Circulating tumour cell enumeration does not correlate with Miller-Payne grade in a cohort of breast cancer patients undergoing neoadjuvant chemotherapy

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Abstract

Background: Detection and enumeration of Circulating Tumour Cells (CTCs) has been evaluated in many cancers such as breast cancer. However, the full prognostic and predictive power of CTCs for cancer cannot currently be harnessed, and the association between pathological complete response in patients receiving neoadjuvant chemotherapy for breast cancer and CTCs is still not clear. The aim of this study was to assess if CTCs could be used to predict pathological response to neoadjuvant chemotherapy in breast cancer patients. **Methods:** 26 patients were recruited, and blood samples taken pre- and post-neoadjuvant chemotherapy. CTCs were isolated using the ScreenCell device and stained using a modified Giemsa stain. CTCs were enumerated by 2 pathologists and classified as single CTCs, doublets clusters/microemboli. Counts were then correlated to the pathological response as measured by the Miller-Payne grading system. The associations between CTCs and clusters and pathological variables were evaluated with χ^2 or ANOVA tests performed in the SPSS 24.0 statistics software. **Results:** 89% of the patients had invasive ductal carcinoma and 11% invasive lobular carcinoma. At baseline 85% of patients had CTCs present and only 4 patients were CTC negative. Median baseline CTC count was 7 (0-161) CTCs per 3mls of whole blood. Post chemotherapy, 58% of the patients had an increase in CTCs. This change in CTC count did not correlate with the Miller Payne grade of response to chemotherapy. No significant association was identified between the number of CTCs and clinical characteristics, including patient age, receptor status, tumour grade, disease type, lymph node metastasis, lymphovascular space invasion, radiological response or clinical or pathological stage. However, we did observe a correlation between pre-treatment CTC counts and body mass index, $p < 0.05$. **Conclusions:** There was no correlation between the pre- and post-chemotherapy total number of CTCs/clusters and the Miller Payne grade. It is not enough to evaluate pathological response for neoadjuvant chemotherapy for breast cancer patients utilising CTCs identified by Giemsa staining alone. Additional characterisation is needed to further characterise CTCs isolated pre- and post-chemotherapy. Long-term follow-up of these patients will determine the significance of CTCs in breast cancer patients undergoing neoadjuvant chemotherapy.

Background

Breast cancer is a major public health issue globally, representing the most common cancer in women and one in ten of all newly diagnosed cancers. It is also the main cause of female cancer death globally, with 2,088,849 cases diagnosed in 2018 and 626,679 deaths [1]. Neoadjuvant chemotherapy (NAC) is now a standard treatment for breast cancer, often shrinking the tumour and allowing a less aggressive surgical approach for the patient [2]. 7-27% of new breast cancers are treated with NAC [3]. Between 10% and 40% of patients, depending on tumour subtype, receiving NAC can have a pathologic complete response (pCR) to chemotherapy [4] as determined by the Miller-Payne grading system [5].

While mortality has decreased dramatically due to earlier diagnosis and advances in treatment, metastatic disease represents the main cause of breast cancer-related morbidity and death [6], and

approximately 20% of breast cancers will experience metastatic relapse. Disease spread via circulating tumour cells (CTCs) in the bloodstream may explain how metastasis occurs [7, 8]. At present, enumeration of CTCs is limited in the clinical setting to predicting clinical outcome [9, 10]. However, future potential applications include their use for both determining and monitoring efficacy of personalised treatment, and predicting and detecting metastases [11, 12]. In order that the full prognostic and predictive power of CTCs is realised, there are a number of issues to examine in terms of limitations in how CTCs are defined, detected and isolated [9], as EpCAM-based detection excludes what is widely considered the most clinically relevant subsets of CTCs. The gold standard CellSearch™ for CTC identification approved by U.S. Food and Drug Administration (FDA) uses fluorescently labelled cytokeratin monoclonal antibodies, the nuclear stain DAPI, and the absence of staining by CD45 (pan-leukocyte stain), resulting in the overall selection of EpCAM⁺, CD8⁺, CD18⁺, CD45⁻ cells [9]. A major disadvantage of a number of CTC detection techniques is that they are dependent on capture based on epithelial marker expression, i.e. EpCAM and cytokeratins [13]. EpCAM is not a universal CTC biomarker [14] and therefore, detection is limited if expression has been downregulated due to epithelial mesenchymal transition (EMT) [15], while circulating tumour cells of mesenchymal origin are also not captured [16]. It is well recognised that CTCs are heterogeneous, with certain subgroups of CTCs harbouring higher metastatic potential. CTC clusters/microemboli have been associated with a worse clinical outcome in breast and lung cancer [17-19]. Current technologies underestimate CTC clusters because few specialized devices exist for the detection of CTC clusters and microemboli [20, 21].

