REF 7900001
16 Test Kit

CellSearch®

Circulating Tumor Cell Kit (Epithelial)

IVD

MENARINI silicon biosystems

INTENDED USE

For *in vitro* diagnostic use.

The CELLSEARCH® Circulating Tumor Cell Kit 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, colorectal or prostate* cancer. The test is to be used as an aid in the monitoring of patients with metastatic breast, colorectal or prostate cancer. Serial testing for CTC should be used in conjunction with other clinical methods for monitoring metastatic breast, colorectal and prostate 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.

**Metastatic prostate cancer patients in this study were defined as having two consecutive increases in the serum marker PSA above a reference level, despite standard hormonal management. These patients are commonly described as having androgen-independent, hormone-resistant, or castration-resistant prostate cancer.*

SUMMARY AND EXPLANATION

Cancer metastasis occurs when cells shed from a primary or metastatic tumor, enter the circulation, and begin to grow in distant locations of the body. Carcinomas are derived from epithelial cells that are not normally found in circulation.[1] The CELLTRACKS® AUTOPREP® System was designed to standardize and automate the sample preparation protocol for use with the CELLSEARCH® Circulating Tumor Cell Kit (CELLSEARCH® CTC Kit). Analysis and enumeration of CTC's are performed using the CELLTRACKS ANALYZER II®, a semi-automated fluorescence microscope. The assay enumerates only those cells that express the Epithelial Cell Adhesion Molecule (EpCAM) and cytokeratins (CK) 8, 18, and/or 19.

PRINCIPLES OF THE PROCEDURE

The CELLSEARCH® Circulating Tumor Cell Kit contains a ferrofluid-based capture reagent and immunofluorescent reagents. The ferrofluid reagent consists of particles 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® 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-.

MATERIALS PROVIDED

- Instructions for Use
- **3.0 mL Anti-EpCAM Ferrofluid:** Contains a suspension of 0.022% magnetic particles 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 CELLSEARCH® Conical Centrifuge Tubes (15 mL) 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)
- CELLSEARCH® Circulating Tumor Cell Control Kit (Catalog #7900003)
- CELLTRACKS® AUTOPREP® Instrument Buffer (Catalog #7901003)
- Horizontal swing out style rotor (i.e. 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

- 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.

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 eight times. 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. Mix the blood in the CellSave Preservative Tube by manually inverting five times. Then remove the rubber stopper.
2. Using a new pipette, transfer 7.5 mL of blood from the CellSave Preservative Tube into a correspondingly labeled 15 mL CELLSEARCH® Conical Centrifuge Tube provided with the CELLSEARCH® CTC Kit.
3. Using a new pipette, add 6.5 mL of Dilution Buffer.
4. Cap the CELLSEARCH® Conical Centrifuge Tube and mix by inversion five times.
5. Centrifuge the sample at 800 x g for a full 10 minutes with the brake off using a swing bucket centrifuge. The 10 minute centrifugation time does not take into account the time required to reach 800 x g. Set the centrifuge brake to "off" or if your centrifuge provides a variable braking feature, set the brake to the lowest brake setting. Centrifuge at room temperature using a room temperature capable centrifuge. Following sample centrifugation, visually inspect each sample tube for separation of plasma and red blood cells.
6. 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®

The CELLTRACKS® AUTOPREP® System dispenses the processed sample into a cartridge ready for analysis using the CELLTRACKS ANALYZER II®. 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® User's Guide for instructions on sample analysis and data review.

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.

Metastatic Colorectal Cancer (MCRC)

A CTC count of **3** 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.

Metastatic Prostate Cancer (MPC)

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

Carryover from a CTC count sample with **5000 or greater** CTCs per 7.5 mL of blood can affect samples subsequently processed on the CELLTRACKS® AUTOPREP® System, including the subsequent batch. If cells are carried over to subsequent samples, the CTC counts of these samples may be erroneously higher than the patient's actual CTC count. **Please refer to the CELLTRACKS® AUTOPREP® User's Guide for further information.**

LIMITATIONS

- Caution: U.S. Federal law restricts this device to sale by or on the order of a physician.
- 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 management procedures.
- 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® CTC 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 1.**

Table 1. 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]

Healthy volunteers, non-malignant colorectal disease

Blood was collected from healthy men and women who were 35 years or older. These healthy volunteers were enrolled at three US centers. For the purposes of this study, two tubes of blood were drawn from each subject and both were evaluated for CTC levels. There were a total of 150 evaluable subjects with either one or two separate 7.5 mL aliquots of blood processed for CTC. Not all evaluable subjects had CTC results available from both tubes. The mean number of circulating tumor cells was 0.0 for both groups of subjects, with standard deviations of 0.1 to 0.2. Of the 284 total samples from healthy volunteers (men and women), zero subjects had more than 3 CTC/7.5 mL. Results are shown in **Table 2**.

