	p63	PSCA
Sarcomas	SMA	S100	CSV	—
Stomach	CDX2	CEA	CK7	—
Thyroid	TTF-1	Thyroglobulin	Calcitonin	CK19
Uterine	CK19	Pax-8	CEA	CK7

Abbreviation: CNS, central nervous system.
[a]The listed organ-specific and subtype-specific markers were evaluated by immunocytochemistry profiling for each cancer type.

indicated markers, irrespective of CD45 status.[15] CTCs were defined as single cells that stained positive for the indicated markers and negative for CD45.

Immunocytochemistry Workflow

Dissociated C-ETACs (single cells) were fixed on slides with 4% paraformaldehyde, pH 6.9, for 20 minutes. Cell permeabilization was achieved with 0.3% Triton X-100 (15 minutes) followed by blocking with 3% bovine serum albumin (30 minutes). Cells were immunostained with primary antibodies (60 minutes), washed with phosphate-buffered saline, pH 7.4, incubated with secondary antibodies (60 minutes), washed with phosphate-buffered saline, and then incubated with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) in the dark (15 minutes). All incubations were at ambient temperature (range, from 20 °C to 25 °C). Positive and negative cell line controls were also processed with each batch of samples. ICC slides were scanned by using the Cell Insight CX7 High-Content Screening platform (Thermo Fisher Scientific USA), which enables nuclear size filters and calibration of intensity thresholds for individual fluorophore-conjugated antibodies. The intensity of each antigen expression was compared with that of batch controls (reference cell lines) (Supporting Table 4). These precautions avoid or eliminate crosstalk in multiplexed analysis with different fluorophore-conjugated antibodies.

Design of Organ-Specific and Subtype-Specific Immunocytochemistry Panels

Where EpCAM-positive, PanCK-positive clusters (C-ETACs) were detected, these clusters were gently dissociated into single cells for ICC profiling to determine the status of OSS markers. We observed that the detection of OSS markers was more efficient and sensitive in single cells than in clusters. Cancer-specific prescreening panels of OSS markers were designed based on publicly available information on antigen markers used in routine HPE or ICC analysis (Table 2). ICC methods were initially developed and optimized on respective control cell lines, which also were used for analytical validation. Details of the control cell lines, antibodies (primary and secondary), and fluorophores are provided in Supporting Table 4. All control cell lines used in this study were procured in the last 3 years. All cell lines were mycoplasma-free.

Combined Prospective and Retrospective Evaluation of Concordance

In a subset of 3509 samples (see Supporting Table 5), C-ETACs were profiled with respective cancer-specific OSS-ICC panels. This subset included 2281 previously diagnosed and pretreated cases in which OSS-ICC findings were retrospectively evaluated for concordance with HPE findings on a foundational biopsy during primary diagnostic workup. Concordance (%) was determined as the proportion of samples in which OSS-ICC findings agreed with prior HPE findings. The remaining 1228 samples formed the prospective, double-blinded evaluation cohort in which OSS-ICC profiling of C-ETACs and HPE of a biopsied tumor tissue sample were conducted concurrently. For all 1228 samples, OSS-ICC panels were selected on the basis of clinician's recommendation of a suspected primary. Findings of HPE and OSS-ICC profiling were masked from each other until all samples had been evaluated. After unblinding, concordance (%) was determined as the proportion of samples in which OSS-ICC findings agreed with recent HPE findings.

In a subset of 229 samples (Supporting Table 6) from metastatic cancers, including samples from 163 previously diagnosed and pretreated patients and from 66 recently diagnosed therapy-naive patients, the ICC profile of C-ETACs was evaluated to determine fidelity in representing the primary organ versus the commonly

TABLE 3. Organ and Subtype-Specific Antibody Panels to Discern Primary From Metastatic Deposits

		Primary		Metastasis	
Primary	Metastasis	Marker 1	Marker 2	Marker 1	Marker 2
Bladder	Brain	Uroplakin-II	GATA3	GFAP	S100
Breast	Brain	GCDFP15	GATA3	GFAP	S100
	Lung	GCDFP15	GATA3	Napsin-A	TTF1
	Liver	GCDFP15	GATA3	Glypican3	HepPar1
Cervix	Brain	p63	CK7	GFAP	S100
Colon	Brain	CDX2	MUC2	GFAP	S100
	Lung	CDX2	MUC2	Napsin-A	TTF1
	Liver	CDX2	MUC2	HepPar1	Glypican3
Head and neck	Brain	p63	HMWCK	GFAP	S100
Kidney	Brain	CA-IX	RCC	GFAP	S100
Liver	Lung	Glypican3	HepPar1	Napsin-A	TTF1
Lung	Brain	Napsin-A	TTF1	GFAP	S100
	Liver	Napsin-A	TTF1	Glypican3	HepPar1
Esophagus	Brain	p63	CK5/6	GFAP	S100
	Lung	p63	CK5/6	Napsin-A	TTF1
Ovary	Brain	WT1	PAX8	GFAP	S100
	Liver	WT1	PAX8	Glypican3	HepPar1
Pancreas	Lung	CA19.9	Maspin	Napsin-A	—
	Liver	CA19.9	Maspin	Glypican3	—
Stomach	Brain	CDX2	CK7	GFAP	S100
	Lung	CDX2	CK7	Napsin-A	TTF1

observed organ(s) of metastases, such as lung, liver, and brain. C-ETACs in this subset of samples were profiled with OSS-ICC markers of the primary organs as well as the organ of metastasis (Table 3) to determine whether the approach accurately discerns the primary organ.

RESULTS

Detection of C-ETACs

C-ETACs were detected in 14,221 of 15,441 samples (sensitivity, 92.1%) of various cancers. C-ETACs were detected in 3623 of 3947 (91.8%) nonmetastatic cases, in 8959 of 9675 (92.6%) metastatic cases, in 5578 of 6025 (92.6%) recently diagnosed therapy-naive cases, and in 8643 of 9416 (91.8%) previously diagnosed pretreated cases. C-ETAC detection rates for each cancer type are provided in Table 4. Figure 1 depicts representative sets of images for ICC profiling of C-ETACs and CTCs for EpCAM, PanCK, and CD45 status. Representative images depicting ICC profiling of C-ETACs for these markers have also been published previously.[15] Among the 700 patients who were diagnosed with benign or other nonmalignant conditions, C-ETACs were detected in 35 individuals (5%). Among the 13,919 asymptomatic individuals who had negative findings on all screening investigations, C-ETACs were undetectable in 13,408 individuals (specificity, 96.3%).

Concordance of OSS-ICC Profiling With HPE Findings

To determine whether ICC profiling can provide accurate representation of histologically relevant information, such as the organ of origin and subtype, we evaluated C-ETACs from a subset of 3509 patient samples. Among the 1228 recently diagnosed and therapy-naive individuals who formed the prospective cohort, OSS-ICC profiling was accurate in 1150 cases (93.6%) and negative or aberrant in 78 cases (6.4%). Among the 2281 previously diagnosed and pretreated patients who formed the retrospective cohort, OSS-ICC profiling was accurate in 2116 cases (92.8%) and negative or aberrant in 164 cases (7.2%). Overall, among the 3509 samples, OSS markers were accurate in 3266 cases (93.1%) and negative or aberrant in 243 cases (6.9%). Cancer-specific concordance of OSS markers is detailed in Table 4. Also among the 3509 samples, OSS marker positivity rates were comparable in CTCs from metastatic (92.4%) and nonmetastatic (94%) samples irrespective of prior treatment status.

In the subset of 229 samples in which ICC profiling of C-ETACs was evaluated for fidelity in determining primary deposits and in discerning primary from metastatic deposits, an overall 96.9% accuracy was determined based on 95.5% accuracy in 66 therapy-naive cases and 97.5% accuracy in 163 pretreated cases (see Supporting Table 6).

TABLE 4. Circulating Tumor Cell Detection Rates (Sensitivity) and Concordance of Organ-Specific and Subtype-Specific Panels With Histopathologic Examination Data (Accuracy)

	CTC Detection Rate, %			OSS Marker Concordance Rate, %		
Cancer Type	Prospective	Retrospective	Overall	Prospective	Retrospective	Overall
Bladder	91.0	96.2	94.7	100.0	98.1	98.5
Breast	92.4	92.5	92.5	95.4	93.4	94.0
CNS	90.0	—	90.0	90.0	—	90.0
Cervix	96.0	86.7	89.8	87.7	88.6	88.3
Colorectum	89.8	93.4	92.4	91.8	92.8	92.6
Gallbladder	97.3	90.1	93.0	100.0	87.9	91.3
Head and neck	92.2	92.8	92.5	97.9	98.5	98.3
Kidney	92.2	95.0	93.5	100.0	100.0	100.0
Liver	91.8	91.8	91.8	94.3	83.3	91.5
Lung	95.8	94.1	95.0	91.8	86.3	89.2
Esophagus	96.8	92.3	94.7	88.4	85.1	86.3
Ovary	86.8	85.6	85.9	96.2	86.2	87.7
Pancreas	96.6	91.8	94.1	100.0	93.0	96.0
Prostate	93.7	97.9	96.0	91.3	96.3	93.0
Sarcoma	94.1	95.3	95.1	100.0	100.0	100.0
Stomach	92.6	95.3	93.8	96.0	97.8	96.8
Thyroid	100.0	94.5	97.0	100.0	100.0	100.0
Unknown primary	89.3	81.5	88.9	—	—	—
Uterine	88.4	88.1	88.2	85.7	77.6	79.7
Other	89.4	85.0	89.3	—	—	—
Overall	92.6	91.8	92.1	93.6	92.8	93.1

Abbreviations: CNS, central nervous system; CTC, circulating tumor cells; OSS, organ and subtype specific.

Among the 35 C-ETAC positive benign cases (out of a total of 700), C-ETACs from 5 samples (0.71% out of 700) were positive for ≥1 OSS marker associated with the organ of suspicion, indicating a possible risk of malignancy. The low OSS positivity rate in benign indicates high specificity of the approach to discern malignant and benign conditions in a particular organ.

C-ETACs detected in asymptomatic individuals were not profiled by ICC.

C-ETAC positivity in individuals with benign conditions and in asymptomatic individuals were conveyed to the referring clinicians for further surveillance, the results of which will be communicated later. Figure 2 and Figure 3 depict representative images of CTCs profiled for OSS markers. Additional images of CTCs profiled for OSS markers are provided in the online Supporting Information (see Supporting Figs. 1-10). Images of C-ETACs profiled for OSS markers have been published previously.[15]

DISCUSSION

With approximately 18 million new cases diagnosed annually,[16] cancer contributes significantly to the global disease burden. HPE of malignant tissue obtained by invasive biopsy is the current standard to determine malignant status in suspected cancer cases as well as for morphologic characterization of subtype, aggressiveness, and grade. Invasive biopsies not only cause pain, discomfort, and anxiety to patients but are also associated with procedural risks, such as hemorrhage, sepsis, and tumor seeding.[17,18] Organ-specific risks pose additional challenges to invasive biopsies. In the lung, percutaneous computed tomography-guided transthoracic needle biopsy is associated with a risk of pneumothorax, leading to lung collapse, pneumonia, and systemic air embolism.[19-22] In the kidney, the risks of biopsy include dysuria, hematuria, hematoma, and arteriovenous fistula.[23,24] Biopsies of the liver and gallbladder are known to be associated with risks such as pneumothorax, hemothorax, bile peritonitis, hemobilia, intrahepatic arteriovenous fistula, and neuralgia.[25] Percutaneous biopsies of the pancreas are associated with risks such as macrohematuria, pancreatitis, exocrine leak, and inadvertent biopsy of other organs.[26,27] Risks associated with prostate biopsies include hematuria, hematospermia, rectal bleeding, vasovagal episodes, urosepsis, and acute urinary retention.[28] Among all biopsies, brain biopsies in individuals who present with intracranial space-occupying lesions are perhaps most daunting because these are associated with risks of intracranial hemorrhage, morbidity, and mortality.[29] In addition to these risks, invasive biopsies may not be possible because of inaccessibility of the tumor or comorbidities.[2,3,5,30]

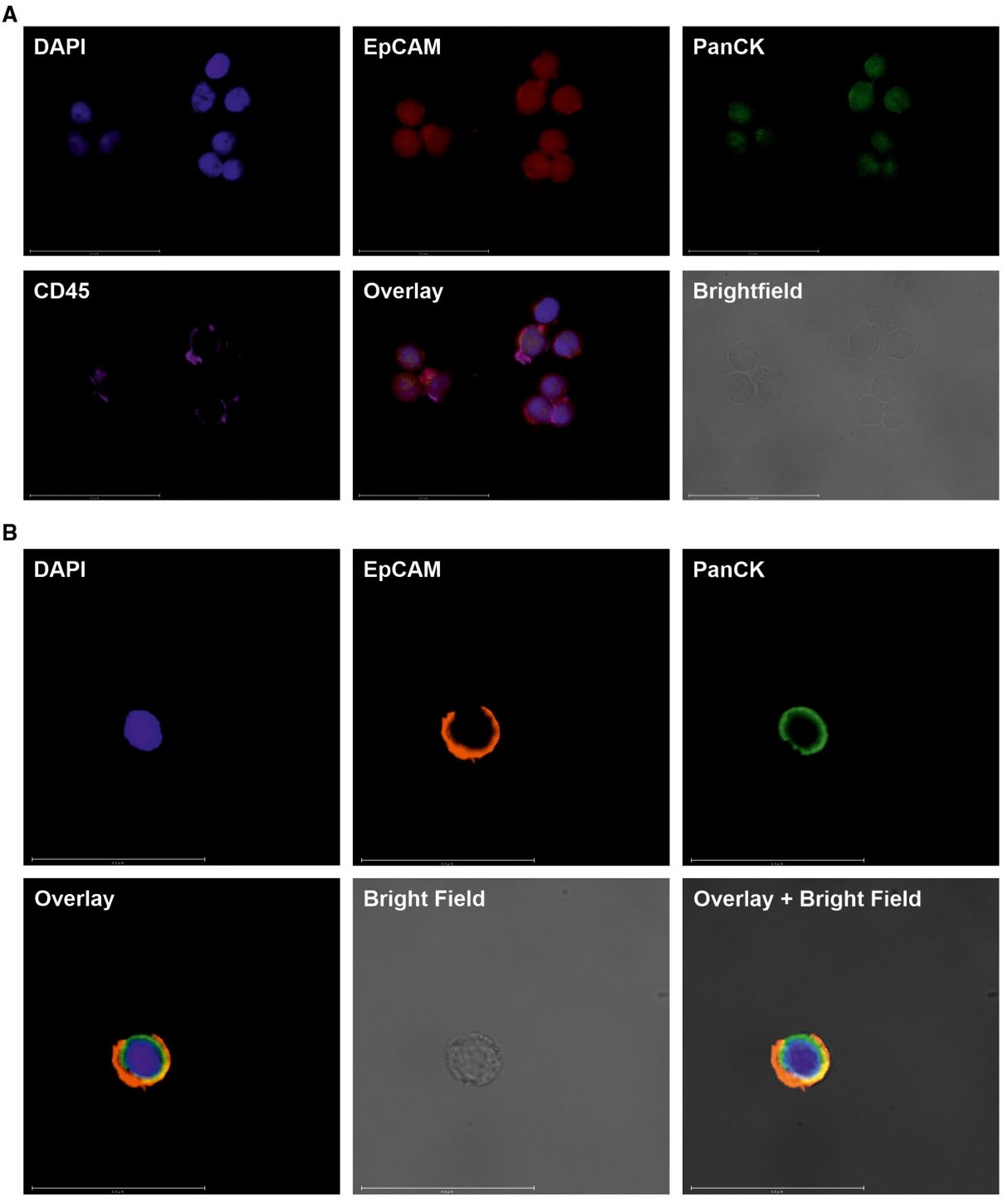

Figure 1. Images depict the identification of circulating ensembles of tumor-associated cells (C-ETACs) and circulating tumor cells (CTCs) by immunocytochemistry profiling. (A) C-ETACs are defined as clusters of ≥3 cells that are positive for epithelial cell adhesion molecule (EpCAM), positive for pan-cytokeratin (PanCK), and irrespective of CD45 status. (B) CTCs are defined as single cells that are positive for EpCAM, positive for PanCK, and negative for CD45. Representative images of C-ETACs and CTCs are shown for 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), EpCAM, and PanCK staining along with a fluorescence overlay, a brightfield image, and a brightfield image with fluorescence overlay.

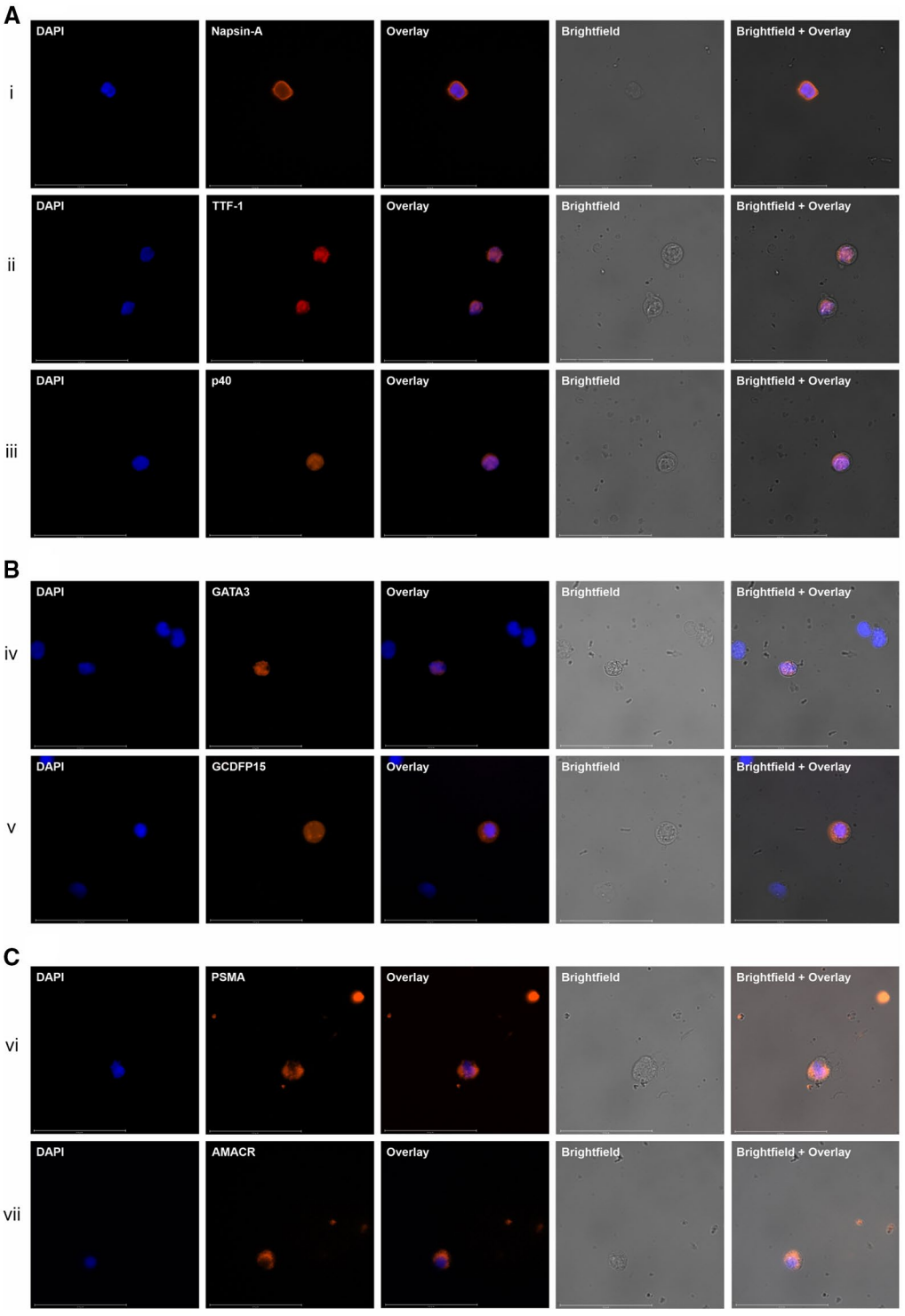

Figure 2. Images depict immunohistochemistry (ICC) profiling of circulating tumor cells (CTCs) for organ-specific and subtype-specific (OSS) markers of carcinomas. Representative images show ICC profiles of CTCs from (A) lung cancers, (B) breast cancers, and (C) prostate cancers. (A) In lung cancer, (i) Napsin-A and (ii) thyroid transcription factor-1 (TTF-1) are specific for adenocarcinoma (AD), whereas (iii) p40 is specific for squamous cell carcinoma (SCC). (B) In breast cancer, (iv) GATA-binding protein 3 (GATA3) and (v) gross cystic disease fluid protein 15 (GCDFP15) are specific for ductal and lobular breast carcinomas. (C) In prostate cancer, (vi) prostate-specific membrane antigen (PSMA) and (vii) α-methylacyl coenzyme A racemase (AMACR) are specific for prostate adenocarcinoma (AD). Each row of images (i-vii) shows 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining, an OSS marker, a fluorescence overlay, a brightfield image, and a brightfield image with fluorescence overlay. All CTCs were negative for CD45 (not depicted). Additional representative ICC profiling images of CTCs from other cancers are provided in the online Supporting Information.

