ORIGINAL ARTICLE

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Analogous detection of circulating tumor cells using the AccuCyte®—CyteFinder® system and ISET system in patients with locally advanced and metastatic prostate cancer

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Introduction: Circulating tumor cells (CTCs) can provide important information on patient's prognosis and treatment efficacy. Currently, a plethora of methods is available for the detection of these rare cells. We compared the outcomes of two of those methods to enumerate and characterize CTCs in patients with locally advanced and metastatic prostate cancer (PCa). First, the selection-free AccuCyte® – CyteFinder® system (RareCyte®, Inc., Seattle, WA) and second, the ISET system (Rarecells Diagnostics, France), a CTC detection method based on cell size-exclusion.

Methods: Peripheral blood samples were obtained from 15 patients with metastatic PCa and processed in parallel, using both methods according to manufacturer's protocol. CTCs were identified by immunofluorescence, using commercially available antibodies to pancytokeratin (PanCK), EpCAM, CD45/CD66b/CD34/CD11b/CD14 (AccuCyte® – CyteFinder® system), and pancytokeratin, vimentin (Vim) and CD45 (ISET system).

Results: The median CTC count was 5 CTCs/7.5 mL (range, 0-20) for the AccuCyte® – CyteFinder® system and 37 CTCs/7.5 mL (range, 8-139) for the ISET system ($P < 0.001$). Total CTC counts obtained for the two methods were correlated ($r = 0.750$, $P = 0.001$). When separating the total CTC count obtained with the ISET system in PanCK+/Vim− and PanCK+/Vim+ CTCs, the total CTC count obtained with the AccuCyte® – CyteFinder® system was moderately correlated with the PanCK+/Vim− CTCs, and strongly correlated with the PanCK+/Vim+ CTCs ($r = 0.700$, $P = 0.004$ and $r = 0.810$, $P < 0.001$, respectively).

Conclusion: Our results highlight significant disparities in the enumeration and phenotype of CTCs detected by both techniques. Although the median amount of CTCs/7.5 mL differed significantly, total CTC counts of both methods were strongly correlated. For future studies, a more uniform approach to the isolation and definition

of CTCs based on immunofluorescent stains is needed to provide reproducible results that can be correlated with clinical outcomes.

KEYWORDS

AccuCyte® – CyteFinder® system, circulating tumor cells, CTCs, ISET system, metastatic prostate cancer

1 | INTRODUCTION

With 161 360 estimated new cases in 2017, prostate cancer (PCa) is the most common cancer in US men and the second most common cause of male cancer death.[1] Unfortunately, there is still no cure for this disease once it has metastasized. Circulating tumor cells (CTCs) are tumor cells that have managed to leave the primary solid tumor in order to enter the peripheral blood, and are thereby involved in the hematogeneous metastatic spread.[2] These cells could provide important information on patient's prognosis and treatment efficacy.[3,4]

At present, a plethora of methods have been developed and are commercially available for the detection and isolation of these rare cells, all with their own pros and cons.[2] The CellSearch® system (Janssen Diagnostic), which uses anti-EpCAM labeled magnetic beads for the positive selection of CTCs, is the only FDA-approved assay to date.[5] It is the most commonly used CTC detection method, mainly because of its repeatability and its proven contribution to predict outcome and progression-free survival in patients with metastatic breast, colorectal, and prostate cancers.[6–8] It is now understood that the expression of epithelial markers varies with tumor type and that the expression of those markers, especially EpCAM, is downregulated by epithelial-to-mesenchymal transition (EMT) to increase the ability of the tumor cells to mobilize, invade, and thus facilitate metastasis.[9,10] Therefore, epithelial markers do not formally establish the metastatic nature of CTCs.[11–14] CTC detection methods that rely on EpCAM expression of the tumor cells, like CellSearch®, fail to detect CTCs with more mesenchymal, and possibly more aggressive, features.[11]

