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Peripheral Blood Circulatory Tumour Cells Enumeration as A Non-Invasive Biomarker in Breast Cancer Diagnosis: A Case-Control Study

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ABSTRACT

Aim and Background: Breast cancer is one of the most prevalent malignancy among women worldwide, and its early detection remains a challenge. Circulating tumour cells (CTCs) are emerging as promising biomarkers for the diagnosis, prognosis, and monitoring of cancer. This study aims to assess the diagnostic performance of CK-positive and HER2-positive CTCs in breast cancer patients and healthy controls.

Methods: This prospective case-control study involved 60 breast cancer patients (cases) and 60 age-matched healthy individuals (controls). Peripheral blood samples were collected prior to any oncological intervention. CTCs were isolated using Ficoll density gradient centrifugation and identified by flow cytometry, using cytokeratin (CK), CD45, and HER2 markers. Immunohistochemistry (IHC) and fluorescence in situ hybridisation (FISH) were performed to assess hormone receptor status (ER, PR, and HER2) in CTCs. All statistical analyses were performed using SPSS 14 software.

Results: Circulating tumour cells (CTCs) were detected in 85% of breast cancer patients, with cytokeratin-positive (CK+) CTCs showing high specificity and sensitivity for breast cancer detection (AUC = 1.000). Mann-Whitney U test results indicated significant differences in CTC counts between breast cancer patients and controls ($p < 0.001$ for both CK and HER2). In HER2-positive breast cancer, the expression of the HER2 marker demonstrated a 100% negative predictive value (NPV) and positive predictive value (PPV), along with a sensitivity of 51.67% and an area under the curve (AUC) of 0.758 for differentiation.

Conclusion: Our study results support the clinical utility of CTCs as non-invasive biomarkers for breast cancer detection and characterisation. Applications of liquid biopsy in precision oncology is valuable, and further large-scale validation studies are warranted to enhance the clinical implementation of CTC analysis in breast cancer management.

Keywords: *Breast cancer, circulating tumour cells, circulating tumour DNA, Liquid biopsy, Oncology*

1. INTRODUCTION

Breast cancer stands as the most frequently diagnosed cancer among women globally, with around 2.3 million new cases reported annually. It is one of the most commonly identified cancers and ranks as the fifth leading cause of cancer-related fatalities (Sung et al., 2020).

Worldwide, breast cancer caused 684,996 fatalities, with the highest mortality rates reaching up to 63% in Asian and African nations. Reports indicate that women diagnosed with breast cancer in high-income nations tend to survive, whereas the same is not true for women in many low- and middle-income countries. This inequality stems from inadequate

screening, delayed diagnosis, and a lack of appropriate treatment facilities in low- and middle-income regions (Ferlay et al., 2020; Ginsburg et al., 2016; Łukasiewicz et al., 2021).

Breast cancer epidemiology in India is distinct from that in the West, where women are typically diagnosed at a median age of 61 years, with a peak age of 60-70 years. In contrast, the maximum age of breast cancer in India is between 40 and 50 years, and a higher proportion of premenopausal women are affected (Mehrotra et al., 2022; Leong et al., 2010; Malvia et al., 2017; Siddiqui et al., 2023).

Moreover, only 1–8% of women in India receive a diagnosis of stage 1 breast cancer, in contrast to 60–70% of women in the United States. The rates of stage IV breast cancer diagnosis in India range from 6% to 24%, compared to just 10% among American women. Additionally, stage III breast cancer affects between 29% and 52% of Indian women. Breast cancer classification has increasingly depended on histopathological features, molecular characterization, and immunohistochemistry (IHC), with invasive lobular carcinoma and invasive ductal carcinoma being the two most common histological subtypes (Malvia et al., 2017; Siddiqui et al., 2023; Bhattacharyya et al., 2020).

The key to reducing breast cancer mortality lies in early detection, timely intervention, and precise monitoring of disease progression. Traditional diagnostic approaches, such as mammography, ultrasound, and tissue biopsy, remain the gold standard for identifying breast tumours and assessing their characteristics. However, these methods are often invasive, uncomfortable for patients, and may not always provide a complete picture of tumour behaviour, especially in cases of minimal residual disease or early metastasis (Duggan et al., 2018).

The idea of using liquid biopsy for cancer diagnosis was first presented in 2010, and since then, it has gained widespread use in cancer detection due to its non-invasive nature, efficiency, and ability to be repeated (Alix et al., 2023; Morgan et al., 2019).