The relationship between the presence of CTCs in the circulation and the response to NAC is currently an area of interest. If CTCs had the ability to predict those patients that have a complete pathological response, this could have a major impact on breast cancer treatment. A recent meta-analysis looking at the utility of CTCs assessed using the CellSearch™ system in non-metastatic breast cancer patients receiving neoadjuvant chemotherapy did not find any correlation with CTC count and response to chemotherapy [22]. A second meta-analysis concluded that CTC count as measured by multiple devices had utility in predicting therapy response in breast cancer [23]. The isolation technologies and characterisation of CTCs is clearly recognised as a limitation in these studies.

This current study focussed on evaluation of all physical forms of CTCs using a non-marker based approach, ScreenCell (Paris, France), pre- and post-neoadjuvant chemotherapy for breast cancer in order to ascertain the utility of CTCs to predict response to neoadjuvant chemotherapy. The ScreenCell size-selective method takes advantage of the larger size of CTCs compared with nucleated blood cells for isolation, with its circular pores of $7.5 \pm 0.36 \mu\text{m}$ randomly distributed throughout the filter with a pore density of 1×10^5 pores/cm² [24]. It avoids the bias introduced by antibodies, and false negatives/positives associated with these methods. Response to chemotherapy was assessed using the Miller-Payne grading system [5] and radiological assessment.

Methods

Patient Recruitment

Blood samples were obtained from 26 patients undergoing treatment for breast cancer at St. James's Hospital, Dublin 8, Ireland between 2015-2016. All patients received informed consent, and the study was approved by the St James's and AMNCH (Adelaide and Meath incorporating the National Children's Hospital) research ethics committee. Patients with a preoperative indication at multidisciplinary team discussion for neoadjuvant chemotherapy followed by surgery were recruited. Patients with Stage 4 disease were excluded. A 3ml blood sample was taken from each patient prior to the initiation of chemotherapy, and following completion of chemotherapy but prior to surgery. Clinicopathological data was collected for each patient including patient age, body mass index (BMI), receptor status, tumour grade, lymphovascular space invasion (LVI), clinical and pathological stage (TNM status) and pathological response (Miller-Payne grade) to chemotherapy. An Allred score was used to determine oestrogen and progesterone status. The Abbott Vysis system was used to assess HER2 status. The molecular subtype was recorded but a limitation of this is the ki-67 is not routinely done in our centre so the differentiation between luminal A and B was not always possible. The characteristics of these patients are shown in Table 1. In addition, a number of other blood parameters assessed as part of the routine clinical care including CA153 (if relevant), haemoglobin, haematocrit, white cell count and platelet count were recorded for analysis.

Blood Processing

Patient blood samples were obtained and stored in K₂ EDTA tubes at 4°C. 3 mls of blood was placed in a 15 ml falcon tube, combined with 4 ml FC₂ buffer, inverted 3 times and incubated for 8 minutes at room temperature (RT). Blood was filtered through the ScreenCell device as per manufacturer's instructions, and 1.6 mls PBS was also passed through the filter to remove any blood on the sides of the device.

The filter was detached from the device to enable downstream manipulation and the underside wiped with a PBS-soaked cotton bud to reduce the number of white blood cells trapped. The filter was placed on tissue paper and 50µl PBS was drawn through twice by gentle application of pressure using tweezers on the metal O-ring. The filter was submerged in 3 ml HistoClear II, and detached from the O-ring using curved-tipped callipers. The filter was placed on a slide and a small right angle cut on the upper left so the upper side of the filter could be identified easily later. The filter was submerged 3-4 times in dH₂O to rinse off excess HistoClear II, and placed on a parafilm-wrapped slide, ensuring that there was no dH₂O trapped between the filter and the parafilm.