Table 2. Results of CELLSEARCH® Circulating Tumor Cell Assay in Control Subjects

Healthy Controls	All Controls			Male Only			Female Only		
	Tube 1	Tube 2	Total	Tube 1	Tube 2	Total	Tube 1	Tube 2	Total
N	149	135	284	68	64	132	81	71	152
CTC Range	0 - 1	0 - 1	0 - 1	0 - 1	0 - 1	0 - 1	0 - 1	0 - 1	0 - 1
Average CTC	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
CTC SD	0 .1	0 .2	0 .1	0 .1	0 .1	0 .1	0 .1	0 .2	0 .2
N (%) ≥ 1 CTC	2 (1%)	4 (3%)	6 (2%)	1 (1%)	1 (2%)	2 (2%)	1 (1%)	3 (4%)	4 (3%)
N (%) ≥ 2 CTC	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)

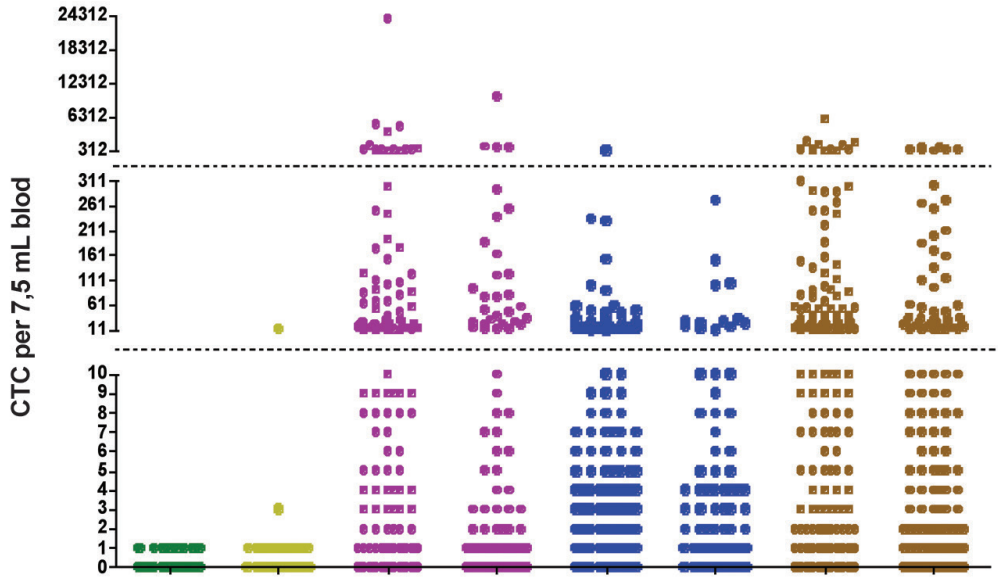
Approximately thirty mL of blood (to increase the probability of detecting cells) was collected into four separate CellSave tubes (minimum of 7.5 mL per tube) from patients undergoing colonoscopy or surgery for benign disease. Up to four 7.5 mL blood samples were evaluated for each subject prior to the procedure. The results are shown below in **Table 3.** Not all evaluable subjects had CTC results available from all four tubes. None of the patients with benign colorectal disease had more than a single circulating tumor cell per 7.5 mL of blood.

Table 3. Results of CELLSEARCH® Circulating Tumor Cell Assay in Subjects with Benign Colorectal Disease

Benign Disease	Blood Drawn Prior to Procedure				
	Tube 1	Tube 2	Tube 3	Tube 4	Total
N	55	55	53	47	210
Range	0 - 1	0 - 1	0 - 1	0 - 1	0 - 1
Average	0.1	0.0	0.0	0.1	0.0
SD	0.2	0.1	0.2	0.3	0.2
% ≥ 1 CTC	3 (5%)	1 (2%)	2 (4%)	4 (9%)	10 (5%)
% ≥ 2 CTC	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)

Figure 1 shows the frequency of CTC in the combined healthy and benign disease subjects, (controls), and in the MBC, MCRC and MPC 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 Breast[1] (MBC), Metastatic Colorectal[2] (MCRC) or Metastatic Prostate Cancer[3] (MPC) before Initiation of a new line of Therapy (Baseline) and ~2-5 weeks After the Initiation of Therapy.



