janssen

CellSearch® Circulating Epithelial Cell Kit (Immunomagnetic capture and fluorescent reagents) Instructions for Use

CTC kit

REF **7900001**

[PRODUCT NAME]

Generic Name: CellSearch® Circulating Epithelial Cell Kit (Immunomagnetic capture and fluorescent reagents)

English name: CellSearch® Circulating Tumor Cell Kit (Epithelial)

[PACKAGING SPECIFICATION]

CellSearch® Circulating Epithelial Cell Kit (Immunomagnetic capture and fluorescent staining) 16 tests/package

[INTENDED USE]

For *in vitro* diagnostic use.

CellSearch® Circulating Epithelial Cell Kit (Immunomagnetic capture and fluorescent staining) is intended for the enumeration of circulating tumor cells (CTC) of epithelial origin (CD45-, EpCAM+, and cytokeratins 8, 18+, and/or 19+) in whole blood. The presence of CTC in the peripheral blood, as detected by the CellSearch® Circulating Tumor Cell Kit, is associated with decreased progression free survival and decreased overall survival in patients treated for metastatic breast cancer. The test is to be used as an aid in the monitoring of patients with metastatic breast cancer. Serial testing for CTC should be used in conjunction with other clinical methods for monitoring metastatic breast cancer. Evaluation of CTC at any time during the course of disease allows assessment of patient prognosis and is predictive of progression free survival and overall survival diagnosed with detection methods (i.e., histology or imaging).

[PRINCIPLES OF THE PROCEDURE]

The CellSearch® Circulating Tumor Cell Kit contains a ferrofluid-based capture reagent and immunofluorescent reagents. The ferrofluid reagent consists of nanoparticles with a magnetic core surrounded by a polymeric layer coated with antibodies targeting the EpCAM antigen for capturing CTC. After immunomagnetic capture and enrichment, fluorescent reagents are added for identification and enumeration of CTC. The fluorescent reagents include the following: anti-CK-Phycoerythrin (PE) specific for the intracellular protein cytokeratin (characteristic of epithelial cells), DAPI, which stains the cell nucleus, and anti-CD45-Allophycocyanin (APC) specific for leukocytes.

The reagent/sample mixture is dispensed by the CellTracks® AutoPrep® System into a cartridge that is inserted into a MagNest® Cartridge Holder. The strong magnetic field of the MagNest® Cartridge Holder attracts the magnetically labeled epithelial cells to the surface of the cartridge. The CellTracks Analyzer II® or CellSpotter® Analyzer automatically scans the entire surface of the cartridge, acquires images and displays any event to the user where CK-PE and DAPI fluorescence are co-located. Images are presented to the user in a gallery format for final classification. An event is classified as a tumor cell when its morphological features are consistent with that of a tumor cell and it exhibits the phenotype EpCAM+, CK+, DAPI+ and CD45-.

[MAIN COMPOSITION]

MATERIALS PROVIDED

- 3.0 mL Anti-EpCAM Ferrofluid: Contains a suspension of 0.022% magnetic nanoparticles conjugated to a mouse monoclonal antibody specific for the cell surface marker EpCAM present on epithelial cells in a buffer containing 0.03% bovine serum albumin (BSA) and 0.05% ProClin™ 300 preservative. (brown cap)

- 3.0 mL Staining Reagent: Contains 0.0006% mouse monoclonal antibodies specific to cytokeratins conjugated to phycoerythrin (PE); 0.0012% mouse anti-CD45 monoclonal antibody conjugated to allophycocyanin (APC) in buffer containing 0.5% BSA and 0.1% sodium azide. (white cap)
- 3.0 mL Nucleic Acid Dye: Contains 0.005% 4', 6-diamidino-2-phenylindole, dihydrochloride (DAPI) and 0.05% ProClin™ 300. (blue cap)
- 3.0 mL Capture Enhancement Reagent: Contains 0.02% proprietary reagent for controlled ferrofluid aggregation, 0.5% BSA, and 0.1% sodium azide in buffer. (clear cap)
- 3.0 mL Permeabilization Reagent: Contains 0.011% proprietary permeabilization reagent and 0.1% sodium azide in buffer. (green cap)
- 3.0 mL Cell Fixative: Contains 25% proprietary fixative ingredients, 0.1% BSA, and 0.1% sodium azide in buffer. (red cap)
- 2 x 110 mL bottle Dilution Buffer: Contains buffer with 0.1% sodium azide.
- 16 Conical Tubes (15 mL swing bucket centrifuge) and Conical Tube Caps
- 16 Cartridges and Cartridge Plugs

MATERIALS REQUIRED, NOT PROVIDED

- CellSave Preservative Tubes (Catalog #7900005)
- CellTracks® AutoPrep® System (Catalog #9541)
- CellTracks Analyzer II® (Catalog #9555) or CellSpotter® Analyzer (Catalog #9525)
- CellSearch® Circulating Tumor Cell Control Kit (Catalog #7900003)
- CellTracks® AutoPrep® Instrument Buffer (Catalog #7901003)
- Swing bucket centrifuge capable of 800 x g
- Test tube racks
- Calibrated micro-pipettors and tips
- Vortex mixer

[WARNINGS AND PRECAUTIONS]

1. For in vitro diagnostic use.
2. Please read the entire contents of these Instructions for Use before testing samples.
3. Caution: Collect blood into a CellSave Preservative Tube only. CTC's are fragile and require preservation for accurate analysis.
4. Caution: All personnel should follow universal precautions and use laboratory safety equipment (i.e., safety glasses, laboratory coat, gloves).
5. Caution: Microbial contamination of reagents can cause erroneous results and should be avoided.
6. Caution: Some of the reagents contain sodium azide as a preservative. If swallowed, seek medical advice immediately. Keep out of reach of children. Keep away from food and drink. Wear suitable protective clothing. Contact with acids liberates very toxic gas. Azide compounds should be flushed with large volumes of water during disposal to avoid deposits in lead or copper plumbing where explosive conditions can develop.
7. Warning: All biological specimens, cartridges and other materials coming into contact with the specimen(s) are considered biohazardous. Handle as if capable of transmitting infection. Treat and dispose of waste using proper precautions and in accordance with local, state, and federal regulations. Never pipette by mouth.
8. Warning: Some of the reagents contain ProClin™ 300 as a preservative.
9. Operator training is required to perform the test procedure.

Following are the Hazards and Precautionary statements:

H317 May cause an allergic skin reaction.

Prevention:

P261 Avoid breathing dust/fume/gas/mist/vapors/spray.

P272 Contaminated work clothing should not be allowed out of the workplace.

P280 Wear protective gloves.

Response:

P333 + P313 If skin irritation or rash occurs: get medical advice/attention.

P362 + P364 Take off contaminated clothing and wash it before reuse.

Disposal:

P501 Dispose of contents/container to an approved waste disposal plant.

For additional information please refer to the Safety Data Sheet on www.cellsearchctc.com

[REAGENT STORAGE AND HANDLING STABILITY]

	Storage Conditions	Stability
Sealed	Refrigerate at 2-8℃	12 Months

- Reagents are supplied ready for use. Store unopened at 2 to 8°C.
- After opening, reagents in the reagent pack should be stored for no longer than 30 days at 2° to 8°C. For storage, opened reagents must be recapped with their unique colored caps using the colors indicated on the reagent tray labels as a guide. This is to ensure cross-contamination of reagents does not occur.
- NOTE: After opening, the dilution buffer bottle, which is not a part of the reagent pack, must be stored at room temperature for no longer than 30 days.
- Protect reagents from heat in excess of 35°C. Do not freeze.
- Bring to room temperature (15 to 30°C) before use.
- Visually inspect the reagent pack for the proper placement of the reagents. Verify that each reagent is in the proper location by matching its unique colored cap with the colors indicated on the label. Refer to the photo for proper placement. If reagents are found to be incorrectly placed or if duplicate bottles are present, do not use the reagent pack and notify Customer Technical Services to arrange for a replacement.
- Protect reagents from exposure to sunlight.
- When properly stored, reagents are stable until the expiration date printed on the reagent container or kit box. Do not use expired reagents.

- The kit components are manufactured and tested as a master lot. Do not mix and match reagents from different kits.

[APPLICABLE INSTRUMENT]

CellTracks AutoPrep System & CellTracks Analyzer II

[TEST PROCEDURE]

Specimen Collection and Preparation

Collection of whole blood into CellSave Preservative Tubes

1. Draw initial samples prior to initiation of a therapy regimen. Subsequent samples can be drawn after the start of a therapy regimen, usually at 3 to 4 week intervals, to follow CTC levels during therapy. If the patient is on doxorubicin therapy, allow at least 7 days following administration of a dose of doxorubicin before blood draw.
2. Collect whole blood aseptically by venipuncture or from a venous port into a CellSave Preservative Tube only.
3. Fill the tube until blood flow stops to ensure the correct ratio of sample to anticoagulant and preservative. Immediately mix by gently inverting the tube. Tube inversion prevents clotting. Inadequate or delayed mixing may result in inaccurate test results.
4. Blood samples may be stored or transported in CellSave Preservative Tube. Please refer to the CellSave Preservative Tube Instructions for Use for process, storage and handling instructions. Do not refrigerate

samples.

CAUTION: Visually inspect each sample for clotting before processing on the CellTracks® AutoPrep® System. Clotted samples should be discarded.

Processing with the CellTracks® AutoPrep® System

1. Transfer 7.5 mL of blood from the CellSave Preservative Tube into a correspondingly labeled 15 mL Conical Tube provided with the CellSearch® Kit.
2. Add 6.5 mL of Dilution Buffer.
3. Cap the Conical Tube and mix by inversion five times.
4. Centrifuge the sample at 800 x g for 10 minutes with the brake off.
5. Process on the CellTracks® AutoPrep® System within 1 hour of the above sample preparation. Refer to the CellTracks® AutoPrep® System User's Guide for full instructions.

Analysis using the CellTracks Analyzer II® or CellSpotter® Analyzer

The CellTracks® AutoPrep® System dispenses the processed sample into a cartridge ready for analysis using the CellTracks Analyzer II® or CellSpotter® Analyzer. The filled cartridge within the MagNest® Cartridge Holder should be allowed to incubate in the dark for a minimum of 20 minutes and analyzed within 24 hours. Please refer to the CellTracks Analyzer II® or CellSpotter® Analyzer User's Guide for instructions on sample analysis and data review.

[REFERENCE VALUES (RANGE)]

The threshold of CTC count detection is 5 CTC/7.5 mL peripheral whole blood for prognosis of patients with metastatic breast cancer. If result of detection is ≥ 5 CTC/7.5 mL, the patient is believed to have unfavorable prognosis. If the result is < 5 CTC/7.5 mL, the patient is believed to have favorable prognosis.

In Single point CTC analyses were performed on control groups of 200 Chinese healthy volunteers and female subjects with non-malignant breast disease, including 101 women with non-malignant breast disease, and 99 women ware healthy volunteers, none of them had more than 5 CTC/7.5 mL. The results are presented in Table 1.

Table 1 CTC determination results of Chinese healthy volunteers and female subjects with non-malignant breast disease

Category	N	Mean # CTC	SD	# Patients with≥ 5 CTC	Min.*	Max.*
Healthy	99	0.05	0.26	0	0	2
Non-malignant breast disease	101	0.09	0.35	0	0	2

[QUALITY CONTROL]

The CellSearch® Circulating Tumor Cell Control Kit (Catalog #7900003) checks the overall system performance, including instrument, reagents and operator technique. A CellSearch® Circulating Tumor Cell Control should be run each day of patient testing or when using a new lot of the CellSearch® CTC Kit. Please refer to the CellSearch® Circulating Tumor Cell Control Kit Instructions for Use and expected values.

[INTERPRETATION OF RESULTS]

Results are reported as the number of <CTC >/ 7.5 mL of blood.

Metastatic Breast Cancer (MBC)

A CTC count of 5 or more per 7.5 mL of blood at any time during the course of the disease is associated with a poor prognosis and is predictive of shorter progression free survival and overall survival.

Precaution: Specimens with more than 5,000 CTC per 7.5 mL of blood accounted for less than 0.03% of those seen in our clinical studies. Sample carryover is of concern when such a high CTC specimen is immediately followed in the CellTracks® AutoPrep® System by a specimen yielding a CTC result in the range 3 to 15 CTC per 7.5 mL of blood. In this case, we recommend obtaining a new blood sample from the low CTC patient and performing a confirmatory CTC analysis. To identify following samples, refer to the CellTracks® AutoPrep® User's Guide section on View Data and

obtain the detailed batch data, including sample and patient identification for each tube in the batch.

[LIMITATIONS]

CellSearch® results should be used in conjunction with all clinical information derived from diagnostic tests (i.e., imaging, laboratory tests), physical examination and complete medical history in accordance with appropriate patient therapeutic regimen

This prognostic study does not demonstrate that any current line of therapy is any more or less effective than any other or no therapy.

CellSearch® results and imaging results are not equivalent in assessing the transition of patients between non progressive disease and progressive disease.

If the patient is on doxorubicin therapy, allow at least 7 days following administration of a dose of therapy before blood draw. The results of a CellSearch® test should be interpreted with caution if samples are drawn within 7 days of administration of doxorubicin therapy.

CTC that do not express EpCAM will not be detected by the CellSearch® test.

CTC that express EpCAM but not cytokeratins 8, 18, and 19 will not be detected by the CellSearch® test.