Through liquid biopsy, various biomarkers are assessed such as circulating tumour DNA (ctDNA), exosomes, microRNAs and circulating tumour cells (CTCs). CTCs are cancer cells that have detached from the primary tumour site and entered the bloodstream, which provides a “snapshot” of the disease in real time (Singhal et al., 2018). The detection and enumeration of CTCs in breast cancer patients provide crucial information about the likelihood of disease progression, therapeutic response, and overall prognosis. CTCs are rare, mostly fewer than 10 cells per millilitre of blood and can vary widely in their molecular characteristics. Therefore, precise, sensitive, and specific methods are necessary to isolate and analyze these cells.

Through the use of flow cytometry, it is possible to assess various markers on single cells at the same time by employing antibodies labeled with fluorescent tags. In breast cancer research, cytokeratin (CK) and HER2/neu are essential markers utilized for the identification of circulating tumour cells (CTCs) (Andreou et al., 2019). Cytokeratins are structural proteins found in epithelial cells, and their presence in peripheral blood is considered a hallmark of epithelial-derived tumour cells. HER2 (human epidermal growth factor receptor 2), on the other hand, is a protein overexpressed in approximately 20–25% of breast cancer cases and is associated with aggressive tumour behaviour and poorer outcomes. Detecting CTCs that express CK and HER2 can help classify breast cancer subtypes, predict response to targeted therapies, and monitor disease progression or recurrence (Alix et al., 2023; Singhal et al., 2018; Andreou et al., 2019).

As there is a growing interest in exploring liquid biopsy technologies and their applications in precision oncology, our current case-control study is proposed to evaluate the diagnostic utility of peripheral blood CTC enumeration using flow cytometry in breast cancer patients. The study will assess CTCs marked by CK and HER2 expression in a cohort of breast cancer patients compared to healthy controls. In an era where personalized medicine and non-invasive diagnostics are at the forefront of cancer care, CTC detection represents a promising advancement and the results of the study will be a valuable addition to this evolving field.

2. MATERIALS AND METHODS

2.1 Study Design and Ethical Considerations

This prospective case-control study was conducted at the Department of Molecular Biology, Medical Oncology and Pathology Department of Aster MIMS Hospital in Calicut, Kerala, India, during the period of 2022 to 2024. The study protocol was reviewed and approved by the Institutional Ethics Committee of Aster MIMS Hospital (Study no: EC/30C2020). All participants provided written informed consent for the study. This study was conducted in full accordance with the principles of the Declaration of Helsinki, ensuring the ethical treatment of all participants, including respect for individual rights, safety, and well-being.

2.2 Sample size

The study included 120 participants, categorised into Group I (Cases, n=60) of histologically confirmed breast cancer patients with positive mammograms and biopsy confirmation and Group II (Controls, n=60), age-matched healthy females with no history of malignancy.

All the participants were undergoing screening or consulting the Oncology and Pathology Department of the study centre. The sample size calculation for this study was performed using the Kelsey, Fleiss, and Fleiss with continuity correction (CC) methods based on the previous studies of Zhu et al. (2022) and Vijay et al (2024) (Kelsey et al., 2010). The minimum sample size required for adequate statistical power (80%) ranged from 44 to 54 participants (cases + controls) to provide a more adequate statistical power, the study expanded the sample size to 60 cases and 60 controls.

Inclusion criteria:

Case definition: Sixty Female patients (≥18 years) consulting the Department of Medical Oncology and Pathology Department of Aster MIMS Hospital and later histologically confirmed breast cancer, positive mammogram and biopsy, and no prior oncological treatment.

Controls definition: Female patients (≥18 years) consulting the Department of Medical Oncology and Pathology Department of Aster MIMS Hospital with no diagnosis or history of malignancy, breast lesions, or any other inflammatory diseases.

Exclusion criteria:

Pregnant or lactating women and patients with autoimmune diseases or chronic infections that might influence CTC levels and cases undergoing pre-surgical neoadjuvant therapy were not included in the study.

Sample collection and analysis

Peripheral blood sampling

Peripheral blood samples (6mL) were obtained from controls and patients with breast cancer at the Department of Medical Oncology before surgical and oncological interventions at the Oncology Division of the study centre. Samples were collected under sterile conditions using EDTA-coated tubes to prevent clotting and processed within 2 hours of collection. If any delay in processing is anticipated, samples were stored at 4°C and analysed within 6 hours.