Giemsa Staining and imaging

200 µl modified Giemsa was applied to the filter and incubated at RT for 10 minutes. Excess stain was removed using tissue paper, before the filter was placed on a clean parafilm-covered slide. 200 µl buffer pH 6.8 was applied and incubated for 2 minutes at RT. Buffer was removed and the washing step was repeated, and the filter was submerged in 3 ml fresh Histoclear II.

To prepare for imaging, 400 µl Histoclear II was applied to a clean, parafilm-covered slide and the filter was placed on it. An additional 20 µl Histoclear II was added, and a coverslip slowly lowered on the slide. Excess Histoclear was absorbed using tissue paper, and the coverslip was secured using clear varnish at the corners. The slides were stored in a humidified chamber and scanned using a NanoZoomer 2.0-RS (Hamamatsu Photonics KK, Japan) at 20X with 9 layer z-stacks of 2µM per stack.

Statistical Analysis

All data were analysed using SPSS 24.0 statistic software (SPSS Inc., Chicago, IL, USA). The associations between CTCs and clinical and pathological variables were evaluated with χ^2 , with $p < 0.05$ indicating significance.

Results

Clinicopathological Data

26 patients were recruited into the study, blood samples were taken prior to neoadjuvant chemotherapy and post neoadjuvant chemotherapy. Breast cancer diagnosis was made following referral by imaging (mammography, ultrasound, magnetic resonance imaging (MRI)) and biopsy. Disease was staged and the presence of metastatic disease assessed via Computerised Tomography (CT)/Thorax, Abdomen, Pelvis (TAP) and bone scan. Clinicopathological details are presented in Table 1 and Table S1. The median age of the cohort was 46 (29-69) years. Median BMI for the cohort was 27 (18-38), with over 50% of the cohort in the overweight/obese category. 89% (23) of the patients were diagnosed with invasive ductal carcinoma (IDC) and 11% of patients (3) were diagnosed with invasive lobular carcinoma (ILC). Patients received neoadjuvant chemotherapy following discussion at a multidisciplinary team meeting. The majority of patients had locally advanced disease with no distant metastasis, while others had a triple positive or triple negative diagnosis with no lymph node metastasis diagnosis prior to treatment. The majority of patients were treated with the ACT chemotherapy regimen, which consists of doxorubicin (Adriamycin) and cyclophosphamide, followed by treatment with paclitaxel (taxane). Patients with human epidermal growth factor receptor 2 (HER2⁺) tumours also received Herceptin®. 1 patient developed neuropathy and did not complete paclitaxel treatment. Response to neoadjuvant chemotherapy was assessed prior to surgery using ultrasound, mammography or MRI. The majority (17 (65%)) of our cohort expressed oestrogen receptor (ER) and progesterone receptor (PR) positive as displayed in Table 1 and Supplementary Table 1. Four (15%) of the cohort expressed HER2 which was confirmed by fluorescence in situ hybridisation (FISH). The predominant molecular subtype in our cohort was ER⁺, PR⁺ and HER2⁻ with 58% (15) of our cohort staining for this subtype. 8% were triple positive and

27% triple negative. Pathological stage was recorded post-surgery. Patient age, body mass index, tumour subtype, receptor status, molecular subtype, tumour grade, clinical stage, pathological stage, LVI, radiological response and pathological response (Miller-Payne grade) to chemotherapy are displayed in Table 1. *(Insert Table 1)*