Interfering Substances:

SK-BR-3 cells spiked into blood samples were exposed to potential interfering substances and compared to untreated controls. Toxic levels (5 times therapeutic index) of the following cancer drugs, over-the-counter drugs, and other exogenous substances were tested: cyclophosphamide, Mitomycin C®, Procrit®, biotin, 5-fluorouracil, methotrexate, tamoxifen citrate, paclitaxel, Arimidex®, acetaminophen, acetylsalicylic acid, caffeine, dextromethorphan, Aredia®, Human Anti-Mouse Antibody (HAMA) type 1, HAMA type 2, Herceptin®, and ibuprofen. No significant differences in SK-BR-3 cell numbers were detected, indicating that these substances do not interfere with the CellSearch® kit.

Samples spiked with toxic levels of doxorubicin resulted in aberrant staining of leukocytes as cytokeratin and CD45 dual positive cells, due to the doxorubicin being a fluorescent compound that is incorporated into nucleated cells. If seen, the staining pattern of all cells being CD45 positive and cytokeratin positive is obvious and easily identified by the operator as a known interference staining profile. If blood is drawn after the recommended 7-day washout period, following doxorubicin infusion, this interference is unlikely to be observed in clinical practice given controlled therapeutic levels and rapid drug clearance.

Potential interference from lipemia was studied by adding Intralipid to samples at a concentration of 2.6%, which corresponds to greater than 1000 mg/dl triglyceride. Samples were lysed to simulate total hemolysis. Bilirubin at 7.4 mg/dL, HAMA 1/HAMA 2 and hematocrit from 18-60% were studied. Lipemia, hemolysis, icterus and a broad range of hematocrit values do not interfere with the CellSearch® test. HAMA 1 and HAMA 2 also do not interfere, indicating that individuals receiving mouse Ig by parenteral routes can be tested successfully with the CellSearch® test.

[EXPECTED VALUES]

Healthy volunteers, non-malignant breast disease, non-malignant other disease

Single point CTC analyses were performed on control groups of 145 healthy volunteers, 101 women with non-malignant breast disease, and 99 women with other non-malignant diseases. Epithelial cells are not expected to be present in the peripheral blood of healthy individuals. Of the 345 total samples from healthy volunteers and women with non-malignant disease, only one subject had more than 5 CTC/7.5 mL. The results are presented in Table 2.

Table 2. Control Subjects

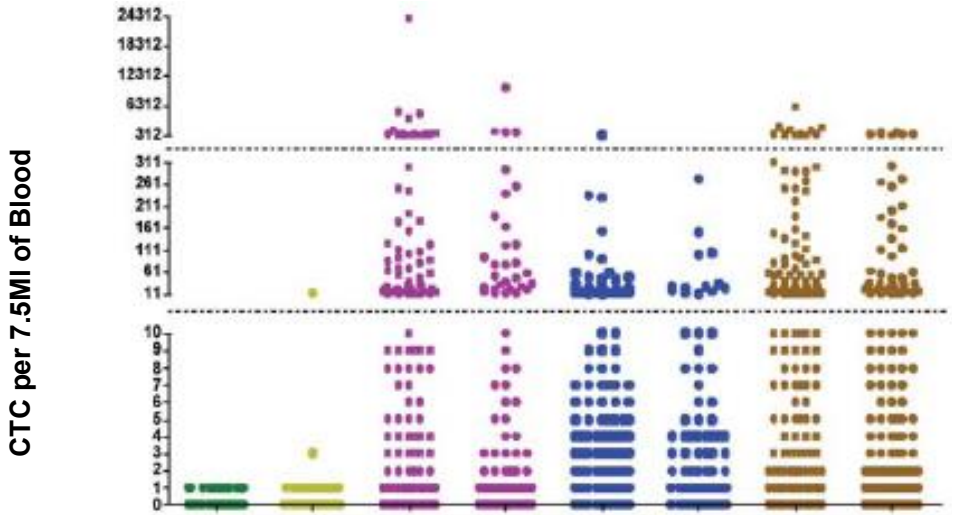
Category	N	Mean # CTC	SD	# Patients with≥ 5 CTC	Min.*	Max.*
Healthy	145	0.1	0.2	0	0	1
Non-malignant breast disease	101	0.2	1.2	1	0	12
Non-malignant other disease	**99**	**0.1**	**0.4**	**0**	**0**	**3**

♦ NCCLS Guideline C28-A2[3]

Figure 1 shows the frequency of CTC in the combined healthy and benign disease subjects, (controls), and in the

MBC patients prior to the initiation of therapy and approximately 1 month after the initiation of therapy.

Figure 1. Frequency of CTC in Controls (Subjects without Cancer) and Patients with Metastatic Breast1 (MBC) before Initiation of a new line of Therapy (Baseline) and 3-5 weeks after the Initiation of Therapy.



	Healthy Controls	Benign Diseases	Baseline MBC Patients 1	3-5 Weeks
# of Case	295	255	177	132
N(%)≥ 1 CTC	10(3%)	19(7%)	125(71%)	73(55%)
N(%)≥ 2 CTC	0(0%)	2(1%)	108(61%)	53(40%)
N(%)≥ 3 CTC	0(0%)	2(1%)	102(58%)	47(36%)
N(%)≥ 4 CTC	0(0%)	1(0%)	94(53%)	42(32%)
N(%)≥ 5 CTC	0(0%)	1(0%)	88(50%)	40(30%)

1. MBC reference population information –Page 13

[PERFORMANCE CHARACTERISTICS]

Recovery

Blood samples from a single healthy donor were pooled and five of six 7.5 mL aliquots were spiked with approximately 1300, 325, 81, 20, and 5 cultured breast cancer cells (SK-BR-3). The sixth tube was unspiked pooled blood and served as a zero point. These samples were processed on the CellTracks® AutoPrep® System with the CellSearch® Circulating Tumor Cell Kit and CTC counts were determined on the CellTracks Analyzer II®. The experiment was repeated for four additional donors. The observed cell counts were plotted against the results of the expected cell count. The results are summarized in Table 3.

Table 3. Percent Detection Estimates.

Expected Tumor Cell Count	Mean Observed Tumor Cell Count	Range of Percent Recovery
1300	1215	91 to 95%
325	308	82 to 101%
81	85	80 to 136%
20	22	95 to 140%
5	7	120 to 200%

To determine the overall, or least squares fit, for the comparison of the observed and expected cell counts across all the data, linear regression analysis was performed. The regression equation for these 30 samples was Y=0.93x + 3.87 with an R2=0.999 (R=0.999). The results of this study indicate that on average, over the tested CTC range, the recovery,as derived from regression analysis, is 93%.

Given the linear response of the tumor cell counts, one would expect the slope of the observed versus expected plot to be 1.0. However, the slope was 0.93. This is because the CellTracks® AutoPrep® System with CellSearch® CTC Kit involves the capture and fluorescent labeling of cells followed by their detection and enumeration by the

CellTracksAnalyzer II®. The loss of cells could therefore be attributed to one of the following possibilities; 1) the recovery of only 93% of the tumor cells spiked into 7.5 mL of blood by the CellTracks® AutoPrep® System, 2) the detection of only 93% of the tumor cells present in the sample chamber by the CellTracks Analyzer II® or 3) a combination of both of these sources of error.

Linearity / Reportable Range

Another way to examine the previous data is to analyze it as a dilution series to evaluate test linearity. We removed the confounding variable of percent recovery by using the observed value of the initial sample in the dilution series (i.e. the first tube) divided by the dilution factors to determine the expected values for the dilution series for each patient sample. Regression of all of these numbers of observed tumor cells versus the numbers of expected tumor cells yielded a slope of 1.007, an intercept of 3.0, and an R2 = 0.990 (R = 0.995). Therefore, once the percent recovery (cell loss) was factored out of the CTC values of each of the initial samples, the analysis of the data demonstrated that the detection of CTC was linear over the reportable range of 0 to 1238 tumor cells.

Limits of Detection

One CTC per 7.5 mL can be detected by the CellTracks Analyzer II® resulting in a limit of detection of 1 CTC in a cartridge. Linear regression shows that on average, 93% of CTC present in a 7.5 mL blood sample are recovered using the CellTracks® AutoPrep® System (see Recovery section). The loss of approximately 7% of the CTC in the sample is not sufficient to reduce the limit of detection of 1 CTC.

Reproducibility:

a. System Reproducibility with CellSearch® Circulating Tumor Cell Control

Three separate CellSearch® Circulating Tumor Cell Control samples were prepared and processed each day for over 30 days, per the long run method of NCCLS guideline EP5-A2. Each single-use sample bottle contains a low and a high concentration of cells from a fixed cell line that have been pre-stained with two different fluorochromes. Summary statistics for the high and low control cells is presented below.

Table 4. Summary of Precision Analyses

	Low	High
N	99	99
Mean cell count	48	969
Total Precision Standard Deviation (ST) % CV	18%	5%

b. System Reproducibility with Patient Samples

Metastatic Breast Cancer (MBC)

A total of 163 duplicate blood samples were collected from 47 metastatic breast cancer patients over the course of the clinical study. These samples were processed at multiple sites to determine the reproducibility of CTC measurements. The regression equation for the comparison of these 163 duplicate samples was Y=0.98x + 0.67, R2=0.99. Figure 2 shows a scatter plot of the duplicate CTC results in blood from MBC patients plotted on a logarithmic scale, with the threshold of 5 CTC indicated by the dashed lines.

Figure 2. Reproducibility of CTC Counts in Duplicate MBC Samples (n=163) with Average of < 5 or ≥ 5 CTC per 7.5 mL of blood.

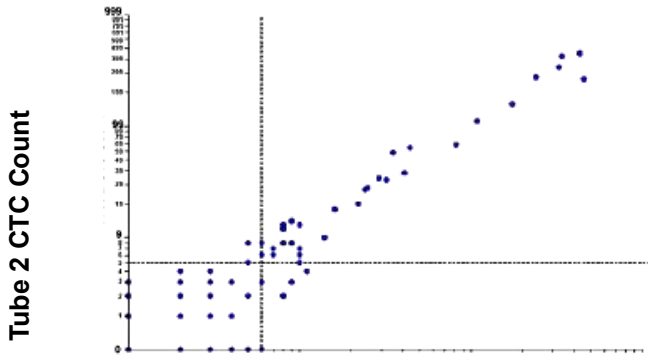


Figure 2 Note: There may be more than one point superimposed over another. For example, on this plot, there are 50 instances (31%) where both tubes had 0 CTC, 18 instances (11%) where Tube 1 had 0 CTC and Tube 2 had 1 CTC, and another 18 instances (11%) where Tube 1 had 1 CTC and Tube 2 had 0 CTC.

The tube-to-tube variation of CTC counts in blood samples from metastatic breast patients is shown in Figure 2. The distribution of infrequent events within a given volume is random. This is best characterized by the Poisson distribution – a mathematical method employed for modeling systems where the probability of an event occurring is very low but the number of opportunities for such an event to occur is large [5].

Note: due to limitations in current detection technology, according to calculation using statistical method, %CV value is 45% when CTC detects 5 cells. Using additional experiments, %CV value is 29% when 5 cells are detected.

[DOMESTIC CLINICAL TRIAL RESULTS]

A multi-center prospective, clinical trial was conducted in China to determine whether the count of CTC predicts disease progression and survival. Metastatic breast cancer patients (N=300) with measurable disease starting a new line of systemic therapy were enrolled. Clinical data were analyzed on an intent-to-treat basis. The patient demographic information is presented in Table 1.

Table 1: Chinese MBC Patients Demographics

Category		N=300 Patients
Age at Baseline	Mean ± Std. Deviation Deviation	49.37 ±9.43
	Median	50.00
	Description of Categories	Number of Subjects (% of total)
Race	Han	293 (97.7%)
	Other	7 (2.3%)
Baseline ECOG Score	0	192 (64.0%)
	1	104 (34.7%)
	2	4 (1.3%)
Disease Site	Viscera	212 (71.1%)
	Bone	89 (29.9%)
ER/PR	+	169 (56.3%)
	-	157 (52.3%)
HER2	Positive	105 (35.0%)
	Negative	157 (52.3%)
	Unknown	38 (12.7%)
Line of Therapy	1st line	122 (40.9%)
	2nd line	83 (27.9%)
	3rd line	66 (22.1%)
	4th line	27 (9.1%)
Type of Therapy	Chemo (Ch)	275 (93.2%)
	Endocrine (En)	58 (19.7%)
	Targeted (Ta)	93 (31.5%)

Baseline CTC count was determined prior to initiation of a new line of therapy. Follow-up CTC counts were determined at the first visit (week 3-4) and the second visit (week 6-8) after the initiation of therapy. Progression Free Survival (PFS) was measured from the time of the blood draw to the date of progression or death (if progression was not observed before) or to the date that the subjects were contacted at the last time (if progression or death has not occurred).

1.1 CTC frequencies

The CTC results were obtained from the follow-up blood draws at baseline and the first visit and the second visit after the initiation of therapy were classified as being favorable (<5 CTC) or unfavorable (≥ 5 CTC).

Of the total MBC patient number of 300, 6 cases were not evaluable at baseline due to the non-effective determination. At the first visit, the determination results of CTC were not obtained from 73 cases, of which 5 cases (1.7%) had non-effective determination, 11 patients (3.7%) died before follow-up visit and 57 cases (19.0%) have not been determined, At the second visit, the determination results of CTC were not obtained from 67 cases, of which 4 cases (1.3%) had non-effective determination, 13 (4.3%) patients died before follow-up visit and 50 cases (16.7%) have not been determined.

Table 2 summarizes the total number and percentage of patients with unfavorable CTC in the PFS analysis of the clinical trial. The reason for difference in the number of patients as shown is the number of patients received blood drawing at each time point and got evaluable CTC data is different.