The RESOLUTE, TrueBlood, GLIOLens and ProState studies were designed to evaluate the feasibility of detection and in vitro ICC profiling of C-ETACs and/or CTCs for noninvasively screening and obtaining diagnostically relevant information in various cancers. On the basis of an evaluation of blood samples from an initial 10,625 samples in the RESOLUTE study, we previously demonstrated that C-ETACs are rare (3.7%) in asymptomatic populations,[15] and detection rates were lower (3%) in individuals who formed the baseline-risk subgroup (no aberrant findings in cancer markers or on low-dose computed tomography, mammography, or Papanicolaou smear). Subsequently, 12,009 additional individuals were enrolled and, among the total 22,634 individuals, 13,919 formed the baseline-risk subgroup, whereas 8715 who had ≥1 aberrant finding formed the elevated-risk subgroup. C-ETACs were detected in 4.5% of the entire population (n = 22,634), which included a 3.7% detection rate in the baseline-risk subgroup and a 5.8% detection rate in the elevated-risk subgroup.

The negative-enrichment approach described previously[15] yielded sufficient C-ETACs to permit meaningful downstream applications. The high detection rates of C-ETACs across the entire cancer cohort were consistent with confirmed diagnoses of cancer, and the baseline detection rates in individuals with benign conditions and in asymptomatic individuals indicated high specificity. Because the detection and yield of C-ETACs were not affected by therapy status, we found this approach suitable for longitudinal evaluations during treatment. The objective of the current study was not to evaluate a quantitative change in C-ETACs based on extent of disease or in response to treatment.

The current approach is primarily intended for symptomatic individuals who have been referred for a biopsy but have not yet undergone the biopsy. Therefore, it is imperative to evaluate the performance characteristics of this approach on blood samples from a similarly biopsy-naive population rather than a postbiopsy population to accurately ascertain its sensitivity. Hence the study population had sufficient representation from suspected cases in which the blood samples were collected before a biopsy. We observed no significant differences in C-ETAC detection rates between biopsy-naive individuals and patients who had undergone a previous diagnostic biopsy or between individuals with metastatic and nonmetastatic disease.

The detection of C-ETACs (or CTCs) offers direct, visual evidence of malignancy; it is comparable to a positive finding of malignancy in HPE on a tumor tissue sample and is effectively an oligobiopsy/microbiopsy without stromal content, necrotic content, or normal tissue. C-ETACs are viable malignant cells shed from a tumor and hence contribute to and retain (a subset of or in total) the overall molecular and functional imprint of the parent tumor.[31] C-ETACs are a source of tumor analytes (proteins, DNA, RNA) as well as CTCs that may be evaluated for diagnostic inference.[32] In the current study, CTCs were present in all C-ETAC–positive cases. A few prior reports have indicated the feasibility of evaluating CTCs for organ or origin markers.[8,33,34] In a subset analysis of 3,509 cancer samples, we observed significant concordance between OSS-ICC profiles of C-ETAC samples and HPE diagnoses in both the prospective and retrospective settings. Likewise, in another subset analysis of 229 samples from patients with metastatic solid organ cancers, we observed that ICC profiles of C-ETACs accurately conveyed the primary cancer type/organ without any interference from OSS markers specific to the organ of metastasis. Thus we observed that C-ETACs retained and faithfully conveyed the molecular and functional characteristics of the tumor tissue of origin, irrespective of metastatic status or prior therapy status, and had minimal intermarker interference.

Prior reports have indicated lower expression of OSS markers in poorly differentiated or undifferentiated tumors as well as in CTCs undergoing epithelial-to-mesenchymal transition.[35] In the current study, OSS markers were negative (undetectable) in C-ETACs from a limited number of samples; this false negativity may be speculatively ascribed to dedifferentiation or epithelial-to-mesenchymal transition. Similarly, detection rates of OSS markers are currently restricted to classical CTCs (CD45-negative). During analysis of our samples, OSS marker positivity was also observed in CD45-positive subpopulations of C-ETACs.

The standard for diagnosis in the current study is HPE of biopsied tumor tissue, the verdict of which (malignant vs benign) determined C-ETAC findings as true-positive or false-positive. Any error in HPE would result in a conflict in diagnosis. Therefore, although the 5% detection rate of C-ETACs in HPE-determined benign cases is undesirable, it falls in the realm of inherent

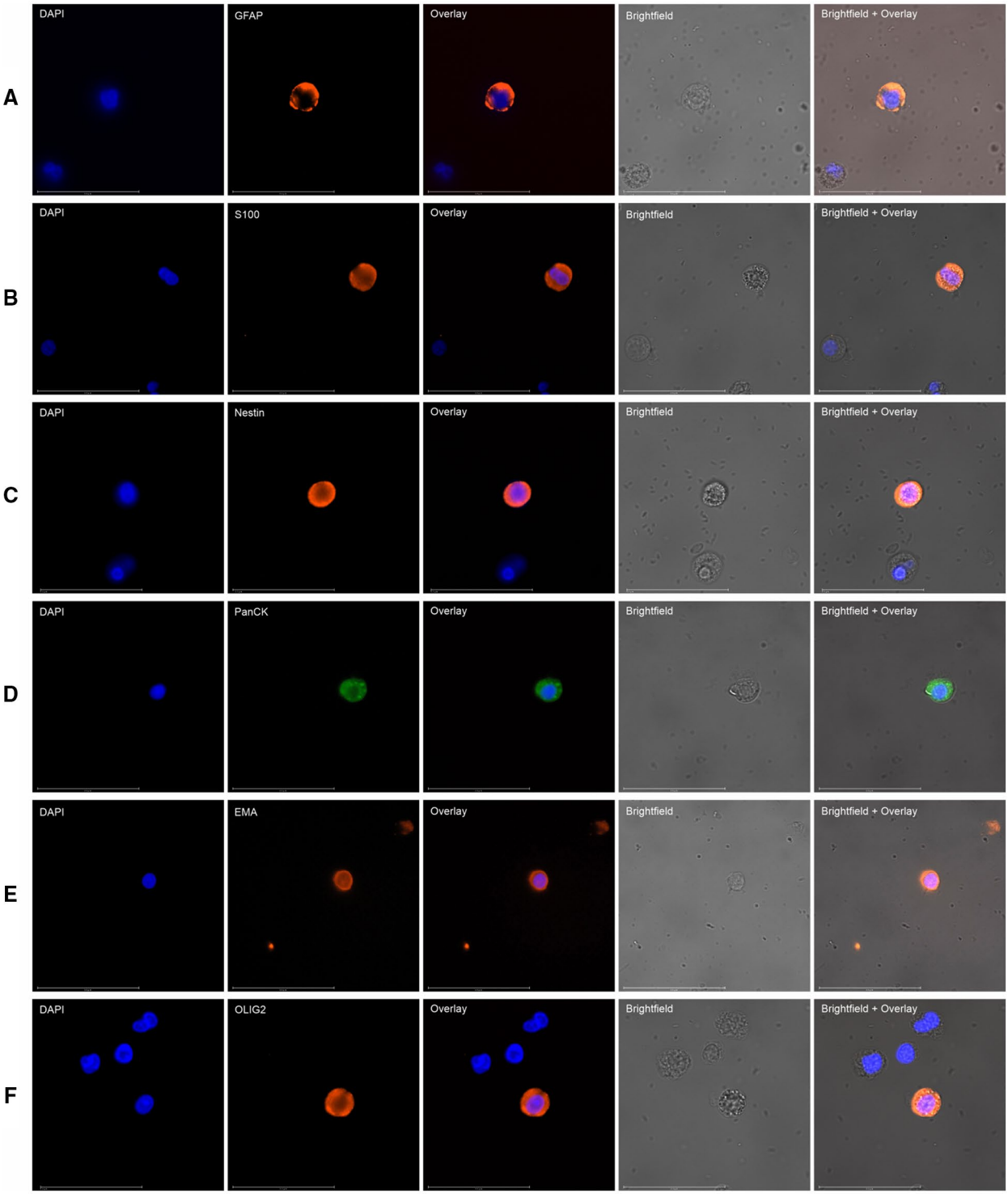

Figure 3. Images depict immunohistochemistry profiling of circulating tumor cells for central nervous system (CNS)-specific markers. The markers used for CNS malignancy were (A) glial fibrillary acidic protein (GFAP), (B) S100, (C) Nestin, (D) pan-cytokeratin (PanCK), (E) epithelial membrane antigen (EMA), and (F) oligodendrocyte transcription factor (OLIG2). Each row of 5 images shows 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining, a CNS-specific marker, a fluorescence overlay, a brightfield image, and a brightfield image with fluorescence overlay. All samples were negative for CD45 (not depicted).

methodological limitations. No morphologic differences were observed between the C-ETACs detected in malignant and benign cases. The 3.7% detection rate of C-ETACs in asymptomatic individuals may represent a risk of malignancy that is currently without clinical or symptomatic manifestation. Individuals in both cases have been advised surveillance.

Image-guided biopsies require specialized infrastructure and highly trained staff, which are generally unavailable at primary care centers and hence necessitate the patient's visit to a secondary or tertiary care facility, which can lead to increased time to diagnosis. By comparison, the current noninvasive approach requires a simple blood draw, which can be fulfilled at any primary health care clinic or even at the patient's home.

It is acknowledged that the total number of biopsies performed every year exceeds the actual number of diagnosed cases; the additional biopsies account for negative (benign cases and false-negatives) and inconclusive findings on subsequent HPE. For example, it has been estimated that benign fibroadenomas account for the majority of all breast masses as well as biopsied lesions, thus adding up to a significantly high rate of negative findings.[36-38] Similarly, it has been estimated that the majority of all enlarged prostate cases are benign enlargements or inflammatory conditions.[39] In the ProState study, we evaluated 140 known cases of prostate cancer, 71 known cases of benign prostate hyperplasia/prostatitis, and 347 symptomatic cases with enlarged prostate suspicious for prostate cancer; of the latter, 111 were eventually diagnosed with prostate cancer, and 236 were diagnosed with benign conditions based on HPE of biopsied tissue. In our analysis of the 347 samples, in which the operator was initially blinded to the findings of HPE, we observed 98.9% overall accuracy for detection of prostate cancer in samples that were positive for at least 1 marker (α-methylacyl coenzyme A racemase/prostate-specific membrane antigen) and 93.1% overall accuracy in discerning prostate cancer from benign conditions. Currently, the only limitation of this approach for prostate cancers is that it has not been validated for concordance with the Gleason score. Among the 236 benign cases, 228 (96.6%) were accurately identified based on the absence of C-ETACs. In a real-world scenario, this represents the number of individuals with benign conditions for whom an unnecessary biopsy can be avoided. We foresee this analysis being used in conjunction with multiparametric magnetic resonance imaging for assessment, to identify patients who have a high probability of cancer and to achieve diagnostic triaging for individuals in whom the diagnosis still needs confirmation by tissue biopsy.

Malignancies of the CNS (brain tumors) are especially challenging because these are associated with significant limitations to biopsy and postbiopsy complications. Brain biopsies are considered especially challenging compared with biopsies of other organs because of the greater risks of morbidity and mortality associated with procedural complications, such as intracranial hemorrhage.[29] Unarguably, a noninvasive diagnostic approach would be most appreciated for CNS malignancies. In the subset of samples from the GlioLENS study, ICC profiling of C-ETACs (with GFAP, S100, Nestin, PanCK, epithelial membrane antigen, and oligodendrocyte transcription factor) helped differentiate CNS malignancies from benign conditions and metastasis from primary carcinoma with 90% and 100% specificity, respectively, and it also ascertained the glial lineage with 90% accuracy.

Often a repeated biopsy may be desirable after suspected false-negative or inconclusive findings on HPE or when progression of disease is suspected in the case of CNS malignancies. However, this may not be advisable or immediately viable because of health risks, expenses, logistical considerations, and delayed diagnosis and treatment. Conversely, inconclusive findings in C-ETAC–based diagnosis merely necessitate another blood draw. Noninvasive approaches that can reduce dependence on invasive biopsies or defer the immediate need for a biopsy could alleviate infrastructural burdens on the health care system.

The objective of the current study was to raise and answer 3 analytical questions with regard to the clinical utility of a C-ETAC–based diagnostic approach for symptomatic individuals presenting at a tertiary cancer care center and who have been advised to undergo an invasive biopsy: 1) whether it is possible to provide a noninvasive diagnosis of cancer with accuracy that is not inferior to that of conventional tissue-based procedures, 2) whether C-ETACs can be used for immunopathologic characterization of the tumor according to the tissue of origin, and 3) whether this approach is suitable and robust for the real-time assessment of tumor dynamics in patients with pretreated cancer. All of these questions are answered affirmatively in light of the study findings. We demonstrate that viable C-ETACs can be obtained in most patients

with cancer and that ICC profiling of these C-ETACs can provide diagnostically relevant information. The strength of the study lies in demonstrating: 1) the ability to detect and harvest C-ETACs in a significant proportion of a large cohort of patients, 2) the ability to detect OSS markers in a majority of samples covering diverse cancer types, and 3) that the approach is feasible in all patients irrespective of extent of disease (metastatic status) and therapeutic status.

Furthermore, and because C-ETACs are probably derived from the leading edge or tumor-budding elements of a growing cancer and have their own evolving transcriptome, future research on molecular profiling of C-ETACs may help unravel the metastatic potential and inherent aggressive nature of the evolving cancer and would be an intuitive addition to existing approaches for the molecular profiling of circulating tumor nucleic acids for diagnostic and treatment purposes.

The current study is based on existing antigen markers that are approved for use in the diagnosis of various solid organ cancers by HPE. Like HPE, the success of C-ETAC–based diagnostic approaches are affected by the inherent limitations of these markers, including detection rates and cross-reactivity. We have not evaluated the interference of ongoing chemotherapy on C-ETAC yields or ICC; a gap of 21 days was ensured as a washout period for patients on systemic therapy before blood collection. Currently, this study is unable to report on melanoma because of lower prevalence rates and an insufficient sample size. Further evaluation of C-ETACs for determining additional parameters, such as Ki-67, grade, and status of therapeutically relevant markers (eg, estrogen receptor, human epidermal growth factor receptor , androgen receptor, programmed death-ligand 1, and neurotrophic tyrosine receptor kinase), is expected to add to the value of this noninvasive approach.

In conclusion, in a large cohort study, we demonstrate for the first time the clinical potential of using C-ETACs for noninvasive diagnostic triaging of suspected cancer cases, particularly in cases unfit for biopsy or in which biopsy is difficult for any reason, and for clinical decision making. The current study goes some way toward that Holy Grail of a simple blood test to detect cancer.

FUNDING SUPPORT

This entire study was self-funded by Datar Cancer Genetics, and no external funding was received for the study.

CONFLICT OF INTEREST DISCLOSURES

Revati Patil, Pradip Fulmali, Vineet Datta, Prashant Kumar, Darshana Patil, and Dadasaheb Akolkar are full-time employees of the study sponsor (Datar Cancer Genetics) and report personal fees from the company during the conduct of the study. The remaining authors made no disclosures.

AUTHOR CONTRIBUTIONS

Darshana Patil, **Prashant Kumar**, and **Dadasaheb Akolkar:** Study design, supervision, data review, advised on results interpretation, and wrote the article. **Revati Patil:** Sample analysis, data review, and advised on results interpretation. **Pradip Fulmali:** Sample analysis and data review. **Tim Crook:** Study design and reviewed the article. **Andrew Gaya:** Study design and reviewed the article. **Nicholas Plowman:** Study design and reviewed the article. **Raymond Page:** Study design and reviewed the article. **Vineet Datta:** Study design and reviewed the article. **Sewanti Limaye:** Study design and reviewed the article. **Anantabhushan Ranade:** Study design and reviewed the article. **Amit Bhatt:** Study design and reviewed the article. All authors read and approved the final version.

REFERENCES

1. Mills SE. Histology for Pathologists. 4th ed. Lippincott Williams & Wilkins; 2012.
2. Hiley CT, Le Quesne J, Santis G, et al. Challenges in molecular testing in non-small-cell lung cancer patients with advanced disease. *Lancet*. 2016;388:1002-1011.
3. Abraham NE, Mendhiratta N, Taneja SS. Patterns of repeat prostate biopsy in contemporary clinical practice. *J Urol*. 2015;193:1178-1184.
4. Walk EE, Yohe SL, Beckman A, et al; College of American Pathologists Personalized Health Care Committee. The cancer immunotherapy biomarker testing landscape. *Arch Pathol Lab Med*. 2020;144:706-724.
5. Shachar SS, Fried G, Drumea K, Shafran N, Bar-Sela G. Physicians' considerations for repeat biopsy in patients with recurrent metastatic breast cancer. *Clin Breast Cancer*. 2016;16:e43-e48.
6. De Rubis G, Rajeev Krishnan S, Bebawy M. Liquid biopsies in cancer diagnosis, monitoring, and prognosis. *Trends Pharmacol Sci*. 2019;40:172-186.
7. Krebs MG, Hou JM, Ward TH, Blackhall FH, Dive C. Circulating tumour cells: their utility in cancer management and predicting outcomes. *Ther Adv Med Oncol*. 2010;2:351-365.
8. Cummings J, Sloane R, Morris K, et al. Optimisation of an immunohistochemistry method for the determination of androgen receptor expression levels in circulating tumour cells. *BMC Cancer*. 2014;14:226.
9. Cristofanilli M, Hayes DF, Budd GT, et al. Circulating tumor cells: a novel prognostic factor for newly diagnosed metastatic breast cancer. *J Clin Oncol*. 2005;23:1420-1430.
10. Danila DC, Heller G, Gignac GA, et al. Circulating tumor cell number and prognosis in progressive castration-resistant prostate cancer. *Clin Cancer Res*. 2007;13:7053-7058.
11. Cohen SJ, Punt CJA, Iannotti N, et al. Relationship of circulating tumor cells to tumor response, progression-free survival, and overall survival in patients with metastatic colorectal cancer. *J Clin Oncol*. 2008;26:3213-3221.
12. Banko P, Lee SY, Nagygyorgy V, et al. Technologies for circulating tumor cell separation from whole blood. *J Hematol Oncol*. 2019;12:48.
13. Gwak H, Kim J, Kashefi-Kheyrabadi L, Kwak B, Hyun KA, Jung HI. Progress in circulating tumor cell research using microfluidic devices. *Micromachines*. 2018;9:353.

