Circulating Tumor Cells may be an Early Diagnostic Marker for Disease Progression in Patients with Breast Carcinoma

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ABSTRACT

Background: Cancer affects millions of people around the world. In Pakistan, breast cancer (BC) is the leading cause of death in women. Once the tumor has established itself, tumor cells shed and enter the blood circulation to metastasize to distant areas of the body. These cells are known as circulating tumor cells (CTCs) and their presence in cancer patients has been documented. The current study was designed to determine CTCs in BC patients.

Methods: This was a descriptive cross-sectional study that consisted of 80 newly diagnosed females with BC. Patients were allotted different stages according to the American Joint Committee on Cancer (AJCC) staging system. Stage I comprised 11 subjects, stage II had 30, stage III 31 and stage IV included 8 subjects. After writing informed consent, 3 ml anticoagulated whole blood was collected from each patient, and transported to the Department of Immunology, University of Health Sciences (UHS), Lahore. CTCs were stained with CD45, CD326, and anticytokeratins 8, 18,

19. CTCs were analyzed by FACS calibur flow cytometer (BD) using BD Cell Quest Pro software. Data was entered and analyzed using SPSS 20.0.

Results: Twenty-three (28.7%) patients had more than 5 CTCs, while 57 (71.3%) had less than 5 CTCs in their blood samples. Mean ± SD of CTCs was high in stage IV of BC (8±7.92) as compared to stage I (1±1.67), stage II (2±2.91) and stage III (4±4.88) and on comparison there was a statistically significant difference between stage I and IV (p=0.002) and between stage II and IV (p=0.003). Mean ± SD of CTCs was high in patients with metastatic disease (8±7.92) as compared to non-metastatic disease (3±3.99) and on comparison the difference was statistically significant (p= 0.002). Mean ± SD of CTCs was high (5±5.89) in patients with family history of carcinomas including BC and other solid tumors compared to patients who did not have family history of cancer (2±3.36) and on comparison, the difference between these groups was statistically significant (p=0.016).

Conclusions: CTCs were higher in stage IV as compared to other stages of BC. CTCs were high in patients with a positive family history of BC compared to subjects without family history of BC. CTCs were higher in patients with metastatic disease as compared to non-metastatic disease.

Keywords: Circulating Tumor Cells (CTCs), Metastatic Breast Cancer (MBC); EpCAM (Epithelial Cell Adhesion Molecule), CK (Cytokeratin)

1. INTRODUCTION

Cancer is defined as an uncontrolled division of abnormal cells in any part of the body. These cancerous growths mostly invade the surrounding tissue and can metastasize to distant sites (1). Cancer is the leading cause of death worldwide. In 2019, global cancer reports suggested approximately 1,762,450 new cases of cancer and 606,880 cancer-related deaths (2). In women, commonly diagnosed cancers include breast cancer (BC), lung cancer, and colorectal cancer, while BC alone accounts for 30% of new cancer cases (2). Worldwide, among women, BC is the most prevalent cancer (3). Pakistan has the highest rate of BC in Asia, where one in every nine Pakistani women is affected by BC (4). BC is of grave concern in Pakistan, as its frequency is alarming, and its mortality rate is the highest among the Asian population (5). In Pakistan, in 2014, BC alone was responsible for 30.8% of all female deaths, whereas mouth and oropharyngeal

cancers caused 7.9%, cervical uterine cancer 5.5%, ovarian cancer 5.1%, and esophageal cancer 4.8% of deaths (6).

During the early process of tumorigenesis in breast tissues, some cells detach from the primary tumor and enter the blood circulation (7). These cells are of epithelial origin, and they have special characteristics resembling the primary tumor cells from which they have been detached (8). These cells are known as circulating tumor cells (CTCs), which have been found in patients with both localized and advanced stages of tumors (7).

A CTC is a cell that has a round or oval shape, a nucleus surrounded by cytoplasm, but lacks the expression of CD45 (lymphocyte common antigen). The average cell size of a CTC varies from 4 μm to ⩾30 μm (10). CTCs can be detected in the peripheral blood of BC patients (9). The diameter of CTCs originating from prostate cancer may be more than 100 μm, while those from small lung cancer may be only 10 μm, whereas BC CTCs may exceed 70 μm (11).