Table 2 PFS OF MBC Patients with CTC Count < 5 or ≥ 5 at each time point

1	2	3	4	5	6
Sampling Time	**N**	**≥5CTC**	**Median PFS in Weeks**		**Log-rank p-value**
			<5CTC	**≥5CTC**	
Baseline	294	115 (39.1%)	42.0	24.9	0.0009
1st visit	227	49 (21.6%)	35.6	24.0	0.0024
2nd visit	233	39 (16.7%)	31.0	2.9	<0.0001

1.2 Progression-free survival (PFS) Analysis of MBC patients

1.2.1 PFS Using Baseline CTC Results

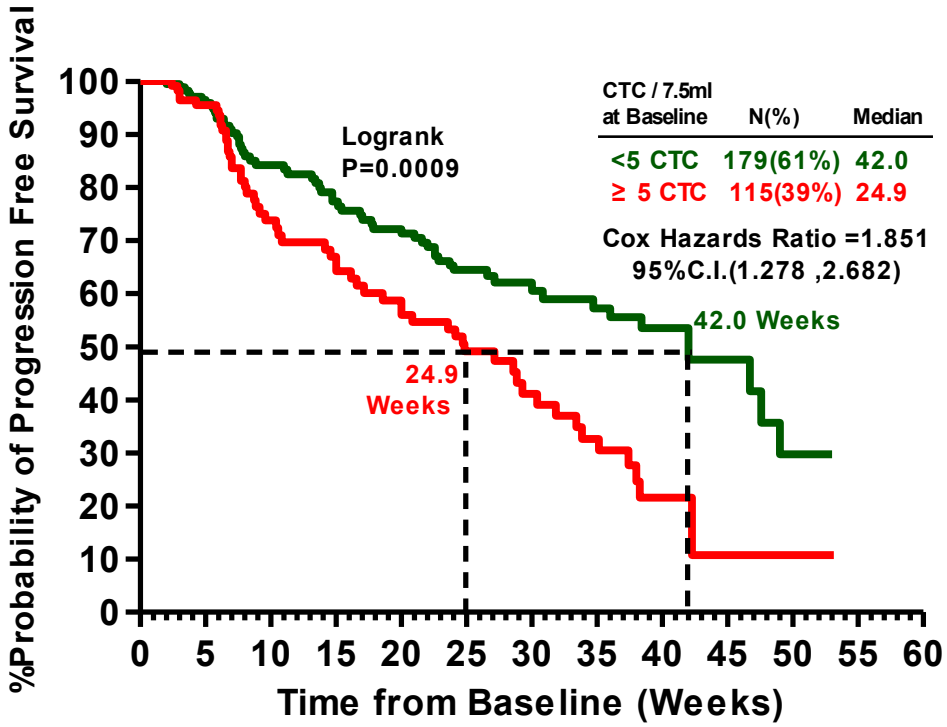
Two hundred and ninety four (294) patients had a baseline CTC result available. For Kaplan-Meier analysis, patients were segmented into two groups based upon their CTC count at baseline:

• The Favorable group (N=179), represented in green, consisted of patients with <5 CTC.

• The Unfavorable group (N=115), represented in red, consisted of patients with ≥ 5 CTC.

Median PFS was longer in the Favorable group compared to the Unfavorable group (42.0 vs 24.9 weeks, respectively). These results are illustrated in **Figure 3**.

Figure 1: Kaplan-Meier Curve of PFS of MBC Patients with <5 or ≥ 5 CTC at Baseline



1.2.2 PFS Using CTC Results at 1st Follow-up Visit

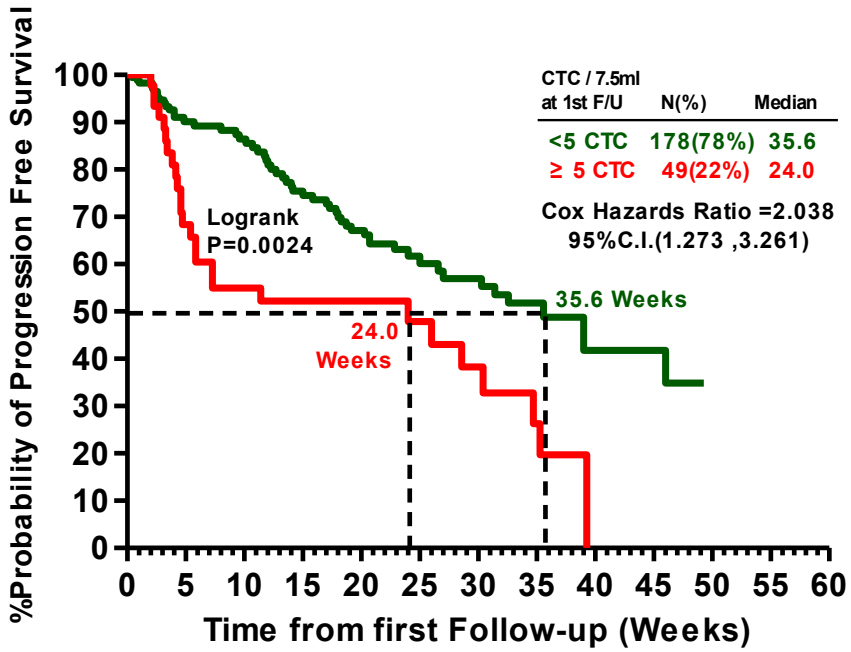
Two hundred and twenty seven (227) patients had CTC result available at the first visit. For Kaplan-Meier analysis, patients were segmented into two groups based upon their CTC count.

- The Favorable group (N=178), represented in green, consisted of patients with <5 CTC,

- The Unfavorable group (N=49), represented in red, consisted of patients with ≥ 5 CTC.

Median PFS was longer in the Favorable group compared to the Unfavorable group (35.6 vs 24.0 weeks, respectively). These results are illustrated in Figure 4.

Figure 2: Kaplan-Meier PFS Curve of MBC Patients with <5 or ≥ 5 CTC at first visit



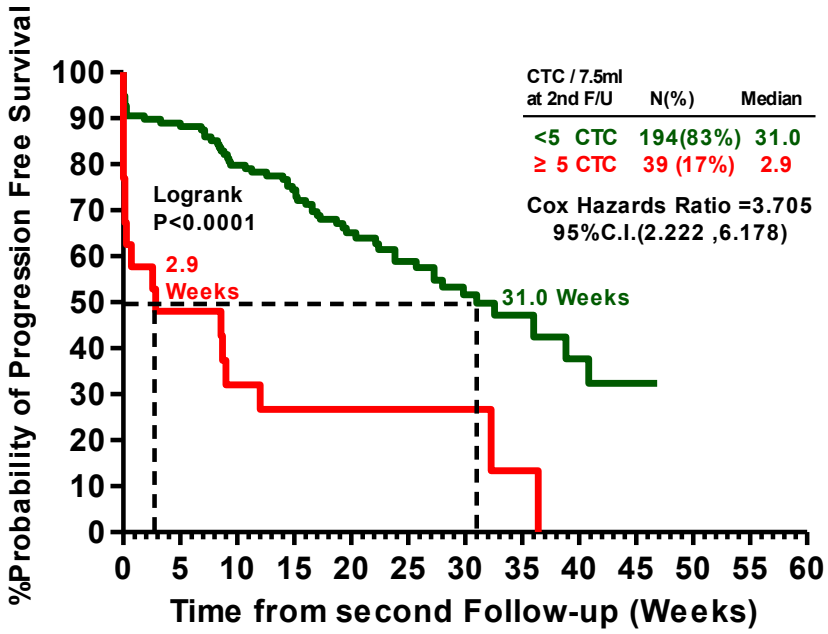
1.2.3 PFS Analysis of CTC Results in the Second Follow-up Visit

Results of CTC determination in the second follow-up visit were obtained from 233 patients with MBC. The patients were segmented into two groups based on CTC values for Kaplan-Meier analysis of survival:

- Favorable group (N=194), represented in green, consisted of patients with a CTC count < 5.
- Unfavorable group (N=39), represented in red, consisted of patients with a CTC count ≥ 5.

As shown in Figure 5, median PFS in favorable group was longer than that in unfavorable group (31.0 weeks vs. 2.9 weeks, respectively).

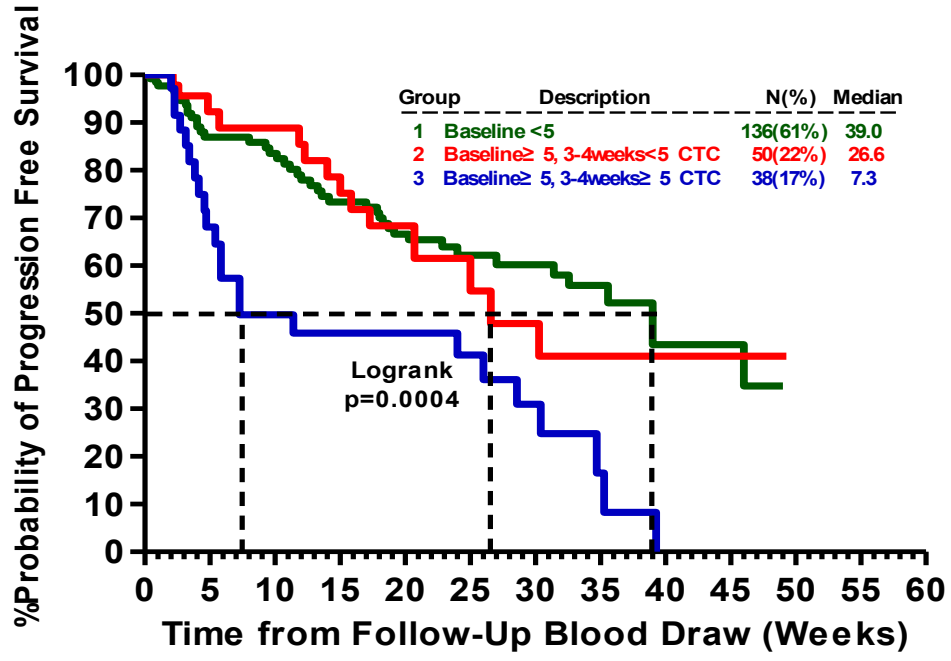
Figure 3: Kaplan-Meier PFS Curve of MBC Patients with <5 or ≥ 5 CTC at second visit



12.4 Reduction or Increase in CTC Predicts Improved or Decreased PFS

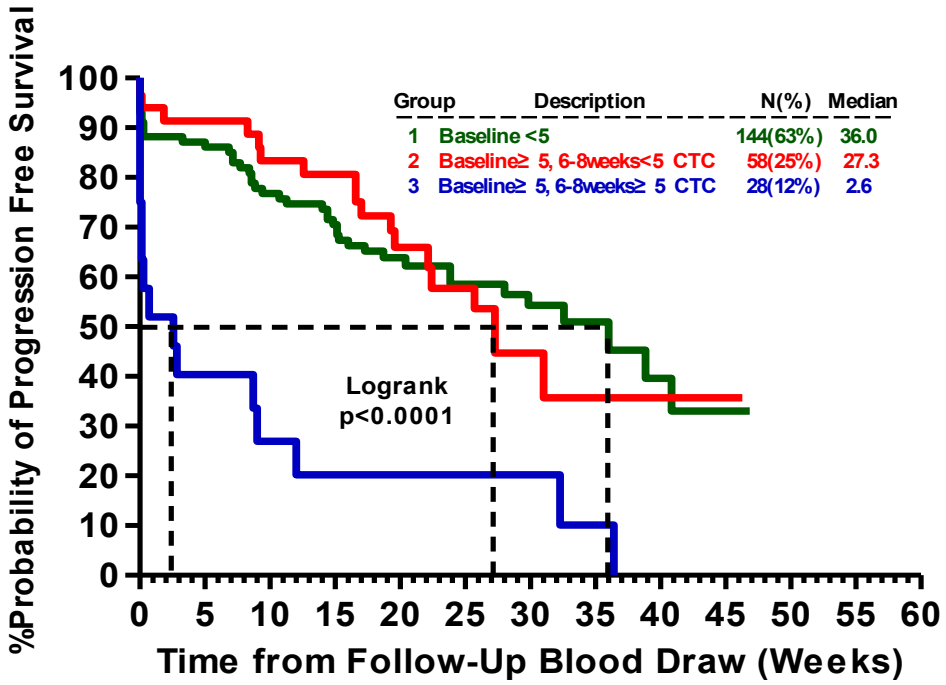
Patients with a baseline CTC results ≥ 5 CTC were further analyzed and segmented into < 5 CTC group (group 2, red) and ≥ 5 CTC group (group 3, blue) based on CTC results in the first follow-up visit. Their median PFS was 26.6 weeks and 7.3 weeks, respectively, with statistically significant difference between survival curves of the groups by logrank test (P < 0.0001). Subjects with a CTC result < 5 CTC at baseline (group 1, green) were compared to these two groups for PFS survival curve, with no statistically significant difference between group 1 and group 2 by lonrank test (P = 0.9453), but difference did exist between group 1 and group 3 (P < 0.0001). Logrank test suggested differences among the 3 groups.

Figure 6: Kaplan-Meier curves of survival in MBC patients with < 5 CTC at baseline and MBC patients with ⩾ 5 CTC at baseline and < 5 CTC or ⩾ 5 CTC at 1st visit.