CTC identification and enumeration

Modified Giemsa staining was used for the identification of CTCs on scanned images of the ScreenCell filters. Two pathologists reviewed the filters and identified CTCs on the basis of morphology, using the following criteria: intact cell, high nuclear:cytoplasmic ratio, hyperchromatic nucleus with coarse chromatin, and the presence of macro-nucleoli. CTC heterogeneity was observed with CTCs being identified as single cells ([Figure 1A](#)), doublets ([Figure 1B](#)) and clusters/microemboli ([Figure 1C, 1D](#)). CTC clusters/microemboli are defined as ≥ 3 CTCs [19] in a spatiotemporal pattern. CTCs were enumerated in the pre- and post- chemotherapy sample and classified according to the CellSearch™ cut-off of <5 or ≥ 5 CTCs; correlations with clinical parameters are shown in Table 1. Detailed CTC counts are displayed in Supplementary Table 1. As only 3mls of blood was used with the ScreenCell device, correlations were also assessed with the equivalent cut-off of 2 CTCs to correct for the volume used with the CellSearch™ device (data not presented). Data was also analysed categorising patients as positive or negative for CTCs (data not presented). *(Insert Figure 1)*

Correlation of CTCs with clinicopathological data

Data for CTC counts are presented in Table 1 and individual counts are detailed in supplementary Table 1. At baseline, 4 patients were CTC-negative. 7 patients had 1-4 CTCs and 15 patients had ≥ 5 CTCs as determined by Total CTC count in Supplementary Table 1. The median baseline number of CTCs was 7 (0-161). Post chemotherapy, 1 patient was CTC-negative, 8 patients had 1-4 CTCs and 17 patients had ≥ 5 CTCs. The median post-chemotherapy number of CTCs was 9.5 (0-300).

No significant association was identified between the number of CTCs (categorised $<$ or ≥ 5 CTCs as per the CellSearch™ studies) in the pre-chemotherapy or post-chemotherapy blood sample and clinical characteristics, such as patient age, receptor status, molecular subtype, tumour grade, disease type, clinical or pathological stage (incorporating tumour size and lymph node metastasis), LVI, radiological response or Miller Payne Grade (Table 1). A significant correlation was observed between BMI and pre-treatment CTC count, $p < 0.05$.

CTC counts were also categorised using 2 CTCs as the cut-off (correcting for the blood volume used) and as positive/negative but no correlation was observed.

CTC counts were correlated with corresponding blood parameters assessed on the same day as part of the routine work-up, including haemoglobin level, haematocrit, white cell count, CA153 if available and platelet count. No correlation was found between CTC count and any of these parameters (data not shown).

The change in total CTCs between the pre- and post-neoadjuvant chemotherapy samples was very variable between patients (Supplementary Figure 1), with some having a decrease of 61 CTCs while others had an increase of 270 CTCs. Overall, 13 (50%) patients experienced an increase in total CTCs, 10 experienced a decrease and 2 had no change.

65% of patients had a good response to neoadjuvant chemotherapy with a Miller Payne grade of 3 or more. Five patients (19%) had a Miller Payne grade of 5, or a complete pathological response to chemotherapy. This did not correlate with CTC counts, either pre- or post- chemotherapy or with the change in CTC numbers.

Correlation of CTC clusters with clinicopathological data

In addition to total CTC counts, the number of CTC clusters were also evaluated and assessed independently for clinical significance. No association was found between the total number of CTC clusters in a pre- or post- chemotherapy sample (also assessed as positive or negative for clusters) and the clinical factors of patient age, BMI, tumour subtype, receptor status, tumour grade, clinical or pathological tumour stage, LVI, radiological response or Miller Payne Grade. However, if we categorise clusters as $<$ or ≥ 2 we do observe a correlation pre-treatment counts and BMI, $p < 0.05$. The number of cells in each cluster was also counted as displayed in supplementary Table 1 but no correlation in this total cell count was observed with clinical parameters.

In relation to other blood parameters, no correlation was seen between CTC cluster counts and haemoglobin level, haematocrit, white cell count, CA153 if available and platelet count.

At baseline, 13 patients were negative for CTC clusters, 5 patients had 1-4 CTC clusters and 8 patients had ≥ 5 CTC clusters. The median baseline number of clusters was 0.5 (0-40). The change in CTC clusters was again very variable between patients (Supplementary Figure 2). Post chemotherapy, 10 patients were negative for CTC clusters, 9 patients had < 5 CTC clusters and 7 patients had > 5 CTC clusters. The median post-chemotherapy CTC cluster value was 1.5 (0-26). Overall, 11 (42%) patients experienced an increase in CTC clusters, 10 had a decrease in CTC clusters and 5 remained unchanged.