Based on this knowledge, this study prospectively compared the performance of two different methods that both do not solely rely on EpCAM. First, the selection-free AccuCyte® – CyteFinder® system (RareCyte, Inc., Seattle, WA), which is able to identify EpCAM negative, as well as EpCAM positive CTCs. This method involves spreading of nucleated cells on positively charged slides, followed by immunofluorescence (IF) staining. An IF imaging microscope is then used to enumerate CTCs.[15,16] The proposed strength of this method is limited loss of CTCs by marker selection or physical overlap between CTCs and other blood components. The second system used in this study is the ISET (Isolation by Size of Epithelial Tumor cells) system (Rarecells Diagnostics, France), which uses size-based separation of CTCs, and thus does not rely on the expression of epithelial markers only, thereby allowing for the identification of CTCs with different phenotypes.

Size-based separation relies on the assumption that circulating tumor cells are larger than the other components of the blood.[17,18] Cells are enriched by blood filtration through filtering membranes with 8 μm pores in diameter and the enriched cells are subsequently stained on the filter and characterized by IF. Distinguishing between both epithelial and mesenchymal-like tumor cells, by using pancytokeratin and vimentin stains, is the proposed strength of this system[19–21] (Table 1). The main aim of this prospective study is to investigate whether samples processed with the AccuCyte® – CyteFinder® system and ISET system give comparable results in terms of number of CTCs detected, and to show the differences in enumeration and phenotyping between both techniques.

2 | MATERIALS AND METHODS

CTC analysis was carried out in parallel on samples obtained from the same patient using both methods. Peripheral blood samples (7.5 mL) from 15 patients with locally advanced and metastatic prostate cancer were drawn into Rarecyte blood collection tubes (Rarecyte Inc, Seattle, WA) and processed within 72 h of collection. For the ISET assay, blood was collected in lavender-topped ethylenediaminetetraacetic acid (EDTA) vacutainers and processed within 6 h of collection. The specimens were collected under a protocol approved by the Johns Hopkins Medicine Institutional Review Board.

2.1 | Enumeration of CTCs by the selection-free AccuCyte® – CyteFinder® system

2.1.1 | Sample processing

The AccuCyte® sample processing system (Rarecyte Inc.) was used to process and transfer the samples to eight positively charged slides. Until staining, slides were stored at −20°C.

2.1.2 | Sample staining

Slides were stained according to manufacturer's protocol, using a previously described protocol.[22] Briefly, on the day of staining slides were first fixed in 10% neutral buffered formalin (Sigma, St. Louis, MO) for 60 min at room temperature. Next, two washes in Tris-buffered saline (Quality Biological Inc., Gaithersberg, MD) were performed to neutralize the slides for excess formalin.

TABLE 1 Differences between both CTC detection methods

Assay characteristics	AccuCyte® – CyteFinder® system	ISET system
CTC isolation strategy	Selection-free	Size-based separation
Markers used in assay	DAPI, PanCK, EpCAM, CD45/CD66b/CD34/CD11b/CD14	DAPI, PanCK, Vimentin, CD45
Able to distinguish between epithelial and mesenchymal-like CTCs	No	Yes
Costs per test	±$170	±$210

Subsequently, slides were put in an antigen retrieval bath for 6 min (Tris-HCl buffer, pH 10, Sigma-Aldrich, St. Louis, MO) to improve accessibility of the antibodies to the tissue antigens. A proprietary custom reagent kit developed by RareCyte was used to perform the staining. This includes 4',6-diamidino-2-phenylindole (DAPI; nuclear stain); anti-pancytokeratin (PanCK; epithelial marker), anti-EpCAM (epithelial marker), and anti-CD45/anti-CD66b (counterstain channel). We modified the assay by adding the following additional counterstain markers: anti-CD14-PE (Biolegend, San Diego, CA; clone M5E2, 1:200), anti-CD34-PE (Biolegend; clone 581, 1:200,), and anti-CD11b-PE (Biolegend; clone M1/70, 1:200). Next, Fluoromount aqueous mounting medium (Sigma-Aldrich) was used to cover-slip the slides and allowed to dry overnight at room temperature (25°C).