Antal fall	295	255	177	132	413	334	219	203
N (%) ≥1 CTC	10 (3%)	19 (7%)	125 (71%)	73 (55%)	196 (47%)	89 (27%)	170 (78%)	123 (61%)
N (%) ≥2 CTC	0 (0%)	2 (1%)	108 (61%)	53 (40%)	138 (33%)	48 (14%)	152 (69%)	106 (52%)
N (%) ≥3 CTC	0 (0%)	2 (1%)	102 (58%)	47 (36%)	108 (26%)	41 (12%)	139 (63%)	93 (46%)
N (%) ≥4 CTC	0 (0%)	1 (0%)	94 (53%)	42 (32%)	90 (22%)	34 (10%)	129 (59%)	86 (42%)
N (%) ≥5 CTC	0 (0%)	1 (0%)	88 (50%)	40 (30%)	75 (18%)	26 (8%)	125 (57%)	80 (39%)

[1]MBC reference population information – Table 1 of the clinical IFU.
[2]MCRC reference population information – Table 12 of the clinical IFU.
[3]MPC reference population information – Table 22 of the clinical IFU.

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 4**.

Table 4. 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 $R^2 = 0.999$ ($R = 0.999$). 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 CELLTRACKS ANALYZER 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 $R^2 = 0.990$ ($R = 0.995$). 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-A[2]. 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 5. Summary of Precision Analyses

	Low	High
N	99	99
Mean cell count	48	969
Total Precision Standard Deviation (S_T) % 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 $Y = 0.98x + 0.67$, $R^2 = 0.99$. **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.

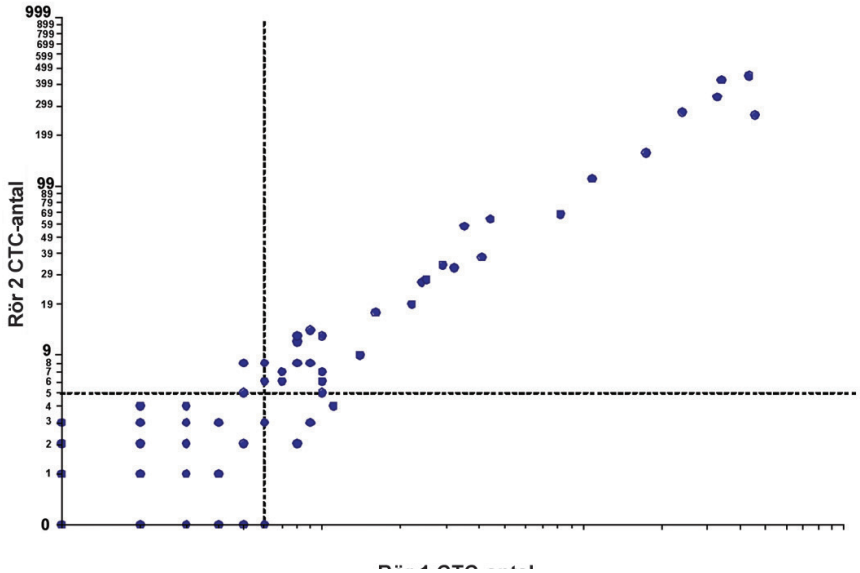


Figure 2 Note: There may be more than one point superimposed over another. For example, on this plot, there are 50 instances (31%) where both tubes had 0 CTC, 18 instances (11%) where Tube 1 had 0 CTC and Tube 2 had 1 CTC, and another 18 instances (11%) where Tube 1 had 1 CTC and Tube 2 had 0 CTC.

Metastatic Colorectal Cancer (MCRC)

A total 1,627 duplicate blood samples were collected from 430 MCRC 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 1,627 duplicate samples was $Y=0.98x + 0.18$, $R^2=0.96$. **Figure 3** shows a scatter plot of the duplicate CTC results in blood from MCRC patients plotted on a logarithmic scale, with the threshold of 3 CTC indicated by the dashed lines.

Figure 3. Reproducibility of CTC Counts in Duplicate MCRC Samples (n=1627) with Average of < 3 or ≥3 CTC per 7.5 mL of blood.