14. Hong B, Zu Y. Detecting circulating tumor cells: current challenges and new trends. *Theranostics*. 2013;3:377-394.
15. Akolkar D, Patil D, Crook T, et al. Circulating ensembles of tumor-associated cells: a redoubtable new systemic hallmark of cancer. *Int J Cancer*. 2020;146:3485-3494.
16. Wu CC, Maher MM, Shepard JA. Complications of CT-guided percutaneous needle biopsy of the chest: prevention and management. *Am J Roentgenol*. 2011;196:W678-W682.
17. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2018;68:394-424.
18. Sennerstam RB, Franzen BSH, Wiksell HOT, Auer GU. Core-needle biopsy of breast cancer is associated with a higher rate of distant metastases 5 to 15 years after diagnosis than FNA biopsy. *Cancer Cytopathol*. 2017;125:748-756.
19. Robertson EG, Baxter G. Tumour seeding following percutaneous needle biopsy: the real story! *Clin Radiol*. 2011;66:1007-1014.
20. Khan MF, Straub R, Moghaddam SR, et al. Variables affecting the risk of pneumothorax and intrapulmonal hemorrhage in CT-guided transthoracic biopsy. *Eur Radiol*. 2008;18:1356-1363.
21. Freund MC, Petersen J, Goder KC, Bunse T, Wiedermann F, Glodny B. Systemic air embolism during percutaneous core needle biopsy of the lung: frequency and risk factors. *BMC Pulm Med*. 2012;12:2.
22. Lang D, Reinelt V, Horner A, et al. Complications of CT-guided transthoracic lung biopsy: a short report on current literature and a case of systemic air embolism. *Wien Klin Wochenschr*. 2018;130(7-8):288-292.
23. Trajceska L, Severova-Andreevska G, Dzekova-Vidimliski P, et al. Complications and risks of percutaneous renal biopsy. *Open Access Maced J Med Sci*. 2019;7:992-995.
24. Lubomirova M, Krasteva R, Bogov B, Paskalev E. Incidence of A-V fistulas after renal biopsy of native and transplanted kidney—two centers experience. *Open Access Maced J Med Sci*. 2015;3:241-244.
25. Machado NO. Complications of liver biopsy—risk factors, management and recommendations. In: Takahashi H, ed. Liver Biopsy. IntechOpen; 2011. Accessed August 29, 2020. https://www.intechopen.com/books/liver-biopsy/complications-of-liver-biopsy-risk-factors-management-and-recommendations
26. Atwell TD, Gorman B, Larson TS, Charboneau JW, Ingalls Hanson BM, Stegall MD. Pancreas transplants: experience with 232 percutaneous US-guided biopsy procedures in 88 patients. *Radiology*. 2004;231:845-849.
27. Klassen DK, Weir MR, Cangro CB, Bartlett ST, Papadimitriou JC, Drachenberg CB. Pancreas allograft biopsy: safety of percutaneous biopsy-results of a large experience. *Transplantation*. 2002;73:553-555.
28. Efesoy O, Bozlu M, Çayan S, Akbay E. Complications of transrectal ultrasound-guided 12-core prostate biopsy: a single center experience with 2049 patients. *Turk J Urol*. 2013;39:6-11.
29. Malone H, Yang J, Hershman DL, Wright JD, Bruce JN, Neugut AI. Complications following stereotactic needle biopsy of intracranial tumors. *World Neurosurg*. 2015;84:1084-1089.
30. Amir E, Miller N, Geddie W, et al. Prospective study evaluating the impact of tissue confirmation of metastatic disease in patients with breast cancer. *J Clin Oncol*. 2012;30:587-592.
31. Germano G, Mauri G, Siravegna G, et al. Parallel evaluation of circulating tumor DNA and circulating tumor cells in metastatic colorectal cancer. *Clin Colorectal Cancer*. 2018;17:80-83.
32. Batth IS, Mitra A, Manier S, et al. Circulating tumor markers: harmonizing the yin and yang of CTCs and ctDNA for precision medicine [published correction appears in *Ann Oncol*. 2019;30:1845]. *Ann Oncol*. 2017;28:468-477.
33. Paoletti C, Muniz MC, Thomas DG, et al. Development of circulating tumor cell-endocrine therapy index in patients with hormone receptor-positive breast cancer. *Clin Cancer Res*. 2015;21:2487-2498.
34. Chou J, Provot S, Werb Z. GATA3 in development and cancer differentiation: cells GATA have it! *J Cell Physiol*. 2010;222:42-49.
35. Qi LN, Xiang BD, Wu FX, et al. Circulating tumor cells undergoing EMT provide a metric for diagnosis and prognosis of patients with hepatocellular carcinoma. *Cancer Res*. 2018;78:4731-4744.
36. Cerrato F, Labow BI. Diagnosis and management of fibroadenomas in the adolescent breast. *Semin Plast Surg*. 2013;27:23-25.
37. Chang DS, McGrath MH. Management of benign tumors of the adolescent breast. *Plast Reconstr Surg*. 2007;120:13e-19e.
38. Lee M, Soltanian HT. Breast fibroadenomas in adolescents: current perspectives. *Adolesc Health Med Ther*. 2015;6:159-163.
39. Miah S, Catto J. BPH and prostate cancer risk. *Indian J Urol*. 2014;30:214-218.

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Article

Accurate Screening for Early-Stage Breast Cancer by Detection and Profiling of Circulating Tumor Cells

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Simple Summary: Detection of breast cancer in the early stages is associated with higher cure rates and better survival, and also requires fewer intensive treatments. Current breast cancer screening via mammography is unsuitable for use among (younger) women with more dense breasts, and also has limitations in its ability to detect aggressive breast cancers. In this research article, we describe a breast cancer detection test that is based on the detection of 'circulating tumor cells' in blood samples. This test can detect breast cancer CTCs with high accuracy across all age groups, hormone receptor subtypes, histological subtypes, and disease grade. In our study, this test detected breast cancer cases and differentiated them from healthy (cancer-free) females as well as those with non-cancerous conditions with high accuracy. This test has negligible risk of false positive findings, as well as high detection rate for early-stage (localized) breast cancer. Clinical adoption of this test can be beneficial in cancer screening as well as in detection of breast cancers in suspected cases.

Abstract: Background: The early detection of breast cancer (BrC) is associated with improved survival. We describe a blood-based breast cancer detection test based on functional enrichment of breast-adenocarcinoma-associated circulating tumor cells (BrAD-CTCs) and their identification via multiplexed fluorescence immunocytochemistry (ICC) profiling for GCDFP15, GATA3, EpCAM, PanCK, and CD45 status. Methods: The ability of the test to differentiate BrC cases (N = 548) from healthy women (N = 9632) was evaluated in a case–control clinical study. The ability of the test to differentiate BrC cases from those with benign breast conditions was evaluated in a prospective clinical study of women (N = 141) suspected of BrC. Results: The test accurately detects BrAD-CTCs in breast cancers, irrespective of age, ethnicity, disease stage, grade, or hormone receptor status.

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Citation: Crook, T.; Leonard, R.; Mokbel, K.; Thompson, A.; Michell, M.; Page, R.; Vaid, A.; Mehrotra, R.; Ranade, A.; Limaye, S.; et al. Accurate Screening for Early-Stage Breast Cancer by Detection and Profiling of Circulating Tumor Cells. *Cancers* **2022**, *14*, 3341. https://doi.org/10.3390/cancers14143341

Academic Editor: David Wong

Received: 24 May 2022
Accepted: 7 July 2022
Published: 9 July 2022

Analytical validation established the high accuracy and reliability of the test under intended use conditions. The test detects and differentiates BrC cases from healthy women with 100% specificity and 92.07% overall sensitivity in a case–control study. In a prospective clinical study, the test shows 93.1% specificity and 94.64% overall sensitivity in differentiating breast cancer cases (N = 112) from benign breast conditions (N = 29). Conclusion: The findings reported in this manuscript support the clinical potential of this test for blood-based BrC detection.

Keywords: breast cancer; screening; circulating tumor cells; immunocytochemistry

1. Background

Breast cancer (BrC) is the most common malignancy, and a leading cause of cancer-related mortality among women globally [1]. Although mammography is the standard of BrC screening in asymptomatic females, there is a need for improved BrC detection which addresses the risks and limitations of mammography, such as radiation exposure, lower specificity in differentiating benign conditions from malignancy, and lower sensitivity for invasive carcinomas, as well as incompatibility with dense breast tissue. Circulating tumor analytes in peripheral blood were evaluated for potential application in more accurate, non-radiological, and non-/minimally invasive screening for breast cancer. Circulating tumor cells (CTCs) are an ideal analyte for detection of cancers, since they are intact malignant cells that harbor the imprint of the parent tumor. CTCs have distinct advantages over nucleic acid fragments or serum antigens, since the latter may also be released by non-malignant cells, and are associated with lower sensitivity and specificity, respectively. There is evidence of sufficient viable CTCs being released into blood even during the early stages of carcinogenesis. In breast cancer, it is reported that angiogenesis commences at the DCIS stage itself, which can facilitate the dissemination of tumor cells [2–4]. Such disseminated tumor cells (DTCs) are reported in bone marrow of 20% to >50% of patients with DCIS or DCIS with microinvasion, respectively [5–7]. Prior studies also indicate high detection rates of CTCs in blood samples of patients with early-stage breast cancers. Using nanostructured coated slides, Krol et al. [8] report 62.5% CTC detection rate for stage I and II BrC. Using filtration-based devices, Reduzzi et al. [9] show a 76% CTC detection rate in early-stage breast cancer. Similarly, Jin et al. [10] use the CytoSorter® CTC capture system, and show 50% and >80% sensitivity in DCIS and stage I/II BrC. Fina et al. report >78% CTC detection rates in early-stage breast cancers using an antigen-independent method [11], and 65% CTC detection rate using antigen-dependent (EpCAM, ERBB2, and EGFR expression) capture, followed by quantitative polymerase chain reaction (qPCR) profiling of targeted gene panel [12]. These studies support the biological plausibility of CTC-based cancer screening approaches. Although CTCs were evaluated for cancer detection, the inability of prior technologies to effectively enrich and harvest sufficient CTCs hindered meaningful downstream applications. Most prior attempts at evaluating CTCs for cancer screening were based on epitope capture using the CellSearch platform, which, while not approved for CTC detection, is frequently used in research. Several prior studies highlight the lower performance of epitope capture, arising due to its inability to efficiently harvest or detect CTCs with lower expression of EpCAM and PanCK, which are the most routinely employed target markers [13–19], with some improvements in sensitivity when epitope capture is used in combination with gene expression profiling [12]. We previously described a novel functional enrichment method with high CTC detection sensitivity, which yields sufficient CTCs for downstream applications, such as immunocytochemistry (ICC) profiling [20,21]. In this manuscript, we describe the validation of this technology for use as a BrC detection test. Findings from our case–control and prospective clinical studies show that the test vastly improves CTC detection sensitivity, even in stage 0 BrC (DCIS), and addresses several limitations of prior CTC-based cancer detection efforts.

2. Methods

2.1. Study Participants and Samples

Samples for method development and validation were obtained from participants in two ongoing observational studies of the sponsor, TRUEBLOOD (http://ctri.nic.in/Clinicaltrials/pmaindet2.php?trialid=31879, accessed on 7 July 2022), and RESOLUTE (http://ctri.nic.in/Clinicaltrials/pmaindet2.php?trialid=30733, accessed on 7 July 2022), the design of which were intended to support the identification and characterization of blood-based malignant-tumor-derived analytes for non-/minimally invasive cancer detection. The TRUEBLOOD study (March 2019—ongoing) enrolls known cases of cancers, as well as individuals with clinical or radiological findings suspected of cancers. The RESOLUTE study (January 2019—ongoing) enrolls asymptomatic adults with no prior diagnosis of cancer, no current symptoms, or findings suspected of cancer and only age associated risk of cancer. Both studies were approved by Datar Cancer Genetics Limited Institutional Ethics Committee (code/registration number—ECR/231/Indt/MH/2015/RR-20), as well as the participating institutes, and were performed in accordance with the Declaration of Helsinki. Fifteen milliliters of peripheral blood were collected from all enrolled study participants in EDTA vacutainers, after obtaining written informed consent. Where possible, tissue samples were also obtained from TRUEBLOOD study participants posted for a biopsy, as per standard of care (SoC) procedures (tissue samples were used for method development). In addition, leftover blood samples from suspected or known (recently diagnosed or pre-treated) cancer patients who availed of the study sponsor's commercial services for cancer management, as well as healthy (asymptomatic) volunteers at the study sponsor's organization, were also obtained after due consent. Blood samples (15 mL) from suspected cases of cancers were collected prior to the patients undergoing an invasive biopsy. All biological samples were assigned alphanumeric barcodes, and stored at 2 °C–8 °C during transport to reach the clinical laboratory within 46 h. Sample blinding avoided systematic differences between groups due to (un)known baseline variables that could affect the test findings, and also eliminated potential biases that could have otherwise arisen due to operator's knowledge of the sample. From the originally collected 15 mL blood samples, a 5 mL aliquot was set aside for processing (CTC enrichment and ICC profiling) as part of clinical studies. The remaining blood samples were used for various method development studies. All samples were processed at the CAP and CLIA-accredited facilities of the study sponsor Datar Cancer Genetics, which also adhere to quality standards ISO 9001:2015, ISO 27001:2013, and ISO 15189:2012. The reporting of observational studies in this manuscript is compliant with STROBE guidelines [22].

2.2. Enrichment of Circulating Tumor Cells from Peripheral Blood

Aliquoted blood samples (5 mL) were processed for the enrichment of CTCs from peripheral blood mononuclear cells (PBMC), as described previously [20,21,23]. Comprehensive details are provided in Supplementary Materials File S1, Figures S1–S9, Tables S1–S15.

2.3. Immunocytochemistry Profiling of CTCs

The process of ICC profiling of CTCs was as described previously [21]. Comprehensive details are provided in Supplementary Materials. Figure 1 is a schema of the test showing the various steps in CTC enrichment, and identification by ICC profiling for various markers. The decision matrix for assigning samples as positive, equivocal, or negative, based on the findings of ICC profiling, is provided in Figure 2. Numerical thresholds for assigning samples as positive or negative were based on the limit of quantitation (LoQ) studies, as described under analytical validation. A 20% margin was defined to include those samples (assigned as equivocal) where the CTC counts may be lower than this threshold, due to ~20% losses observed during storage and transport (as explained in the section on analyte stability under analytical validation in the Supplementary Materials).

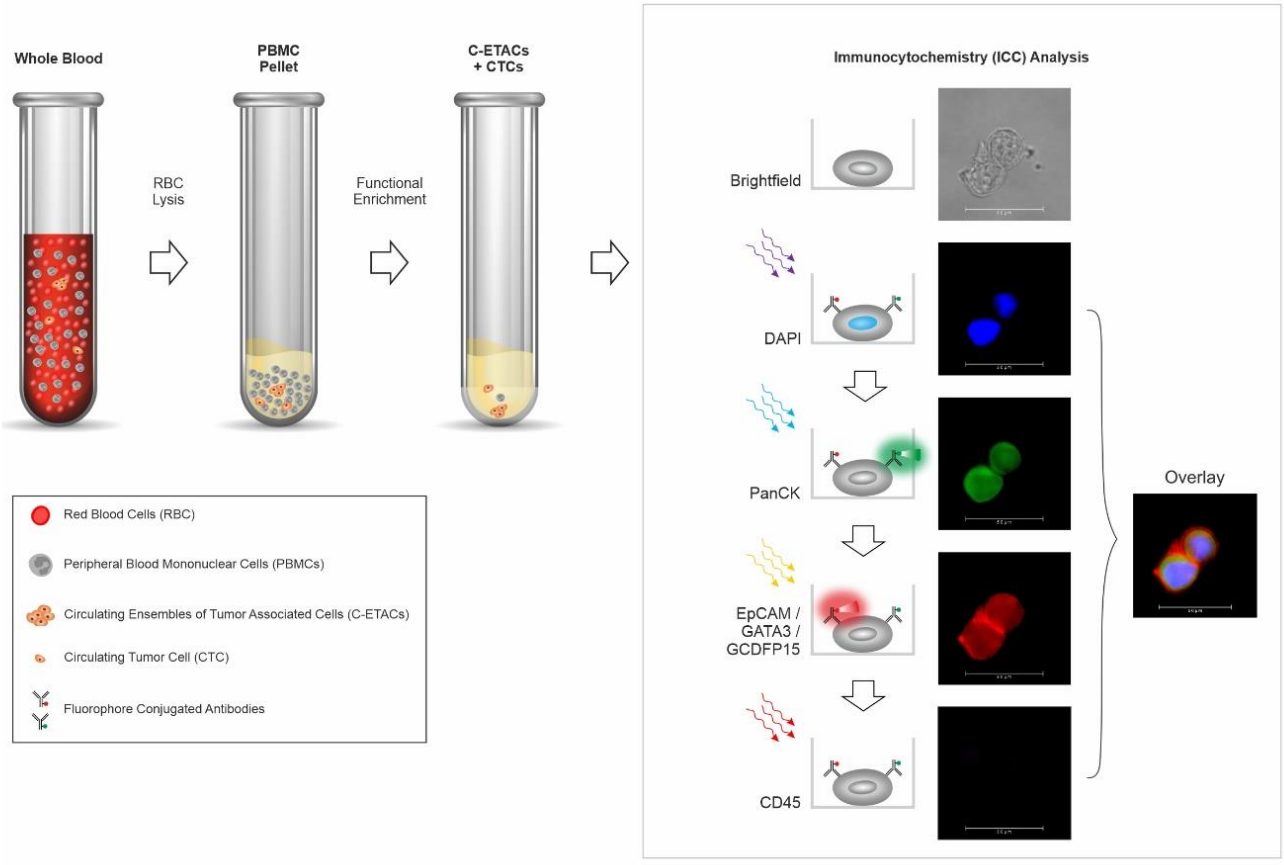

Figure 1. Schema of test. Functional enrichment of circulating tumor cells (CTCs) is achieved using a cell culture medium that is cytotoxic towards all non-malignant cells, and permits survival of tumor-derived malignant cells. Peripheral blood mononuclear cells (PBMC) isolated from whole blood are treated with the medium for 120 h, after which the surviving cells and cell clusters are harvested and evaluated by multiplexed immunocytochemistry (ICC) profiling, to determine presence of breast-adenocarcinoma-associated CTCs (BrAD-CTCs), which are identified as CD45-negative cells that express GATA3, GCDFP15, and EpCAM in combination with PanCK.