2.1.3 | Slide scanning

The CyteFinder® imaging system (Rarecyte Inc.) was used to scan the stained slides (magnification objective 10X) with the following exposure times: DAPI – 0.020 s; PanCK – 0.025 s; CD45/CD66b/CD11b/CD14/CD34 (counterstain channel) – 0.050 s; EpCAM – 0.100 s. Candidate CTCs were identified from top ranked cells by a single trained technician and the report was always reviewed by a second technician. Candidate CTCs were called positive if they exhibited the following criteria: a DAPI-positive nucleus with a diameter ≥4 µm; positivity for the cytokeratin stain, AND/OR positivity for the EpCAM stain, and lacking of signal in the counterstain channel.

2.2 | Enumeration of CTCs by the size-based approach ISET System

2.2.1 | Sample processing

Using previously described protocols by Rarecells Diagnostics, blood samples were processed and filtered using the ISET method.[18,23] In short, an isolation buffer was prepared by combining three buffer samples provided by Rarecells Diagnostics with ultra-filtered water and was brought to a pH between 7.2 and 7.4 with 1 M/L sodium hydroxide. Ten milliliters of blood was diluted using 90 mL of buffer

(10-fold dilution) and formaldehyde was added. The solution was then filtrated by the Rarecells Device using a disposable cartridge (Rarecells Block) containing a filter with 8 µm-sized pores. After filtration, membranes were stored in the dark at −20°C until further staining and analysis.

2.2.2 | Sample staining

Immunofluorescence of the ISET membranes was performed using a previously published protocol with commercially available conjugated antibodies.[24] In short, membranes were re-hydrated using 1× tris-buffered saline after which 0.2% Triton was added to permeabilize cell membranes. After the Triton was removed, a 5% milk-based blocking solution with immunofluorescent antibodies to pancytokeratin (Abcam, Alexa Fluor 488, 1:100), CD45 (Bioss, Alexa Fluor 647, 1:100), and vimentin (Abcam, Alexa Fluor 594, 1:100) was used to incubate the membranes. Membranes were washed a final time with 1× tris-buffered saline before being fixed on a positively charged slide with DAPI (Life Sciences). Slides were allowed to dry overnight in the dark at 4°C.

2.2.3 | Slide scanning

Analysis of slides with an IF microscope was done manually at 20× magnification. With the Nikon NIS Elements imaging program (version 4.20.02, 64-bit), different exposure times and wavelengths were used to detect staining with DAPI (0.6 sec milliseconds), pancytokeratin (1.0 s), vimentin (0.8 s), and CD45 (3.0 s). Identified CTCs were counted by a single user and images under each wavelength were taken and stored. An epithelial CTC (PanCK+/Vim− CTC) was defined as a cell with a DAPI-positive nucleus, cytoplasmic labeling for pancytokeratin and no expression of CD45. A mesenchymal-like CTC (PanCK+/Vim+ CTC) was defined as a cell with a DAPI-positive nucleus, cytoplasmic labeling for pancytokeratin and vimentin and no expression of CD45. PanCK+ and PanCK+/Vim+ CTCs were counted and presented in a mutually exclusive fashion.

2.2.4 | Data analysis/statistical methods

Since CTC levels in patients were non-normally distributed, CTC counts were presented as medians with corresponding ranges. Linear regression plots were computed for CTC counts obtained by the AccuCyte® – CyteFinder® and ISET techniques. The Spearman test was subsequently used to assess the correlation of CTC counts determined by both techniques. In addition, the Mann-Whitney U-test was used to compare the differences in median CTC count of both detection methods. All statistical analyses were performed using the SPSS software (version 24; SPSS, Chicago, IL).

3 | RESULTS

3.1 | Patient characteristics

All 15 patients included in this study had histologically confirmed diagnosis of locally advanced or metastatic adenocarcinoma (IA) of the

prostate. Each of the 15 patients had blood collected for CTC isolation and characterization by both the AccuCyte® – CyteFinder® system and the ISET system. The average age of the patients was 64.1 years (range, 49-78 yrs), while median PSA was 10.3 ng/mL (range, 2.2-360). Patients had either a relapse of PCa diagnosed years before and were to start chemo- and/or hormone therapy, or had a documented detectable prostate PSA after primary treatment (radical prostatectomy or radiation) before receiving additional therapy. Prior adjuvant treatment of any type for metastatic disease was permitted.