Metastasis of a malignant tumor takes place when it becomes motile and invasive (12). During metastasis, tumor cells, either singly or in the form of clusters, detach from the primary tumor or penetrate the surrounding tissue to enter or extravasate into the blood and/or lymphatic channels (13). Millions of CTCs are continuously released from the primary tumor, but their rate of release is still unknown. Only a few tumor cells escape immune surveillance and systemic therapy to reach secondary organs and develop metastasis (14, 15). CTCs start to appear in the early stages of tumor progression, and later they may settle in distant organs of the body. This colonization leads to metastasis. Liquid biopsy can be used to assess tumor invasiveness and its response to the given treatment (16). CTCs derived from epithelial neoplasms can be identified by the expression of EpCAM (Epithelial Cell Adhesion Molecule) and CK (Cytokeratin) (17). The expression of EpCAM and CK is used as biomarkers for CTC identification in the epithelial phase (18, 19).

Detection of CTCs in the blood is a less invasive procedure compared to incisional or excisional biopsies. It is an early predictive biomarker that can help clinicians in selecting the best possible treatment for patients and therefore minimizing the risks of advanced stages of the disease (20).

Numbers of CTC vary with different histological grades, stages, and invasiveness of BC. The numbers of CTCs are also affected by different therapies. Multiple trials have reported that variation in the numbers of CTCs during treatment plays an important role in predicting the outcome of the treatment, thus signifying the importance of CTCs in predicting the progression of the disease (20).

In metastatic BC (MBC) patients, a change in the level of CTC in response to certain treatments provides prognostic information. Hayes et al. (21) found that reduced CTC counts in MBC patients after initial therapy were associated with a prognosis similar to that of CTC-negative patients. Pachmann et al. (22) confirmed that treatment had an effect on CTC counts. The higher levels of CTCs in non-metastatic primary BC patients declined after treatment and were associated with a better prognosis compared to those patients who did not respond to the therapy and showed no change in CTC counts. Pierga et al. (23) found a significant correlation between CTCs before neoadjuvant therapy and reduced disease-free survival (DFS), but no correlation was found between the persistence of CTCs post-neoadjuvant therapy and tumor response. A correlation between the changes in the number of CTCs and an objective response to therapy, as assessed by serial imaging, was reported by Nakamura et al. (24).

Riethdorf et al. (25) showed no correlation between the presence of CTCs before neoadjuvant therapy and the tumor's response to certain therapies, nor did the changes in CTCs indicate the effectiveness of treatment. Another study suggested that the presence of CTCs plays a role in predicting local and distant tumor recurrences but had no correlation with the reduction in primary tumor size (26).

There are a few ongoing clinical trials, such as the “STIC CTC” trial (NCT01710605) and the “CirCe01” trial (NCT01349842), aimed at examining the utility of CTCs in BC patients and their response to treatment. Results from clinical trials on CTCs are still insufficient to guide their use; therefore, further studies are needed to determine their role in improving anticancer treatment strategies (27). Although a few studies have estimated the number of CTCs (7, 25, 26), their results remain controversial. Therefore, this study was planned to enumerate the levels of CTCs in female BC patients, which may help in understanding their role in BC progression and treatment.

2. MATERIAL AND METHODS

This was a descriptive cross-sectional study that included 80 female BC patients newly diagnosed based on tissue biopsy. BC patients undergoing anticancer therapy and patients with cancers other than breast cancer were excluded. According to the American Joint Committee on Cancer (AJCC) staging system, patients were categorized into different stages (28). Stage I included 11 subjects, Stage II had 30, Stage III consisted of 31, and Stage IV comprised 8 subjects. After obtaining written informed consent, 3 mL of whole blood was collected from each patient in an anticoagulant vial and transported to the Department of Immunology, University of Health Sciences (UHS), Lahore.

Histopaque (Sigma-Aldrich, USA) was used to isolate PBMCs from whole EDTA-treated blood. After PBMC isolation, cell surface staining of CTCs was performed. A 100-µL sample of the mononuclear cell suspension was added into each tube (test sample and blank). In tube 1, 10 µL of CD326 PE, CD45 PerCP, and anticytokeratins 8, 18, and 19 FITC (Miltenyi Biotec Inc., Lindbergh Street, Auburn, CA, USA) were added, whereas no antibody was added to tube 2. To avoid erythrocyte contamination, 2 mL of BD FACS lysing solution was added to lyse red blood cells. The pellet was re-suspended in 500 µL of sheath fluid and immediately analyzed using a BD FACS Calibur flow cytometer (BD PharmingenTM, USA).