Correlation of CTCs with clinical outcome

We were unable to assess the prognostic potential of the CTC counts in our study to date as very few of the patients have presented with a recurrence. Only observational data can be presented at this stage. As outlined in Supplementary Table 1, following a 3-year follow-up, 21 patients are currently alive with no

evidence of disease (ANED), 3 patients have had a recurrence (AWD) and 2 patients have died of disease (DOD). Of the 2 patients that died of disease, both had an increase in CTCs following chemotherapy, 1 had no CTCs at baseline. Of the 3 patients that had a recurrence, 1 had a decrease in CTCs post chemo, 1 had no change in CTCs between the pre and post chemo sample and the 3rd had an increase in CTCs (in particular in clusters). At this stage it would seem that the presence of CTCs post chemotherapy will have some prognostic potential, but it is not possible to reliably say this at this point. It is our intention to review this data for a 5-year follow-up and beyond if necessary.

Discussion

This study did not show any benefit for CTC counts prior to treatment or prior to surgery in assessing pathological response to neoadjuvant chemotherapy in breast cancer patients. It is likely that more in depth analysis of CTCs will unlock their true potential in the clinic but currently many limitations exist in terms of their isolation and characterisation.

CTCs were detected in 85% of patients in this study prior to treatment, which is higher than some of the quoted studies in the literature which vary from 31% to 61% [22, 23]. However, many of the published studies used the CellSearch™ system, which we know underestimates CTC numbers due to its reliance on the presence of EpCAM. In addition, some meta-analyses published [22, 23] have included early breast cancer patients whereas in this study we focussed on those with locally advanced disease (including some triple positive/negative with N0 disease n=6) who were undergoing NAC followed by surgery, a cohort we would expect to be higher CTC traffickers. The GeparQuattro trial did focus on a neoadjuvant cohort using the CellSearch™ system and found prognostic ability in pre-treatment CTC counts for HER2 positive and triple negative patients using a cut-off of 2 CTCs but similar to our findings this prognostic ability was independent of the primary tumour response [25]. The patient population selected and the ScreenCell isolation device used may explain the higher percentage of patients having CTCs at the outset.

Significant heterogeneity was observed in the CTC phenotypes isolated, single cells, doublets and clusters. At baseline, clusters were isolated in 50% of our patient cohort. This is slightly higher than recently reported by Vetter et al, who found 35% of their cohort had clusters present prior to treatment [26], using the Parsortix microfluidic device. Studies using the CellSearch™ system report cluster rates around 20% [27]. Current technologies significantly under-call the number of CTC clusters because few specialised devices exist for their detection. The definition of clusters also varies in the literature from >1 CTC to >2 or 3 cells [19]. In this study, we define clusters as ≥ 3 CTCs but we have also conducted analyses using the other cut-offs of >1 cell and we did not find any significance with our clinicopathological details or response to neoadjuvant chemotherapy. Clusters are more aggressive than single CTCs [18, 19] and a lot of effort is now focussed on methods for isolation and characterisation of CTC clusters [20, 21]. We have previously reported the importance of platelets in cancer metastasis, which are a major component of these clusters and microemboli [28] and may explain the increased aggressiveness observed. In addition, we have shown that the platelet cloak can inhibit immune surveillance by NK cells enabling the CTCs to establish metastasis [29].

We did not observe any relationship between CTC counts and clinicopathological details such as patient age, receptor status, tumour grade, disease type, tumour size, LVI, lymph node metastasis radiological response or Miller Payne Grade for our patient cohort. We did see a correlation between BMI and pre-treatment CTC counts with higher BMI patients having a higher count. BMI has been shown to mediate the prognostic significance of CTCs in inflammatory breast cancer [30]. In a recent publication, patient-derived xenograft (PDX) model, tumours grown in the presence of obesity-altered adipose stem cells in SCID/beige mice had increased circulating HLA1⁺ human cells as well as increased numbers of CD44⁺CD24⁻ cancer stem cells in the peripheral blood, the authors concluded leptin produced by obesity-altered adipose stem cells promotes metastasis. Others have found a negative association with BMI and CTC counts using the CellSearch device [31, 32]. Further work is needed to assess this correlation between CTCs and BMI. Most studies including two meta-analyses [22, 23], did not observe any correlation with clinicopathological details but they did find CTC counts to have prognostic ability. The prognostic potential of CTC counts in our study has not yet been statistically assessed due to the low number of patients, presenting with recurrences but it is our intention to carry out a 5 year follow up on these patients. It does, however, seem like the presence of CTCs post chemotherapy will have some prognostic potential.