Buffy Coat Preparation and Staining Protocol

Whole blood (6 mL) was collected in EDTA tubes and mixed with 3 mL of HistoPrep®, followed by centrifugation at 2000 RPM for 20 minutes. The buffy coat layer, rich in lymphocytes and potential circulating tumour cells (CTCs), was carefully extracted and washed three times with phosphate-buffered saline (PBS). The final pellet was resuspended in PBS. For flowcytometry each tube received 20 µL of PerCP-conjugated antibody and 5 µL of HER2-APC-conjugated antibody, along with 100 µL of the respective sample. Samples were gently vortexed and incubated in the dark for 20 minutes.

Tissue sample collection

Biopsy samples were collected from breast cancer patients using core-needle biopsy. Histological grading was performed based on the Scarff-Bloom-Richardson grading system, and tumor classification was done using the AJCC TNM system. All 60 breast cancer patients in Group I (Case) had positive mammograms and biopsy confirmations.

Circulatory tumour cell (CTC) isolation and characterisation

Flow cytometry for CTC

Multiple flow cytometric analysis was performed using a BD FACSCanto II (BD Biosciences) equipped with FACSDiva software. The markers used are listed in table 1.

Table 1: Fluorescently labelled antibodies used for identifying and characterizing circulating tumour cells (CTCs) by flow cytometry. The table includes the specific clones, associated fluorophores, and the working volumes per sample.

Marker	Clone	Fluorophore	Volume
Cytokeratin (CK)	AE1/AE3	FITC (Fluorescent isothiocyanate)	20µL
CD45	HI30	APC (Allophycocyanin)	20µL
HER2/neu	24D2	PerCP (Peridinin-chlorophyll-protein)	5µL

*A minimum of 100,000 events per sample were analyzed

Statistical Analysis

All statistical analyses were performed using SPSS 14 software. Descriptive statistics were reported in, Mean ± standard deviation (SD) for normally distributed data, and for categorical variables, frequency and percentage distributions were used. A two-tailed p-value < 0.05 was considered statistically significant. Chi-square tests were used for categorical variables. Mann-Whitney U tests were used to compare different groups.

3. RESULT

A total of 120 participants were included in the study, consisting of age-matched 60 breast cancer patients (cases) and 60 healthy controls. Among cancer patients, 31 cases had primary tumours in the left breast, 28 cases in the right breast, and 1 case had tumours in both breasts. Metastasis was present in 7 cases, while 53 patients were non-metastatic. The TNM stage distribution revealed 9 patients in stage I, 24 in stage II, 22 in stage III, and 1 patient each in stage IV and stage V, while 3 cases had unspecified stages. The histological types of breast cancer included invasive carcinoma (NST) (n = 50), ductal carcinoma in situ (DCIS) (n = 3), invasive lobular carcinoma (n = 3), papillary carcinoma (n = 3), and mixed invasive carcinoma (n = 1). Lymph node involvement was observed in 25 out of 60 patients (41.6%) (See Table 3 and Figures 1 to 5).

	Case (N=60)	Control (N=60)	
Age	54.3 ± 9.5	52.1 ± 7.8	
CTC count (CK+ cells)	225 ± 85	3 ± 12	p < 0.001
HER2 +CTC count	2.77 ± 1.5	0	p < 0.001

Table 3: Represents the clinical and histopathological characteristics of cases and controls. The average number of CTCs positive for cytokeratin (CK+) was 225, with a range from 107 to 951. In the control, the average number of CK+ CTCs was 3, with a range of 0 to 76, and the average number of HER2-positive cells per patient was 2.77± 1.5, with a range of 1 to 7.

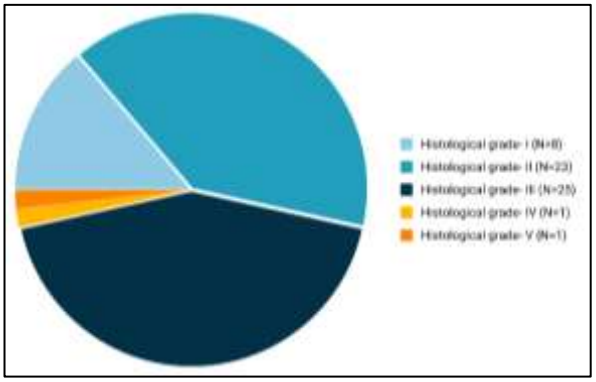

Figure 5: Shows that the majority of tumors were Grade II (38.33%) and Grade III (41.67%), indicating moderate-to-high tumor aggression. Two patients' staging data were not recorded.