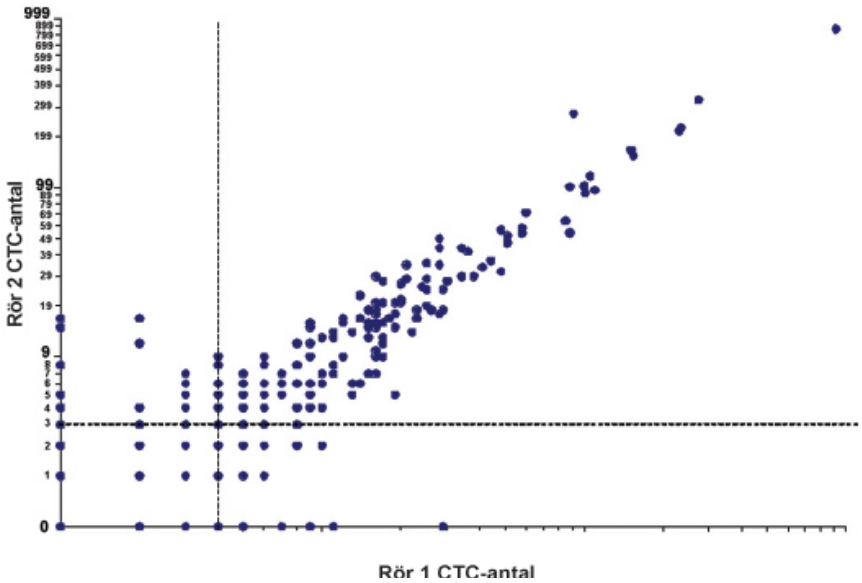


Figure 3 Note: There may be more than one point superimposed over another. For example, on this plot, there are 975 instances (60%) where both tubes had 0 CTC, 116 instances (7%) where Tube 1 had 0 CTC and Tube 2 had 1 CTC, and another 109 instances (7%) where Tube 1 had 1 CTC and Tube 2 had 0 CTC.

The tube-to-tube variation of CTC counts in blood samples from metastatic breast and colorectal cancer patients is shown in **Figures 2 & 3**. The distribution of infrequent events (such as tumor cells) within a given volume is random and independent of cell or disease type. 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]. For tubes with very few prostatic CTC it is reasonable to expect variation in results similar to what is depicted in **Figures 2 & 3**. Because the two previous studies in MBC and MCRC patients showed almost identical results, a tube-to-tube comparison of CTC counts in blood samples from metastatic prostate cancer patients was not performed during the CELLSEARCH® CTC prostate clinical trial. However, results of an independent study using CELLSEARCH® technology conducted at the Memorial Sloan-Kettering Cancer Center demonstrated no systematic site-to-site or tube-to-tube variation in CTC counts across a range of 0 to 1192 CTC per tube in patients with metastatic prostate cancer [4].

BIBLIOGRAPHY

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).

REF 7900001
16 Test Kit

CellSearch®

Circulating Tumor Cell Kit (Epithelial)

CLINICAL TRIAL RESULTS

Table of Contents

1 Metastatic Breast Cancer (MBC) Patients 5
1.1 CTC frequencies 5
1.2 Progression Free Survival (PFS) Analysis of MBC Patients 6
1.2.1 PFS Using Baseline CTC Results 6
1.2.2 PFS Using Follow-up CTC Results 6
1.2.3 Reduction or Increase in CTC Predicts Improved or Decreased PFS 7
1.3 Overall Survival (OS) Analysis of MBC Patients 8
1.3.1 OS Analysis Using Baseline CTC Results 8
1.3.2 OS Using Follow-up CTC Results 8
1.3.3 Reduction or Increase in CTC Predicts Improved or Decreased OS 9
1.3.4 Univariate Cox Regression Analysis in MBC Patients 10
1.3.5 Multivariate Cox Regression Analysis in MBC Patients 10
1.4 Use of CTC to Monitor Clinical Status of Metastatic Breast Cancer 12
1.4.1 Relationship between survival, CTC, and disease assessment by imaging 12
1.4.2 CTC 12
1.4.3 Imaging 12
1.4.4 Relationship between survival to imaging and CTC 13
1.4.5 Concordances between CTC and Radiological Monitoring 15
1.4.6 CTC as an Adjunct to Imaging 17
1.5 Variability of CTC and Radiological Assessments 17
1.5.1 CTC 17
1.5.2 Imaging 18
2 Metastatic Colorectal Cancer (MCRC) Patients 18
2.1 CTC frequencies 19
2.2 Progression Free Survival (PFS) Analysis of MCRC Patients 20
2.2.1 PFS Using Baseline CTC Results 20
2.2.2 PFS Using Follow-up CTC Results 20
2.2.3 Reduction or Increase in CTC Predicts Improved or Decreased PFS 21
2.3 Overall Survival (OS) Analysis of MCRC Patients 22
2.3.1 OS Analysis Using Baseline CTC Results 22
2.3.2 OS Using Follow-up CTC Results 23
2.3.3 Reduction or Increase in CTC Predicts Improved or Decreased OS 23
2.3.4 Univariate Cox Regression Analysis in MCRC Patients 24
2.3.5 Multivariate Cox Regression Analysis in MCRC Patients 25
2.4 Use of CTC to Monitor Clinical Status of Metastatic Colorectal Cancer 25
2.4.1 Relationship between survival, CTC, and disease assessment by imaging 25
2.4.2 CTC 26
2.4.3 Imaging 26
2.4.4 Relationship between survival to imaging and CTC 26
2.4.5 Concordances between CTC and Radiological Monitoring in MCRC Patients 28
2.4.6 CTC as an Adjunct to Imaging 29
3 Metastatic Prostate Cancer (MPC) Patients 30
3.1 CTC frequencies 31
3.2 Progression Free Survival (PFS) Analysis of MPC Patients 32
3.2.1 PFS Using Baseline CTC Results 32
3.2.2 PFS Using Follow-up CTC Results 32
3.2.3 Reduction or Increase in CTC Predicts Improved or Decreased PFS 33