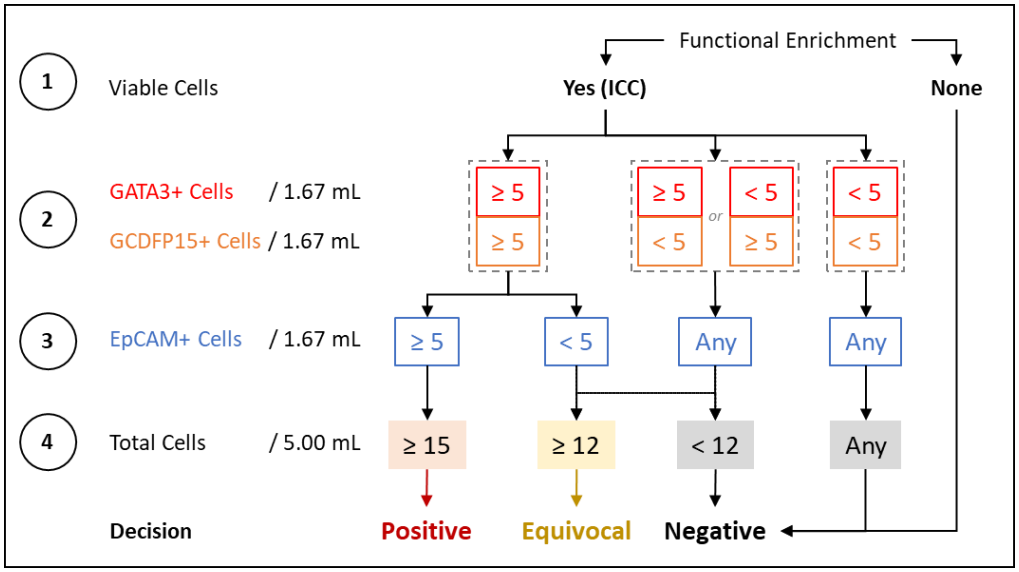

Figure 2. Decision matrix for classifying samples. The detection threshold for breast-adenocarcinoma-associated CTCs (BrAD-CTCs) is ≥ 15 PanCK cells/5 mL, which is constituted by the detection of ≥ 5 GATA3+, PanCK+, and CD45-cells, along with ≥ 5 GCDFP15+, PanCK+, and CD45- cells, as well as ≥ 5 EpCAM+, PanCK+, and CD45-cells in the respective aliquots. Depending on the number of each type of marker positive cells, samples are marked as positive, equivocal or negative. The decision matrix bestows priority to GATA3 and GCDFP15 over EpCAM while classifying samples to ensure specificity for BrAD over other epithelial malignancies where EpCAM+ cells may be detected but breast-specific markers would be absent. Thus, while the test can detect EpCAM+, PanCK+, and CD45-cells, which may be present in various epithelial malignancies, it specifically reports only BrAD-CTCs.

2.4. Method Development and Optimization

Comprehensive details of method development and optimization studies are provided in the Supplementary Materials.

2.5. Analytical Validation

Analytical validation established the performance characteristics of the test with standard analyte (SKBR3 cells), spiked into healthy donor blood to generate various dilutions (cell densities). These dilutions were processed as per the described procedures (proprietary differentially cytotoxic medium treatment and ICC profiling) to determine the yield of spiked cells. Comprehensive details of analytical validation studies are provided in the Supplementary Materials.

2.6. Case–Control Clinical Study

The ability of the test to discern/identify BrC from asymptomatic individuals was initially ascertained and established in a case–control study with 548 females who were recently diagnosed, therapy naïve cases of BrC, and 9632 healthy females with no prior diagnosis of any cancer, no current suspicion of any cancer, and with BIRADS-I on a mammogram, i.e., no evidence of breast cancer (Supplementary Table S1). The detailed inclusion and exclusion criteria are provided in Supplementary Table S2. Supplementary Figure S1 is a schema of the overall design of clinical studies. Initially, samples in the asymptomatic cohort were randomized into training and test sets in a 70%:30% ratio. The BrC cases were first segregated by stage (0–IV), and the samples per stage were then assigned to training and test sets in a 70%:30% ratio. The training set samples (384 BrC and 6742 cancer-free females) was initially evaluated, with the analysts unblinded to the status of the samples, to determine the concordance between the clinical status and the interpretation of the marker status based on the decision matrix. Then the blinded test set,

comprising of 164 BrC and 2890 cancer-free females' samples, was evaluated to determine the performance characteristics. Subsequently, all training and test samples (BrC and healthy) were shuffled, and a random 30% of samples (with stage-wise for cancer) were selected for analysis as test set iteration 2. This shuffling step was repeated to obtain 20 iterations of the test set. From these iterative 20 sets, median and range of sensitivity, specificity, and accuracy were determined. With about 160 cancer samples (cases) in the test set, and 92% expected sensitivity (better than 85%), the power of the study for determination of sensitivity is expected to be about 0.84. Similarly, with about 2792 asymptomatic samples (controls) in the test set, and an expected specificity of 99.99% (better than 99.8%), the power of the study for determination of specificity is expected to be about 0.90.

2.7. Prospective Clinical Study

The performance characteristics of the test were next ascertained and established in a prospective blinded study of 141 individuals with clinical symptoms or radiological findings, who were referred for a biopsy due to suspicion of breast cancer (Supplementary Table S3). The detailed inclusion and exclusion criteria are provided in Supplementary Table S4. Supplementary Figure S1 is a schema of the overall design of clinical studies. All participants provided blood sample prior to the biopsy. The sponsor was blinded to the diagnosis, i.e., the findings of the histopathological examination (HPE). Samples were prospectively accrued in this study until 24 samples were each obtained for stage 0, I, and II, 20 samples were each accrued for stage III and IV, and 30 samples were accrued for individuals with benign findings. With about 110 cancer cases (across all stages), and an expected sensitivity of 93% (better than 85%), this study design has a power of 0.83. Clinical status of samples (cancer/benign) was revealed to sponsors only after sample analysis was complete and test findings shared with the clinical study investigator. From these samples, performance characteristics, including sensitivity, specificity, and accuracy, were determined, with equivocal findings considered as positive and as negative, respectively.

2.8. Molecular Concordance Study

In a combined subset of 61 samples from the case–control and prospective cohorts, where matched tumor tissue and blood samples were available, a molecular concordance study was performed. Tumor tissue DNA (ttDNA) was isolated, and profiled by next-generation sequencing (NGS) using the Ion Proton platform and the Comprehensive Ampliseq Multi (409)-gene Cancer Panel. Simultaneously, PBMCs were isolated from the matched blood samples, and used for CTC enrichment. On the 5th day, genomic DNA (gDNA) isolated from all surviving cells was evaluated by a ddPCR assay specific to the driver mutation on a BioRad QX200 platform. Concordance between tumor tissue and CTCs was determined as the proportion of the latter where the corresponding gene variant was detected by ddPCR.

3. Results

3.1. Method Development and Optimization

The method development and optimization studies show the viability of multiplexed fluorescence analysis of markers with minimal or no cross-interference of markers, as well as the ability to detect CTCs with much lower marker expression than primary tumor cells or reference cell lines. Additionally the study also shows the capability of the test in detecting CTCs, irrespective of patient age, ethnicity, cancer stage, tumor grade, subtype, or hormone receptor status. The findings of the method development and optimization are provided in the Supplementary Materials.

3.2. Analytical Validation

The analytical validation studies establish the analyte stability, and also demonstrate the high sensitivity and specificity of the test, as well as significant linear characteristics in addition to high precision. The sensitivity of the test is not adversely affected by presence

of potentially interfering substances, or by controlled variations to operating parameters. The findings of analytical validation that establish these performance characteristics of the test are provided in the Supplementary Materials. The summary of the analytical validation studies is provided in Table 1.

Table 1. Summary of analytical validation studies. The summary of findings of the analytical validation studies indicate that the rest provides consistent, accurate, and reproducible results, with little or no interference from routine endogenous or exogenous factors when samples are obtained, stored, and processed under the recommended conditions.

	EpCAM, PanCK, CD45	GATA3, PanCK, CD45	GCDFP15, PanCK, CD45	Overall
Analyte stability	48 h			
Recovery [1]	94.6%	86.4%	88.6%	89.9%
Limit of detection	1 cell/mL			
Linear range	1–64 cells/mL			
Linearity	$R^2 \geq 0.98$	$R^2 \geq 0.98$	$R^2 \geq 0.98$	$R^2 \geq 0.98$
Sensitivity	96.0% (86.3%–99.5%)	98.0% (89.4%–99.9%)	94.0% (83.5%–98.8%)	94.0% (83.5%–98.8%)
Specificity	100.0% (88.4%–100.0%)	100.0% (88.4%–100.0%)	100.0% (88.4%–100.0%)	100.0% (88.4%–100.0%)
Accuracy	97.5% (91.3% to 99.7%)	98.8% (93.2% to 99.9%)	96.3% (89.4%–99.2%)	96.3% (89.4%–99.2%)
Precision	CV = 4.6%	CV = 3.9%	CV = 3.8%	CV = 4.1%
Robustness	CV < 5%			

[1] Above 10 cells/5 mL as determined from the linearity experiment. Values within parentheses represent 95% CI.

3.3. Case–Control Clinical Study

We evaluated the performance characteristics of the test in two clinical studies. In the case–control cross-validation study, the median stage-wise sensitivities are as follows: 70% for stage 0, 89.36% for stage I, 95.74% for stage II, 100% for stage III, 100% for stage IV, and 92.07% overall. In the absence of any positive or equivocal findings in the control (cancer-free and asymptomatic) cohort, the specificity of the test (cancer versus healthy) is 100%. Cancer samples (cases) with equivocal findings are considered as positive for determination of sensitivity and accuracy. Table 2 provides the specificity, as well as median of stage-wise and cumulative sensitivity and accuracy across the 20 iterations. Details of this iteration analysis are provided in Supplementary Table S5. Sensitivity and accuracy are also determined with samples with equivocal findings being considered as negative. These findings are presented in Supplementary Table S6, which also indicates the stage-wise and cumulative range of sensitivity and accuracy.

Thresholds for sample positivity are determined from the limit of quantitation (LoQ) in the analytical validation study (Supplementary materials). Lower thresholds are considered sub-optimal and not evaluated. Increasing the thresholds leads to a decrease in the sensitivity of the test for the detection of cancer samples, but with no gain in specificity. Since GATA3+ or GCDFP15+ cells are already undetectable in samples from asymptomatic (healthy) individuals, increasing the thresholds for these markers has no benefit to the specificity (which is already at 100%).

Table 2. Summary of clinical validation studies. The table provides the summary of both clinical validation studies. The stringent cross-validation design of the case–control (cancer versu. healthy) study yields a range of sensitivities and accuracies, the median of which are reported along with the 95% confidence interval (CI) for the median. Cancer samples (cases) with equivocal findings are considered as positive for determination of sensitivity and accuracy. The prospective clinical study evaluates the performance of the test among a cohort of symptomatic cases who were eventually diagnosed with breast cancer, or benign conditions of the breast. In this study, benign samples with equivocal findings are considered as false positives for determination of specificity and accuracy. Additional analyses are provided in Supplementary Tables S5–S8.

	Case–Control Study, Cancer vs. Asymptomatic Specificity: 100.00% (95% CI: 99.87%–100.00%)		**Prospective Study, Cancer vs. Benign Specificity: 93.10% (95% CI: 77.23%–99.15%)**	
	Sensitivity	**Accuracy**	**Sensitivity**	**Accuracy**
Cumulative	92.07% 95% CI: 91.12%–93.03%	99.57% 95% CI: 99.34%–99.81%	94.64% 95% CI: 88.70%–98.01%	94.33% 95% CI: 89.13%–97.52%
Stage 0	70.00% 95% CI: 34.75%–93.33%	99.90% 95% CI: 99.70%–99.98%	87.50% 95% CI: 67.64%–97.34%	90.57% 95% CI: 79.34%–96.87%
Stage I	89.36% 95% CI: 76.90%–96.45%	99.81% 95% CI: 99.60%–99.94%	95.83% 95% CI: 78.88%–99.89%	94.34% 95% CI: 84.34%–98.82%
Stage II	95.74% 95% CI: 85.46%–99.48%	99.91% 95% CI: 99.75%–99.99%	95.83% 95% CI: 78.88%–99.89%	94.34% 95% CI: 84.34%–98.82%
Stage III	100.0% 95% CI: 88.43%–100.00%	100.0% 95% CI: 99.87%–100.00%	95.00% 95% CI: 75.13%–99.87%	93.88% 95% CI: 83.13%–98.72%
Stage IV	100.0% 95% CI: 88.43%–100.00%	100.0% 95% CI: 99.87%–100.00%	100.00% 95% CI: 83.16%–100.00%	95.92% 95% CI: 86.02%–99.50%

3.4. Prospective Clinical Study

The second study was an independently conducted, blinded prospective study. Of the total 141 individuals from whom samples were collected, there are 112 breast cancer cases (stages 0–IV), and 29 cases of various benign breast conditions. There are no samples with equivocal findings in the cancer cohort, hence, the overall sensitivity is 94.6%, with stage-wise sensitivities of 87.5% for stage 0, 95.8% for stage I, 95.8% for stage II, 95.0% for stage III, and 100% for stage IV. Two samples with equivocal findings were diagnosed with benign conditions of the breast. In the absence of follow-up data indicating if these cases were indeed subsequently diagnosed with breast cancer, the samples are considered as false positives (worst-case scenario), based on the specificity of the test (cancer vs. benign), which is determined to be 93.1%. When samples with equivocal findings are considered as negative, the specificity of the test (cancer vs. benign) is 100% (best-case scenario). The sample-wise details of the prospective validation cohort findings are provided in Supplementary Table S7. The stage-wise and cumulative sensitivity and accuracy for both these scenarios are provided in Supplementary Table S8.

Thresholds for sample positivity in this study are similarly determined from the limit of quantitation (LoQ) in the analytical validation study (Supplementary materials). Lower thresholds are not evaluated. Among the 29 individuals with benign breast conditions, there are 2 cases with equivocal findings. While higher thresholds may improve the specificity in the benign cohort, they have an adverse effect on the sensitivity for the detection of cancers. In evaluating symptomatic individuals suspected of breast cancer (diagnostic triaging), sensitivity is prioritized to avoid false negatives and improve detection. Hence, greater thresholds to improve specificity (at the cost of sensitivity) are not evaluated.

3.5. Molecular Concordance Study

We identified a subset of 61 samples where driver mutations (allele frequency >0.14) are detected by NGS in tumor tissue; for variants detected in 53 samples, a specific TaqMan

ddPCR assay is available. A CTC-enriched fraction from these samples is used for gDNA isolation, which, in turn, is evaluated by a ddPCR assay specific to the driver mutation on a BioRad QX200 platform. Variants in ttDNA detected by NGS are also detected by ddPCR in 81.1% of CTCs, indicating significant concordance (Supplementary Table S9).

4. Discussion

We describe a blood test for BrC detection in asymptomatic women based on multiplexed fluorescence ICC profiling of CTCs in peripheral blood. The test can accurately determine the presence of CTCs in BrC irrespective of stage, grade, subtype, age, ethnicity, or hormone receptor status (Supplementary materials). Analytical validation establishes high sensitivity, specificity, precision, and robustness, in addition to non-interference from endogenous and exogenous factors (Supplementary materials). Two separate clinical studies establish 100% specificity (cancer vs. asymptomatic), with 92–94% overall sensitivity and 70–87% stage 0 sensitivity (Table 2). The test can differentiate samples from cancer patients and healthy individuals with high (100%) specificity, and can also identify individuals with benign conditions with ≥93% specificity. Our test has (a) high sensitivity, especially for early stages including DCIS, for more effective detection of cancers at localized stages, which are amenable to curative resection, and (b) high specificity, so that the vast majority of cancer-free individuals do not undergo additional unnecessary procedures. Our test offers compelling advantages over screening mammography and is, hence, a strong candidate for non-invasive BrC screening in asymptomatic women.

Presently, any benefits of standard mammography screening are largely in populations aged 50 years and above who have a higher age-associated cancer risk [24–30]. Standard 2D digital mammography is reported to have 73–87.3% sensitivity and 86–96% specificity [31–33]. The low accuracy of screening mammography is noted in younger women, particularly those aged below 40 years [34]. In addition, challenges associated with screening mammography are the high rates of false positives (7–12% at first mammogram [35] and 50–60% after ten yearly mammograms [36]). Besides, mammography also has a lower sensitivity for invasive cancers (76–85%) than DCIS (83.0–94.3%) [32,33]. Prior studies also suggest a modest association between radiation exposure in mammograms, and elevated risk of cancer in BRCA1 mutation carriers [37].

A longitudinal study of screening mammograms in 69,025 women reports 705 cases of screen-detected BrC (SBC) and 206 cases of interval BrC (IBC, not detected by mammography) [38]; the latter are more likely to be of high-grade, as well as have higher mortality than SBC. Niraula et al. encourage a re-evaluation of the concept of population-based screening mammography, and recommend exploring strategies beyond conventional screening mammography. In support, the 2018 data available at the US National Centre for Health Statistics, Centres for Disease Control and Prevention indicates that approximately 27.1% of women in the age group 50 to 74 years default on SoC mammography [39]. Improved BrC screening and risk-mitigation strategies are, hence, vital to improve compliance and BrC detection.

Recent efforts at developing non-invasive cancer screening technologies focus on a multi- or a pan-cancer approach. Notably, GRAIL's Galleri introduced the pan-cancer screening test based on methylation profiling in ctDNA [40]. However, the Galleri test has very low sensitivity (<10–16%) for stage I BrC [41,42], with no data on its ability to detect DCIS. Similarly, the CancerSEEK test, based on simultaneous evaluation of serum proteins and gene variants, has ~40% cumulative sensitivity for early stage and overall ~30% sensitivity for BrC [43]. Purposeful screening for early cancer detection necessitates sensitivity for early stages (0–II), which is not demonstrated by these tests. To the best of our knowledge, there are no other platforms with high sensitivity and specificity for early stages (0–II) of BrC.

The present test is based on detection of BrAD-CTCs, which are ubiquitously found in the blood of patients with an underlying breast cancer, and are undetectable in healthy individuals [20,21]. We show that functionally enriched BrAD-CTCs differentiate be-

tween breast cancer samples and samples from benign breast conditions, as well as from asymptomatic women with no underlying breast cancer, with high specificity. Obtaining numerically sufficient BrAD-CTCs is akin to a non-invasive biopsy of the breast tumor without stromal or other non-tumor content. Since the present test is based on detection of BrAD-CTCs, which represent the hematogenous phase of carcinomas associated with a higher risk of progression/metastasis, it is likely that the test has a higher sensitivity for detecting those sub-populations of DCIS, where the risk of progression is higher.

Traditionally, epitope capture with Anti-EpCAM is the preferred method for CTC enrichment. However, several studies demonstrate the poor CTC capture/detection rate of this platform [15,16,44]. It would be pertinent to mention that, although technologies like CellSearch are frequently mentioned in research pertaining to cancer detection, these are not approved for the detection of cancers based on CTCs. Hence, the limitations of these technologies for cancer detection must be critically understood and proactively addressed to improve CTC and cancer detection; our test was developed on such a working hypothesis. The label- and size-agnostic functional CTC enrichment technique in our test is immune to the limitations of epitope capture platforms and, hence, may offer a more realistic CTC detection rate. In our test, marker expression is determined by a sensitive high-content-screening (HCS) system, with standardized thresholds to minimize false negatives. The detection thresholds of the test accommodate CTCs with significantly lower marker expression (as compared to tumor cells or reference cell lines), such as those undergoing epithelial to mesenchymal transition [45,46].