2.1. Flow Cytometric Analysis

Cells were analyzed for the phenotypic expressions by *FACS CALIBUR* (BD USA) flow cytometer using Cell Quest Pro software and 1000,000 events were acquired.

To enumerate CTCs, mononuclear cells expected to contain CTCs population were selected by drawing gate (R1) on the FSC and SCC dot plot (Figure 2- A). The gated population was further analyzed on dot plots with CD45 on x-axis and CD 326 on Y-axis. A second gate was drawn on the population with positive expression of CD326 (negative for CD45) (Figure 2 –B). The second gated population (R2) was next analyzed with Anticytokeratins 8, 18, 19 on the x-axis and CD 326 on the Y-axis (Figure 2-C). Cells showing double positive expressions for both CD326 and

Anticytokeratins 8, 18, 19 were counted by quadrant analyzer and expressed as number of events.

Data was entered and analyzed using SPSS 20.0 version. Mean ± SD was used for quantitative variables. Qualitative variables were expressed as frequencies, percentages, and graphs. Student *t*-test was used to compare CTCs based on metastases and family history. One-way Anova and post hoc Tukey test was used to compare CTCs among different stages of disease. A *p*-value of ≤ 0.05 was considered as statistically significant.

3. RESULTS

The characteristics of the selected subjects are shown in Table 1. The mean ± SD age of the subjects was 45.94 ± 12.1 years, while the mean ± SD of CTCs was 3.37 ± 4.75. Twenty-three patients had greater than 5 CTCs, while 57 patients had less than 5 CTCs. The family history of carcinoma, including breast and other solid tumors, was reported in 34 patients. According to AJCC (Hortobagyi et al., 2017), patients were allotted different stages based on severity of their disease. Stage I comprised of 11, stage II had 30, stage III had 31 and stage IV included 8 patients. Metastases was reported in 8 out of 80 patients.

As shown in Table 2 a higher mean ± SD of CTCs was observed in patients with a family history of BC (5 ± 5.89) compared to those without a family history of BC (2 ± 3.36), and the difference was statistically significant (p = 0.016). A statistically significant difference was also observed among different stages of the disease (p = 0.001). The mean ± SD of CTCs was highest in stage IV (8 ± 7.92) of BC patients compared to stage I (1 ± 1.67), stage II (2 ± 2.91), and stage III (4 ± 4.88). Significant differences were found between stage I and IV (p = 0.002) and stage II and IV (p = 0.003). A graphical representation of Circulating Tumor Cells in Different Stages of Disease is also shown in Figure 1 below. The mean ± SD of CTCs was also higher in patients with metastatic disease (8 ± 7.92) compared to non-metastatic disease (3 ± 3.99), and the difference was statistically significant (p = 0.002) (Table 2).

Table 1: Demographic Characteristics of Studied Population

Variables		**Values**
Age (mean±SD) years		45.94 ±12.0
Circulating Tumor Cells n (%),	<5	57 (71.3) / 3.37±4.75

mean±SD	≥5	23 (28.7)/ 3.37±4.75
Family History n (%)	Yes	34 (42.5)
	No	46(57.5)
Stage n (%)	I	11 (13.8)
	II	30 (37.5)
	III	31 (38.8)
	IV	8 (10)
Metastases n (%)	Yes	8(10)
	No	72 (90)

Table 2: Mean± SD and Comparison of Number of Circulating Tumor Cells, Family History, Stages of Breast Carcinoma and Metastasis among the Study Groups

Group Variable	Group	Mean ± SD of CTCs	*p*-value
Family History n=80	Yes (n=34)	5±5.89	0.016*[1]
	No (n=46)	2±3.36	
Stage	I	1±1.67	0.001*[1] 0.839[2] 0.085[3] 0.002*[4] 0.147[5] 0.003*[6] 0.120[7]
	II	2±2.91	
	III	4±4.88	
	IV	8±7.92	
Metastases	Yes (n=8)	8±7.92	0.002*[1]
	No (n=72)	3±3.99	

**Note: ***$p \leq 0.05$=statically significant

[1]Comparison among the Groups, [2]Comparison between Stage I and II, [3]Comparison between Stage I and III, [4]Comparison between Stage I and IV, [5] Comparison between Stage II and III, [6]Comparison between Stage II and IV, [7]Comparison between Stage III and IV.