Ten out of 15 men had metastatic disease, of which nine had bone metastases and one had pulmonary metastases. None of the patients had brain metastases. Six of the metastatic patients received androgen deprivation therapy (two patients received abiraterone (#10, #12), two patients received bicalutamide in combination with Lupron (#1, #7), one patient received degarelix and had undergone previously a trial with pembrolizumab (#11), and one patient received enzalutamide and completed previously a trial with Sipuleucel T and a trial with UCSF ipilimumab (#2), and four patients were about to start androgen deprivation therapy with/or without taxotere (#6, #8, #13, #15).

Five out of 15 men had locally advanced disease; of which four were about to start androgen deprivation therapy with/or without taxotere (#3, #4, #5, #9,) and one patient had recently started trelstar and was in cycle 2 of chemotherapy with taxotere (#14).

Table 2 shows the clinicopathological characteristics of the patients, as well as their respective CTC counts obtained by both techniques.

3.2 | Circulating tumor cell detection

The median CTC count was 5 CTCs/7.5 mL (range, 0-20) for the AccuCyte® – CyteFinder® system and 37 CTCs/7.5 mL (range, 8-139) for the ISET system. When assessing differences in median CTC counts between both systems with the Mann-Whitney U-test, the median total CTC count of 5 CTCs/7.5 mL for the AccuCyte® – CyteFinder® system was significantly lower than the 37 CTCs/7.5 mL found with the ISET system ($P < 0.001$). However, total CTC counts obtained with the two methods were highly correlated ($r = 0.750$, $P = 0.001$) (Figure 1A).

When separating the total CTC count found with the ISET system in PanCK+/Vim− (epithelial CTCs) and PanCK+/Vim+ CTCs (mesenchymal-like CTCs), the median CTC count was 30 CTCs/7.5 mL (range, 8-124) for the PanCK+/Vim− CTCs and 8 CTCs/7.5 mL (range, 0-15) for the PanCK+/Vim+ CTCs. The amount of 5 CTCs/7.5 mL of CTCs identified by the AccuCyte® – CyteFinder® system was significantly lower than the median 30 CTCs/7.5 mL of PanCK+/Vim- CTCs ($P < 0.001$) identified by the ISET assay, but did not differ significantly from the found median 8 CTCs/7.5 mL of PanCK+/Vim+ CTCs ($P = 0.870$). Furthermore, the total CTC count obtained with the AccuCyte® – CyteFinder® system was moderately correlated with the PanCK+/Vim− CTCs, and strongly correlated with the PanCK+/Vim+ CTCs ($r = 0.700$, $P = 0.004$ and $r = 0.810$, $P < 0.001$, respectively) (Figure 1B-C).

Of the 15 patients, 8 (53%) had CTC counts ≥5/7.5 mL according to the AccuCyte® – CyteFinder® system, while all 15 (100%) had CTC counts ≥5/7.5 mL using the ISET system. When breaking down the CTC count of the ISET system per phenotype, all 15 patients (100%) had ≥5/7.5 mL epithelial CTCs, while 9 (60%) had ≥5/7.5 mL mesenchymal-like CTCs. One patient was found to have 0 CTCs by the AccuCyte® – CyteFinder® system, while four patients had no mesenchymal-like CTCs detected by the ISET system.