The potential benefits of the test include early BrC detection, especially in asymptomatic women who decline guideline-recommended screening mammography, as well as in asymptomatic women for whom the guidelines may not recommend routine screening mammography (e.g., those below 50 years of age). The high (>90%) cumulative sensitivity at stage 0-II indicates a <10% risk of missing these localized cases where the disease has not spread to other organs, and where the 5-year survival rate is ~99%. For the <10% cases that are not detected at local stages, subsequent detection at stage III (regional spread) with >95% reported sensitivity is still associated with ~86% 5-year survival. The test also has a significantly higher sensitivity for invasive carcinomas (all stages) than has been reported for screening mammography [32,33], and can potentially mitigate risks of IBC (this is yet to be prospectively established).

The high specificity of the test translates into a negligible risk of false positives in women without breast cancer. In our case–control study, false positive findings (of BrAD-CTCs) are not observed in blood samples from asymptomatic women with no suspicious findings (BIRADS I) on mammography. The absence of false positive findings in these samples may be attributed to the stringent criteria for (a) BrAD-CTC enrichment, which is based on a hallmark characteristic of cancer, as well as for (b) BrAD-CTC detection, which is based on the positive expression of GATA3 and GCDFP15 in addition to EpCAM and PanCK. There are limited or no risks associated with use of the test, since it is non-invasive and is performed on a venous blood draw of 5 mL of peripheral blood.

The strength of our study stems from the use of an adequately powered sample size and the avoidance of overfitting, since the findings of the iterative validation study agree well with that of the training set.

The test has certain limitations in the context of a universal BrC screening. The sensitivity of the test is lowest for stage 0 disease. However, this does not present any increased risk of false negatives as compared to screening mammography. Since individuals with potentially false negative findings would not be deprived of standard mammography screening, it would not add to the pre-existing risk of the individual. While there is virtually no risk of false positives, the detection of BrAD-CTCs may be construed as false positives in individuals where the malignancy may not be immediately evident on a standard screening mammogram or in a biopsy (as observed in individuals with benign findings in the prospective validation cohort). This risk may be mitigated by use of a more diagnostically relevant imaging modality or follow-up among individuals with positive test findings.

Minor non-(Adeno) carcinoma subtypes of breast cancers are not detected by this test. The test has not been evaluated in a prospective large cohort study with the intent to test asymptomatic population. Finally, as inherent to any cancer screening test, our test could result in over-diagnosis and over-treatment.

5. Conclusions

We describe a blood-based, non-invasive test that detects breast-AD-associated CTCs with high specificity and sensitivity. The test presents a superior alternative to mammography screening of asymptomatic women for BrC detection. Approximately 38 million mammograms are performed every year in the US [47] of which ~280,000 (~0.75%) of cases are diagnosed with BrC [1,48,49]. Similarly, of the ~16 million mammograms performed annually in Europe [50], ~500,000 [1] (~3.1%) are diagnosed with BrC. Our test has the potential to minimize the need for mammography screening in individuals with positive findings who could be referred for standard assessments, including diagnostic imaging and work up leading to a final confirmed diagnosis. The test may also minimize the need for screening mammography in individuals with negative findings. The test can, thus, improve the accuracy of breast cancer detection.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cancers14143341/s1, reference [21,23,51]. File S1: Supplementary materials methods and findings. Figure S1. Design of Clinical Studies Figure S2. Detection Thresholds. Figure S3. Expression of GATA3 (A) and GCDFP15 (B) in various CTCs. Figure S4. Age-group and Marker Expression on CTCs. Figure S5. Marker Expression on CTCs from Ductal and Lobular Subtypes. Figure S6. Grade and Marker Expression on CTCs. Figure S7. Hormone Receptor Status and Marker Expression on CTCs. Figure S8. Stage and Marker Expression on CTCs. Figure S9. Ethnicity and Marker Expression on CTCs. Figure S10: Analytical Validation: Linearity. Table S1. Demographics of Case Control Validation Cohort. Table S2. Eligibility Criteria for Case Control Study. Table S3. Demographics of Prospective Validation Cohort. Table S4. Eligibility Criteria for Prospective Study. Table S5. Validation Set Analysis. Table S6. Expanded findings of the Case Control Clinical Validation Study. Table S7. Prospective Validation Cohort findings. Table S8. Expanded findings of the Prospective Clinical Validation Study. Table S9. Findings of TaqMan ddPCR Assays. Table S10. Demographics of Cohort with Benign or Inflammatory Breast Conditions. Table S11. Analytical Validation: Stability and Recovery of Spiked Cells. Table S12. Analytical Validation Stability and Recovery of CTCs in Clinical Samples. Table S13. Analytical Validation: Sensitivity, Specificity, Accuracy. Table S14. Analytical Validation: Precision. Table S15. Analytical Validation: Guard Banding Studies for Robustness. Table S16. Analytical Validation: Impact of Potentially Interfering Substances.

Author Contributions: Conceptualization, T.C., R.L., K.M., A.T., M.M., R.P., A.V., R.M., A.R., S.L., D.P., D.A., V.D., P.F., S.S., A.S. and R.D.; data curation, S.A. and A.S.; formal analysis, D.P., D.A., P.F., S.A. and A.S.; funding acquisition, R.D.; investigation, T.C., D.P., D.A., V.D. and R.D.; methodology, T.C., R.L., K.M., A.T., M.M., R.P., A.V., R.M., A.R., S.L., D.P., D.A., V.D., P.F., S.A., S.S., A.S. and R.D.; project administration, D.P., D.A., V.D. and P.F.; resources, T.C., D.P., D.A., V.D., P.F., S.A. and R.D.; software, D.P., D.A., S.A., A.S. and R.D.; supervision, T.C., D.P., D.A., V.D., P.F., A.S. and R.D.; validation, D.P., D.A., P.F., S.A., A.S. and R.D.; visualization, D.P., D.A., P.F., S.A., A.S. and R.D.; writing—original draft, A.S. and R.D.; writing—review and editing, T.C., R.L., K.M., A.T., M.M., R.P., A.V., R.M., A.R., S.L., D.P., D.A., V.D., P.F., S.A., S.S., A.S. and R.D. All authors have read and agreed to the published version of the manuscript.

Funding: No external funding was obtained for this study. The entire study was funded by the study sponsor (DCG).

Institutional Review Board Statement: Clinical studies referred to in this manuscript were approved by Datar Cancer Genetics Limited Institutional Ethics Committee (code/registration number—ECR/231/Indt/MH/2015/RR-20). Both studies were performed in accordance with the Declaration of Helsinki. The present manuscript does not contain any personal or identifiable information or data of any participant.

Informed Consent Statement: Informed consent was obtained from all study participants involved in the studies referenced in this manuscript. All study participants also consented for publication of de-identified biological data.

Data Availability Statement: All relevant data are included in the manuscript and the Supplementary Information files.

Acknowledgments: The authors are grateful to the staff of the study sponsor (DCG) for their contributions in managing various clinical, operational, and laboratory aspects of the study.

Conflicts of Interest: Timothy Crook, Robert Leonard, Alastair Thompson, Michael Michell, Ashok Vaid, Ravi Mehrotra, Anantbhushan Ranade, and Sewanti Limaye have no competing interests. Kefah Mokbel and Raymond Page are fractional stock holders of the Study Sponsor. Darshana Patil, Dadasaheb Akolkar, Vineet Datta, Pradip Fulmali, Sachin Apurwa, Stefan Schuster, and Ajay Srinivasan are employees of the Study Sponsor. Rajan Datar is the founder of the Study Sponsor.

References

1. Sung, H.; Ferlay, J.; Siegel, R.L.; Laversanne, M.; Soerjomataram, I.; Jemal, A.; Bray, F. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J. Clin.* **2021**, *71*, 209–249. [CrossRef]
2. Guidi, A.J.; Fischer, L.; Harris, J.R.; Schnitt, S.J. Microvessel density and distribution in ductal carcinoma in situ of the breast. *J. Natl. Cancer Inst.* **1994**, *86*, 614–619. [CrossRef]
3. Teo, N.B.; Shoker, B.S.; Jarvis, C.; Martin, L.; Sloane, J.P.; Holcombe, C. Vascular density and phenotype around ductal carcinoma in situ (DCIS) of the breast. *Br. J. Cancer* **2002**, *86*, 905–911. [CrossRef] [PubMed]
4. Heffelfinger, S.C.; Yassin, R.; Miller, M.A.; Lower, E. Vascularity of proliferative breast disease and carcinoma in situ correlates with histological features. *Clin. Cancer Res.* **1996**, *2*, 1873–1878.
5. Sänger, N.; Effenberger, K.E.; Riethdorf, S.; Van Haasteren, V.; Gauwerky, J.; Wiegratz, I.; Strebhardt, K.; Kaufmann, M.; Pantel, K. Disseminated tumor cells in the bone marrow of patients with ductal carcinoma in situ. *Int. J. Cancer* **2011**, *129*, 2522–2526. [CrossRef] [PubMed]
6. Gruber, I.V.; Hartkopf, A.D.; Hahn, M.; Taran, F.-A.; Staebler, A.; Wallwiener, D.; Brucker, S.Y.; Hanke, J.; Fehm, T. Relationship Between Hematogenous Tumor Cell Dissemination and Cellular Immunity in DCIS Patients. *Anticancer Res.* **2016**, *36*, 2345–2351.
7. Hosseini, H.; Obradović, M.M.S.; Hoffmann, M.; Harper, K.L.; Sosa, M.S.; Werner-Klein, M.; Nanduri, L.K.; Werno, C.; Ehrl, C.; Maneck, M.; et al. Early dissemination seeds metastasis in breast cancer. *Nature* **2016**, *540*, 552–558. [CrossRef] [PubMed]
8. Krol, I.; Schwab, F.D.; Carbone, R.; Ritter, M.; Picocci, S.; De Marni, M.L.; Stepien, G.; Franchi, G.M.; Zanardi, A.; Rissoglio, M.D.; et al. Detection of clustered circulating tumour cells in early breast cancer. *Br. J. Cancer* **2021**, *125*, 23–27. [CrossRef] [PubMed]
9. Reduzzi, C.; Di Cosimo, S.; Gerratana, L.; Motta, R.; Martinetti, A.; Vingiani, A.; D'Amico, P.; Zhang, Y.; Vismara, M.; Depretto, C.; et al. Circulating Tumor Cell Clusters Are Frequently Detected in Women with Early-Stage Breast Cancer. *Cancers* **2021**, *13*, 2356. [CrossRef]
10. Jin, L.; Zhao, W.; Zhang, J.; Chen, W.; Xie, T.; Wang, L.; Fan, W.; Xie, S.; Shen, J.; Zheng, H.; et al. Evaluation of the diagnostic value of circulating tumor cells with CytoSorter® CTC capture system in patients with breast cancer. *Cancer Med.* **2020**, *9*, 1638–1647. [CrossRef]
11. Fina, E.; Reduzzi, C.; Motta, R.; Di Cosimo, S.; Bianchi, G.; Martinetti, A.; Wechsler, J.; Cappelletti, V.; Daidone, M.G. Did circulating tumor cells tell us all they could? The missed circulating tumor cell message in breast cancer. *Int. J. Biol. Markers* **2015**, *30*, e429–e433. [CrossRef] [PubMed]
12. Fina, E.; Cleris, L.; Dugo, M.; Lecchi, M.; Ciniselli, C.M.; Lecis, D.; Bianchi, G.V.; Verderio, P.; Daidone, M.G.; Cappelletti, V. Gene signatures of circulating breast cancer cell models are a source of novel molecular determinants of metastasis and improve circulating tumor cell detection in patients. *J. Exp. Clin. Cancer Res.* **2022**, *25*, 78. [CrossRef] [PubMed]
13. Spizzo, G.; Fong, D.; Wurm, M.; Ensinger, C.; Obrist, P.; Hofer, C.; Mazzoleni, G.; Gastl, G.; Went, P. EpCAM expression in primary tumour tissues and metastases: An immunohistochemical analysis. *J. Clin. Pathol.* **2011**, *64*, 415–420. [CrossRef]
14. Soysal, S.D.; Muenst, S.; Barbie, T.; Fleming, T.; Gao, F.; Spizzo, G.; Oertli, D.; Viehl, C.T.; Obermann, E.C.; Gillanders, W.E. EpCAM expression varies significantly and is differentially associated with prognosis in the luminal B HER2^{+}, basal-like, and HER2 intrinsic subtypes of breast cancer. *Br. J. Cancer* **2013**, *108*, 1480–1487. [CrossRef] [PubMed]
15. Rao, C.G.; Chianese, D.; Doyle, G.V.; Miller, M.C.; Russell, T.; Sanders, R.A.J.; Terstappen, L.W.M.M. Expression of epithelial cell adhesion molecule in carcinoma cells present in blood and primary and metastatic tumors. *Int. J. Oncol.* **2005**, *27*, 49–57. [CrossRef] [PubMed]
16. de Wit, S.; Manicone, M.; Rossi, E.; Lampignano, R.; Yang, L.; Zill, B.; Rengel-Puertas, A.; Ouhlen, M.; Crespo, M.; Berghuis, A.M.S.; et al. EpCAM(high) and EpCAM(low) circulating tumor cells in metastatic prostate and breast cancer patients. *Oncotarget* **2018**, *9*, 35705–35716. [CrossRef] [PubMed]
17. Adams, D.L.; Stefansson, S.; Haudenschild, C.; Martin, S.S.; Charpentier, M.; Chumsri, S.; Cristofanilli, M.; Tang, C.-M.; Alpaugh, R.K. Cytometric characterization of circulating tumor cells captured by microfiltration and their correlation to the CellSearch® CTC test. *Cytometry A* **2015**, *87*, 137–144. [CrossRef] [PubMed]

18. Deng, G.; Herrler, M.; Burgess, D.; Manna, E.; Krag, D.; Burke, J.F. Enrichment with anti-cytokeratin alone or combined with anti-EpCAM antibodies significantly increases the sensitivity for circulating tumor cell detection in metastatic breast cancer patients. *Breast Cancer Res.* **2008**, *10*, R69. [CrossRef] [PubMed]
19. Sheng, Y.; Wang, T.; Li, H.; Zhang, Z.; Chen, J.; He, C.; Li, Y.; Lv, Y.; Zhang, J.; Xu, C.; et al. Comparison of analytic performances of Cellsearch and iFISH approach in detecting circulating tumor cells. *Oncotarget* **2017**, *8*, 8801–8806. [CrossRef] [PubMed]
20. Akolkar, D.; Patil, D.; Crook, T.; Limaye, S.; Page, R.; Datta, V.; Patil, R.; Sims, C.; Ranade, A.; Fulmali, P.; et al. Circulating ensembles of tumor-associated cells: A redoubtable new systemic hallmark of cancer. *Int. J. Cancer* **2020**, *146*, 3485–3494. [CrossRef] [PubMed]
21. Gaya, A.; Crook, T.; Plowman, N.; Ranade, A.; Limaye, S.; Bhatt, A.; Page, R.; Patil, R.; Fulmali, P.; Datta, V.; et al. Evaluation of circulating tumor cell clusters for pan-cancer noninvasive diagnostic triaging. *Cancer Cytopathol.* **2021**, *129*, 226–238. [CrossRef] [PubMed]
22. von Elm, E.; Altman, D.G.; Egger, M.; Pocock, S.J.; Gøtzsche, P.C.; Vandenbroucke, J.P. The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) Statement: Guidelines for reporting observational studies. *Int. J. Surg.* **2014**, *12*, 1495–1499. [CrossRef] [PubMed]
23. Crook, T.; Gaya, A.; Page, R.; Limaye, S.; Ranade, A.; Bhatt, A.; Patil, S.; Kumar, P.; Patil, D.; Akolkar, D. Clinical utility of circulating tumor-associated cells to predict and monitor chemo-response in solid tumors. *Cancer Chemother. Pharmacol.* **2021**, *87*, 197–205. [CrossRef] [PubMed]
24. World Health Organization. *WHO Position Paper on Mammography Screening*; World Health Organization: Geneva, Switzerland, 2014; ISBN 978-92-4-150793-6.
25. Siu, A.L. Screening for Breast Cancer: U.S. Preventive Services Task Force Recommendation Statement. *Ann. Intern. Med.* **2016**, *164*, 279–296. [CrossRef]
26. NCCN Guidelines: Breast Cancer Screening and Diagnosis version 1. 2021. Available online: https://www.nccn.org/professionals/physician_gls/pdf/breast-screening.pdf (accessed on 25 July 2021).
27. Cardoso, F.; Kyriakides, S.; Ohno, S.; Penault-Llorca, F.; Poortmans, P.; Rubio, I.T.; Zackrisson, S.; Senkus, E. Early breast cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann. Oncol.* **2019**, *30*, 1194–1220. [CrossRef]
28. National Guideline Alliance (UK). *Early and Locally Advanced Breast Cancer: Diagnosis and Management*; National Guideline Alliance: London, UK, 2018; ISBN 978-1-4731-3008-1.
29. Wöckel, A.; Festl, J.; Stüber, T.; Brust, K.; Stangl, S.; Heuschmann, P.U.; Albert, U.-S.; Budach, W.; Follmann, M.; Janni, W.; et al. Interdisciplinary Screening, Diagnosis, Therapy and Follow-up of Breast Cancer. Guideline of the DGGG and the DKG (S3-Level, AWMF Registry Number 032/045OL, December 2017)-Part 1 with Recommendations for the Screening, Diagnosis and Therapy of Breast C. *Geburtshilfe Frauenheilkd* **2018**, *78*, 927–948. [CrossRef]
30. Schünemann, H.J.; Lerda, D.; Quinn, C.; Follmann, M.; Alonso-Coello, P.; Rossi, P.G.; Lebeau, A.; Nyström, L.; Broeders, M.; Ioannidou-Mouzaka, L.; et al. Breast Cancer Screening and Diagnosis: A Synopsis of the European Breast Guidelines. *Ann. Intern. Med.* **2020**, *172*, 46–56. [CrossRef]
31. Abdullah, P.; Alabousi, M.; Ramadan, S.; Zawawi, I.; Zawawi, M.; Bhogadi, Y.; Freitas, V.; Patlas, M.N.; Alabousi, A. Synthetic 2D Mammography Versus Standard 2D Digital Mammography: A Diagnostic Test Accuracy Systematic Review and Meta-Analysis. *Am. J. Roentgenol.* **2021**, *217*, 314–325. [CrossRef]
32. Salim, M.; Dembrower, K.; Eklund, M.; Lindholm, P.; Strand, F. Range of Radiologist Performance in a Population-based Screening Cohort of 1 Million Digital Mammography Examinations. *Radiology* **2020**, *297*, 33–39. [CrossRef]
33. Lehman, C.D.; Wellman, R.D.; Buist, D.S.M.; Kerlikowske, K.; Tosteson, A.N.A.; Miglioretti, D.L. Diagnostic Accuracy of Digital Screening Mammography With and Without Computer-Aided Detection. *JAMA Intern. Med.* **2015**, *175*, 1828–1837. [CrossRef]
34. McNeil, C. Screening Mammograms in Younger Women Have Low Accuracy and Detect Few Cancers. *JNCI J. Natl. Cancer Inst.* **2010**, *102*, 841–842. [CrossRef]
35. Nelson, H.D.; Fu, R.; Cantor, A.; Pappas, M.; Daeges, M.; Humphrey, L. Effectiveness of Breast Cancer Screening: Systematic Review and Meta-analysis to Update the 2009 U.S. Preventive Services Task Force Recommendation. *Ann. Intern. Med.* **2016**, *164*, 244–255. [CrossRef] [PubMed]
36. Hubbard, R.A.; Kerlikowske, K.; Flowers, C.I.; Yankaskas, B.C.; Zhu, W.; Miglioretti, D.L. Cumulative probability of false-positive recall or biopsy recommendation after 10 years of screening mammography: A cohort study. *Ann. Intern. Med.* **2011**, *155*, 481–492. [CrossRef] [PubMed]
37. Goldfrank, D.; Chuai, S.; Bernstein, J.L.; Ramon, Y.; Cajal, T.; Lee, J.B.; Alonso, M.C.; Diez, O.; Baiget, M.; Kauff, N.D.; et al. Effect of mammography on breast cancer risk in women with mutations in BRCA1 or BRCA2. *Cancer Epidemiol. Biomark. Prev.* **2006**, *15*, 2311–2313. [CrossRef]
38. Niraula, S.; Biswanger, N.; Hu, P.; Lambert, P.; Decker, K. Incidence, Characteristics, and Outcomes of Interval Breast Cancers Compared With Screening-Detected Breast Cancers. *JAMA Netw. Open* **2020**, *3*, e2018179. [CrossRef]
39. Health, United States, 2019—Data Finder. Available online: https://www.cdc.gov/nchs/data/hus/2019/033-508.pdf (accessed on 26 July 2021).
40. Locke, W.J.; Guanzon, D.; Ma, C.; Liew, Y.J.; Duesing, K.R.; Fung, K.Y.C.; Ross, J.P. DNA Methylation Cancer Biomarkers: Translation to the Clinic. *Front. Genet.* **2019**, *10*, 1150. [CrossRef]