Some studies have assessed correlation of CTC counts with complete pathological response. The method of measuring the pCR is not detailed in all studies. In this study, the CTC count before and after chemotherapy was correlated with radiological findings and also to the pathological score of Miller Payne grading. There was no correlation between CTC counts and this score, 19% of the patients in our cohort had a pCR and all of these still had CTCs following neoadjuvant chemotherapy. Other studies report a decline in CTC counts post neoadjuvant treatment [33] while some report an increase [34]. While CTCs were present post treatment, we do not know the metastatic potential of these CTCs and this is needed to unlock the true potential of CTCs in the clinic. In our study, we observed an increase of 58% in CTCs and 42% in CTC clusters in the patients post chemotherapy which is similar to the observations in a prostate cancer study where 42% of patients had an increase in CTCs post treatment [35]. There may be many reasons for the increase in CTC counts/clusters observed post treatment. This may be explained by the rate at which the various tumour subtypes shed tumour cells into the circulation. The chemotherapy may have caused the vessels to become leaky and shed more cells into the vasculature. It may be that the chemotherapy has selected for a clonal population of stem like cells that have survived the chemotherapy. Other mechanisms that may explain this increased number include autophagy, anikis, expansion of pro-metastatic variants or a potential drive towards dormancy, which would be of interest for future studies. Clearly, further work is needed on assessing the viability and molecular characterisation of the CTCs that remain post treatment. We hypothesise that CTC clusters are more likely to be viable than single CTCs. While these reasons may explain the findings in the cohort of patients who still have residual disease, it does not explain the situation for those who have had a complete pathological response. It suggests a differential response between the primary tumour and the CTCs and is similar to the findings in the Geparquattro trial [18]. Follow-up of these patients is needed to determine the long-term significance of these CTCs. One study has reported the detection of CTCs 8-22

years out from treatment, despite no clinical evidence of disease [36]. It is not known if this represents tumour dormancy and persistence of disseminated disease in the bone marrow as suggested by some investigators [37, 38] or whether a proportion of these patients will go on to develop metastatic disease. This stresses the need for molecular characterisation and long term follow up of patients.

Many groups are now focussing on characterising and dissecting the metastatic potential of CTCs. Our group has established a CTC-5 program which allows us to merge a morphology image of the isolated CTCs with an immunofluorescent profile for the same cells which will give us a better insight into their biology (manuscript in prep). A focus on single cell genomics and cluster dissection may reveal mechanisms of how the cells in a cluster co-operate and metastasise, enabling CTCs to have a more proactive role in the clinic. Unlocking the full potential of the liquid biopsy to monitor treatment response in breast cancer will allow us ultimately to deliver a more personalised medicine approach for cancer patients, improving therapeutic outcomes.

Conclusions

CTC enumeration is not sufficient to predict which breast cancer patients should undergo surgery following neoadjuvant chemotherapy. Additional characterisation of CTCs and CTC clusters is needed to assess the true potential of CTCs in this cohort of patients. Long-term follow-up of patients is needed to assess the significance of CTC counts.

Abbreviations

AMNCH: Adelaide and Meath incorporating the National Children's Hospital

ANED: Alive with no evidence of disease

AWD: Alive with disease

BL: Baseline

BMI: Body mass index

CK: Cytokeratin

CT: Computerised Tomography

CTCs: Circulating tumour cells

DOD: Died of disease

EMT: Epithelial-mesenchymal transition

EpCAM: Epithelial cell adhesion molecule

ER: Oestrogen receptor

FISH: Fluorescence in situ hybridisation

HER2: Human epidermal growth factor receptor 2

HR: Hazard ratio

IDC: Invasive ductal carcinoma

ILC: Invasive lobular carcinoma

MRI: Magnetic resource imaging

NAC: Neoadjuvant Chemotherapy

OS: Overall survival

pCR: Pathological complete response

PR: Progesterone receptor

RT: Room temperature

TNBC: Triple-negative breast cancer

Declarations

Ethics approval and consent to participate

The study was approved by the St James's and AMNCH (Adelaide and Meath incorporating the National Children's Hospital) research ethics committee. All included patients provided written informed consent for participation.