Patients with a baseline CTC results ≥ 5 CTC were further segmented into < 5 CTC group (group 2, red) and ≥ 5 CTC group (group 3, blue) based on CTC results in the second follow-up visit. Their median PFS was 27.3 weeks and 2.6 weeks, respectively, with statistically significant difference between survival curves of the groups by logrank test (P < 0.0001). Subjects with a CTC result < 5 CTC at baseline (group 1, green) were compared to these two groups for PFS survival curve, with no statistically significant difference between group 1 and group 2 by logrank test (P = 0.9453), but difference did exist between group 1 and group 3 (P < 0.0001). Logrank test suggested differences among the 3 groups.

Figure 5: Kaplan-Meier curves of survival in MBC patients with < 5 CTC at baseline and MBC patients with ⩾ 5 CTC at baseline and < 5 CTC or ⩾ 5 CTC at 2nd visit.



1.2.5 Univariate cox regression analysis in MBC patients

Univariate Cox regression was used to analyze how well other factors predicted PFS in subjects with MBC, as shown in Figure 7. The results show number of treatment lines influence PFS in MBC subjects. Among these factors, CTC was the strongest prognosis predictor of PFS, and CTCs at baseline and in the first and the second follow-up visits were all predictive.

Table 3 Univariate Cox Regression Analysis in MBC Patients

	Categories	Number of MBC Patients	PFS	
			HR	95%CI
Age	Years	300	0.992	0.973, 1.011
ER/PR	+ -	292	0.830	0.562, 1.225
HER2	+ -	262	1.212	0.822, 1.786
ECOG	2 vs. 1/0	300	1.057	0.260, 4.288
Line of Therapy	≥2 vs. 1	298	1.592	1.066, 2.378
Disease Free Survival	Years	254	0.944	0.884, 1.008
Baseline CTC	≥5 <5	294	1.851	1.278, 2.682
CTC at First Visit	≥5 <5	227	2.038	1.273, 3.261
CTC at Second Visit	≥5 <5	233	3.705	2.222, 6.178

Some important clinical factors that may affect the prognosis of subjects are included in the multivariate Cox regression analyses model. Disease Free Survival and CTC were shown as the independent prognosis predictors of

PFS and CTC counts were found to be stronger predictor of PFS than Disease Free Survival. CTC counts at baseline, the first visit and second visit were predictive power of PFS (Table 4)

Table 4: PFS Multivariate Cox Regression Analysis in MBC Patients

	Categories	Number of MBC Patients	PFS	
			HR	95%CI
Age	Years	219	0.997	0.975, 1.020
ER/PR	+ -		0.943	0.571, 1.558
HER2	+ -		1.040	0.651, 1.662
ECOG	2 vs. 1/0		0.352	0.047, 2.626
Line of Therapy	≥2 vs. 1		1.173	0.737, 1.869
Disease Free Survival	Years		0.890	0.811, 0.977
Baseline CTC	≥5 <5		1.943	1.255, 3.008
Age	Years	166	0.997	0.969, 1.026
ER/PR	+ -		1.091	0.561, 2.120
HER2	+ -		1.079	0.603, 1.932
ECOG	2 vs. 1/0		0.000	0.000, NA
Line of Therapy	≥2 vs. 1		0.814	0.461, 1.438
Disease Free Survival	Years		0.880	0.781, 0.992
CTC at First Visit	≥5 <5		2.377	1.301, 4.342
Age	Years	171	1.006	0.977, 1.036
ER/PR	+ -		1.249	0.649, 2.405
HER2	+ -		1.133	0.658, 1.951
ECOG	2 vs. 1/0		0.000	0.000, NA
Line of Therapy	≥2 vs. 1		1.010	0.579, 1.765
Disease Free Survival	Years		0.891	0.799, 0.993
CTC at Second Visit	≥5 <5		3.713	1.957, 7.043

1.3 Comparison of CTC results and imaging assessment

CTC results and imaging assessment results were obtained in the second follow-up visit after effective treatment in 233 MBC patients, see table 5 below.

Table 5: Comparison of CTC results and efficacy assessment by imaging in the second follow-up visit after treatment in MBC patients

Efficacy assessment by imaging	CTC results	
	Favorable prognosis (<5 CTC)	Unfavorable prognosis (>5 CTC)
Non-progressive disease (S / PR / CR)	178	25
Progressive disease (PD)	16	14

Positive % agreement: 0.47, 95% CI [0.29, 0.65]

Negative % agreement: 0.88, 95% CI [0.83, 0.93]

Overall % agreement: 0.83, 95% CI [0.78, 0.88]

OR: 6.23, 95% CI [2.72, 14.3]

CTC results and efficacy assessment results by imaging were obtained in the first follow-up visit after effective treatment in 227 patients with MBC, see table 6 below

Table 6: Comparison of CTC results and efficacy assessment results by imaging in the first follow-up visit after treatment in MBC patients

Efficacy assessment by imaging	**CTC results**	
	Favorable prognosis (<5 CTC)	**Unfavorable prognosis (>5 CTC)**
Non-progressive disease (S / PR / CR)	164	34
Progressive disease (PD)	14	15

Positive % agreement: 0.47, 95% CI [0.34, 0.70]

Negative % agreement: 0.83, 95% CI [0.78, 0.88]

Overall % agreement: 0.79, 95% CI [0.74, 0.84]

OR: 5.17, 95% CI [2.28, 11.7]

CTC results in the second and the first follow-up visits after treatment agreed well with currently available treatment monitoring tool, that is, efficacy assessment by imaging. Currently in clinical practice, efficacy assessment by imaging is generally conducted in the second follow-up visit after treatment, as imaging changes in the tumor are barely noticeable in the first follow-up visit. Therefore, CTC determination in the first follow-up visit can provide even earlier information on clinical efficacy. Positive agreement rate is lower than negative agreement rate. Further studies in these patients are needed to confirm whether CTC change appears earlier than imaging changes. CTC should be used in combination with imageology and other monitoring methods to evaluete patients.

[CLINICAL TRIAL RESULTS ABROAD]

Metastatic Breast Cancer (MBC) Patients

A multi-center prospective, clinical trial was conducted to determine whether the count of CTC predicts disease progression and survival. Metastatic breast cancer patients (N=177) with measurable disease starting a new line of therapy were enrolled. Clinical data were analyzed on an intent-to-treat basis. The patient demographic information is presented in **Table 1**.

Table 1: MBC Patient Demographics

Category		N=177 Patients
Age at Baseline	Mean ± Std. Deviation	58 ±13
	Median	58
	Description of Categories	Number of Subjects (% of total)
Age at Baseline	1	26 (15%)
	2	92 (52%)
	3	26 (15%)
	4	20 (11%)
	Unknown	13 (7%)
Race	***White***	153 (86%)
	Black	14 (8%)
	Hispanic	7 (4%)
	Unknown	3 (2%)
Baseline ECOG Score	0	82 (46%)
	1	72 (41%)

	2	18 (10%)
	Unknown	5 (3%)
– **Disease Site**	Visceral	152 (86%)
	Bone	153 (86%)
ER/PR	+	121 (68%)
	-	54 (31%)
	Unknown	2 (1%)
HER2	0	91 (51%)
	1+	12 (7%)
	2+	18 (10%)
	3+	27 (15%)
	Unknown	29 (17%)
Line of Therapy	1st line	82 (46%)
	2nd line	26 (15%)
	≥ 3rd line	67 (38%)
	Unknown	2 (1%)
Type of Therapy	Chemo (Ch)	74 (42%)
	Endocrine (En)	45 (25%)
	Targeted (Ta)	9 (5%)
	Ch/En	10 (6%)
	Ch/Ta	23 (13%)
	En/Ta	7 (4%)
	Ch/En/Ta	2 (1%)
	Miscellaneous	2 (1%)
	Unknown	5 (3%)

Baseline CTC count was determined prior to initiation of a new line of therapy. Follow-up CTC counts were determined after the initiation of therapy at approximately 3 to 4 weeks intervals. For the baseline analyses, Progression Free Survival (PFS) was measured from the time of the baseline blood draw to the diagnosis of progression by CT scans and/or clinical signs and symptoms, and Overall Survival (OS) was measured from the time of baseline blood draw to the time of death. For the follow-up analyses, PFS was measured from the time of the follow-up blood draw to diagnosis of progression or death, and OS was measured from the time of the follow-up blood draw to the date of death.

1.4 CTC frequencies

The CTC results were obtained from the follow-up blood draws at 3-5 weeks, 6-8 weeks, 9-12 weeks, and 13-20 weeks after the initiation of therapy were classified as being favorable (<5 CTC) or unfavorable (≥ 5 CTC). If more than one CTC result was obtained within any of the designated follow-up time points, the CTC result from the blood draw furthest from the baseline blood draw was used.

Of the total MBC patient number of 177, 23 were not evaluable at first follow-up. Of these 23 patients, ten patients died before a follow-up blood draw could be obtained, nine patients progressed prior to a follow-up blood draw, and four were lost to follow-up. Notably, each of the ten patients who died had ≥ 5 to extremely high CTC counts at baseline (CTC counts 9, 11, 15, 24, 111, 126, 301, 1143, 4648 and 23618). Of the 154 patients available for follow-up, 132, 99, 129, and 85 patients had a blood draw at 3-5, 6-8, 9-14, and 15-20 weeks after initiation of therapy, respectively.

Table 3 summarizes the total number and percentage of patients with unfavorable CTC in the clinical trial for Overall Survival that differs from the numbers and percentages of patients for Progression Free Survival shown in **Table 10**. The difference in the number of patients at each time point between the two tables is due to the progression or death of some patients prior to the blood draw. The difference in the number of patients within the tables is due to the difference of the number of patients with blood draws and evaluable CTC results at each time point.

1.5 Progression Free Survival (PFS) Analysis of MBC Patients

15.1 PFS Using Baseline CTC Results

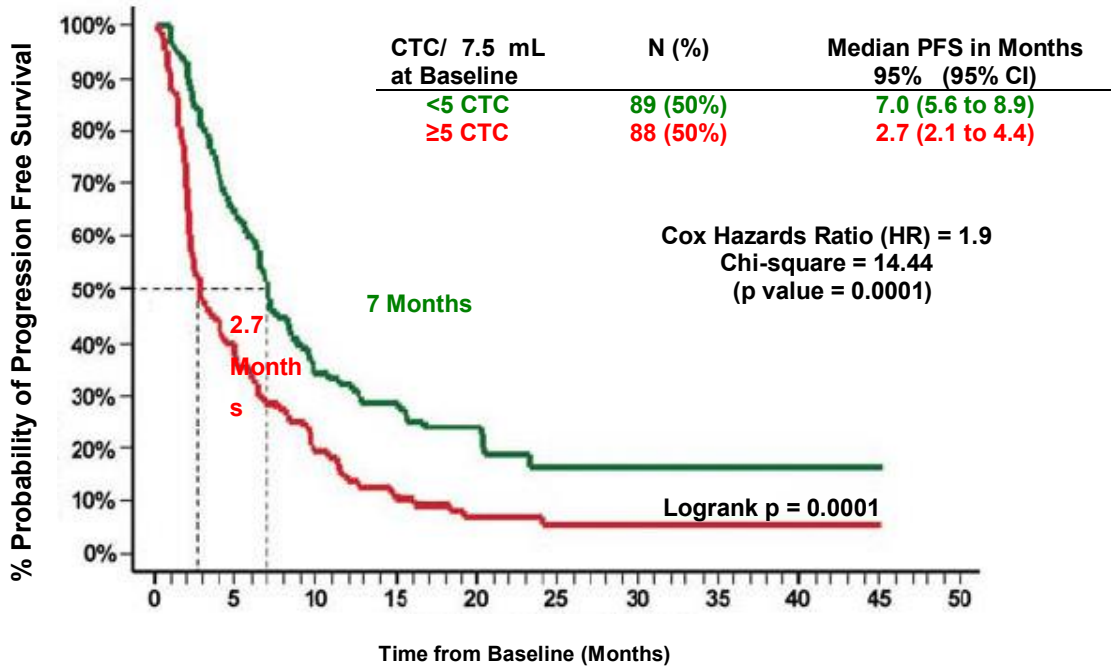
All 177 MBC patients had a baseline CTC test performed. For Kaplan-Meier analysis, patients were segmented into

two groups based upon their CTC count at baseline:

- The Favorable group (N=89), represented in green, consisted of patients with < 5 CTC.
- The Unfavorable group (N=88), represented in red, consisted of patients with ≥ 5 CTC.

Median PFS was longer in the Favorable group compared to the Unfavorable group (7.0 vs. 2.7 months, respectively). These results are illustrated in Figure 1.

– **Figure 1: PFS of MBC Patients with <5 or ≥ 5 CTC at Baseline (N=177).**



1.5.2 PFS Using Follow-up CTC Results

For Kaplan-Meier analysis, MBC patients were segmented into two groups based upon their CTC count at each of the various follow-up blood draws. Both patient groups at each of the different follow-up blood draw times after initiation of therapy for PFS are illustrated in Figure 2. PFS times were calculated from the time of each blood draw, and any patient showing evidence of progression prior to a particular blood draw was excluded from the analysis of that and all subsequent follow-up blood draws. Figure 2 illustrates the ability of CTC in patients with < 5 and ≥ 5 CTC 3-5 weeks, 6-8 weeks, 9-14 weeks, and 15-20 weeks after the initiation of therapy to predict time to clinical progression in 177 patients with metastatic breast cancer.

- The Favorable group, represented in olive green, blue, purple, and cyan, consisted of patients with <5 CTC,
- The Unfavorable group, represented in brown, black, grey, and orange, consisted of patients with ≥ 5 CTC.