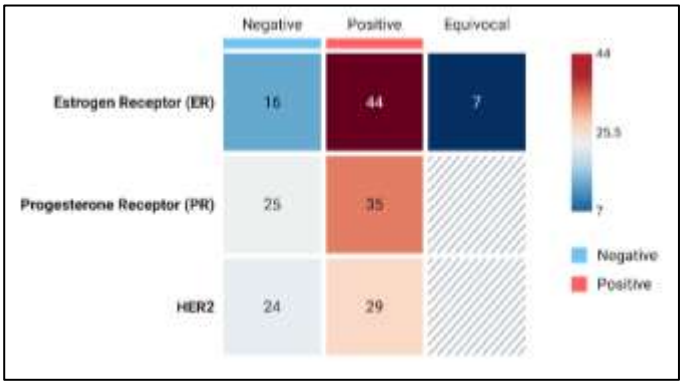

Figure 6: Heatmap showing the distribution of hormonal marker expression (HER2, ER, PR) in breast cancer patients. The expression status is categorised as Positive, Negative, or Equivocal (HER2 only). Colour intensity reflects the number of patients in each category, with darker shades indicating higher frequencies.

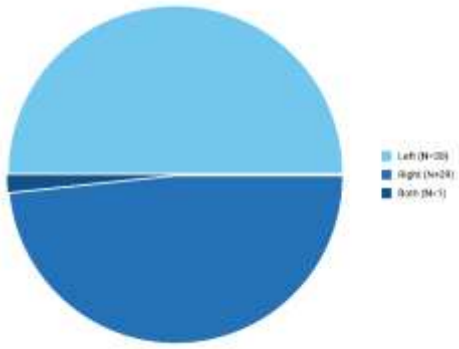

Figure 1: Showing almost equal breast involvement distribution the left (50%, N=30) and right (48.33%, N=29) breasts, with one patient (1.67%, N=1) having bilateral breast cancer.

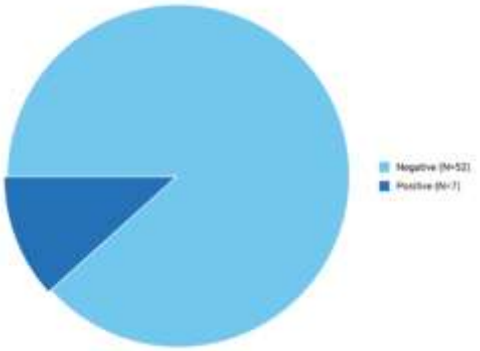

Figure 2: Represents metastasis in 11.67% (N=7) of cases, while 88.33% remained non-metastatic at the time of sample collection.

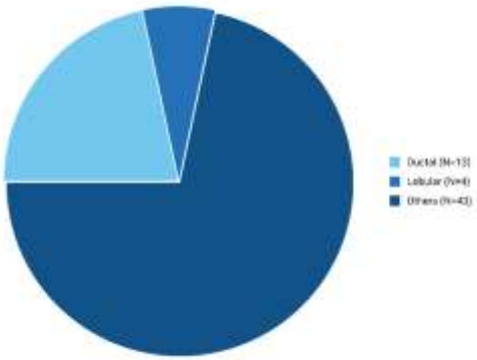

Figure 3: Showing histological subtypes of breast cancer in cases with invasive carcinoma was the predominant subtype (76.67%), while ductal carcinoma accounted for 21.67% of cases.

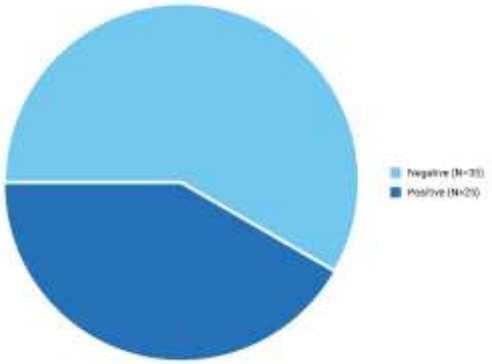

Figure 4: Shows that that lymphovascular invasion distribution was present in 41.67% of cases.