Consent for publication

All included patients provided written informed consent; no patient identifier is included in the publication.

Availability of data and material

The datasets used and analysed during the current study are available from the corresponding author on request.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

SOT, CS, BF, JK and JOL were involved in the conception and design of the study took part in acquisition of data, analysis and interpretation of data, and drafting and reviewing of the manuscript. YH was involved in acquisition of data, analysis and interpretation of the data and drafting and reviewing of the manuscript. MF, BM, MW, MG, CM, TK were involved in the interpretation of the data and manuscript revision. COB was involved in optimising scanning of all images. CR was responsible for consent and follow up of patients. AB was involved in processing of samples and manuscript revision. DM was involved in data analysis and interpretation of the data and characterisation of CTCs. EC and SMcG were responsible for identification of patients, recruitment, interpretation of the data and manuscript revision.

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Tables

Table 1. Association between pre-chemotherapy and post-chemotherapy CTC counts and clinical characteristics.

Characteristics	Patients n (%)	Pre-chemo Patients n (%)	CTCs Patients n (%)	P Value	Post-chemo Patients n (%)	CTCs Patients n (%)	P value
		<5	≥5		<5	≥5	
Patient Cohort	26 (100.0%)	11 (42%)	15 (58%)		9 (35%)	17 (65%)	
Age (median 46 years)							
<46	13 (50.0%)	5 (19.2%)	8 (30.8%)	0.691	3 (11.5%)	9 (34.6%)	0.68
≥46	13 (50.0%)	6 (23.1%)	7 (26.9%)		5 (19.2%)	8 (30.8%)	
BMI							
< 25	12 (46.2%)	8 (30.8%)	4 (15.4%)	0.02*	4 (15.4%)	8 (30.8%)	0.899
≥25	14 (53.8%)	3 (11.5%)	11 (42.3%)		5 (11.5%)	9 (34.6%)	
Subtype							
Ductal	23 (88.5%)	9 (34.6%)	14 (53.8%)	0.364	7 (26.9%)	16 (61.5%)	0.215
Lobular	3 (11.5%)	2 (7.7%)	1 (3.8%)		2 (7.7%)	1 (3.8%)	
Receptor Status							
ER+/PR+	17 (65.4%)	8 (30.8%)	9 (34.6%)	0.286	7 (26.9%)	10 (38.5%)	0.139
ER-/PR-	8 (30.8%)	2 (7.7%)	6 (23.1%)		1 (3.8%)	7 (26.9%)	
ER+/PR-	1 (3.8%)	1 (3.8%)	0 (0.0%)		1 (3.8%)	0 (0.0%)	
HER2+	4 (15.4%)	2 (7.7%)	2 (7.7%)	0.735	2 (7.7%)	2 (7.7%)	0.482
HER2-	22 (84.6%)	9 (34.6%)	13 (50.0%)		7 (26.9%)	15 (57.7%)	
TNBC	7 (26.9%)	2 (7.7%)	5 (19.2%)	0.39	1 (3.8%)	6 (23.1%)	0.186
Non TNBC	19 (73.1%)	9 (34.6%)	10 (38.5%)		8 (30.8%)	11 (42.3%)	
Molecular Subtype							
Luminal-A-like [#]	15 (57.7%)	7 (26.9%)	8 (30.8%)	0.548	6 (23.1%)	9 (34.6%)	0.339
Luminal-B (Her2+)	3 (11.5%)	2 (7.7%)	1 (3.8%)		2 (7.7%)	1 (3.8%)	
Basal like/TNBC	7 (26.9%)	2 (7.7%)	5 (19.2%)		1 (3.8%)	6 (23.1%)	
Her2 Enriched	1 (3.8%)	0 (0.0%)	1 (3.8%)		0 (0.0%)	1 (3.8%)	
Grade							
Grade 1	2 (7.7%)	0 (0.0%)	2 (7.7%)	0.155	0 (0.0%)	2 (7.7%)	0.372
Grade 2	16 (61.5%)	9 (34.6%)	7 (26.9%)		7 (26.9%)	9 (34.6%)	
Grade 3	8 (30.8%)	2 (7.7%)	6 (23.1%)		2 (7.7%)	6 (23.1%)	
Clinical Stage							
T1	2 (7.7%)	1 (3.8%)	1 (3.8%)	0.763	0 (0.0%)	2 (7.7%)	0.177
T2	10 (38.5%)	5 (19.2%)	5		2	8	