3.3 Overall Survival (OS) Analysis of MPC Patients....34
3.3.1 OS Analysis Using Baseline CTC Results....34
3.3.2 OS Using Follow-up CTC Results....35
3.3.3 Reduction or Increase of CTC Predicts Improved or Decreased OS....35
3.3.4 Univariate Cox Regression Analysis in MPC Patients....36
3.3.5 Multivariate Cox Regression Analysis in MPC Patients....37
3.4 Use of CTC to Monitor Clinical Status of Metastatic Prostate Cancer Patients....38
3.4.1 Relationship between survival, CTCs and disease assessment by PSA....38
3.4.2 Concordances between CTC and PSA Changes in MPC Patients....40
3.4.3 CTC Levels and PSA Reduction Combined to Predict OS in MPC Patients....41

List of Tables

Table 1: MBC Patient Demographics....5
Table 2: Progression Free Survival (PFS) for MBC patients with <5 or ≥5 CTC at different time points....7
Table 3: Overall Survival (OS) for MBC patients with <5 or ≥5 CTC at different time points....9
Table 4: Univariate Cox Regression Analysis in MBC Patients....10
Table 5: Progression Free Survival Multivariate Cox Regression Analysis in MBC Patients....11
Table 6: Overall Survival Multivariate Cox Regression Analysis in MBC Patients....12
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....14
Table 8: MBC Patient-Wise Comparison of CTC and Imaging....16
Table 9: MBC Observation-Wise Comparison of CTC and Imaging....16
Table 10: MBC Patient-Wise Comparison of CTC and Imaging....17
Table 11: Variability of Radiological and CTC Assessments in MBC Patients....18
Table 12: MCRC Patient Demographics....19
Table 13: Exclusions from Progression Free and Overall Survival Analyses in MCRC Patients....19
Table 14: Progression Free Survival (PFS) for MCRC patients with <3 or ≥3 CTC at different time points....21
Table 15: Overall Survival (OS) for MCRC patients with <3 or ≥3 CTC at different time points....23
Table 16: Univariate Cox Regression Analysis in MCRC Patients....24
Table 17: Multivariate Cox Regression Analysis in MCRC Patients....25
Table 18: OS of MCRC Patients with CTC assessment approximately one month after the initiation of therapy and within one month of the radiological assessment....27
Table 19: MCRC Patient-Wise Comparison of CTC and Imaging....28
Table 20: MCRC Observation-Wise Comparison of CTC and Imaging....29
Table 21: MCRC Patient-Wise Comparison of CTC and Imaging....29
Table 22: MPC Patient Demographics....31
Table 23: Exclusions from PFS and OS Analyses in MPC Patients....32
Table 24: Progression Free Survival (PFS) for MPC patients with <5 or ≥5 CTC at different time points....33
Table 25: Overall Survival (OS) for MPC patients with <5 or ≥5 CTC at different time points....35
Table 26: Univariate Cox Regression Analysis in MPC Patients....37
Table 27: Multivariate Cox Regression Analysis in MPC Patients....38
Table 28: Comparison of Median OS between Favorable and Unfavorable CTC and PSA Reduction Groups....40
Table 29: MPC Patient-Wise Comparison of CTC and 30% PSA Reduction at 2-5 Weeks....41
Table 30: MPC Patient-Wise Comparison of CTC and 30% PSA Reduction at 13-20 Weeks....41
Table 31: MPC Observation-Wise Comparison of CTC and 30% PSA Reduction....41