Hormonal Marker Profile

Immunohistochemistry (IHC) was performed on all breast cancer tissue samples to assess the expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2/neu). Of the 60 patients, 29 were HER2-positive, 24 were HER2-negative, and 7 had equivocal HER2 results. ER was positive in 44 cases and PR in 35 cases. Negative ER and PR expression were observed in 16 and 25 cases, respectively. HER2 equivocal cases were further evaluated using fluorescence in situ hybridization (FISH), which confirmed 4 as HER2-positive and 3 as HER2-negative (See Figure 6).

Circulating Tumor Cell (CTC) Detection by Flow Cytometry

Circulating tumour cells were isolated from peripheral blood using density gradient centrifugation followed by immunofluorescent staining and flow cytometric analysis. CTCs were defined as cells expressing cytokeratin (CK^+), negative for leukocyte marker CD45 ($CD45^-$), and, where applicable, HER2-positive ($HER2^+$). Among the breast cancer group, 85% of patients had detectable CK^+ CTCs, with counts ranging from 107 to 951 and an average count of 225± 85 cells. In contrast, healthy controls showed minimal CK^+ cells, with an average of 3± 12 cells (range 7–76), and none tested positive for HER2 or CK above diagnostic thresholds. $HER2^+$ CTCs were identified in 31 out of 60 breast cancer patients (60.67%), with a mean $HER2^+$ cell count of 2.77± 1.5 (range 1–7). Notably, no healthy individual showed HER2 expression. Flow cytometric plots demonstrate differential expression patterns of CD45, CK, and HER2 between cases and controls (see Figure 7).

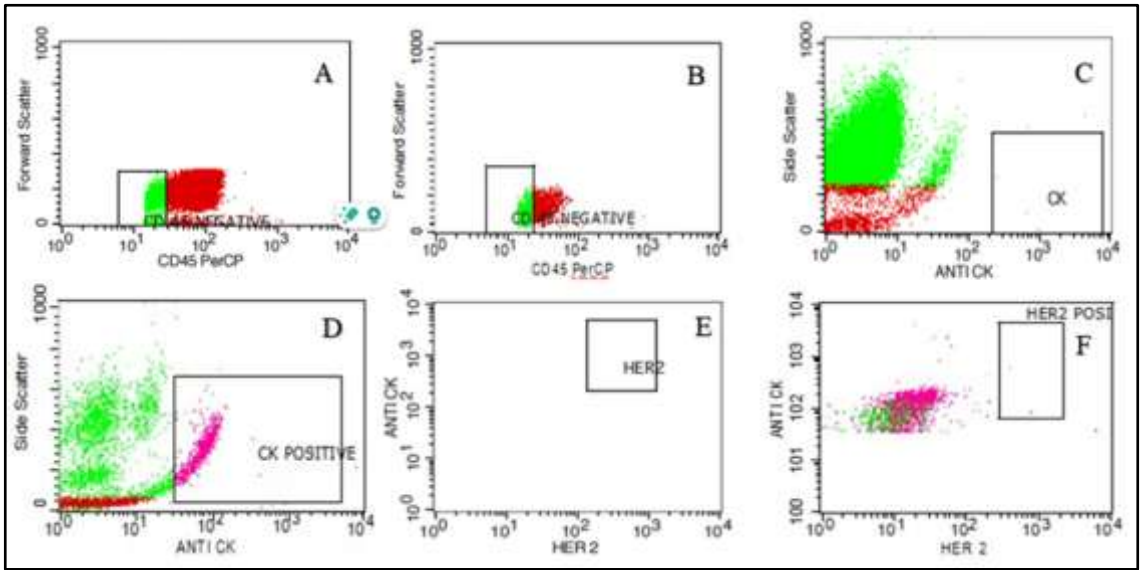

Figure 7. Flow cytometric analysis of CD45, CK, and HER2 expression in peripheral blood mononuclear cells (PBMCs) from healthy and breast cancer patients. (A-B) CD45 expression in PBMCs from healthy (A) and breast cancer (B) patients [allophycocyanin-conjugated CD45–Per CP]. (C-D) Cytokeratin (CK) expression in PBMCs from healthy (C) and breast cancer (D) patients [fluorescent isothiocyanate-conjugated CK FITC]. (E-F) HER2 expression in PBMCs from healthy (E) and breast cancer (F) patients [peridinin-chlorophyll-protein – conjugated Her2 -APC]. Isotype controls were used for background staining. PerCP, peridinin chlorophyll protein; FITC, fluorescein isothiocyanate; APC, allophycocyanin.