TABLE 2 Clinicopathological characteristics of the patient cohort with their respective CTC counts

Pat.	Age*	Histology	Gleason score	TNM stage	PSA	AccuCyte® – CyteFinder® system CTCs‡	ISET CTCs‡	ISET PanCK +/Vim- CTCs‡	ISET PanCK +/Vim+ CTCs‡
P1	52	IA	X*	T2N1M1	4.6	10	37	26	11
P2	68	IA	4 + 3 = 7	T3aN1M1	5.0	0	8	8	0
P3	75	IA	5 + 4 = 9	T2cN1M0	360.0	4	52	45	7
P4	75	IA	4 + 5 = 8	T3bN0M0	2.2	20	139	124	15
P5	51	IA	4 + 4 = 8	T1cN1M0	58.3	10	49	41	8
P6	68	IA	4 + 4 = 8	T3aN1M1	43.0	8	23	23	0
P7	64	IA	4 + 4 = 8	T3aN1M1	43.9	10	37	26	11
P8	73	IA	4 + 4 = 8	T2cN1M1	11.8	4	30	30	0
P9	78	IA	4 + 5 = 9	T3aN0M0	10.3	13	68	53	15
P10	63	IA	4 + 3 = 7	T3bN0M1	5.0	2	19	15	4
P11	68	IA	5 + 4 = 9	T3aN1M1	8.0	5	46	38	8
P12	49	IA	4 + 4 = 8	T3aN1M1	19.7	6	44	33	11
P13	52	IA	5 + 4 = 9	T1cN1M1	23.0	1	8	8	0
P14	61	IA	4 + 5 = 9	T2bN1M0	3.8	1	16	12	4
P15	64	IA	4 + 4 = 8	T3bN1M1	20.0	5	38	30	8

Pat., Patient; CTC, Circulating Tumor Cells; Age*, at moment of CTCs analysis; X*, Tumor cannot be reliably graded due to prominent treatment effect; TNM, tumor node metastasis; PSA, Prostate specific antigen (ng/mL), measured at the moment of CTC analysis; ‡, CTCs counted per 7.5 mL of blood.
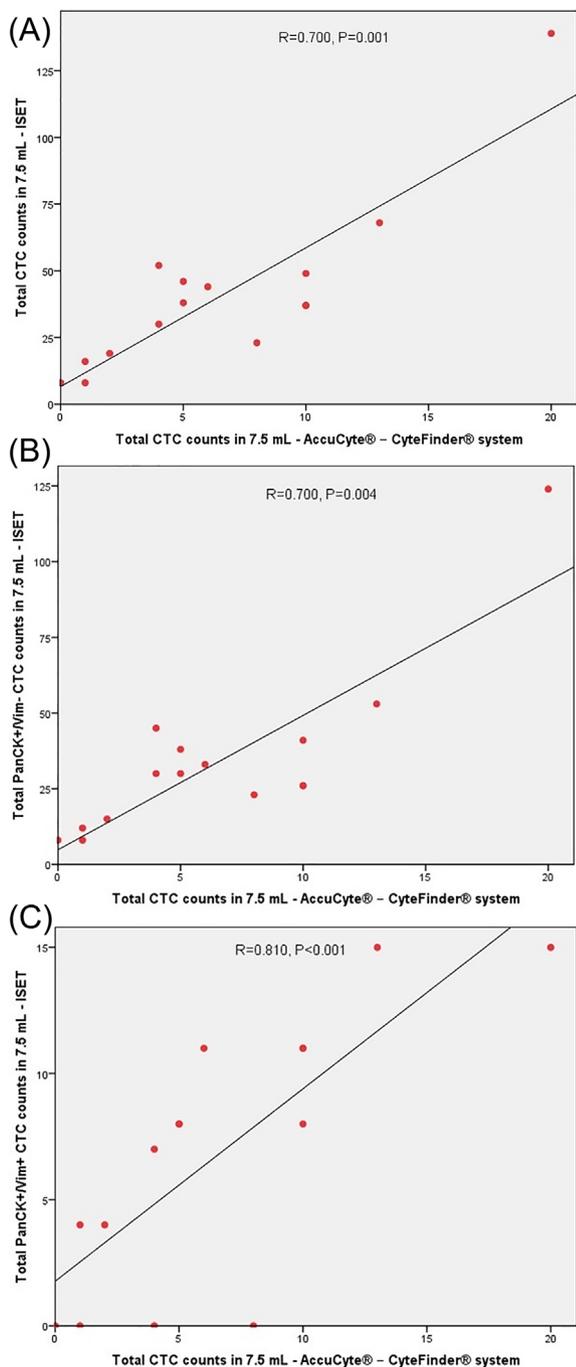
FIGURE 1 Linear regression plots of CTC counts by the AccuCyte® – CyteFinder® system and ISET. (A) Total CTC counts by both methods. (B) Total CTC counts by the AccuCyte® – CyteFinder® system and CK+/Vim- CTC counts by ISET. (C) Total CTC counts by the AccuCyte® – CyteFinder® system and CK+/Vim + CTC counts by ISET