– **Figure 2: PFS of MBC Patients with <5 or ≥ 5 CTC at different times of Follow-Up**

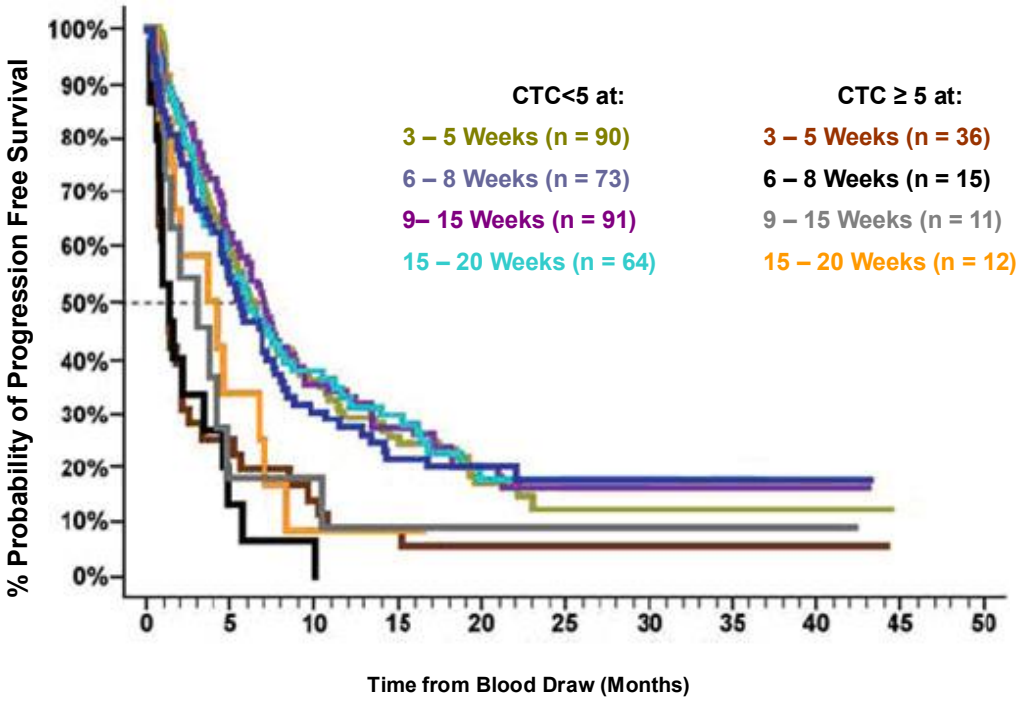


Table 2 summarizes the results of the PFS analysis using the CTC levels and a threshold of ≥ 5 CTC/7.5 mL at each of the different blood draw time points.

Table 2: Progression Free Survival (PFS) for MBC patients with <5 or ≥ 5 CTC at different time points

1	2	3	4	5	6
Sampling Time After Treatment Initiation	**N**	**≥5CTC**	**Median PFS in Months (95% CI)**		**Log-rank p-value**
			<5CTC	**≥5CTC**	
Baseline	117	88 (50%)	7.0 (5.6 to 8.9)	2.7 (2.1 to 4.4)	0.0001
3-5 Weeks	126	36 (29%)	6.1 (4.7 to 8.6)	1.3 (0.7 to 2.1)	<0.0001
6-8 Weeks	88	15 (17%)	5.6 (4.5 to 7.6)	1.4 (0.6 to 3.4)	0.0001
9-14 Weeks	102	11 (11%)	7.0 (5.1 to 8.8)	3.0 (0.9 to 4.8)	0.0251
15-20 Weeks	76	12 (16%)	5.9 (3.8 to 8.7)	3.6 (0.7 to 7.0)	0.0610

As illustrated in **Figure 2** and **Table 2**, patients with elevated CTC (≥ 5 CTC/7.5 mL whole blood) at any of the time points had a much higher likelihood of rapid progression than did those with <5 CTC. Table 10 column 4 shows the median PFS times for those patients with <5 CTC ranged from 5.6 to 7.0 months and were substantially longer than the median PFS times for those patients with ≥ 5 CTC, which ranged from 1.3 to 3.6 months (column 5).

1.5.3 Reduction or Increase in CTC Predicts Improved or Decreased PFS

Elapsed PFS times were calculated from the baseline blood draw. For Kaplan-Meier analysis (**Figure 3**), MBC patients were segmented into four groups based upon their CTC counts at baseline, 3-5 weeks, 6-8 weeks, 9-14 weeks, and 15-20 weeks after the initiation of therapy:

- Group 1 (green curve), 83 (47%) patients with <5 CTC at all blood draw time points. Five (6%) of these patients only had a baseline blood draw while two (2%) had a single blood draw between their first and last blood draw that had ≥ 5 CTC;
- Group 2 (blue curve), 38 (21%) patients with ≥ 5 CTC prior to the initiation of therapy but who had decreased to <5 CTC at the time of their last blood draw;
- Group 3 (orange curve), 17 (10%) patients with <5 CTC at an early draw (baseline, 3-5 weeks, and/or 6-8 weeks) but who increased to ≥ 5 CTC at the time of their last blood draw;
- Group 4 (red curve), 39 (22%) patients with ≥ 5 CTC at all blood draw time points. Ten (26%) of these patients only had a baseline blood draw;

– **Figure 3: A Reduction in CTC Below 5 After the Initiation of Therapy Predicts Longer PFS in MBC Patients**

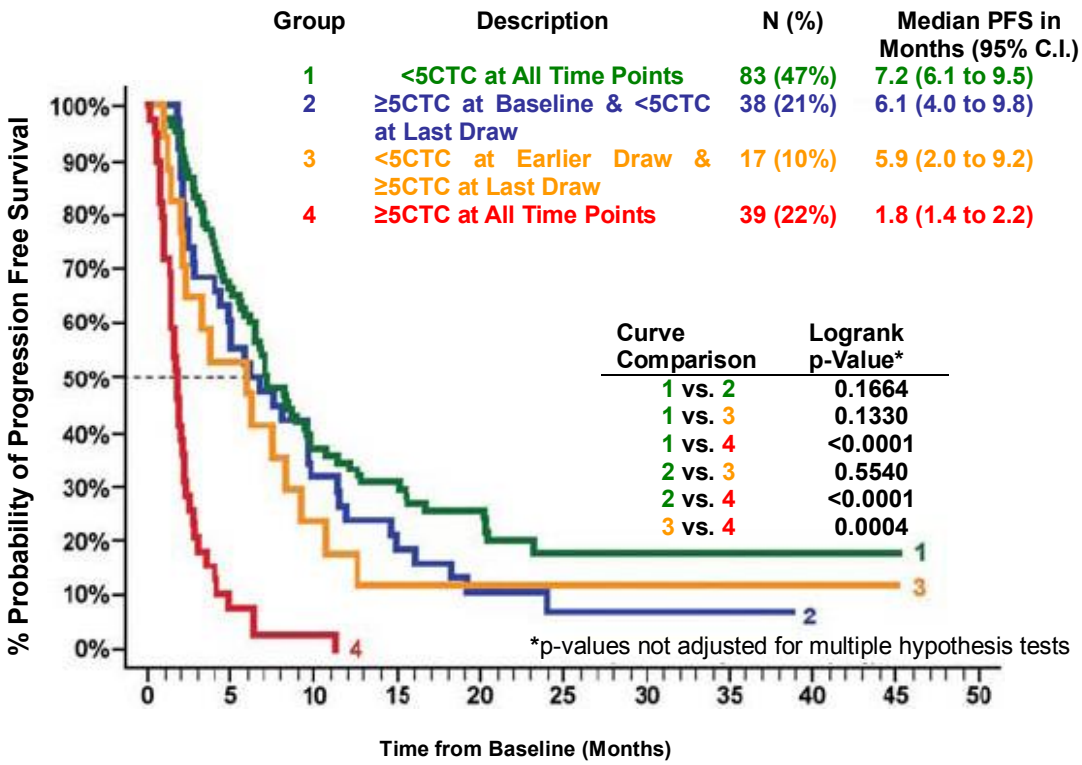


Figure 3 shows that MBC patients with ≥ 5 CTC at all time points (Group 4) had the shortest median PFS, which was significantly different compared to the median PFS of Group 3, Group 2, and Group 1. Differences between the curves for the other groups in this figure were not significant.

1.6 Overall Survival (OS) Analysis of MBC Patients

1.6.1 OS Analysis Using Baseline CTC Results

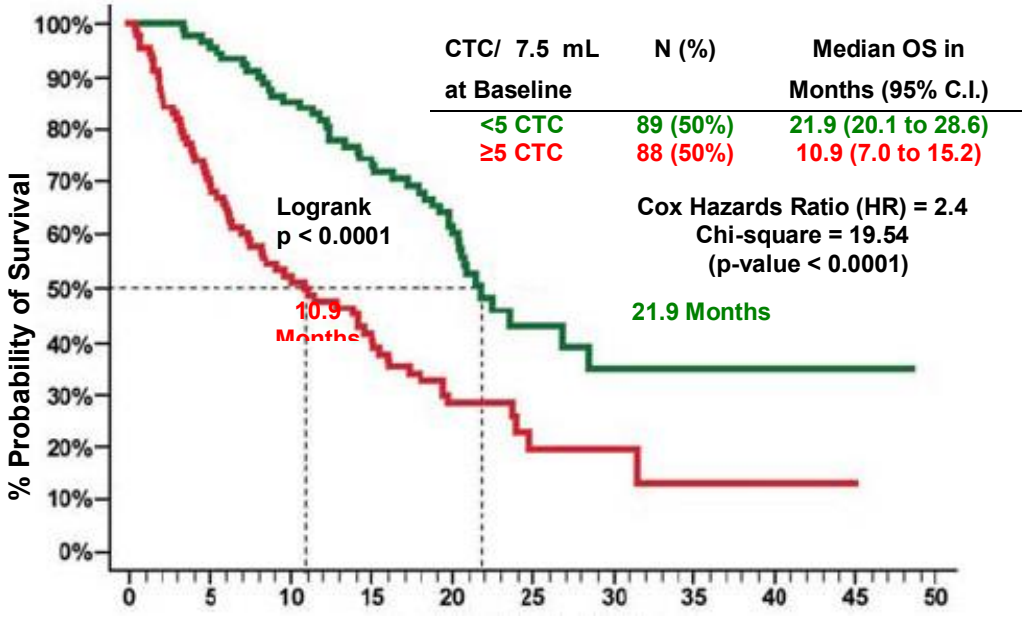
Death occurred in 109 (62%) of the 177 MBC patients, with a mean follow-up time for the 68 (38%) patients still alive of 22.7 ± 9.4 months (median = 21.1, range = 4.4 – 48.6). At the time of these analyses, 44 (49%) of 89 patients from Favorable group (<5 CTC at baseline) compared to 65 (74%) of 88 from Unfavorable group (≥ 5 CTC at baseline) had died.

For Kaplan-Meier analysis, patients were segmented into two groups based upon their CTC count at baseline:

- The Favorable group (N=89), represented in green, consisted of patients with <5 CTC.
- The Unfavorable group (N=88), represented in red, consisted of patients with ≥ 5 CTC.

Median OS was significantly longer in the Favorable group compared to the Unfavorable group (21.9 vs. 10.9 months, respectively). These results are illustrated in Figure 4.

– **Figure 4: OS of MBC Patients with <5 or ≥ 5 CTC at Baseline (N=177).**



1.6.2 OS Using Follow-up CTC Results

The Kaplan-Meier analyses of both MBC patient groups at each of the different follow-up blood draw times after initiation of therapy are illustrated in **Figure 5**. This figure illustrates the ability of CTC in MBC patients with <5 and ≥ 5 CTC 3-5 weeks, 6-8 weeks, 9-14 weeks, and 15-20 weeks after the initiation of therapy to predict time from the blood draw time point to death in 177 patients with metastatic breast cancer. OS times were calculated from the time of each blood draw.

- The Favorable group, represented in olive green, blue, purple, and cyan, consisted of patients with <5 CTC,
- The Unfavorable group, represented in brown, black, grey, and orange, consisted of patients with ≥ 5 CTC.

– **Figure 5: OS of MBC Patients with <5 or ≥ 5 CTC at different times of Follow-Up.**

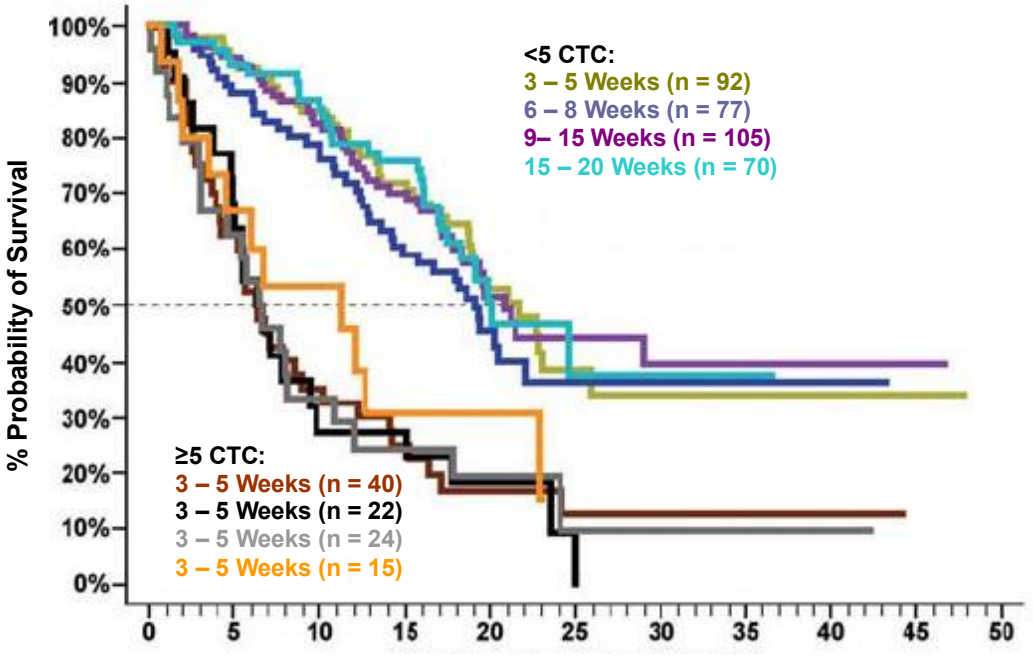


Table 3 summarizes the results of the OS analysis using the CTC levels and a threshold of ≥ 5 CTC/7.5 mL at each of the different blood draw time points.