Diagnostic Performance Analysis of CK and HER2 CTCs

To evaluate the diagnostic utility of CTC markers, cutoff values for CK^+ cells were analyzed. A CK threshold of 107 cells yielded 100% sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV), confirming its high diagnostic accuracy. Lower thresholds (e.g., 76 cells) still maintained excellent sensitivity and specificity (See Figure 8). The complete diagnostic performance across various CK cutoffs is provided in Table 3. For $HER2^+$ CTCs, a cutoff value of ≥1 cell achieved a sensitivity of 51.67%, with both PPV and NPV at 100%, indicating that although HER2 is highly specific, its sensitivity is limited (Table 4).

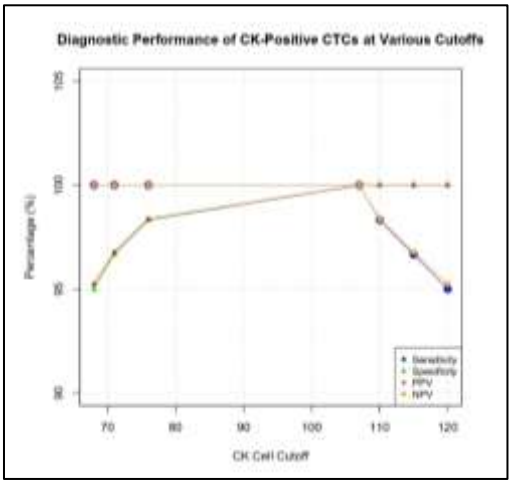

Figure 8: The line plot showing the diagnostic performance of CK-positive CTCs at various cutoff values. At 107 CK cells, all metrics reach 100%, confirming it as the optimal cutoff. As the cutoff increases beyond 107, sensitivity and NPV gradually decrease. Specificity and PPV remain consistently high (100%) from 107 onward.

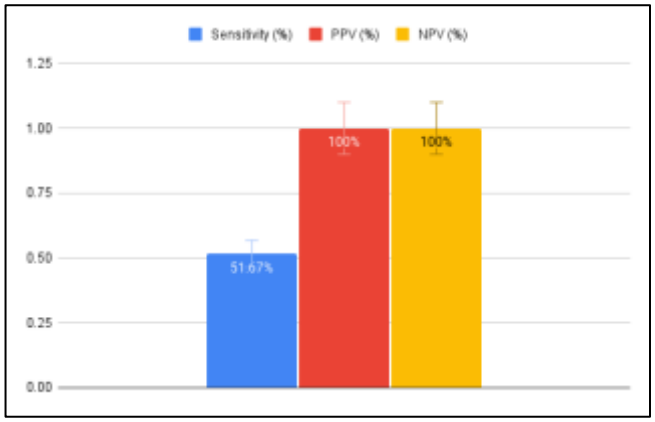

Figure 9: The bar chart visualizing the diagnostic performance of HER2-positive CTCs at a cutoff of ≥1 cell. Sensitivity is moderate at 51.67%, indicating that $HER2^+$ CTCs are detected in about half of true breast cancer cases. PPV and NPV are both 100%, highlighting that when $HER2^+$ CTCs are detected, they are highly predictive of cancer, and their absence effectively rules it out.

Statistical comparison between breast cancer patients and controls was performed using the Mann-Whitney U test. Results demonstrated statistically significant differences for both CK and HER2 CTC counts ($p < 0.001$), as shown in Table 5.

Receiver operating characteristic (ROC) curve analysis further validated the diagnostic value of these markers. The area under the ROC curve (AUC) for CK was 1.0, indicating perfect discrimination between breast cancer and control groups. HER2 yielded an AUC of 0.758, supporting its specificity but highlighting limited sensitivity. An additional derived ratio variable showed an AUC of 0.719. Complete AUC values are presented in Table 6. The ROC coordinate analysis provided optimal cutoff points for clinical application. For CK cells, the ideal diagnostic threshold was identified at 91.5 cells, providing 100% sensitivity and 0% false positives. For HER2, diagnostic sensitivity decreased progressively at higher thresholds. Full ROC coordinates are detailed in Table 7.