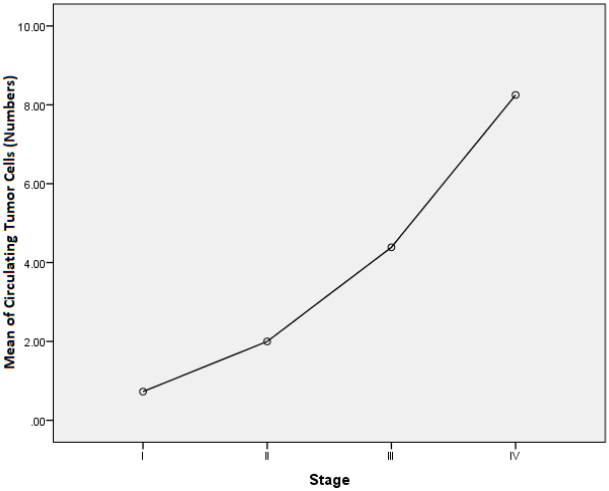

Figure 1: Graphical Representation of Circulating Tumor Cells in Different Stages of Disease

Graphical representation of circulating tumor cells in different stages of disease is given in Figure 1. Stages are plotted on x-axis whereas on y-axis means CTCs are shown. The trend above represents the increased number of CTCs from stage I to IV.

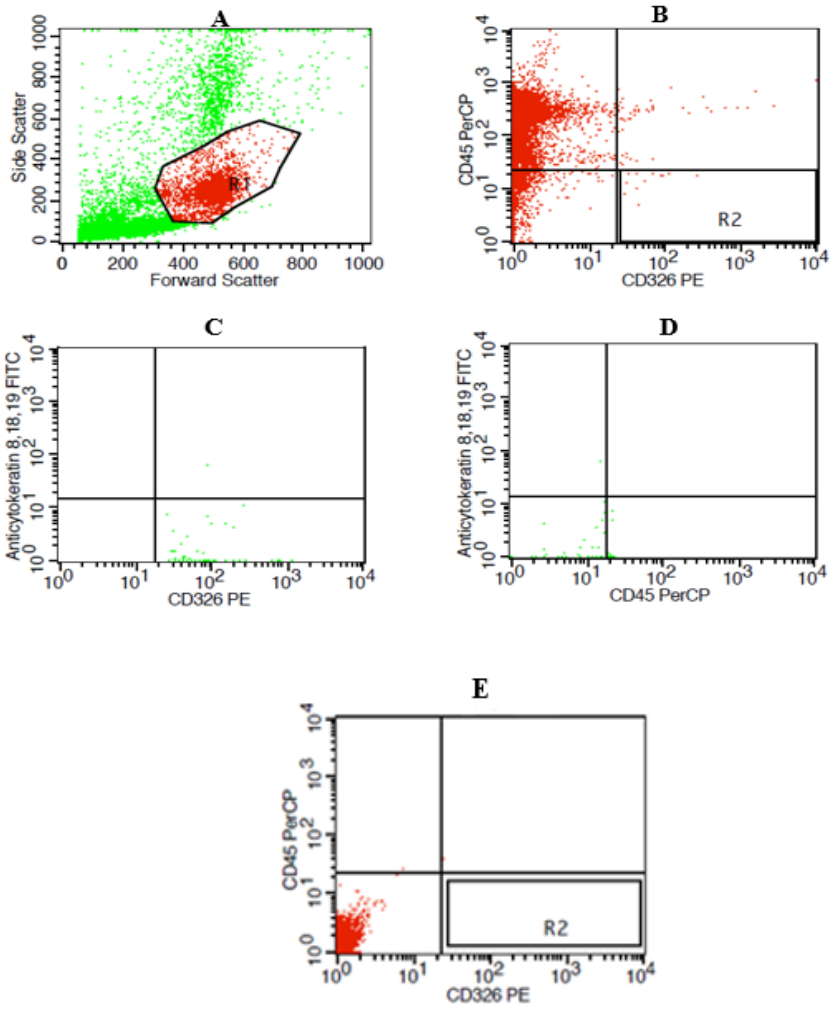

Figure 2: Identification of Circulating Tumor Cells

Dot-plot (Figure 2) represent five quadrant images observed by flow cytometric analysis**. Dot-Plot A:** The analysis demonstrated the physical properties of cells. GATE R1 represents mononuclear cells. **B:** R2 Gate was applied on CD326-positive cells that lacked CD45 expression. **C:** Cells with double-positive

(anticytokeratins and CD 326 positive) expression were selected from the second quadrant. **D:** The desired population was screened for anticytokeratin-positive expression. **E:** Sample blank.