Table 3: Overall Survival (OS) for MBC patients with <5 or ≥ 5 CTC at different time points

1	2	3	4	5	6
Sampling Time After Treatment Initiation	**N**	**≥5 CTC**	**Median OS in Months (95% CI)**		**Log-rank p-value**
			<5CTC	**≥5 CTC**	
Baseline	177	88 (50%)	21.9 (20.1 to 28.6)	10.9 (7.0 to 15.2)	<0.0001
3-5 Weeks	132	40 (30%)	21.7 (18.8 to 25.9)	6.2 (4.1 to 8.9)	<0.0001
6-8 Weeks	99	22 (22%)	19.1 (14.2 to 22.1)	6.3 (4.8 to 9.8)	0.0001
9-14 Weeks	129	24 (19%)	20.8 (17.8 to ≥45)	6.4 (3.0 to 10.9)	<0.0001
15-20 Weeks	85	15 (18%)	20.1 (17.1 to ≥ 35)	11.3 (2.0 to 22.9)	0.0021

1.6.3 Reduction or Increase in CTC Predicts Improved or Decreased OS

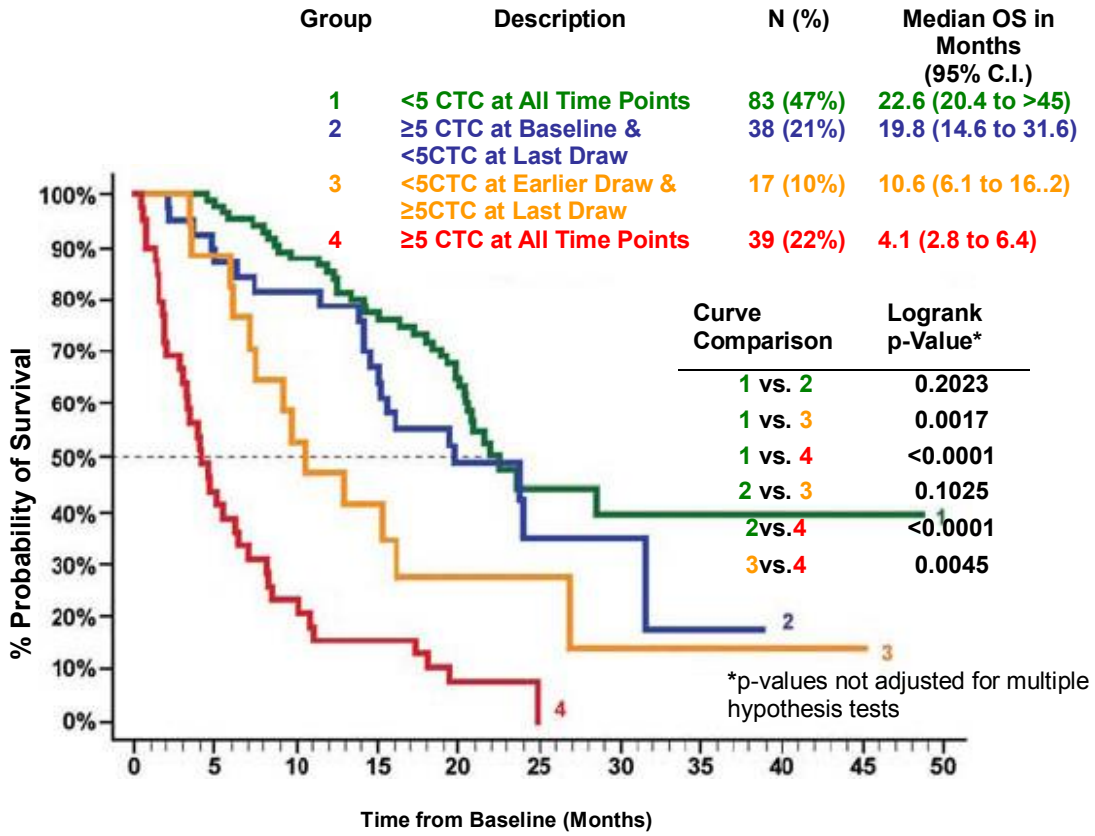
Elapsed OS times were calculated from the baseline blood draw. For Kaplan-Meier analysis (**Figure 6**), patients were segmented into four groups based on their CTC counts at baseline, 3-5 weeks, 6-8 weeks, 9-14 weeks, and 15-20 weeks after the initiation of therapy:

- Group 1 (green curve), 83 (47%) patients with <5 CTC at all blood draw time points. Five (6%) of these patients only had a baseline blood draw while two (2%) had a single blood draw between their first and last blood draw that had ≥ 5 CTC;
- Group 2 (blue curve), 38 (21%) patients with ≥ 5 CTC prior to the initiation of therapy but who had decreased to <5 CTC at the time of their last blood draw;
- Group 3 (orange curve), 17 (10%) patients with <5 CTC at an early draw (baseline, 3-5 weeks, and/or 6-8 weeks) but who increased to ≥ 5 CTC at the time of their last blood draw;
- Group 4 (red curve), 39 (22%) patients with ≥ 5 CTC at all blood draw time points. Ten (26%) of these patients only had a baseline blood draw.

Figure 6 shows that MBC patients who exceed the threshold of 5 CTC at any point after the initiation of therapy had a much higher likelihood of shorter overall survival. Patients with ≥ 5 CTC at all time points (Group 4) had the shortest median OS, which was significantly different compared to the median OS of Group 3, Group 2, and Group 1. The difference in the median survival between Group 3 and Group 1 was also significant, and although the median OS for Group 3 was shorter compared to Group 2, the difference was not statistically significant. Figure 6 also shows that patients who have ≥ 5 CTC at baseline but eventually decrease to <5 CTC after the initiation of therapy have approximately the same risk of death as those patients who never exceed the 5 CTC threshold.

– **Figure 6: A Reduction in CTC Below 5 After the Initiation of Therapy Predicts Longer OS whereas an Increase in**

CTC Count to 5 or above Predicts Shorter OS in MBC Patients.



As illustrated in **Figure 6** and columns 4 & 5 in **Table 3** , MBC patients with ≥ 5 CTC at any of the time points had a much higher probability of dying sooner than did those with <5 CTC. The median OS times for those patients with <5 CTC ranged from 19.1 to 21.9 months and were substantially longer than the median OS times for those patients with ≥ 5 CTC, which ranged from 6.2 to 11.3 months.

1.6.4 Univariate Cox Regression Analysis in MBC Patients

The following parameters were analyzed using univariate Cox regression analysis to evaluate association with PFS and OS: patient age (continuous), stage of disease at diagnosis (1-4), time to metastasis (continuous), ECOG status before initiation of a new line of therapy (0-2), ER/PR status (+/-), HER2/neu status (0-3+), line of therapy (≥ 2nd or 1st), type of therapy (chemo only or hormonal / combination), baseline CTC count (≥ 5 or <5 CTC/7.5 mL), and follow-up CTC counts 3-5 weeks, 6-8 weeks, 9-14 weeks, and 15-20 weeks after the initiation of therapy (≥ 5 or <5 CTC/7.5 mL). **Table 4** shows the results of this analysis and presents the Cox hazard ratio (HR) and associated p-value (Wald test of Z statistic) as well as the number of patients in each evaluation.

Table 4: Univariate Cox Regression Analysis in MBC Patients

Parameter	Categories		Number of MBC Patients	PFS Risk from Baseline		PFS Risk from Baseline	
	Positive	Negative		HR	p-value	HR	p-value
Age at Baseline Blood Draw	**Age in Years**		175	0.99	0.173	0.99	0.375
PFS Risk from Baseline	**4 vs. 3 vs. 2 vs. 1**		164	0.97	0.723	1.00	0.969
ER/PR	**Positive**	**Negative**	175	0.84	0.327	0.53	0.002
Her-2/neu	**3+ vs. 2+ vs. 1+ vs. 0**		148	0.91	0.207	0.93	0.422
Baseline ECOG Status	**2 vs. 1 vs. 0**		172	1.14	0.307	1.64	0.001
Time to Metastasis	**Time in Years**		175	0.97	0.048	0.95	0.018

Line of Therapy	≥2**nd**	1**st**	175	1.55	0.007	1.91	0.001
Type of Therapy	**Chemo Only**	H / C and/or I	172	1.97	<0.001	2.22	<0.001
Baseline CTC Number	≥5	<5	177	1.85	<0.001	2.36	<0.001
3 - 5 Week CTC Number	≥5	<5	132	2.52	<0.001	3.30	<0.001
6 - 8 Week CTC Number	≥5	<5	99	3.57	<0.001	2.87	<0.001
9 - 14 Week CTC Number	≥5	<5	129	2.89	<0.001	3.64	<0.001
15 - 20 Week CTC Number	≥5	<5	85	1.86	0.041	2.85	0.004

H/C/ and/or I – Hormonal or Targeted therapy alone or Combination of Hormonal and/or Chemo and/or Targeted therapy

1.6.5 Multivariate Cox Regression Analysis in MBC Patients

Multivariate Cox regression analyses were conducted in MBC patients to evaluate the independent predictive power of CTC count by adjusting for the effects of the known important clinical factors that are statistically significant in the univariate analyses. CTC were found to be strong predictors of PFS (**Table 5**) and OS (**Table 6**).

Table 5: Progression Free Survival Multivariate Cox Regression Analysis in MBC Patients

Variable	N	HR	P **value**
Baseline CTC (<5 vs ≥5)	172	1.69	0.001
Time to Metastasis (year)		0.98	0.154
Line of Therapy (1st vs.≥2nd)		1.52	0.013
Type of Therapy (Hormonal/Other vs. Chemo Only)		1.74	0.001
3-5 week follow-up CTC (<5 vs ≥5)	132	2.32	<0.001
Time to Metastasis (year)		0.97	0.166
Line of Therapy (1st vs. ≥2nd)		1.68	0.008
Type of Therapy (Hormonal/Other vs. Chemo Only)		1.50	0.060
6-8 week follow-up CTC (<5 vs ≥5)	99	2.92	<0.001
Time to Metastasis (year)		0.93	0.023
Line of Therapy (1st vs. ≥2nd)		1.36	0.175
Type of Therapy (Hormonal/Other vs. Chemo Only)		1.90	0.005
9-14 week follow-up CTC (<5 vs ≥5)	129	2.23	0.002
Time to Metastasis (year)		0.97	0.170
Line of Therapy (1st vs. ≥2nd)		1.48	0.061
Type of Therapy (Hormonal/Other vs. Chemo Only)		1.73	0.007
15-20 week follow-up CTC (<5 vs. ≥5)	85	1.58	0.140
Time to Metastasis (year)		0.96	0.064
Line of Therapy (1st vs. ≥2nd)		1.80	0.018
Type of Therapy (Hormonal/Other vs. Chemo Only)		1.66	0.049

Table 6: Overall Survival Multivariate Cox Regression Analysis in MBC Patients

Variable	N	HR	P value
Baseline CTC (<5 vs ≥5)	170	2.62	<0.001
ER/PR (Negative vs Positive))		0.57	0.016
Baseline ECOG Status (2 vs 1 vs 0)		1.58	0.001
Time to Metastasis (Year)		0.97	0.078
Line of Therapy (1st vs. ≥2nd)		2.33	<0.001
Type of Therapy (Hormonal/Other vs. Chemo Only)		1.78	0.006
3-5 week follow-up CTC (<5 vs. ≥5)	130	3.78	<0.001
ER/PR (Negative vs Positive))		0.51	0.020
Baseline ECOG Status (2 vs 1 vs 0)		1.69	0.001
Time to Metastasis (year)		0.96	0.142
Line of Therapy (1st vs. ≥2nd)		2.30	0.001
Type of Therapy (Hormonal/Other vs. Chemo Only)		1.72	0.026
6-8 week follow-up CTC (<5 vs. ≥5)	99	2.88	0.001
ER/PR (Negative vs Positive))		0.58	0.062
Baseline ECOG Status (2 vs 1 vs 0)		1.32	0.173
Time to Metastasis (year)		0.94	0.135
Line of Therapy (1st vs. ≥2nd)		2.51	0.001
Type of Therapy (Hormonal/Other vs. Chemo Only)		2.33	0.003
9-14 week follow-up CTC (<5 vs. ≥5)	129	4.14	<0.001
ER/PR (Negative vs Positive))		0.39	0.002
Baseline ECOG Status (2 vs. 1 vs. 0)		1.57	0.016
Time to Metastasis (year)		0.98	0.388
Line of Therapy (1st vs. ≥2nd)		2.21	0.003
Type of Therapy (Hormonal/Other vs. Chemo Only)		2.28	0.003
15-20 week follow-up CTC (<5 vs. ≥5)	85	3.44	0.006
ER/PR (Negative vs Positive))		0.38	0.024
Baseline ECOG Status (2 vs 1 vs 0)		1.33	0.321
Time to Metastasis (year)		0.94	0.150
Line of Therapy (1st vs. ≥2nd)		3.43	0.001
Type of Therapy (Hormonal/Other vs. Chemo Only)		1.67	0.166

1.7 Use of CTC to Monitor Clinical Status of Metastatic Breast Cancer

1.7.1 Relationship between survival, CTC, and disease assessment by imaging

Radiological imaging is one of the primary means of determining disease status and response to therapy in metastatic breast cancer patients. To establish the relationship of clinical status as determined by imaging to CTC, CTC measured at two different time points and imaging results were compared 1) to the true clinical endpoint overall survival and 2) to each other.