41. Liu, M.C.; Oxnard, G.R.; Klein, E.A.; Swanton, C.; Seiden, M. V Sensitive and specific multi-cancer detection and localization using methylation signatures in cell-free DNA. *Ann. Oncol.* **2020**, *31*, 745–759. [CrossRef]
42. Klein, E.A.; Richards, D.; Cohn, A.; Tummala, M.; Lapham, R.; Cosgrove, D.; Chung, G.; Clement, J.; Gao, J.; Hunkapiller, N.; et al. Clinical validation of a targeted methylation-based multi-cancer early detection test using an independent validation set. *Ann. Oncol. Off.* **2021**, *32*, 1167–1177. [CrossRef]
43. Cohen, J.D.; Li, L.; Wang, Y.; Thoburn, C.; Afsari, B.; Danilova, L.; Douville, C.; Javed, A.A.; Wong, F.; Mattox, A.; et al. Detection and localization of surgically resectable cancers with a multi-analyte blood test. *Science* **2018**, *359*, 926–930. [CrossRef]
44. Sieuwerts, A.M.; Kraan, J.; Bolt, J.; van der Spoel, P.; Elstrodt, F.; Schutte, M.; Martens, J.W.M.; Gratama, J.-W.; Sleijfer, S.; Foekens, J.A. Anti-epithelial cell adhesion molecule antibodies and the detection of circulating normal-like breast tumor cells. *J. Natl. Cancer Inst.* **2009**, *101*, 61–66. [CrossRef]
45. Aktas, B.; Tewes, M.; Fehm, T.; Hauch, S.; Kimmig, R.; Kasimir-Bauer, S. Stem cell and epithelial-mesenchymal transition markers are frequently overexpressed in circulating tumor cells of metastatic breast cancer patients. *Breast Cancer Res.* **2009**, *11*, R46. [CrossRef] [PubMed]
46. Krawczyk, N.; Meier-Stiegen, F.; Banys, M.; Neubauer, H.; Ruckhaeberle, E.; Fehm, T. Expression of stem cell and epithelial-mesenchymal transition markers in circulating tumor cells of breast cancer patients. *BioMed Res. Int.* **2014**, *2014*, 415721. [CrossRef] [PubMed]
47. MQSA National Statistics. Available online: https://www.fda.gov/radiation-emitting-products/mqsa-insights/mqsa-national-statistics (accessed on 17 September 2021).
48. John, M. Eisenberg Center for Clinical Decisions and Communications Science Core-Needle Biopsy for Breast Abnormalities. Available online: https://www.ncbi.nlm.nih.gov/books/NBK368367/ (accessed on 7 July 2022).
49. Cancer Stat Facts: Female Breast Cancer. Available online: https://seer.cancer.gov/statfacts/html/breast.html (accessed on 17 September 2021).
50. Peintinger, F. National Breast Screening Programs across Europe. *Breast Care* **2019**, *14*, 354–358. [CrossRef] [PubMed]
51. CLSI. Evaluation of Linearity of Quantitative Measurement Procedures-Table 3. Deviations from True Ratios for Different Percentages of ADL; EP06Ed2; 2020. Available online: https://clsi.org/standards/products/method-evaluation/documents/ep06/ (accessed on 24 May 2022).

Received: 9 July 2022 | Revised: 11 January 2023 | Accepted: 16 January 2023

DOI: 10.1002/cam4.5649

RESEARCH ARTICLE

Cancer Medicine Open Access WILEY

Accurate prostate cancer detection based on enrichment and characterization of prostate cancer specific circulating tumor cells

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Abstract

Background: The low specificity of serum PSA resulting in the inability to effectively differentiate prostate cancer from benign prostate conditions is a persistent clinical challenge. The low sensitivity of serum PSA results in false negatives and can miss high-grade prostate cancers. We describe a non-invasive test for detection of prostate cancer based on functional enrichment of prostate adenocarcinoma associated circulating tumor cells (PrAD-CTCs) from blood samples followed by their identification by immunostaining for pan-cytokeratins (PanCK), prostate specific membrane antigen (PSMA), alpha methyl-acyl coenzyme-A racemase (AMACR), epithelial cell adhesion molecule (EpCAM), and common leucocyte antigen (CD45).

Methods: Analytical validation studies were performed to establish the performance characteristics of the test using VCaP prostate cancer cells spiked into healthy donor blood (HDB). The clinical performance characteristics of the test were evaluated in a case–control study with 160 known prostate cancer cases and 800 healthy males, followed by a prospective clinical study of 210 suspected cases of prostate cancer.

Results: Analytical validation established analyte stability as well as acceptable performance characteristics. The test showed 100% specificity and 100% sensitivity to differentiate prostate cancer cases from healthy individuals in the case control study and 91.2% sensitivity and 100% specificity to differentiate prostate cancers from benign prostate conditions in the prospective clinical study.

Conclusions: The test accurately detects PrAD-CTCs with high sensitivity and specificity irrespective of stage, serum PSA or Gleason score, which translates into low risks of false negatives or overdiagnosis. The high accuracy of the test could offer advantages over PSA based prostate cancer detection.

KEYWORDS
circulating tumor cells, detection, diagnosis, immunocytochemistry, non-invasive, prostate cancer, screening

1 | INTRODUCTION

Prostate cancer is globally the second most common malignancy and the seventh highest cause of cancer-related mortality among men.[1] Detection of prostate cancer at advanced stages is associated with significant morbidity and mortality as well as reduced survival, while early-stage prostate cancer detection is associated with higher cure rate and improved survival (~99%, 5-year[2]). At present, evaluation of serum prostate specific antigen (PSA) is part of the standard diagnostic work-up in symptomatic cases[3] but less suitable for prostate cancer screening in asymptomatic males due to low specificity[4] and significant risk of false positivity[5] which leads to overdiagnosis and overtreatment.[6] In addition, there is a risk of false negatives, especially in advanced undifferentiated prostate cancers which may have lower PSA levels.[7] More sensitive and specific methods which can provide for more effective prostate cancer detection are required to reduce morbidity and mortality from this disease.[8]

Circulating tumor analytes in blood have received attention for non-radiological, non-invasive detection of prostate cancer.[9] Apart from serum tumor antigens, circulating tumor nucleic acids have been evaluated for prostate cancer detection but have reported limitations in sensitivity for localized prostate cancer.[10] Circulating tumor cells (CTCs) are viable tumor derived cells in circulation, the molecular and functional evaluation of which may be comparable to that of the tumor tissue from which they originate.[11] CTC evaluations are not prone to the limitations in sensitivity and specificity associated with circulating tumor nucleic acids or serum tumor antigens. Prior studies support the ubiquity of CTCs in prostate cancer, especially in early-stage (localized) disease; disseminated tumor cells (DTCs) released during early stages of prostate cancer are known to remain dormant in the bone marrow and result in metastatic recurrence.[12] In a study of bone marrow aspirates from 533 preoperative prostate cancer cases with localized disease (T2-4, N0), DTCs were detected in 380 cases (71.3%), irrespective of pathologic stage, Gleason grade, or PSA.[13] Another study reported CTCs in 19 (79%) of 24 treatment naïve localized prostate cancers.[14] A third study reported >90% sensitivity in 20 known prostate cancer cases and 92.6% specificity in 27 asymptomatic men undergoing prostate cancer screening.[15] A fourth study on pre-operative blood from 86 prostate cancer cases reported 38.4%–62.7% CTC detection rates using CellSearch, CellCollector, and EPISPOT individually, and 80.2%[16] when used together. In a fifth study, using a hybrid microfluidic-imaging along with PSA immunostaining, 38–222 CTCs were reported per mL in recently diagnosed cases of localized prostate cancer.[17] In a sixth study, using near-infrared dyes and EpCAM immunostaining, up to 439 CTCs per mL of blood (mean: 25 CTCs/mL; median: 10 CTCs/mL) were observed in a cohort of patients with localized prostate cancer.[18] The above studies provide evidence for the plausibility of CTC-based prostate cancer detection. Other studies have also shown the inability of existing technology platforms to efficiently enrich and harvest sufficient CTCs. Most prior reports on CTCs in cancer are based on epitope capture using epithelial cell adhesion molecule (EpCAM) followed by immunostaining for cytokeratins (CK). A critical limitation of this approach is its acknowledged inability to effectively enrich and detect CTCs where the expression of target biomarkers such as EpCAM and CK can be significantly lower[19–23] than tumor tissue or reference cell lines. Further, the expression of EpCAM and CK (as well as any other markers) may be even lower in CTCs undergoing epithelial to mesenchymal transition (EMT).[24]

We have previously described a novel functional CTC enrichment process which yields numerically sufficient CTCs for further applications.[25] We have also shown that CTCs thus enriched from blood of patients with prostate cancer are positive for expression of PSMA, AMACR, EpCAM, and PanCK as determined by fluorescence immunocytochemistry (ICC).[26] This multi-marker CTC profiling has high specificity for adenocarcinomas (AD) which represent the vast majority (~92%) of prostate cancers.[27] The test uses standardized fluorescence intensity (FI) thresholds for detection of marker positive cells, optimized to detect CTCs with a wide range of marker expression, especially those with significantly lower marker expression than tumor derived cells or PrC cell lines. In this manuscript, we report the method development as well as analytical and clinical validation of this test for prostate cancer detection.

2 | METHODS

2.1 | Study participants and samples

Biological samples used for method development, analytical validation, and clinical validation as described in this manuscript were obtained from participants in the following observational studies: TRUEBLOOD (http://ctri.nic.in/Clini

caltrials/pmaindet2.php?trialid=31879), ProState (http://ctri.nic.in/Clinicaltrials/pmaindet2.php?trialid=31713), and RESOLUTE (http://ctri.nic.in/Clinicaltrials/pmaindet2.php?trialid=30733). The TRUEBLOOD study (Mar 2019–ongoing) enrolls patients diagnosed with various solid organ cancers or benign (non-malignant) conditions as well as suspected cancer cases. The ProState study (Mar 2019–ongoing) enrolls patients diagnosed with prostate cancers as well as symptomatic males suspected of prostate cancer. The RESOLUTE study (Jan 2019–ongoing) enrolls adults with neither prior diagnosis nor current symptoms suspected of cancer. All studies were approved by the Ethics Committees of the participating institutes as well as the sponsor (Datar Cancer Genetics, DCG) and are performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from all study participants prior to enrolment and sample collection. Fifteen milliliters of peripheral blood were collected from all participants in EDTA vacutainers. Tumor tissue samples were obtained from TRUEBLOOD and ProState study participants who were referred for a biopsy as per Standard of Care (SoC), where such tissue sample was already available. Blood samples were also collected, after obtaining informed consent, from healthy (asymptomatic) volunteers, diagnosed cancer patients, and suspected cases who were not a part of either of the above studies but had availed of the sponsor's services. Blood samples were collected prior to the patients undergoing an invasive biopsy where the same had been advised. Blood and tissue samples were stored under refrigeration (2°C–8°C) during transport to reach the clinical laboratory within 46 h. All samples were identity masked by using blood collection vacutainers with a 10-digit alphanumeric code. All samples were processed at the CAP and CLIA accredited facilities of the Study Sponsor, which also adhere to quality standards ISO 9001:2015, ISO 27001:2013, and ISO 15189:2012. The reporting of observational studies in this manuscript is compliant with STROBE guidelines.[28]

2.2 | Isolation of primary tumor derived cells

The isolation of primary tumor derived cells (TDCs) from an excised tumor (malignant/benign) was performed as described previously[25] and is also explained in Supplementary Materials.

2.3 | Enrichment of circulating tumor cells from peripheral blood

Blood samples were processed for red blood cell (RBC) lysis and isolation of peripheral blood mononuclear cells (PBMC), following which CTCs were enriched from PBMCs as described previously.[26,29] The process is also explained in Supplementary Materials.

2.4 | Immunocytochemistry profiling of CTCs

Immunocytochemistry (ICC) profiling of CTC was performed as described previously[26] and is also provided in Supplementary Materials. A schema showing the various steps of the process including CTC detection and ICC profiling is depicted in Figure 1. The decision matrix for sample classification ("Positive," "Equivocal," or "Negative") based on abundance of each type of marker positive cells is provided in Figure 2. These cut-offs were based on the Limits of Blank, Detection and Quantitation (LoB, LoD, and LoQ) as determined in the analytical validation studies. The Equivocal classification was assigned to include those samples with up to 20% lower CTC count than the positivity threshold due to losses during storage and transport (as explained in the section on Analyte Stability under analytical validation).

Samples with Equivocal classification were considered positive for the purpose of prostate cancer detection by the test.

2.5 | Method development and optimization

Comprehensive details of method development and optimization studies as well as their findings are provided in the Supplementary Materials.

2.6 | Analytical validation

Analytical validations were performed by determining the recovery of reference human prostate cancer cell line (VCaP) spiked into healthy donor blood samples. VCaP reference cells were spiked at various densities as per the design of and requirement for each validation parameter (specified in Supplementary Materials) into healthy donor blood samples, which were processed as per the test for enrichment of CTCs (spiked cells) and immunocytochemistry. The spike-recovery study design was applicable for validation of analyte stability (and recovery), linearity, limit of detection, limit of quantitation, limit of blank, sensitivity, specificity, accuracy, precision, and interference. Comprehensive details of study design, observations, and inferences are provided in Supplementary Materials.

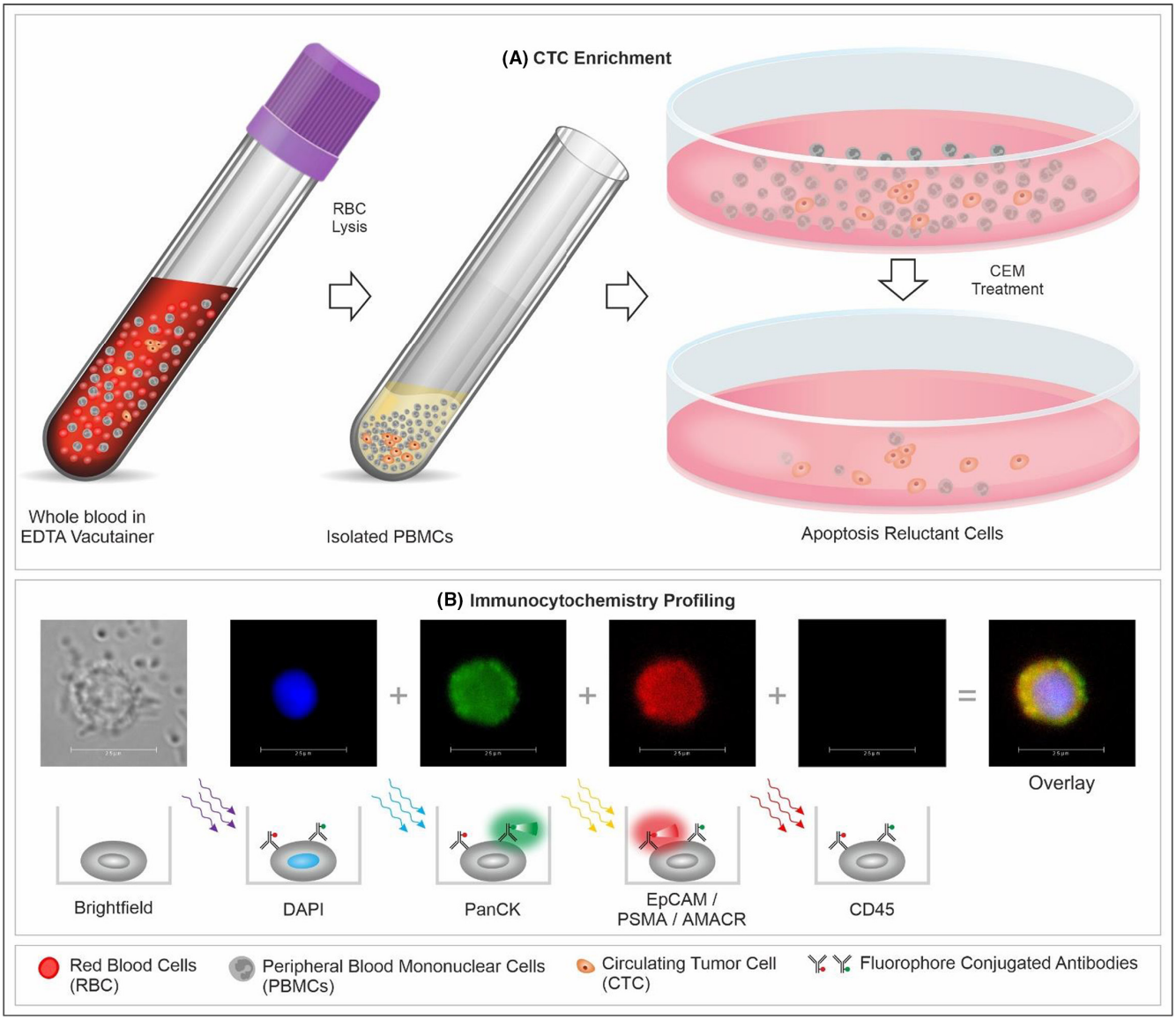

FIGURE 1 Schema of Test. Functional enrichment of CTCs is achieved using a proprietary CTC enrichment medium (CEM) that eliminates all non-malignant cells and permits tumor derived malignant cells to survive. Subsequently, the multiplexed immunocytochemistry (ICC) evaluates and identifies PrAD-CTCs based on positivity of the indicated markers.