4. DISCUSSION

High mean ± SD of CTCs was detected in subjects with a family history of cancer, including BC and other solid tumors (5±5.89), as compared to subjects without family history of cancer (2±3.36), and on comparison, there was a statistically significant difference between the groups (p= 0.016). This study agrees with Ried et al. (29), who detected CTCs in a total of 542 subjects that included 277 solid and blood cancer patients and 265 asymptomatic subjects. Asymptomatic participants had a family history of carcinoma, including solid and blood cancers (CA), and were enrolled to screen for CTCs. CTCs were present in all cancer patients, while 132 asymptomatic patients also had CTCs. These patients developed cancerous lesions within 0.5–10 months of follow-up. Cancer risk assessment was based on the number of CTCs/ml. They proposed a low CTC count, i.e., <3 CTC/ml, as being associated with a mild potential of malignancy, a moderate potential with 3–20 CTC/ml, and a high risk with >20 CTC/ml.

The findings of the current study are also in agreement with Ilie et al. (30), who detected CTCs in 3% of patients with chronic obstructive pulmonary disease. Within 1–4 years of CTC screening, these noncancerous patients developed lung cancer. The present study is in agreement with Castro et al. (31), who screened CTCs in 3,388 healthy people without any history of cancer. They detected 1–10 CTCs in 107 subjects, where 82% had a family history of cancer, 12% were heavy cigarette smokers, 4.5% had raised PSA levels, and 1.5% had chronic HCV or HBsAg infection. They concluded that CTCs can be detected in healthy people with an increased risk of developing cancer, and CTC screening can be important for early cancer detection.

In the current study, the mean ± SD of CTCs in BC with stage IV was higher as compared to other stages of BC, and on comparison, there was a statistically significant difference between stage IV and stage I (p= 0.002), and between stage II and stage IV (p= 0.003), which suggested an increased number of CTCs with the progression of BC. The findings are in accordance with Hu et al. (7), who suggested low levels (<5) of CTCs in BC patients in stage I, II, and

III and high levels (⩾5) in stage IV, while on comparison, there was a statistically significant difference (p= 0.033). The present study is also in agreement with Maestro et al. (32), who screened 438 subjects with localized and metastatic BC for CTCs. CTCs (⩾2/7.5ml of blood) were present in 61.5% of patients with metastatic tumors and 13% of patients with localized disease. On comparison, the difference between the two groups was statistically significant (p= <0.001). The present study does not align with Hofman et al. (33), who screened 208 patients (44% were in stage I, 25% in stage II, 28% in stage III, and 6% in stage IV) with non-small cell lung cancer (NSCLC). They found no correlation between CTCs and disease stages. The probable reason could be differences in patient selection criteria. The participants included in Hofman et al. (33) study were post-operative and/or on chemotherapy, whereas the current study comprised pretreatment subjects.

In the present study, the mean ± SD of CTCs was higher in patients with metastatic disease (8±7.92) as compared to those with non-metastatic disease (3±3.99), and on comparison, the difference was statistically significant (p= 0.002). The current study agrees with Hu et al. (7), who enumerated CTCs in 45 BC patients. They included 25 patients with metastasis and 20 patients without metastasis, and on comparison, there was a significant difference (p= 0.002). The present study is also in agreement with Nolé et al. (34), who detected CTCs in 80 metastatic BC patients and found 49 subjects with ⩾5 CTCs. The presence of ⩾ 5 CTCs was associated with bone metastases (p= 0.03). The present study is also in agreement with De Giorgi et al. (35), who included 195 metastatic BC patients. They reported elevated levels of CTCs in patients with bone metastases as compared to those without metastases (p= 0.012). The present study is also in agreement with Giuliano et al. (36), who enumerated CTCs in 492 pretreated advanced BC patients. A high number of CTCs was associated with metastatic sites, with a statistically significant difference (p= 0.007).