List of Figures

Figure 1: PFS of MBC Patients with <5 or ≥ 5 CTC at Baseline (N=177) 6
Figure 2: PFS of MBC Patients with <5 or ≥ 5 CTC at different times of Follow-Up 7
Figure 3: A Reduction in CTC Below 5 After the Initiation of Therapy Predicts Longer PFS in MBC Patients 8
Figure 4: OS of MBC Patients with <5 or ≥ 5 CTC at Baseline (N=177) 8
Figure 5: OS of MBC Patients with <5 or ≥ 5 CTC at different times of Follow-Up. 9
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 10
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) 14
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) 15
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) 15
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 17
Figure 11: PFS of MCRC Patients with <3 or ≥ 3 CTC at Baseline (N=413) 20
Figure 12: PFS of MCRC Patients with <3 or ≥ 3 CTC at different times of Follow-Up 21
Figure 13: A Reduction in CTC Below 3 After the Initiation of Therapy Predicts Longer PFS in MCRC Patients 22
Figure 14: OS of MCRC Patients with <3 or ≥ 3 CTC at Baseline (N=413) 22
Figure 15: OS of MCRC Patients with <3 or ≥ 3 CTC at different times of Follow-Up. 23
Figure 16: A Reduction in CTC Below 3 After the Initiation of Therapy Predicts Longer OS whereas an Increase in CTC Count to 3 or above Predicts Shorter OS in MCRC Patients 24
Figure 17: Correlation of Radiological and CTC Assessment with OS: OS of MCRC Patients with NPD or PD at 1st Follow-Up Imaging Study (N=402) 27
Figure 18: Correlation of Radiological and CTC Assessment with OS: OS of MCRC Patients with <3 or ≥ 3 CTC at 1st Follow-Up after Initiation of Therapy (N=320) 27
Figure 19: Correlation of Radiological and CTC Assessment with OS: OS of MCRC Patients with <3 or ≥ 3 CTC within ±1 Month of 1st Follow-Up Imaging Study or Death (N=364) 28
Figure 20: OS of MCRC Patients in Groups 1, 2, 3 and 4 using CTC 3-5 Weeks after Initiation of Therapy (n=320) and the Disease Status Determined at the 1st Follow-Up Imaging Study 30
Figure 21: PFS of MPC Patients with <5 or ≥ 5 CTC at Baseline (N = 219). 32
Figure 22: PFS of MPC Patients with <5 or ≥ 5 CTC at different times of Follow-Up 33
Figure 23: A Reduction in CTC Below 5 After the Initiation of Therapy Predicts Longer PFS in MPC Patients 34
Figure 24: OS of MPC Patients with <5 or ≥ 5 CTC at Baseline (N = 219) 34
Figure 25: OS of MPC Patients with <5 or ≥ 5 CTC at different times of Follow-Up. 35
Figure 26: 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 MPC Patients 36
Figure 27: OS of MPC Patients 2-5 weeks after the Initiation of Therapy 39
Figure 28: OS of MPC Patients 6-8 weeks after the Initiation of Therapy 39
Figure 29: OS of MPC Patients 9-12 weeks after the Initiation of Therapy 39
Figure 30: OS of MPC Patients 13-20 weeks after the Initiation of Therapy 40
Figure 31: CTC Levels and PSA Changes Combined to Predict OS 2-5 Weeks (Panel A), 6-8 Weeks (Panel B), 9-12 Weeks (Panel C), and 13-20 Weeks (Panel D) After the Initiation of Therapy 42