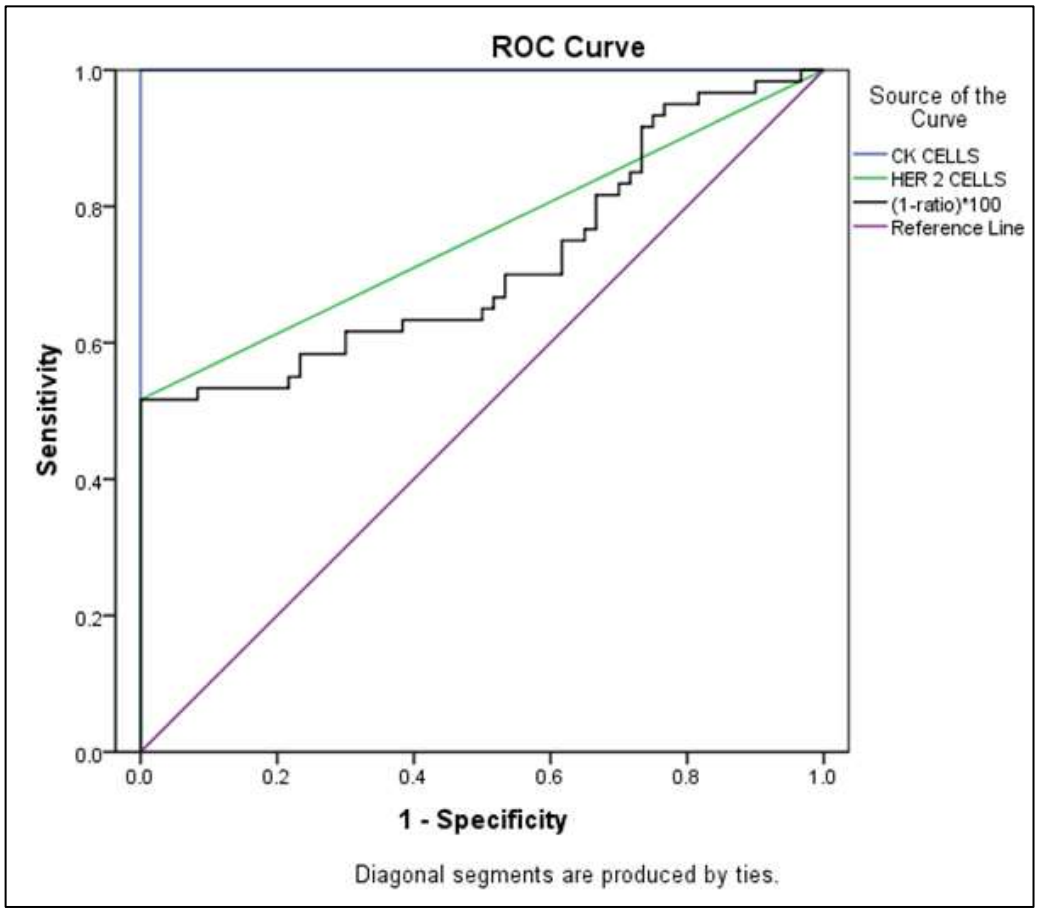

Figure 9. ROC curves for CK and HER2 Circulating Tumor Cells (CTCs). Receiver Operating Characteristic (ROC) curves were generated to assess the diagnostic performance of CK^+ and $HER2^+$ CTCs in differentiating breast cancer patients from healthy controls. The area under the curve (AUC) for CK^+ cells was 1.0, indicating perfect discrimination. The optimal cutoff value for CK CTCs, determined by the ROC curve, was 90 cells. $HER2^+$ CTCs showed a moderate diagnostic profile, with an AUC of 0.758. The reference line represents a non-informative classifier (AUC = 0.5). These findings were supported by the Mann-Whitney U test, which confirmed statistically significant differences in CTC counts between groups ($p < 0.001$ for both CK and HER2

4. DISCUSSION

CTC enumeration has been widely studied as a biomarker for breast cancer progression and prognosis. In our study, 85% of breast cancer patients had CK+ CTC in the sample, compared to a significantly lower count in healthy controls. The high specificity and sensitivity observed in our study further validate CK+ CTCs as a reliable biomarker for early disease detection. Moreover, HER2-positive CTCs were detected in 51.6% of the cases and were not detected in healthy controls.