5. CONCLUSION

CTCs were higher in stage IV of BC as compared to other stages of BC and on comparison there was statistically significant difference between stage I and IV and between stage II and IV. CTCs were higher in patients with family history of BC compared to subjects without family history of BC and on comparison there was statistically significant difference between them.

CTCs were high in patients with metastatic disease as compared to non-metastatic disease and on comparison the difference was statistically significant.

6. LIMITATIONS

While our study provides compelling data on the diagnostic accuracy of VIA and Pap smear, it is limited by its single center design, which may not reflect all demographics in Pakistan. Furthermore, histopathology was used as the sole confirmatory method, which may limit generalizability, as VIA results could vary by examiner experience and lighting conditions.

REFERENCES

1. Tariq R, Huma S, Zaka Butt M, *et al*. Risk factors leading to high prevalence of breast cancer. Journal of Applied Pharmacy. 2013;63(8):1075-1078.
2. Siegel R, Miller K, Jemal A. Cancer statistics, 2019. CA: A Cancer Journal for Clinicians. 2019;69(1):7-34.
3. Ferlay J, Shin H, Bray F, *et al*. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. International Journal of Cancer. 2010;127(12):2893-2917.
4. Asif H, Sultana S, Akhtar N, *et al*. Prevalence, Risk Factors and Disease Knowledge of Breast Cancer in Pakistan. Asian Pacific Journal of Cancer Prevention. 2014;15(11):4411-4416.
5. Naveed S, Kamal A, Shamshad D, *et al*. Frequency and Risk Factors of Breast Cancer in Karachi, Pakistan. DHR International Journal of Medical Sciences. 2014;5(1).
6. Who.int. (2014). [online] Available at: https://www.who.int/cancer/country-profiles/pak_en.pdf [Accessed 2 Mar. 2019].
7. Hu Y, Fan L, Zheng J, *et al*. Detection of circulating tumor cells in breast cancer patients utilizing multiparameter flow cytometry and assessment of the prognosis of patients in different CTCs levels. Cytometry. Part A: The Journal of the International Society for Analytical Cytology. 2010;77(3): 213–219.

8. Ring A, Smith I and Dowsett M. Circulating tumour cells in breast cancer. The Lancet Oncology. 2004;5(2):79-88.
9. Cristofanilli M, Hayes DF, Budd GT, *et al*. Circulating tumor cells: A novel prognostic factor for newly diagnosed metastatic breast cancer. Journal of Clinical Oncology. 2005; 23(7): 1420–1430.
10. Allard WJ, Matera J, Miller MC, *et al*. Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with non-malignant diseases. Clinical Cancer Research. 2004;15: 6897-6904.
11. Harouaka R, Nisic M and Zheng S. Circulating Tumor Cell Enrichment Based on Physical Properties. Journal of Laboratory Automation. 2013;18(6): 455-468.
12. Hanahan D and Weinberg R. Hallmarks of Cancer: The Next Generation. Cell. 2011; 144(5): 646-674.
13. Joosse S, Gorges T and Pantel K. Biology, detection, and clinical implications of circulating tumor cells. EMBO Molecular Medicine. 2014;7(1):1-11.
14. AF, Groom AC and MacDonald IC. Dissemination and growth of cancer cells in metastatic sites. Nat Rev Cancer. 2002;2: 563–572.
15. Kang Y and Pantel K. Tumor cell dissemination: emerging biological insights from animal models and cancer patients. Cancer Cell. 2013;23: 573–581.
16. Khoo LB, Lee SC, Kumar P. *et al*. Expansion of circulating breast cancer cells predicts response to anti- cancer therapy. Oncotarget. 2015;6(17): 15578-15593.
17. Mikolajczyk DS, Millar LS, Tsinberg P. *et al*. Detection of EpCAM-Negative and Cytokeratin-Negative Circulating Tumor Cells in Peripheral Blood. Journal of Oncology. 2011;1-10.
18. Cohen S, Punt C, Iannotti N, *et al*. Relationship of Circulating Tumor Cells to Tumor Response, Progression-Free Survival, and Overall Survival in Patients with Metastatic Colorectal Cancer. Journal of Clinical Oncology. 2008;26(19): 3213-3221.
19. Giuliano, M, Giordano A, Jackson S, *et al*. Circulating tumor cells as prognostic and predictive markers in metastatic breast cancer patients

receiving first-line systemic treatment. Breast Cancer Research. 2011; 13(3): 67-80.