2.7 | Case control clinical study

The ability of the Test to detect PrAD cases and differentiate PrAD cases from asymptomatic males was established in a case control study with pre-biopsy blood samples from 160 recently diagnosed, therapy naïve cases of PrAD and samples from 800 healthy ("asymptomatic") males aged 49 years and above with neither prior diagnosis nor current suspicion of cancer and with serum PSA ≤0.5 ng/mL. The inclusion and exclusion criteria for this study are provided in Table S8. The asymptomatic cohort was randomized into Training, Test, and Validation Sets in a 60%:20%:20% ratio. The PrAD cases were first segregated by extent of disease as Localized (confined to primary site), Regional (spread to regional lymph nodes), and Distant (metastasized to distal lymph nodes or other organs) for which survival is known.[2] Subsequently, the stratified cohorts were assigned to Training and Test Sets in a 60%:20%:20% ratio. The Training Set samples comprising of 96 PrAD and 480 healthy males' samples was first evaluated with the analysts unblinded to the status of the samples. Next the blinded Test Set comprising of 32 PrAD and 160 healthy males' samples was evaluated prior to blinded evaluation of the 32 PrAD and 160 healthy males' samples in the Validation Set. Subsequently all Training, Test, and Validation set samples (PrAD and healthy) were shuffled and random 20% samples (extent-wise for PrAD) were selected for analysis as Validation Set Iteration 2. This shuffling step was repeated to obtain 20 iterations

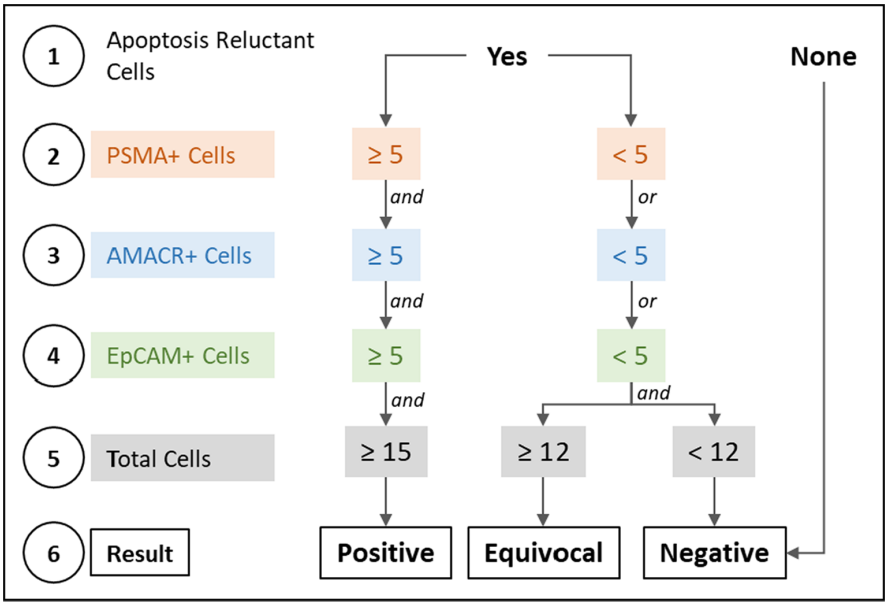

FIGURE 2 Decision matrix for classifying samples. The detection threshold for PrAD-CTCs is ≥15 PanCK cells/5 mL, which is constituted by the detection of ≥5 PSMA+ cells, ≥5 AMACR+ cells and ≥5 EpCAM+ cells in the respective aliquots. Priority is given to PSMA and AMACR over EpCAM while classifying samples as "Positive" to ensure specificity for PrAD over other epithelial malignancies where EpCAM+ cells may be detected.

of the Validation Set from which median and range of Sensitivity, Specificity, and Accuracy were reported. The iterative random sampling permitted diverse scenarios with respect to relative proportion of samples with true positive (TP), false negative (FN), true negative (TN), and false positive (FP) findings thus yielding a range of sensitivities and specificities, the median of which was reported. This design eliminates risks of overfitting due to sample enrichment in the Validation Set.

With about 160 cancer samples (cases) and a 90% expected sensitivity (better than 80%), the power of the study for determination of sensitivity is expected to be about 0.95. Similarly, with about 800 asymptomatic samples (controls) in the test set and an expected specificity of 99.9% (better than 99.0%), the power of the study for determination of specificity is expected to be about 0.97. The design of the clinical study is provided in Figure S1.

2.8 | Prospective clinical study

The performance characteristics of the test were established in a prospective clinical study of blood samples from 210 males with enlarged prostate and urological symptoms who were suspected of PrAD. Additional considerations for deciding the requirement for a prostate biopsy included suspicious findings in digital rectal examination (DRE), ultrasonography (USG), or serum PSA (≥4 ng/mL); in 78 cases, elevated serum PSA was not observed and the indication for a biopsy was based on either DRE or USG in addition to the symptoms. The inclusion and exclusion criteria for this study are provided in Table S10. All participants provided 5 mL blood sample prior to undergoing a prostate biopsy. The findings of the histopathological examination (HPE) and the final diagnosis (cancer or benign) were initially blinded to the sponsor and unmasked only after completion of sample analysis. The concordance between test findings and HPE diagnosis was used to determine Sensitivity, Specificity, and Accuracy. With about 60 cancer cases and an expected sensitivity of 90% (better than 75%), this study design has a power of 0.85. The design of the clinical study is provided in Figure S1.

2.9 | Molecular concordance studies

In a subset of 20 PrC cases a molecular concordance study was performed on matched tumor tissue and blood samples. Tumor Tissue DNA (ttDNA) was isolated and used for next-generation sequencing (NGS) profiling using the Ion Proton Platform and the Oncomine Comprehensive Assay v3 Panel to identify gene variants with loss of tumor suppression or gain of oncogenic function which have been previously reported to be significant in/associated with prostate cancer. PBMCs isolated from blood samples were treated with the CEM for CTC enrichment. Genomic DNA (gDNA) was isolated from apoptosis reluctant (surviving) cells and evaluated by a ddPCR assay specific to the detected gene variant on a BioRad QX200 platform. Concordance between tumor tissue and CTCs was determined as the proportion of the latter where the corresponding gene variant was detected by ddPCR.

Tissue samples from the same 20 patients were also evaluated by fluorescence in situ hybridization (FISH) as per manufacturer's protocol for TMPRSS2-ERG fusion. In samples where tissue was positive for this variation, enriched and harvested CTCs were also evaluated by FISH for the same biomarker.

3 | RESULTS

3.1 | Method development

The method development studies showed the viability of multiplexed fluorescence ICC for detection of PrAD-CTCs with a wide range of EpCAM, PanCK, AMACR, and PSMA expression levels (Figure S2), as well as other key aspects including specificity of marker combination to prostate cancer (Figure S3), absence of PrAD CTCs in benign prostate conditions (Table S1), and the ability of the test to detect CTCs irrespective of patient age (Figure S4), serum PSA levels (Figure S5), Gleason Score (Figure S6), or extent of disease (Figure S7). Comprehensive details are provided in Supplementary Materials.

3.2 | Analytical validation

Table 1 is a summary of all the findings of the analytical validation study. Analytical validation established analyte stability (Tables S2 and S3), demonstrated high sensitivity, and specificity of the test (Table S4), significant linear characteristics (Figure S8), high precision (Table S5), and no loss of sensitivity in presence of potentially interfering substances (Table S6). Comprehensive details are provided in Supplementary Materials.

TABLE 1 Findings of analytical validation studies

	EpCAM, PanCK, CD45	PSMA, PanCK, CD45	AMACR, PanCK, CD45	Overall
Analyte stability	48 h			
Recovery[a]	97.2%	94.4%	94.4%	91.7%
Limit of detection	<1 cell/mL			
Linear range	1–256 cells/mL			
Linearity	$R^2 \geq 0.99$	$R^2 \geq 0.99$	$R^2 \geq 0.99$	$R^2 \geq 0.99$
Sensitivity	95.0%	92.5%	92.5%	92.5%
Specificity	100.0%	100.0%	100.0%	100.0%
Accuracy	97.1%	95.7%	95.7%	95.7%
Precision	CV ≤ 9%	CV ≤ 6%	CV ≤ 6%	CV ≤ 9%
Robustness	CV < 10%			

Note: The analytical validation studies established that the Test provides consistent, accurate, and reproducible results with no interference from endogenous or exogenous factors when samples are obtained, stored, and processed under the recommended conditions.

[a]Above 10 cells/5 mL as determined from the Linearity experiment. Values within parentheses represent 95% CI.

3.3 | Clinical studies

The performance characteristics of the test were established in two clinical studies. The demographics of the study cohorts are provided in Tables S7 and S9 and the inclusion/exclusion criteria are provided in Tables S8 and S10. Both studies were conducted in a South Asian cohort with <0.005% reported prostate cancer incidence,[30] and also where the prostate cancer risk in asymptomatic males is significantly lower than the <7% reported among Caucasians with ≤0.5 ng/mL serum PSA[31,32] most of whom are also expected to be clinically insignificant prostate cancer.[31,33] Due to this low probability of an underlying prostate cancer in healthy subjects, they were a suitable "control" population. Further, the selection of such a control population is also more ethical since it would be unethical to perform a biopsy on asymptomatic individuals for the sole purpose of ruling out prostate cancer for this study. The Case Control Study had a stringent, blinded, iterative cross-validation design which minimized the risk of overfitting. In this study, the median sensitivity was 100% for local, regional and for metastatic disease as well as overall (Table 2). Figure 3 is a graphical representation of the extent-wise sensitivities in the Training and Test Sets as well as the 20 iterations of the Validation sets. The break-up of Positive, Negative, and Equivocal findings in each these sets are provided in Table S11. In absence of any positive or equivocal findings in the asymptomatic cohort, the specificity of the test (cancer v/s healthy) was 100%.

In the second (prospective) clinical study with 210 symptomatic males, 68 (32.4%) were eventually diagnosed with PrAD and 142 (67.6%) were diagnosed with benign prostate conditions. There were no positive or equivocal findings among those diagnosed with benign prostate conditions. Hence the specificity of the test (cancer v/s benign) was 100%. Among the 68 cancer cases, the Test assigned 56 samples as positive, six as equivocal and six as negative (Table S12), yielding a sensitivity of 91.2% since equivocals were considered as positive (Table 2). Equivocals were considered as positive for higher PrC detection sensitivity. In the clinical setting, considering equivocals as positive may lead to reduced specificity for PrC (as compared to other cancers where either PSMA or AMACR may be positive), however this improves the chances for detection of PrC or other cancers in such patients who undergo follow-up investigations. Further, considering equivocals as positive did not decrease the specificity of the test to differentiate PrC cases from asymptomatic individuals. In the prospective study, the sensitivity of the test was observed to correlate positively with Gleason Scores and PSA levels (where available) (Table S13). Among the 68 cancer cases in the prospective cohort were 10 cases with PSA < 10 ng/mL. Of these 10 cases, four were clinically significant with histological grade

TABLE 2 Findings of clinical studies

	Case control study: cancer v/s asymptomatic Specificity: 100.0% (95% CI: *97.7%–100.0%*)		Prospective study: cancer v/s benign Specificity: 100.0% (95% CI: *97.4%–100.0%*)	
	Sensitivity	**Accuracy**	**Sensitivity**	**Accuracy**
Cumulative	100.0% 95% CI: *89.1%–100.0%*	100.0% 95% CI: 98.*1%–100.0%*	91.2% 95% CI: *81.8%–96.7%*	97.14% 95% *CI: 93.9%–98.9%*
Local	100.0% 95% CI: *79.4%–100.0%*	100.0% 95% CI: *97.9%–100.0%*	75.0% 95% CI: 50.9%–91.3%	96.9% 95% CI: 92.9%–98.9%
Regional	100.0% 95% CI: *97.7%–100.0%*	100.0% 95% CI: *97.8%–100.0%*	85.7% 95% CI: 42.1%–99.6%	99.3% 95% CI: 96.3%–99.9%
Distal	100.0% 95% CI: *97.7%–100.0%*	100.0% 95% CI: *97.8%–100.0%*	100.0% 95% CI: 90.8%–100.0%	100.0% 95% CI: 97.9%–100.0%

Note: The Stage-wise and overall performance characteristics of the Test were determined from 20 iterations of the Validation Set in the Case Control Study as well as from the Prospective Study.

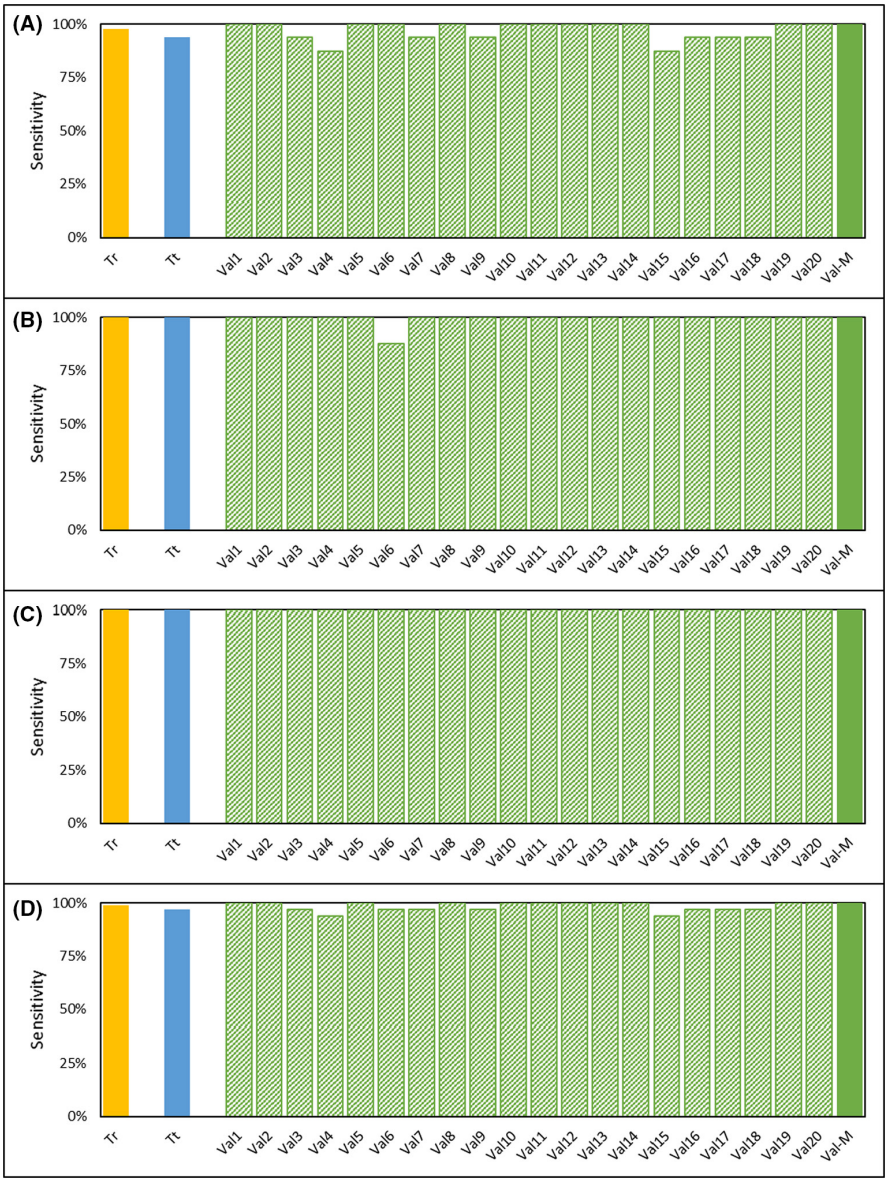

FIGURE 3 Observed Sensitivity in the Case Control Study. The test initially classifies samples as Positive, Equivocal or Negative based on the Decision Matrix provided in Figure 2. Samples with Equivocal findings are considered as Positive for the purpose of reporting and determination of Sensitivity. Each panel depicts the observed sensitivities in the Training set (Tr, solid orange), Test set (Tt, solid blue), the 20 iterations of the Validation set (Val1-Val20, green pattern) as well as the median Sensitivity in the Validation set (Val-M, solid green). The four panels depict findings based on extent of cancer, that is, Local (A), Regional (B), Distal (C), and Overall (D). Table S11 provides a break-up of the number of Positive, Equivocal, and Negative findings in each of the above sets.

3 ($n = 1$, Gleason score 4+3) or 4 ($n = 3$, Gleason score 8). The Test was able to detect 75% of these cases.

3.4 | Molecular concordance studies

Among the 20 tumor samples tested, driver mutations with allele frequency were detected in 15 samples by NGS profiling of tumor tissue DNA using the Oncomine Comprehensive Assay v3 Panel on the Ion Proton Platform. Among these 15 patient samples, a specific TaqMan ddPCR assay was available for variants detected in 12 cases. Genomic DNA was isolated from enriched CTCs and evaluated by ddPCR assays for the corresponding driver mutation (detected on ttDNA by NGS) on a BioRad QX200 platform. Variants in ttDNA detected by NGS were also detected by ddPCR in nine (75%) CTCs (Table S14). A subset of four PrAD cases were identified where the tissue was positive for TMPRSS2-ERG fusion by FISH. The CTC enriched fraction from these four samples was evaluated by FISH and the TMPRSS2-ERG fusion was detected in three cases (75%). Overall, the orthogonal concordance studies appeared to confirm that the CTCs detected by the Test originated from the same prostate malignancy. The 75% concordance was considered satisfactory considering clonal diversity in tumor cells and CTCs.

4 | DISCUSSION

We describe a blood test for Prostate cancer detection based on multiplexed fluorescence ICC profiling of CTCs functionally enriched from a 5 mL blood sample. The test detected Prostate cancer with high sensitivity irrespective of age, serum PSA level, Gleason score, or the extent of disease. Analytical validation ascertained accuracy and reliability of the test. The case control cross-validation study demonstrated 100% specificity as well as 100% sensitivity across all stages of Prostate cancer. The subsequent prospective clinical validation study demonstrated 91.2% Sensitivity and 100% Specificity in the real world setting for detecting Prostate cancer and differentiating prostate cancer from benign prostate conditions. The Test has high sensitivity for all stages, including early stages as well as high specificity to minimize the risk of false positives. The performance characteristics of the test support its potential clinical utility in Prostate cancer detection.

Serum PSA which is evaluated during standard prostate cancer diagnostic work up in symptomatic men is often assessed as part of elective prostate cancer screening in asymptomatic males.[34,35] However, PSA testing has lower specificity and is associated with a high false positive rate, for example ~66%.[5] Other PSA-based tests such as %-free PSA,[36] [−2]pro-PSA (p2PSA),[37] and Prostate Health Index (PHI)[38] with documented sensitivity/specificity trade-off[36,39,40] are currently not recommended or approved for routine prostate cancer screening. The inverse relationship between specificity and sensitivity of PSA and PSA-based tests[40] implies inefficient triaging where a significant proportion of individuals who do undergo a prostate biopsy based on these tests may actually be free from prostate cancer. Based on the limitations of serum PSA evaluations alone to provide meaningful insight into prostate cancer detection, Thompson et al. suggested that *"PSA levels should no longer be referred to as "normal" or "elevated" but should be incorporated into a multivariable risk assessment to provide individualized risk information for decision making"*.[41] Among other non-invasive (blood-based) approaches, a pan-cancer detection test based on methylation profiling in cfDNA reported very low sensitivity (~10%) for localized Prostate cancer.[42,43] While the above tests have been utilized for prostate cancer screening, other tests have been described for triaging of suspected cases so as to improve the specificity of PrC detection and minimize the risk of overdiagnosis. The 4Kscore Test is a follow-up blood test after an abnormal PSA and/or digital rectal exam (DRE) to determine the probability of aggressive prostate cancer.[44] The ExoDx™ Prostate Test is a urine-based test to determine the probability of clinically significant prostate cancer in men with PSA 2–10 ng/mL ("gray zone") who are considering an initial biopsy.[45] A recent study by Hugosson et al. demonstrated that the avoidance of systematic biopsy in favor of MRI-directed targeted biopsy in males with elevated serum PSA levels led to a significant decrease in the risk of overdiagnosis but led to delayed detection of intermediate-risk PrC in some patients.[46]

Our test is based on detection of CTCs, which are ubiquitous in blood of patients with an underlying solid organ cancer[29] and unlikely in the blood of individuals without an underlying malignancy as well as those with other non-malignant or inflammatory conditions. CTCs are hence an ideal analyte to differentiate individuals with and without an underlying malignant condition with high specificity and sensitivity. The risks associated with use of the test are only marginal since it is non-invasive, requiring only a 5 mL peripheral blood sample. The potential benefits of the test include more effective detection of Prostate cancer and reduced requirement for biopsies in symptomatic males. The strengths of our study include (a) use of adequately powered sample sizes, (b) sample blinding to eliminate bias, (c) an iterative cross-validation design intended to eliminate risk of over-fitting, and (d) a prospective study in a real-world setting. The analytical and clinical validations described in this manuscript provide tangible evidence of the test performance which supports

the hypothesis (design) as well as the intended use of the test. The high specificity translates into an exceedingly low risk of false positives in individuals with benign prostate conditions which eliminates or significantly reduces risks of overdiagnosis or overtreatment in these individuals.