1.7.2 CTC

Previous data has shown that MBC patients with ≥ 5 CTC / 7.5 mL of blood at any succeeding follow-up visit after the initiation of therapy had a higher likelihood of progressive disease and decreased overall survival compared to patients with <5 CTC / 7.5 mL of blood. The CTC results obtained at the first follow-up after the initiation of therapy as well as the CTC results obtained within ± one month of the imaging study were classified as <5 CTC and ≥ 5 CTC. If more than one CTC value was obtained within ± one month of the imaging study, the CTC result obtained closest to

the date of the imaging study was used.

1.7.3 Imaging

All imaging sites were in compliance with Digital Imaging and Communications in Medicine (DICOM) standards. Using standardized digital images, two expert radiologists (readers), working individually and blinded to clinical information, classified each follow-up disease assessment (total of 231 imaging studies) from 138 patients with measurable disease as indeterminate (I), stable disease (S), partial response (PR), or progressive disease (PD) according to World Health Organization (WHO) bi-dimensional criteria. Measurable disease was defined as the .presence of at least one lesion ≥ 2cm in its longest dimension.

Readers identified up to eight lesions per patient per time point by describing the longest dimension of the lesion and the longest perpendicular dimension. These two dimensions were multiplied and the "cross product" was reported. Summed measurements for the cross products were calculated, and percent change from the previous time point was determined. Although all patients had measurable disease, non-measurable lesions (still detectable by radiology) were included in the determination of patient status as described in the WHO guidelines. Progressive disease was defined as a >25% increase in the sum of all lesions or appearance of a new measurable or non-measurable lesion. Partial response was defined as a decrease in the sum of all lesions of ≥ 50% and no new lesions.

Radiology interpretations from the two expert radiologists were classified as followed:

- S and PR were considered to both reflect non-progressive disease (NPD)
- PD was considered to reflect progressive disease
- In situations where one of the radiologists rendered a classification of Indeterminate (I) but the other radiologist rendered a classification of S, PR or PD, the classification of the latter radiologist was used for comparison to CTC (n=11)
- When both radiologists rendered a classification of Indeterminate (I), then the data was not used in the comparison to CTC (n=3)
- A third independent radiologist adjudicated disagreements between the two primary readers regarding PD and NPD (n=27)
- In situations where the third independent radiologist rendered a classification of Indeterminate (I), the data was not used in the comparison to CTC (n=2)
- In serial imaging studies, radiology results that were less than one month from a previous tabulated observation were not used (n=1).

1.7.4 Relationship between survival to imaging and CTC

Separate Kaplan-Meier analyses were performed to compare the overall survival of MBC patients in the Favorable (<5 CTC) and Unfavorable (≥ 5 CTC) groups using CTC results at two different time points and the first follow-up imaging study. Using results from the first follow-up imaging studies performed 10.1 ± 5.1 weeks (median = 9.0 weeks) after initiation of therapy (i.e. the baseline blood draw), the median survival of the 96 (70%) patients determined by imaging to have NPD was 23.8 months (95% CI 20.4 to 28.6) (Figure 14, Table 15). For the 42 (30%) patients determined by imaging to have PD, the median survival was 12.9 months (95% CI 7.1 to 19.3).

For CTC at the first follow-up blood draw, performed 4.3 ± 2.5 weeks (median = 4.0 weeks) after initiation of therapy, the median survival of 104 (75%) patients with Favorable CTC results (<5 CTC) was 21.9 months (95% CI 20.4 to 26.9) (Figure 15, Table 15). Thirty-four (34) patients (25%) with Unfavorable CTC results (≥ 5 CTC) had a median survival of 8.3 months (95% CI 5.9 to 15.1).

To determine if CTC assessments performed closer to the time of the imaging results in similar survival prospects compared to CTC assessments done approximately 4 weeks after the initiation of therapy, only those patients with CTC assessments performed within ± one month of the first follow-up imaging study (9.9 ± 5.1 weeks, median = 8.8 weeks, after the initiation of therapy) were analyzed (Figure 16, Table 15). One hundred and thirty four (134) of the 138 patients (97%) had CTC assessments within one month of the first follow-up imaging study. The median survival of 105 (78%) patients with Favorable CTC results was 21.9 months (95% CI 19.9 to 31.6). For 29 (22%) patients with Unfavorable CTC results, the median survival was 8.5 months (95% CI 5.5 to 15.1). These data show that CTC assessments at both time points provide similar results to imaging conducted approximately 9 weeks after the initiation of therapy (*Clin. Cancer Res. Vol 12: 6403-6409, November 2006*).

Table 7: OS of MBC Patients with CTC assessment approximately one month after the initiation of therapy and within one month of the radiological assessment

	N	Median Survival & (95% CI) Months
Imaging	138	
Favorable (NPD)	96 (70%)	23.8 (20.4 - 28.6)
Unfavorable (PD)	42 (30%)	12.9 (7.1 -19.3)
1st follow-up CTC	138	
Favorable (<5)	104 (75%)	21.9 (20.4-26.9)
Unfavorable (≥5)	34 (25%)	8.3 (5.9-15.1)
CTC (±1 Month of Imaging)	134*	
Favorable (<5)	105 (78%)	21.9 (19.9-31.6)
Unfavorable (≥5)	29 (22%)	8.5 (5.5-15.1)

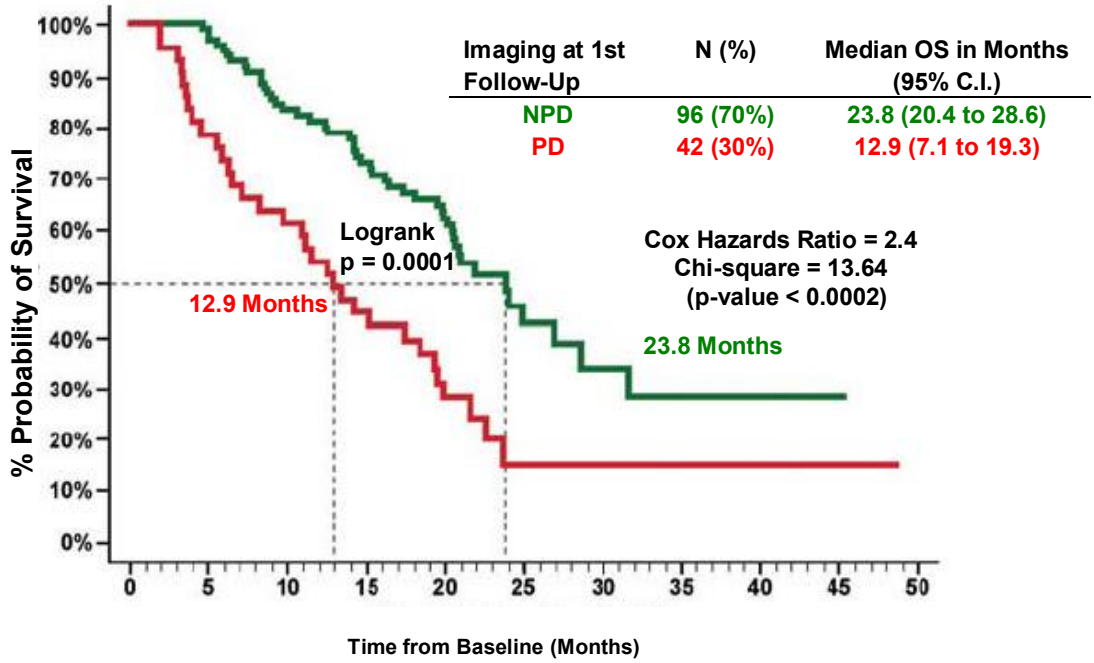
*134 /138 patients had CTC assessments performed within (±) 1 month of Imaging.

- **Figure 7: Correlation of Radiological and CTC Assessment with OS: OS of MBC Patients with NPD or PD at 1st Follow-Up Imaging Study (N=138)**

	N	Median Survival & (95% CI) Months
Imaging	138	
Favorable (NPD)	96 (70%)	23.8 (20.4 - 28.6)
Unfavorable (PD)	42 (30%)	12.9 (7.1 -19.3)
1st follow-up CTC	138	
Favorable (<5)	104 (75%)	21.9 (20.4-26.9)
Unfavorable (≥5)	34 (25%)	8.3 (5.9-15.1)
CTC (±1 Month of Imaging)	134*	
Favorable (<5)	105 (78%)	21.9 (19.9-31.6)
Unfavorable (≥5)	29 (22%)	8.5 (5.5-15.1)

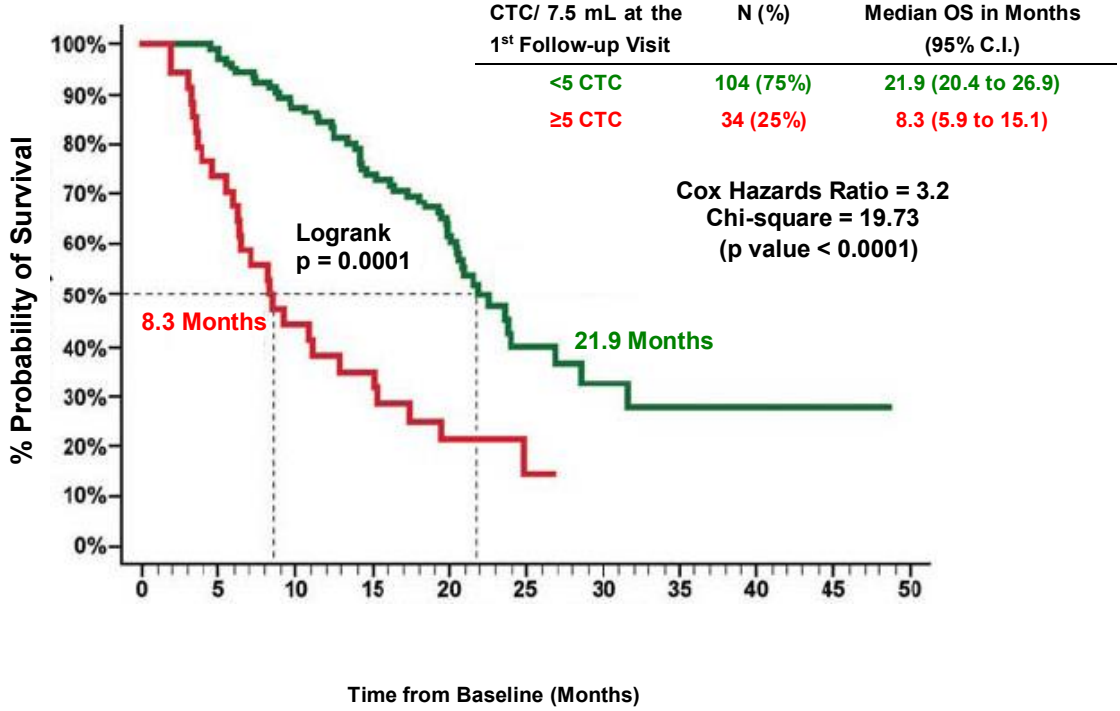
*134 /138 patients had CTC assessments performed within (±) 1 month of Imaging.