4 | DISCUSSION

The main aim of this prospective study was to investigate whether samples processed with the AccuCyte® – CyteFinder® system and ISET system give comparable results in terms of number of CTCs detected. Our results highlight significant disparities in the enumeration and

phenotype of CTCs detected by both techniques. Although the median amount of CTCs/7.5 mL differed significantly, total CTC counts were strongly correlated in these two methods, despite that they rely on both different approaches as well as different molecular markers.

The clinical implications of CTCs have been reported abundantly in the literature. Enumeration of CTCs can help in determining the prognosis of a patients' disease in the castration-resistant setting and improve risk-stratification to avoid overtreatment and prolong cancer survival. Further characterization of CTCs can additionally guide in the different therapeutic options based on their phenotypic and molecular characteristics.[7,8,25–27] Furthermore, the isolation and characterization of CTCs play a very important role in understanding their biological, as well as their clinical relevance. Over the past few years a plethora of different assays for the detection, enumeration, and isolation of CTCs in blood have been developed. Unfortunately, very few studies have been performed that compare the outcome of these assays with different approaches and there is substantial variability in the rates of positive samples using existing detection techniques.[28] A lack of uniformity in used technologies and definitions might hinder the implementation of CTC measurement in clinical routine practice.

Methods that use the physical property "cell size" for the detection of CTCs, like the ISET system, rely on the general assumption that circulating tumor cells are larger than the other components found in the blood of a patient.[18] However, this type of enrichment has some important limitations, as this assumption is not based on the size of actual CTCs in humans, but largely on measurements of cells from cell lines in culture. A significant portion of CTCs in samples from patients have the same size or are sometimes even smaller than the white blood cells in a blood sample (Figure 2). Furthermore, it has been reported that these very small CTCs are associated with worse disease outcome,[29] emphasizing the importance of including these cell in CTC analysis. Also, it has been reported that CTC detection methods based on cell size may miss between 20% and 50% of CTCs.[30]

An important strength of the ISET system, however, is that it allows for different IF stains to be used on the isolated CTCs. In this manner, different phenotypic subpopulations, such as epithelial and mesenchymal-like CTCs, can be identified, characterized, and enumerated (Figure 3). Several studies in a variety of cancers have shown an association between the presence of mesenchymal-like CTCs in the circulation and worse cancer prognosis.[24,31,32] For PCa in particular, it has been reported that the number of PanCK−/Vim+ CTCs is correlated with disease burden, tumor aggressiveness, and worse overall survival. Furthermore, Xu et al showed that the combination of PanCK+/Vim+ CTCs and PSA level improved the prediction of cancer metastases better than other subtypes of CTCs (AUC 0.921 and 95%CI: 0.858-0.985). Identification of CTCs with a mesenchymal-like phenotype could thus further help monitor and predict cancer progression.[33] Future prospective studies, with a larger number of patients will hopefully elucidate whether the presence of these different CTC phenotypes is correlated with different clinical outcomes.