Although the test has high performance characteristics for Prostate cancer detection, we note the following potential limitations of the test. Non-(adeno)-carcinoma types which account for <8% of Prostate cancer are not detected by this test. The sensitivity for the detection was lower (~75%) for localized Prostate cancer in the prospective study. However, these false negatives would not add to pre-existing risks since the lower sensitivity for localized cancers can be partially mitigated by the higher sensitivity for subsequent detection at regional stage which has a comparable 5-year survival.

The risk stratification of prostate cancer includes serum PSA level, clinical stage and Gleason score; a Gleason score of >8 is considered an independent predictor of high-risk disease with increased rates of treatment failures and poorer outcomes. While test is not intended to provide information on, or correlate with, the Gleason score, it can detect high-grade/aggressive prostate cancers where early detection is vital for more effective clinical management. As can be seen from the findings in the prospective clinical study, a significant advantage of the test is its ability to detect clinically significant prostate cancers (histological grade 3 or 4) in patients with low serum PSA.

The prospective study had a lower representation of early-stage disease since it was conducted in a population where prostate cancer is typically detected at advanced stages; of the 68 patients diagnosed with prostate cancer, only 20 (30%) had localized disease ($T_{1\text{-}3}N_0M_0$). Since this was an anticipated limitation, the design of the case control study pre-emptively addressed this challenge by having a higher representation of samples from patients with localized disease; of the 160 patients with prostate cancer, 80 (50%) had localized disease ($T_{1\text{-}3}N_0M_0$).

In 78 cases in the prospective cohort, the decision to perform a prostate biopsy despite unremarkable serum PSA (<4 ng/mL) was based on clinical findings/DRE/USG. While this proportion would appear to be higher, they represent standard approaches in India (study location) based on the observations that 15% of symptomatic males with PSA <4 ng/mL are diagnosed with prostate cancer[47] and that most prostate cancers in India are diagnosed at advanced stages. Notably a prior retrospective cohort analysis reported that 67% of patients were referred for a prostate biopsy at a tertiary centre in Ireland based on abnormal DRE alone.[48]

There would appear to be a minimal risk of overdiagnosis from detection of low-grade (lower risk) prostate cancers which account for up to 66% of all prostate cancers.[49] However, since up to 40% of patients initially diagnosed with low-risk prostate cancer demonstrate pathological progression over time,[50] detection of low-grade prostate cancers can benefit from active surveillance.[51]

5 | CONCLUSION

The high sensitivity and specificity of the test enables prostate cancer detection and differentiation from benign prostate conditions (or healthy individuals) and presents significant advantages over PSA based approaches. The test has potential to reduce the need for invasive biopsies and thus significantly mitigates risks of overdiagnosis and overtreatment. The potential benefits of the test are compelling and support the need for further prospective large cohort clinical studies to determine the performance characteristics of the test for detection of prostate cancer, especially localized disease.

AUTHOR CONTRIBUTIONS

Sewanti Limaye: Conceptualization (equal); methodology (equal); writing – review and editing (equal). **Simon Chowdhury:** Conceptualization (equal); writing – review and editing (equal). **Nitesh Rohatgi:** Conceptualization (equal); writing – review and editing (equal). **Anantabhushan Ranade:** Conceptualization (equal); methodology (equal); writing – review and editing (equal). **Nelofer Syed:** Conceptualization (equal); writing – review and editing (equal). **Johann Riedemann:** Conceptualization (equal); writing – review and editing (equal). **Darshana Patil:** Conceptualization (equal); methodology (equal); project administration (equal); supervision (equal); writing – review and editing (equal). **Dadasaheb Akolkar:** Conceptualization (equal); investigation (equal); methodology (equal); project administration (equal); supervision (equal); writing – review and editing (equal). **Vineet Datta:** Conceptualization (equal); methodology (equal); resources (equal); supervision (equal); writing – review and editing (equal). **Shoeb Patel:** Data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); validation (equal). **Rohit Chougule:** Data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); validation (equal). **Pradyumna Shejwalkar:** Data curation (equal); investigation (equal); validation (equal). **Kiran Bendale:** Conceptualization (equal); investigation (equal); methodology (equal); project administration (equal); validation (equal). **Sachin Apurwa:** Formal analysis (equal); methodology (equal); software (equal); validation (equal); visualization (equal). **Stefan Schuster:** Conceptualization (equal); resources (equal);

writing – review and editing (equal). **Jinumary John:** Data curation (equal); formal analysis (equal); validation (equal); visualization (equal); writing – original draft (equal); writing – review and editing (equal). **Ajay Srinivasan:** Data curation (equal); formal analysis (equal); validation (equal); visualization (equal); writing – original draft (equal); writing – review and editing (equal). **Rajan Datar:** Conceptualization (equal); funding acquisition (equal); investigation (equal); methodology (equal); project administration (equal); resources (equal); supervision (equal); visualization (equal); writing – original draft (equal); writing – review and editing (equal).

ACKNOWLEDGMENTS

The authors acknowledge the invaluable contributions from all study participants including patients and healthy volunteers and their families/caretakers, caregivers, and staff of all study sites. Contributions of members from the sponsor organization including Dr. Raymond Page, Dr. Revati Patil, Dr. Pooja Fulmali, Mr. Pradeep Fulmali, Ms. Archana Adhav, Dr. Sudha Murthy, and Dr. Chirantan Bose are acknowledged for managing various clinical, scientific, and operational aspects of the study.

FUNDING INFORMATION

No external funding was obtained for this study. The entire study was funded by the Study Sponsor (DCG).

CONFLICT OF INTEREST STATEMENT

SL, SC, NR, AR, NS, JR have no conflicts of interest to declare. DP, DA, VD, SP, RC, PS, KB, SA, SS, JJ, and AS are in employment of the Study Sponsor (DCG). RD is the founder of the Study Sponsor.

PRECIS FOR TABLE OF CONTENTS

Enrichment and characterization of circulating tumor cells (CTC) can aid more effective prostate cancer detection. CTC based prostate cancer detection can reduce false negatives and potentially eliminate false positives.

DATA AVAILABILITY STATEMENT

All relevant data are included in the manuscript and its Supplementary Information file.

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REFERENCES

1. Sung H, Ferlay J, Siegel RL, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2021;71:209-249. doi:10.3322/caac.21660
2. Cancer Stat Facts: Prostate Cancer. Natl Cancer Inst Surveillance, Epidemiol End Results Progr n.d. Accessed April 9, 2022. https://seer.cancer.gov/statfacts/html/prost.html
3. Prostate-Specific Antigen (PSA) Test. Natl Cancer Inst n.d. Accessed November 16, 2021. https://www.cancer.gov/types/prostate/psa-fact-sheet/
4. Holmström B, Johansson M, Bergh A, Stenman U-H, Hallmans G, Stattin P. Prostate specific antigen for early detection of prostate cancer: longitudinal study. *BMJ*. 2009;339:b3537. doi:10.1136/bmj.b3537
5. Kilpeläinen TP, Tammela TLJ, Roobol M, et al. False-positive screening results in the European randomized study of screening for prostate cancer. *Eur J Cancer*. 2011;47:2698-2705. doi:10.1016/j.ejca.2011.06.055
6. Loeb S, Bjurlin MA, Nicholson J, et al. Overdiagnosis and overtreatment of prostate cancer. *Eur Urol*. 2014;65:1046-1055. doi:10.1016/j.eururo.2013.12.062
7. Mahal BA, Aizer AA, Efstathiou JA, Nguyen PL. Association of very low prostate-specific antigen levels with increased cancer-specific death in men with high-grade prostate cancer. *Cancer*. 2016;122:78-83. doi:10.1002/cncr.29691
8. D'Amico AV, Whittington R, Malkowicz SB, et al. Biochemical outcome after radical prostatectomy, external beam radiation therapy, or interstitial radiation therapy for clinically localized prostate cancer. *JAMA*. 1998;280:969-974. doi:10.1001/jama.280.11.969
9. Campos-Fernández E, Barcelos LS, de Souza AG, Goulart LR, Alonso-Goulart V. Research landscape of liquid biopsies in prostate cancer. *Am J Cancer Res*. 2019;9:1309-1328.
10. Hennigan ST, Trostel SY, Terrigino NT, et al. Low abundance of circulating tumor DNA in localized prostate cancer. *JCO Precis Oncol*. 2019;3. doi:10.1200/PO.19.00176
11. Labib M, Kelley SO. Circulating tumor cell profiling for precision oncology. *Mol Oncol*. 2021;15:1622-1646. doi:10.1002/1878-0261.12901
12. van der Toom EE, Verdone JE, Pienta KJ. Disseminated tumor cells and dormancy in prostate cancer metastasis. *Curr Opin Biotechnol*. 2016;40:9-15. doi:10.1016/j.copbio.2016.02.002
13. Morgan TM, Lange PH, Porter MP, et al. Disseminated tumor cells in prostate cancer patients after radical prostatectomy and without evidence of disease predicts biochemical recurrence. *Clin Cancer Res*. 2009;15:677-683. doi:10.1158/1078-0432.CCR-08-1754
14. Fizazi K, Morat L, Chauveinc L, et al. High detection rate of circulating tumor cells in blood of patients with prostate cancer using telomerase activity. *Ann Oncol*. 2007;18:518-521. doi:10.1093/annonc/mdl419
15. Ried K, Tamanna T, Matthews S, Eng P, Sali A. New screening test improves detection of prostate cancer using circulating tumor cells and prostate-specific markers. *Front Oncol*. 2020;10:582. doi:10.3389/fonc.2020.00582
16. Kuske A, Gorges TM, Tennstedt P, et al. Improved detection of circulating tumor cells in non-metastatic high-risk prostate cancer patients. *Sci Rep*. 2016;6:39736. doi:10.1038/srep39736
17. Stott SL, Lee RJ, Nagrath S, et al. Isolation and characterization of circulating tumor cells from patients with localized and metastatic prostate cancer. *Sci Transl Med*. 2010;2:25ra23. doi:10.1126/scitranslmed.3000403

18. Shao C, Liao C-P, Hu P, et al. Detection of live circulating tumor cells by a class of near-infrared heptamethine carbocyanine dyes in patients with localized and metastatic prostate cancer. *PLoS One*. 2014;9:e88967. doi:10.1371/journal.pone.0088967
19. Spizzo G, Fong D, Wurm M, et al. EpCAM expression in primary tumour tissues and metastases: an immunohistochemical analysis. *J Clin Pathol*. 2011;64:415-420. doi:10.1136/jcp.2011.090274
20. Rao CG, Chianese D, Doyle GV, et al. Expression of epithelial cell adhesion molecule in carcinoma cells present in blood and primary and metastatic tumors. *Int J Oncol*. 2005;27:49-57.
21. de Wit S, Manicone M, Rossi E, et al. EpCAM(high) and EpCAM(low) circulating tumor cells in metastatic prostate and breast cancer patients. *Oncotarget*. 2018;9:35705-35716. doi:10.18632/oncotarget.26298
22. Adams DL, Stefansson S, Haudenschild C, et al. Cytometric characterization of circulating tumor cells captured by microfiltration and their correlation to the CellSearch(®) CTC test. *Cytometry A*. 2015;87:137-144. doi:10.1002/cyto.a.22613
23. Sheng Y, Wang T, Li H, et al. Comparison of analytic performances of Cellsearch and iFISH approach in detecting circulating tumor cells. *Oncotarget*. 2017;8:8801-8806. doi:10.18632/oncotarget.6688
24. Paterlini-Brechot P, Benali NL. Circulating tumor cells (CTC) detection: clinical impact and future directions. *Cancer Lett*. 2007;253:180-204. doi:10.1016/j.canlet.2006.12.014
25. Crook T, Gaya A, Page R, et al. Clinical utility of circulating tumor-associated cells to predict and monitor chemo-response in solid tumors. *Cancer Chemother Pharmacol*. 2021;87:197-205. doi:10.1007/s00280-020-04189-8
26. Gaya A, Crook T, Plowman N, et al. Evaluation of circulating tumor cell clusters for pan-cancer noninvasive diagnostic triaging. *Cancer Cytopathol*. 2021;129:226-238. doi:10.1002/cncy.22366
27. Iczkowski KA. Adenocarcinoma. PathologyOutlinesCom n.d. Accessed November 15, 2021. https://www.pathologyoutlines.com/topic/prostateadenoNOS.html
28. von Elm E, Altman DG, Egger M, Pocock SJ, Gøtzsche PC, Vandenbroucke JP. The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) Statement: guidelines for reporting observational studies. *Int J Surg*. 2014;12:1495-1499. doi:10.1016/j.ijsu.2014.07.013
29. Akolkar D, Patil D, Crook T, et al. Circulating ensembles of tumor-associated cells: a redoubtable new systemic hallmark of cancer. *Int J Cancer*. 2020;146:3485-3494. doi:10.1002/ijc.32815
30. Cancer Today: Population Fact Sheets. Glob Cancer Obs n.d. Accessed May 9, 2022. https://gco.iarc.fr/today/data/factsheets/populations/356-india-fact-sheets.pdf
31. Carter HB. Prostate cancers in men with low PSA levels—must we find them? *N Engl J Med*. 2004;350:2292-2294. doi:10.1056/NEJMe048003
32. Thompson IM, Pauler DK, Goodman PJ, et al. Prevalence of prostate cancer among men with a prostate-specific antigen level ≤4.0 ng per milliliter. *N Engl J Med*. 2004;350:2239-2246. doi:10.1056/NEJMoa031918
33. McNeal JE, Bostwick DG, Kindrachuk RA, Redwine EA, Freiha FS, Stamey TA. Patterns of progression in prostate cancer. *Lancet (London, England)*. 1986;1:60-63. doi:10.1016/s0140-6736(86)90715-4
34. Grossman DC, Curry SJ, Owens DK, et al. Screening for prostate cancer: US Preventive Services Task Force Recommendation Statement. *JAMA*. 2018;319:1901-1913. doi:10.1001/jama.2018.3710
35. PSA Testing: Prostate Cancer. Natl Heal Serv (NHS), UK n.d. Accessed November 16, 2021. https://www.nhs.uk/conditions/prostate-cancer/psa-testing/
36. Catalona WJ, Partin AW, Slawin KM, et al. Use of the percentage of free prostate-specific antigen to enhance differentiation of prostate cancer from benign prostatic disease: a prospective multicenter clinical trial. *JAMA*. 1998;279:1542-1547. doi:10.1001/jama.279.19.1542
37. Abrate A, Lughezzani G, Gadda GM, et al. Clinical use of [−2] proPSA (p2PSA) and its derivatives (%p2PSA and Prostate Health Index) for the detection of prostate cancer: a review of the literature. *Korean J Urol*. 2014;55:436-445. doi:10.4111/kju.2014.55.7.436
38. Loeb S, Catalona WJ. The Prostate Health Index: a new test for the detection of prostate cancer. *Ther Adv Urol*. 2014;6:74-77. doi:10.1177/1756287213513488
39. Wolf AMD, Wender RC, Etzioni RB, et al. American Cancer Society guideline for the early detection of prostate cancer: update 2010. *CA Cancer J Clin*. 2010;60:70-98. doi:10.3322/caac.20066
40. Fuchsova R, Topolcan O, Windrichova J, et al. PHI in the early detection of prostate cancer. *Anticancer Res*. 2015;35:4855-4857.
41. Thompson IMJ, Leach RJ, Ankerst DP. Focusing PSA testing on detection of high-risk prostate cancers by incorporating patient preferences into decision making. *JAMA*. 2014;312:995-996. doi:10.1001/jama.2014.9680
42. Liu MC, Oxnard GR, Klein EA, Swanton C, Seiden MV. Sensitive and specific multi-cancer detection and localization using methylation signatures in cell-free DNA. *Ann Oncol*. 2020;31:745-759. doi:10.1016/j.annonc.2020.02.011
43. Klein EA, Richards D, Cohn A, et al. Clinical validation of a targeted methylation-based multi-cancer early detection test using an independent validation set. *Ann Oncol*. 2021;32:1167-1177. doi:10.1016/j.annonc.2021.05.806
44. Tutrone R, Donovan MJ, Torkler P, et al. Clinical utility of the exosome based ExoDx Prostate(IntelliScore) EPI test in men presenting for initial Biopsy with a PSA 2-10 ng/mL. *Prostate Cancer Prostatic Dis*. 2020;23(4):607-614. doi:10.1038/s41391-020-0237-z
45. Parekh DJ, Punnen S, Sjoberg DD, et al. A multi-institutional prospective trial in the USA confirms that the 4Kscore accurately identifies men with high-grade prostate cancer. *Eur Urol*. 2015;68(3):464-470. doi:10.1016/j.eururo.2014.10.021
46. Hugosson J, Månsson M, Wallström J, et al. GÖTEBORG-2 trial investigators. Prostate cancer screening with PSA and MRI followed by targeted biopsy only. *N Engl J Med*. 2022;387(23):2126-2137. doi:10.1056/NEJMoa2209454
47. Fenton JJ, Weyrich MS, Durbin S, Liu Y, Bang H, Melnikow J. Prostate-specific antigen-based screening for prostate cancer: evidence report and systematic review for the US Preventive Services Task Force. *JAMA*. 2018;319:1914-1931. doi:10.1001/jama.2018.3712
48. Walsh AL, Considine SW, Thomas AZ, Lynch TH, Manecksha RP. Digital rectal examination in primary care is important for early detection of prostate cancer: a retrospective cohort analysis

study. *Br J Gen Pract J R Coll Gen Pract*. 2014;64:e783-e787. doi:10.3399/bjgp14X682861

49. Pierorazio PM, Walsh PC, Partin AW, Epstein JI. Prognostic Gleason grade grouping: data based on the modified Gleason scoring system. *BJU Int*. 2013;111:753-760. doi:10.1111/j.1464-410X.2012.11611.x
50. Klotz L, Zhang L, Lam A, Nam R, Mamedov A, Loblaw A. Clinical results of long-term follow-up of a large, active surveillance cohort with localized prostate cancer. *J Clin Oncol*. 2010;28:126-131. doi:10.1200/JCO.2009.24.2180
51. Preston MA, Feldman AS, Coen JJ, et al. Active surveillance for low-risk prostate cancer: need for intervention and survival at 10 years. *Urol Oncol*. 2015;33(383):e9-e16. doi:10.1016/j.urolonc.2015.04.015

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Limaye S, Chowdhury S, Rohatgi N, et al. Accurate prostate cancer detection based on enrichment and characterization of prostate cancer specific circulating tumor cells. *Cancer Med*. 2023;00:1-12. doi:10.1002/cam4.5649