1 Metastatic Breast Cancer (MBC) Patients

A multi-center prospective, clinical trial was conducted to determine whether the number of CTC predicted disease progression and survival. Metastatic breast cancer patients with measurable (N=177) 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 Median	58 ± 13 58
	Description of Categories	Number of Subjects (% of total)
Stage	1 2 3 4 Unknown	26 (15%) 92 (52%) 26 (15%) 20 (11%) 13 (7%)
Race	White Black Hispanic Unknown	153 (86%) 14 (8%) 7 (4%) 3 (2%)
Baseline ECOG Score	0 1 2 Unknown	82 (46%) 72 (41%) 18 (10%) 5 (3%)
Disease Site	Visceral Bone	152 (86%) 153 (86%)
ER/PR	+ - Unknown	121 (68%) 54 (31%) 2 (1%)
HER2	0 1+ 2+ 3+ Unknown	91 (51%) 12 (7%) 18 (10%) 27 (15%) 29 (17%)
Line of Therapy	1st line 2nd line ≥ 3rd line Unknown	82 (46%) 26 (15%) 67 (38%) 2 (1%)
Type of Therapy	Chemo (Ch) Endocrine (En) Targeted (Ta) Ch/En Ch/Ta En/Ta Ch/En/Ta Miscellaneous Unknown	74 (42%) 45 (25%) 9 (5%) 10 (6%) 23 (13%) 7 (4%) 2 (1%) 2 (1%) 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 week 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 time of death.

1.1 CTC frequencies

The CTC results 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 timepoints, 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 2**. The difference in the number of patients at each time point *between* the two tables is due to the progression of some patients prior to the blood draw. The difference in the number of patients *within* the tables is due to the number of patients with blood draws and evaluable CTC results at each time point.

1.2 Progression Free Survival (PFS) Analysis of MBC Patients

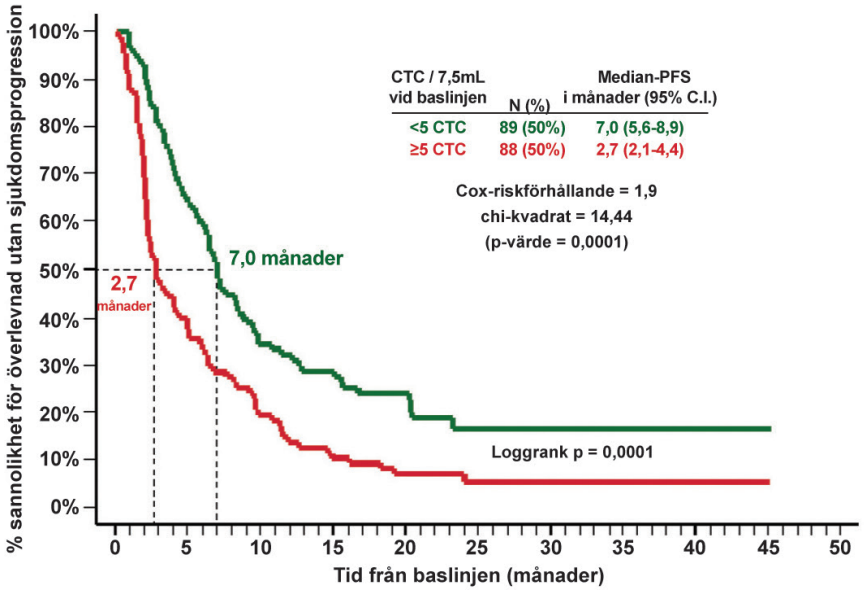
1.2.1 PFS Using Baseline CTC Results

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 significantly 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).



1.2.2 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

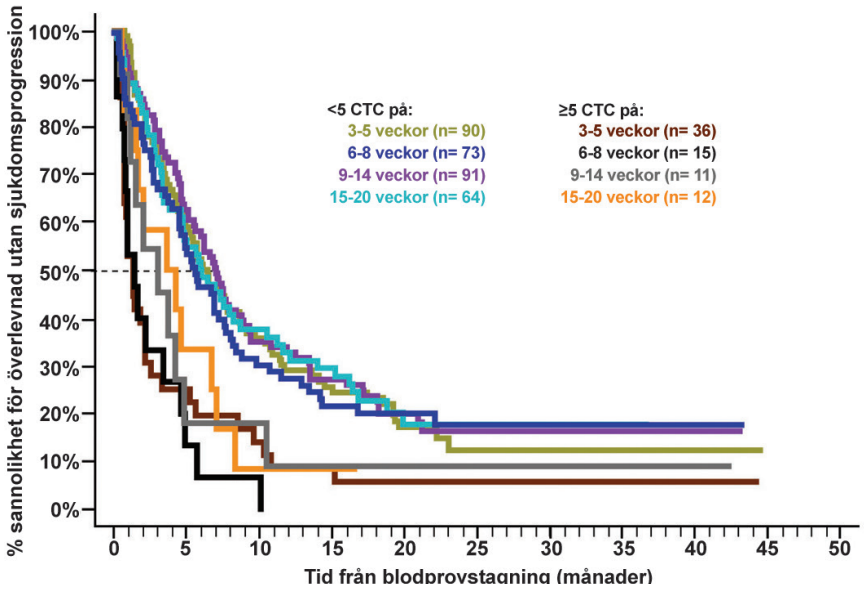


Table 2 summarizes the results of the PFS analysis using the CTC levels and a threshold of ≥ 5 CTC/7.5 mL at each of the different blood draw time points.