In contrast, the AccuCyte® – CyteFinder® system only uses epithelial markers, and is thus not able to establish the potential mesenchymal-like nature of specific CTCs. The strength of the novel
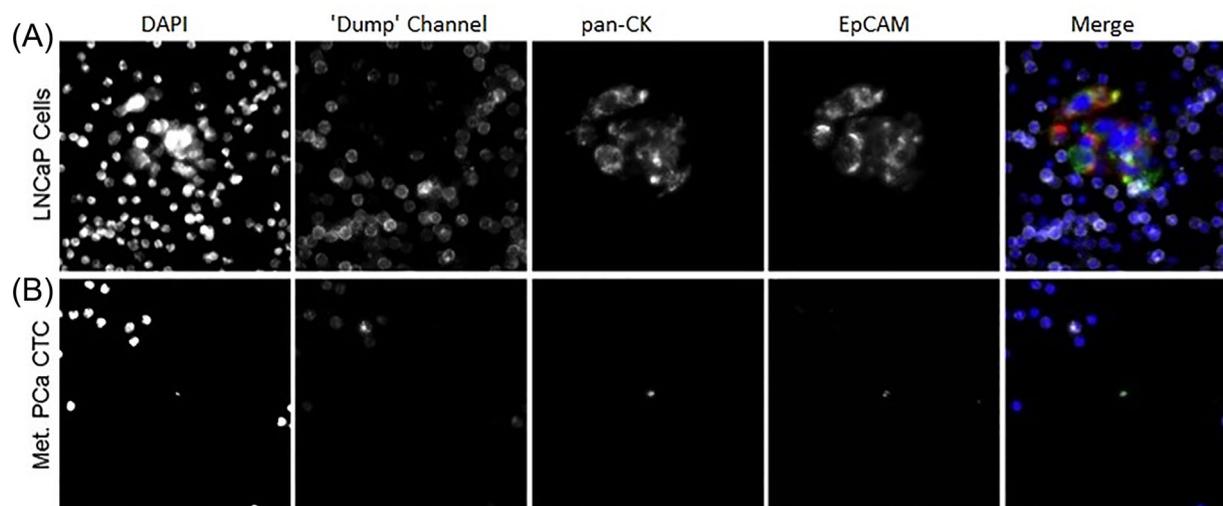
FIGURE 2 Limitations to size-based CTC enrichment: patient CTCs vs Cell Lines. (A) Example of a spiked LNCaP cell in peripheral blood of a healthy donor, processed with the AccuCyte® – CyteFinder® system; LNCaP cell much larger than the white blood cells. (B) Example of a CTC in peripheral blood a patient with metastatic PCa, processed with the AccuCyte® – CyteFinder® system; CTC even smaller than the white blood cells

selection-free AccuCyte® – CyteFinder® system on the other hand, is the idea that "no cell is left behind," due to the limited loss of CTCs by marker selection or physical overlap between CTCs and other blood components. Although the AccuCyte® – CyteFinder® system does not distinguish between epithelial or mesenchymal-like cancer cells, it is able to identify both EpCAM-negative, as well as EpCAM-positive CTCs.

The ISET system identified a significant higher amount of CTCs in the same blood sample when compared to the AccuCyte® – CyteFinder® system. An important difference between both detection assays that can possibly explain this disparity is the difference in the counterstain panel used, since the counterstain of the ISET only