20. Harami SFM, Wisinski KB and Beebe DJ. Circulating Tumor Cells in Metastatic Breast Cancer: A Prognostic and Predictive Marker. J Patient Cent Res Rev 2015;1(2): 85–92.
21. Hayes DF, Cristofanilli M, Budd GT, *et al*. Circulating tumor cells at each follow- up time point during therapy of metastatic breast cancer patients predict progression-free and overall survival. Clin Cancer Res. 2006;12(14): 4218–4224.
22. Pachmann K, Camara O, Kavallaris A, *et al*. Monitoring the response of circulating epithelial tumor cells to adjuvant chemotherapy in breast cancer allows detection of patients at risk of early relapse. Journal of Clinical Oncology. 2008;26(8):1208–15.
23. Pierga, J.Y., Bidard, F.C., Mathiot, *et al.* Circulating Tumor Cell Detection Predicts Early Metastatic Relapse After Neoadjuvant Chemotherapy in Large Operable and Locally Advanced Breast Cancer in a PhaseII Randomized Trial. Clinical Cancer Research. 2008;14(21): 7004–7010.
24. Nakamura S, Yagata H, Ohno S, *et al*. Multi- center study evaluating circulating tumor cells as a surrogate for response to treatment and overall survival in metastatic breast cancer. Breast Cancer. 2010;17(3):199–204.
25. Riethdorf S, Müller V, Zhang L, *et al*. Detection and HER2 expression of circulating tumor cells: prospective monitoring in breast cancer patients treated in the neoadjuvant GeparQuattro trial. Clinical Cancer Research. 2010;16(9):2634–45.
26. Boutrus RR, Abi Raad RF, Kuter I, *et al*. Circulating tumor cells as predictors of response and failure in breast cancer patients treated with preoperative chemotherapy. Int J Biol Markers.2013;28(1):17–23.
27. Bidard F, Proudhon C, Pierga J. Circulating tumor cells in breast cancer. Molecular oncology. 2016;10(3): 418-430.
28. Hortobagyi G, Connolly J, D'Orsi C, *et al*. Breast. AJCC Cancer Staging Manual. 2017;589-636.

29. Ried K, Eng P. and Sali A. Screening for Circulating Tumour Cells Allows Early Detection of Cancer and Monitoring of Treatment Effectiveness: An Observational Study. Advances in Cancer Prevention. 2017;18(8): 2275–2285.
30. Ilie M, Hofman V, Long-Mira E, *et al*. "Sentinel" Circulating Tumor Cells Allow Early Diagnosis of Lung Cancer in Patients with Chronic Obstructive Pulmonary Disease. PLoS ONE. 2014;9(10):111597.
31. Castro J, Sanchez L, Nuñez M, *et al*. Screening Circulating Tumor Cells as a Noninvasive Cancer Test in 3388 Individuals from High-Risk Groups (ICELLATE2). Disease Markers.2018;2018:1-5.
32. Maestro LM, Sastre J, Rafael SB, *et al*. Circulating tumor cells in solid tumor in metastatic and localized stages. Anticancer research. 2009;29(11):4839-4843.
33. Hofman V, Ilie MI, Long E, et al. Detection of circulating tumor cells as a prognostic factor in patients undergoing radical surgery for non-small-cell lung carcinoma: comparison of the efficacy of the CellSearch Assay™and the isolation by size of epithelial tumor cell method. Int J Cancer. 2011;129(7):1651–1660
34. Nolé F, Munzone E, Zorzino L, *et al*. Variation of circulating tumor cell levels during treatment of metastatic breast cancer: prognostic and therapeutic implications. Annals of Oncology. 2008;19(5):891-897.
35. De Giorgi U, Valero V, Rohren, E, *et al*. Circulating tumor cells and bone metastases as detected by FDG-PET/CT in patients with metastatic breast cancer. Annals of Oncology. 2009;21(1): 33-39.
36. Giuliano M, Giordano A, Jackson S, *et al*. Circulating tumor cells as early predictors of metastatic spread in breast cancer patients with limited metastatic dissemination. Breast Cancer Research. 2014;16(5).