– **Figure 7: Correlation of Radiological and CTC Assessment with OS: OS of MBC Patients with NPD or PD at 1st Follow-Up Imaging Study (N=138)**



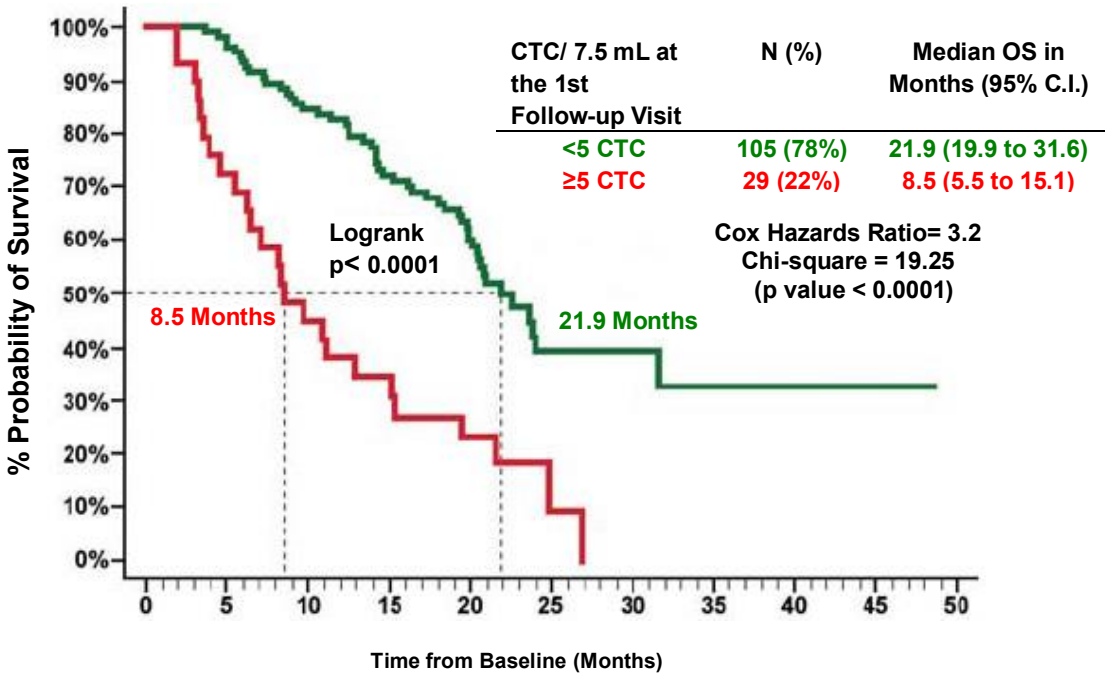
Imaging at 1st Follow-Up	N (%)	Median OS in Months (95% C.I.)
NPD	96 (70%)	23.8 (20.4 to 28.6)
PD	42 (30%)	12.9 (7.1 to 19.3)

– **Figure 8: Correlation of Radiological and CTC Assessment with OS: OS of MBC Patients with <5 or ≥ 5 CTC at 1st Follow-Up after Initiation of Therapy (N=138)**



CTC/ 7.5 mL at the 1st Follow-up Visit	N (%)	Median OS in Months (95% C.I.)
<5 CTC	104 (75%)	21.9 (20.4 to 26.9)
≥5 CTC	34 (25%)	8.3 (5.9 to 15.1)

– **Figure 9: Correlation of Radiological and CTC Assessment with OS: OS of MBC Patients with <5 or ≥ 5 CTC within ± 1 Month of 1st Follow-Up Imaging Study (N=134)**



1.7.5 Concordances between CTC and Radiological Monitoring

As noted above, imaging studies are a major component of the current standard of care for determining disease progression and response to treatment in the metastatic breast cancer setting. To further support the effectiveness of CTC in making these clinical assessments (*Clin. Cancer Res. Vol 12: 6403-6409, November 2006*), two-by-two tabulations of concordant and discordant observations between CTC and radiological imaging were constructed using the previously described criteria.

Using only the 1st follow-up imaging study, the radiological response at this visit was compared with the CTC results obtained within ± one month of this imaging study. A total of 134 of the 138 MBC patients (97%) had CTC results that met this criterion. The result of this "patient-wise" comparison between CTC and imaging is shown in **Table 8**.

Table 8: MBC Patient-Wise Comparison of CTC and Imaging

Response at 1st Follow-Up Imaging Study	CTC within ± 1 Month of Imaging		Total
	<5CTC/7.5mL	≥5CTC/7.5mL	
Non-Progressive Disease	85	9	**94**
Progressive Disease	20	20	**40**
Total	**105**	**29**	**134**

Measurement	Estimate	Lower 95%	Upper 95%
Positive % Agreement	50%	34%	66%
Negative % Agreement	90%	83%	96%
Positive Predictive Value	69%	49%	85%
Negative Predictive Value	81%	72%	88%
Overall Agreement	78%	70%	85%
Odds Ratio	9.4	3.4	26.8

Using all of the follow-up imaging studies performed after the initiation of therapy on the 138 MBC patients that rendered useable radiological response results (n=225), these results were then compared to CTC results obtained within ± one month of the imaging study. A total of 219 of the 225 (97%) imaging studies had CTC results meeting this criterion. The result of this "observation-wise" comparison between CTC and imaging is shown in **Table 9**.

Table 9: MBC Observation-Wise Comparison of CTC and Imaging

Response at All Follow-Up Imaging Studies	CTC within ± 1 Month of Imaging		Total
	<5 CTC/7.5mL	≥5 CTC/7.5 mL	
Non-Progressive Disease	151	16	**167**
Progressive Disease	30	22	**52**
Total	**181**	**38**	**219**

Measurement	Estimate	Lower 95%	Upper 95%
Positive % Agreement	42%	29%	57%
Negative % Agreement	90%	85%	94%
Positive Predictive Value	58%	41%	74%
Negative Predictive Value	83%	77%	89%
Overall Agreement	79%	73%	84%
Odds Ratio	6.9	3.0	15.8

In serial observations, only a minority of the transitions for imaging results between non progressive disease and progressive disease coincided with a matching transition of CTC counts between <5 and ≥ 5 CTC / 7.5 mL.

Because the prognostic value of the CTC results at an earlier time-point was equivalent to that of the CTC results at the time of imaging (Figure 8 and Figure 9), a patient-wise comparison using results from only the 1st follow-up imaging study, performed approximately 9 weeks after the initiation of therapy, and the CTC results obtained at the 1st follow-up, performed approximately 4 weeks after initiation of therapy, was constructed. All 138 MBC patients had CTC results meeting this criterion. The result of this "patient-wise" comparison between CTC at an earlier time point and imaging is shown in **Table 10**.

Table 10: MBC Patient-Wise Comparison of CTC and Imaging

Response at 1st Follow-Up Imaging Study	CTC at 1st Follow-Up		Total
	<5CTC/7.5mL	≥5CTC/7.5mL	
Non-Progressive Disease	84	12	**96**
Progressive Disease	20	22	**42**
Total	**104**	**34**	**138**

Measurement	Estimate	Lower 95%	Upper 95%
Positive % Agreement	52%	36%	68%
Negative % Agreement	88%	79%	93%
Positive Predictive Value	65%	46%	80%
Negative Predictive Value	81%	72%	88%
Overall Agreement	77%	69%	84%
Odds Ratio	7.7	3.0	19.9

1.7.6 CTC as an Adjunct to Imaging

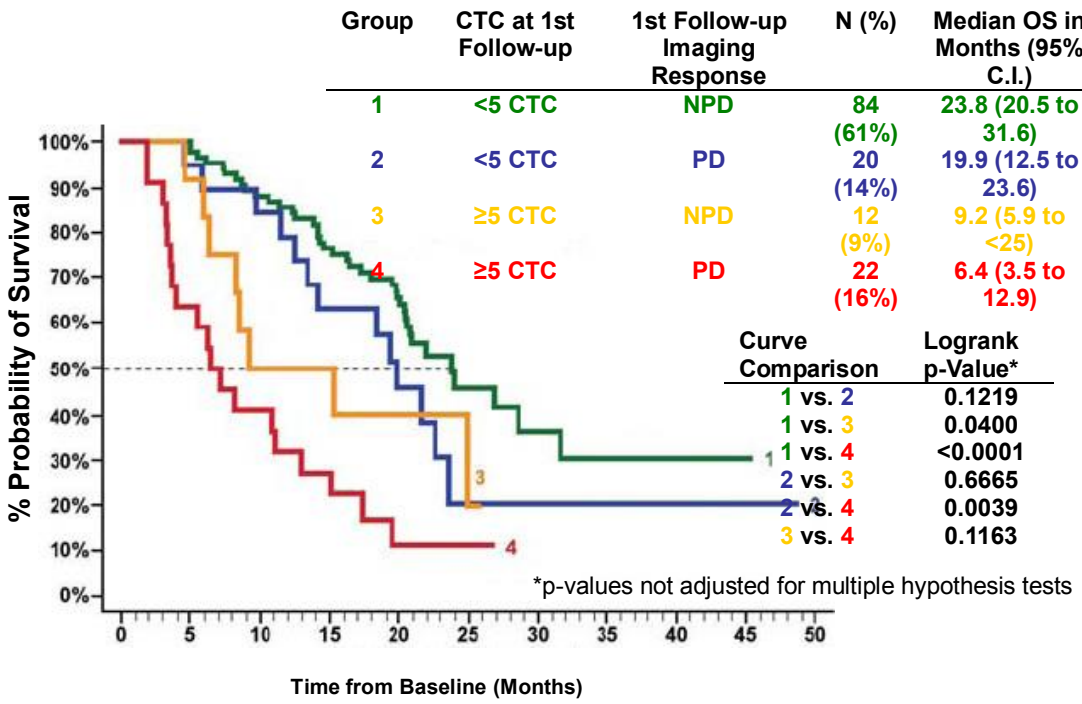
While the overall agreement between CTC and imaging was good (approximately 78%), there was disagreement in approximately 22% of the MBC patients. As the information from CTC assessments is intended to be used in conjunction with other diagnostic modalities to make treatment decisions, CTC assessment at 1st follow-up (approximately 4 weeks after the initiation of therapy) and imaging in the following groups were compared to OS to determine which of the discordant results better reflected the prognosis of the patient (**Figure 10**):

- Group 1 (green curve), 84 (61%) patients with <5 CTC at 1st follow-up and NPD;
- Group 2 (blue curve), 20 (14%) patients with <5 CTC at 1st follow-up and PD;
- Group 3 (orange curve), 12 (9%) patients with ≥ 5 CTC at 1st follow-up and NPD;

- Group 4 (red curve), 22 (16%) patients with ≥ 5 CTC at 1st follow-up and PD.

In this study, CTC determination is a strong independent predictor of overall survival. The study results indicated that the combination of CTC and radiological assessments provides the most accurate assessment of prognosis.

– **Figure 10: OS of MBC Patients in Groups 1, 2, 3 and 4 using the 1st Follow-up CTC assessment after Initiation of Therapy (n=138) and the Disease Status Determined at the 1st Follow-Up Imaging Study**



1.8 Variability of CTC and Radiological Assessments

1.8.1CTC

Inter-reader variabilities for the CTC counts at the first follow-up blood draw was determined by counting the number of instances where the operator at the testing site was not in concordance with the central laboratory in classifying a sample as ≥ 5 CTC versus <5 CTC. In a subset of 71 patients, two tubes of blood were drawn and processed, and the classification of ≥ 5 CTC versus <5 CTC in each of the two tubes as determined by the site as well as by the central laboratory was compared.

1.8.2 Imaging

Inter-reader variability was determined by comparing the radiological interpretations of the two radiologists, classified as NPD vs. PD. Intra-reader variability was calculated by comparing the radiological interpretations of the two radiologists in a subset of patients where each radiologist determined the response at three separate sittings, each sitting separated by a minimum of one week. Imaging segments of later assessments in these 138 MBC patients and CTC assessments before initiation of therapy and at later follow-ups were studied also.

Table 11: Variability of Radiological and CTC Assessments in MBC Patients

	N	Radiology NPD vs. PD Disagreement	N	CTC/7.5 mL <5 vs ≥5 Disagreement
Inter-reader				
1st Follow-Up	132	11.4%	138	0.7%
Any Follow-Up	217	13.4%	695	1.0%
Intra-reader				
1st Follow-Up				
Reader 1 (radiology)	24	25.0%	-	-
Reader 2 (radiology)	22	9.1%	-	-
Any Follow-Up				
Reader 1 (radiology)	30	20.0%	-	-
Reader 2 (radiology)	28	10.7%	-	-
CTC Tube to Tube				
1st Follow-Up	-	-	71	5.6%
Any Follow-Up	-	-	403	5.5%

Table 11 shows that the inter-reader variability of the radiological determinations were significantly higher in both the first follow-up disease assessment and in all subsequent disease follow-up assessments when compared to the inter-reader variability of the CTC counts in the same groups (Fisher's $P<0.001$).

In cases where CTC and radiological assessment were discordant, CTC provided the most accurate assessment of prognosis.

[REFERENCES]

1. Cancer Biology, 3rd edition, Ray Ruddon 1995
2. NCCLS Approved Guideline EP5-A, “Evaluation of Precision Performance of Clinical Chemistry Devices”.
3. NCCLS Approved Guideline C28-A2, “How to Define and Determine Reference Intervals in the Clinical Laboratory”.
4. Shaffer DR, Leversha MA, Danila DC, Lin O, Gonzalez-Espinoza R, Gu B, Anand A, Smith K, Maslak, P, Doyle GV, Terstappen LWMM, Lilja H, Heller G, Fleisher M and Scher HI. “Circulating Tumor Cell Analysis in Patients with Progressive Castration-Resistant Prostate Cancer”, Clinical Cancer Research, Vol 13 No.7: 2023-2029, (2007).
5. Tibbe A.G.J, Miller C.M and Terstappen LWMM “Statistical Considerations for Enumeration of Circulating Tumor Cells”, Cytometry Part A 71a:154-162 (2007).

[BASIC INFORMATION]

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China Agent: Johnson & Johnson Medical (Shanghai) Ltd.
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China Agent Contact Info. 400 920 8790
Date of Manufacture: Refer to product package
Date of Expiration: Refer to product package

[MEDICAL DEVICE CERTIFICATE NO./ PRODUCT TECHNICAL REQUIREMENT NO.]

CFDA(I)20153403566

[APPROVAL DATE AND REVISION DATE OF INSTRUCTIONS FOR USE]

2017.03.08

[SYMBOLS GLOSSARY]

The following symbols might be used in the label printing of the product.

	Date of Manufacture	IVD	*In vitro* diagnostic medical device		Caution, consult accompanying documents
	Use by YYYY-MM-DD or YYYY-MM		Contains sufficient for < n > tests		Manufacturer
LOT	Batch code		Temperature limitation	EC REP	Authorized representative in the European Community
SN	Serial number		Consult instructions for use		Contains sufficient for < n > tests
REF	Catalog number		Biological risks		Warning