consists of the most commonly utilized marker CD45. The AccuCyte® – CyteFinder® system, on the other hand, uses additional markers to common cells of hematopoietic stem cell and endothelial cell lineage to create a comprehensive panel to demarcate cells other than CTCs. This panel consists of CD11b and CD14 (both expressed on macrophages),[34,35] CD34 (a cell surface glycoprotein expressed on blood- and bone marrow-derived progenitor cells, especially hematopoietic and endothelial cells),[36] and CD66b (an activation marker for human granulocytes).[37] It is therefore possible that the samples processed with the ISET system identified cells as CTCs that would have been excluded as CTCs on the AccuCyte® – CyteFinder® system due to the additional markers in counterstains. Based on our data, it seems that,
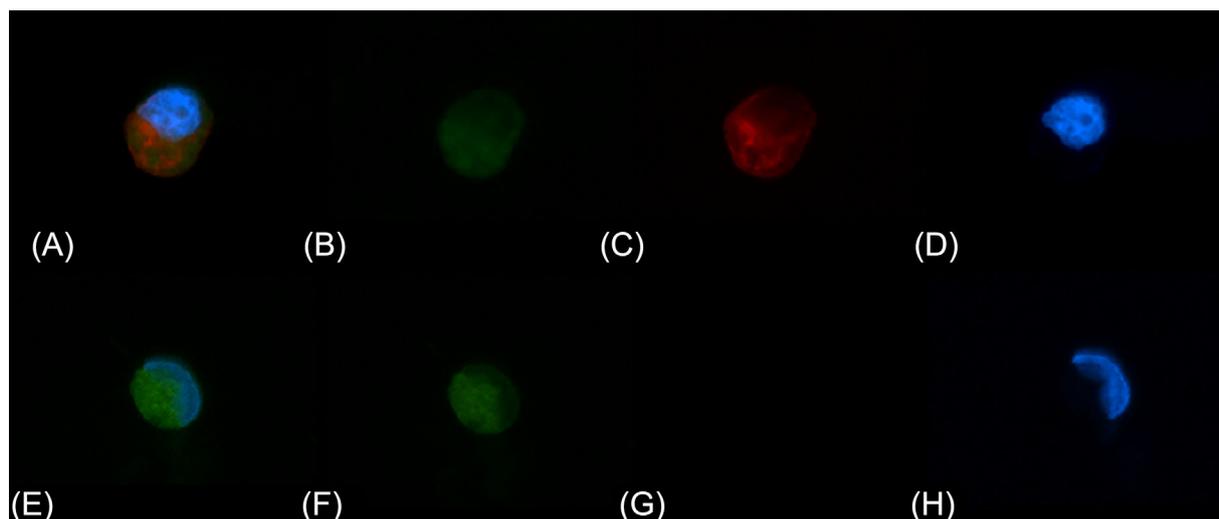
FIGURE 3 Expression of epithelial and mesenchymal markers in CTCs. Epithelial/mesenchymal phenotype (A), positive for pancytokeratin (B), vimentin (C) and DAPI (D), and epithelial phenotype (E), positive pancytokeratin (F), and DAPI (H), and negative for vimentin (G). All immunofluorescent images under 20X magnification (Nikon Eclipse Ti-E microscope)

despite the theoretical loss of potential tumor cells with the size-based enrichment method, the expanded "dump" channel of the AccuCyte® – CyteFinder® system has a higher influence on the total number of detected CTCs. However, as with all CTC detection methods, the process of the ISET and AccuCyte® – CyteFinder® assays both included different steps during which CTCs could also have been lost; for example, during the antigen retrieval bath and during the sequential washes in between the different immunostaining steps. The current study does not allow pinpointing of the specific cause for the found differences in CTC counts between the two systems. Future studies comparing different CTC isolation methods could shed further light on the cause of this found difference.

This study had several limitations worthy mentioning. First, because of the relatively small and heterogeneous patient cohort, the current study was not powered to investigate the relationship between CTC counts and clinicopathological variables and survival outcomes. A large cohort of prostate cancer patients would allow for correlation with clinical outcomes that would potentially reveal associations not appreciated by the current study. However, the main aim of this prospective study was to investigate whether samples processed with the AccuCyte® – CyteFinder® system and ISET system give comparable results in terms of number of CTCs detected, and to show the differences in enumeration and characterization between both techniques. For this purpose, the sample size of included patients was sufficient to show that although significant differences in absolute numbers of detected CTCs exist between the two platforms, the total counts show high correlation. Second, as outlined above, the performed experiments did not allow us to formally establish the underlying mechanisms in both isolation methods that caused the differences found in the identified number of CTCs.

In conclusion, our results highlight significant disparities in the enumeration and phenotype of CTCs detected by the AccuCyte® – CyteFinder® system and the ISET system. Although the median amount of CTCs/7.5 mL differed significantly, total CTC counts of both methods were strongly correlated. For future studies, a more uniform approach to the isolation and definition of CTCs based on immunofluorescent stains are needed to provide reproducible results that can be correlated with clinical outcomes.

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CONFLICTS OF INTEREST

Author K.J.P. is a consultant for Celsee Diagnostic and is on the board of directors for Curis, Inc.

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