			(19.2%)		(7.7%)	(30.8%)	
T3	14 (53.8%)	5 (19.2%)	9 (34.6%)		7 (26.9%)	7 (26.9%)	
Lymph Node Mets Pre							
Yes	20 (76.9%)	8 (30.8%)	12 (46.2%)	0.664	8 (30.8%)	12 (46.2%)	0.292
Not identified	6 (23.1%)	3 (11.5%)	3 (11.5%)		1 (3.8%)	5 (19.2%)	
Radiological Response							
No response	1 (3.8%)	1 (3.8%)	0 (0.0%)	0.274	0 (0.0%)	1 (3.8%)	0.674
Partial	18 (69.2%)	6 (23.1%)	12 (46.2%)		7 (26.9%)	11 (42.3%)	
Complete	7 (26.9%)	4 (15.4%)	3 (11.5%)		2 (7.7%)	5 (19.2%)	
Pathological Stage							
Tis,T0-T1	16 (61.5%)	6 (23.1%)	10 (38.5%)	0.53	4 (15.4%)	12 (46.2%)	0.192
T2-T3	10 (38.5%)	5 (19.2%)	5 (19.2%)		5 (19.2%)	5 (11.5%)	
Lymph Node Metastasis Path							
Yes	16 (61.5%)	7 (26.9%)	9 (34.6%)	0.851	6 (23.1%)	10 (38.5%)	0.696
Not identified	10 (38.5%)	4 (15.4%)	6 (23.1%)		3 (11.5%)	7 (26.9%)	
LVI							
Yes	6 (23.1%)	4 (15.4%)	2 (7.7%)	0.285	3 (11.5%)	3 (11.5%)	0.574
NI	15 (57.7%)	6 (23.1%)	9 (34.6%)		4 (15.4%)	11 (42.3%)	
NA	5 (19.2%)	1 (3.8%)	4 (15.4%)		2 (7.7%)	3 (11.5%)	
Miller Payne Grade							
1	1 (3.8%)	1 (3.8%)	0 (0.0%)	0.113	0 (0.0%)	1 (3.8%)	0.959
2	8 (30.8%)	6 (23.1%)	2 (7.7%)		3 (11.5%)	5 (11.5%)	
3	9 (34.6%)	2 (3.8%)	7 (26.9%)		3 (11.5%)	6 (23.1%)	
4	3 (11.5%)	1 (3.8%)	2 (7.7%)		1 (3.8%)	2 (7.7%)	
5	5 (19.2%)	1 (3.8%)	4 (15.4%)		2 (7.7%)	3 (11.5%)	

*P Value < 0.05

Luminal A and B were not differentiated in all cases as Ki-67 is not performed routinely in our centre.

BMI (Body Mass Index), NA (Non applicable), LVI (Lymphovascular invasion), NI (Not identified), ER (Oestrogen receptor), PR (Progesterone receptor), HER2 (Epidermal growth factor receptor), TNBC (Triple negative breast cancer).

Lymph Node Mets Pre (Lymph node metastasis identified on pre-treatment biopsy or imaging).

Figures

Figure 1

Multiple CTC physical forms were isolated from breast cancer patients. CTCs were enumerated by 2 pathologists and classified as (A) single cells, (B) doublets, (C+D) clusters / microemboli.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [FigureS1CTCCountChange.png](#)
- [BreastCancerSupplementaryTable1revised.xlsx](#)
- [FigureS2CTCClusterchange.png](#)