In the previous studies of Xenidis et al. (2013), Franken et al. (2012), and Bidard et al. (2012), they detected CTCs in 43.5%, 19 %, and 23% of the cases, respectively. These studies also concur with our findings on the efficacy of CTCs as an indicator of treatment and are associated with a favourable clinical outcome of patients with detectable CTCs before chemotherapy.

The HER2 status is another critical factor in determining the prognosis and treatment options for breast cancer (Gupta et al., 2022). In our study, 51.67% of the patients had HER2-positive circulating tumour cells (CTCs). Among the HER2-positive patients, 96.77% had invasive carcinoma, and 50% showed signs of lymphovascular invasion. These findings suggest a correlation between HER2 positivity and disease aggressiveness. Notably, HER2-positive CTCs were not detected in healthy controls. This heterogeneity is also observed in other studies, where they also reported the resistance of these cases to treatment options (Costanza et al., 2015; Nitta et al., 2016).

In clinical practice, HER2-positive CTCs could be useful for identifying patients with HER2-positive breast cancer, which is known to respond well to HER2-targeted therapies such as trastuzumab and pertuzumab (Xu et al., 2024; Jagosky et al., 2021). However, due to the moderate sensitivity observed in our study (51.67%), HER2-positive CTCs may not be as effective in early detection or monitoring compared to CK-positive CTCs. Further optimization of detection methods or combination with other biomarkers will improve the clinical application of HER2-positive CTCs.

The study findings provide strong evidence for the diagnostic utility of CK-positive CTCs and HER2-positive CTCs in breast cancer. Along with these certain other limitations of our study also need to be addressed. Although we included 120 participants, a larger cohort would provide a more robust assessment of the sensitivity and specificity of CTCs in various subtypes of breast cancer, especially those with less aggressive characteristics. Larger sample sizes could also help validate the cutoff thresholds for CK-positive CTCs and HER2-positive CTCs across different populations. Moreover, this is a single central study which may limit the generalizability of the results to other geographical regions or healthcare settings. Multi-centre studies with diverse patient populations are needed in future. In addition to this, we have employed a cross-sectional study design, which limits our understanding of the patients, longitudinal studies are needed to evaluate the role of CTCs in monitoring treatment response and detecting minimal residual disease after surgery or chemotherapy.

The detection of CTCs using flow cytometry is highly dependent on technical factors such as sample quality and processing time. Variations in these factors may affect the accuracy and reproducibility of results. Future research should focus on addressing the above-discussed limitations and also consider optimizing CTC isolation and detection methods to minimize variability and improve consistency.

The findings of this study pave the way for future investigations aimed at optimizing CTC detection methods and expanding their clinical applications in breast cancer management. The study contributes valuable data that will support multicenter trials, longitudinal monitoring, and the development of multi-biomarker panels to improve the early detection, monitoring, and treatment of breast cancer. Additionally, understanding the biological role of CTCs and tumor progression mechanisms will enable the development of novel therapeutic strategies targeting circulating tumour cells, ultimately improving patient outcomes and prognosis.

5. CONCLUSION

Circulating tumour cells (CTCs) are potential non-invasive biomarkers for the detection and characterisation of breast cancer. Our findings support the integration of liquid biopsy techniques—CTC enumeration into routine breast cancer diagnostics, offering a minimally invasive alternative for early detection, prognostic assessment, and personalized therapy selection. These advancements hold significant potential for improving patient outcomes and facilitating precision oncology in breast cancer management.

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Author declaration statement

All the Authors have read and approved the final version of the manuscript. They warrant that the article is the author's original work, hasn't received prior publication and isn't under consideration for publication

Credit authorship contribution statement

Ramya Ravindran: Conceptualization; Data curation; writing –original draft; formal analysis.

Ramadas Naik: Conceptualization, writing-review, editing, and formal analysis.

Kavitha KP: Writing- review, editing, formal analysis.

Vipin Viswanath: Data curation; Writing –review, editing.

Conflict of interest

The authors state that there is no conflict of interest

Ethics approval

The study was approved by the Institutional Ethics Committee of MIMS, Kerala (IEC Reg. EC/NEW/INST/2019/406 & ECR/301/Inst/KL/2013/RR-19).

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