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 Tx Initiation	**N**	**≥5 CTC**	**Median PFS in Months (95% CI)**		**Log-rank p-value**
			<5 CTC	**≥5 CTC**	
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 2** 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).

1.2.3 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

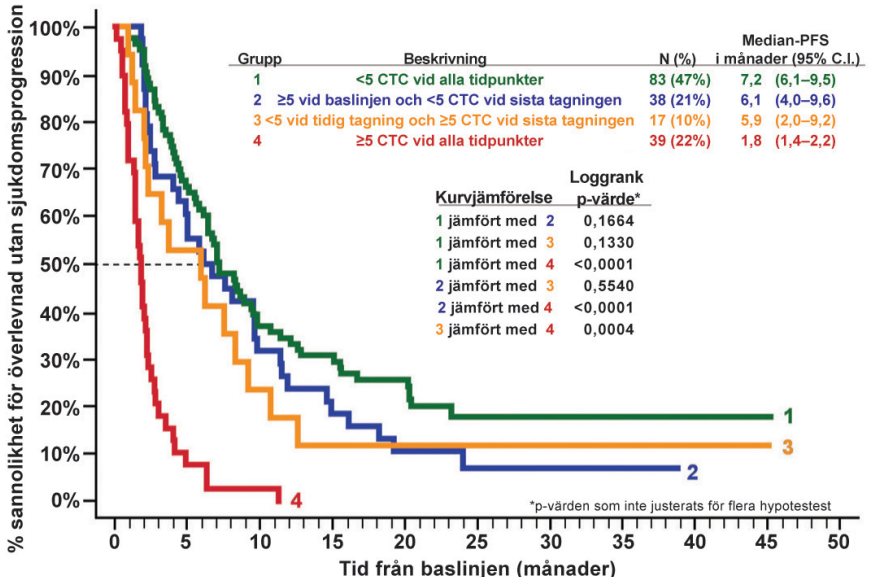


Figure 3 shows that MBC patients with ≥ 5 CTC at all time points (**Group 4**) had the shortest median PFS, which was significantly different compared to the median PFS of **Group 3**, **Group 2**, and **Group 1**. Differences between the curves for the other groups in this figure were not significant.

1.3 Overall Survival (OS) Analysis of MBC Patients

1.3.1 OS Analysis Using Baseline CTC Results

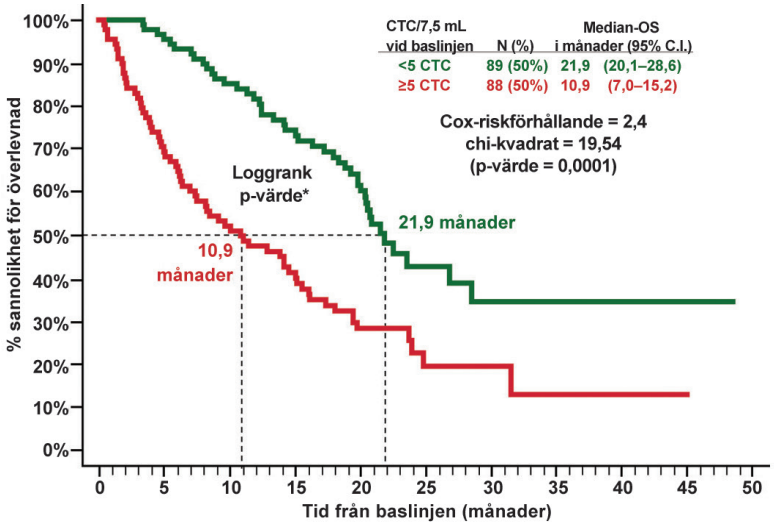
Death occurred in 109 (62%) of the 177 MBC patients, with a mean follow-up time for the 68 (38%) patients still alive of 22.7 ± 9.4 months (median = 21.1, range = 4.4 – 48.6). At the time of these analyses, 44 (49%) of 89 patients from Favorable group (<5 CTC at baseline) compared to 65 (74%) of 88 from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



1.3.2 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 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.

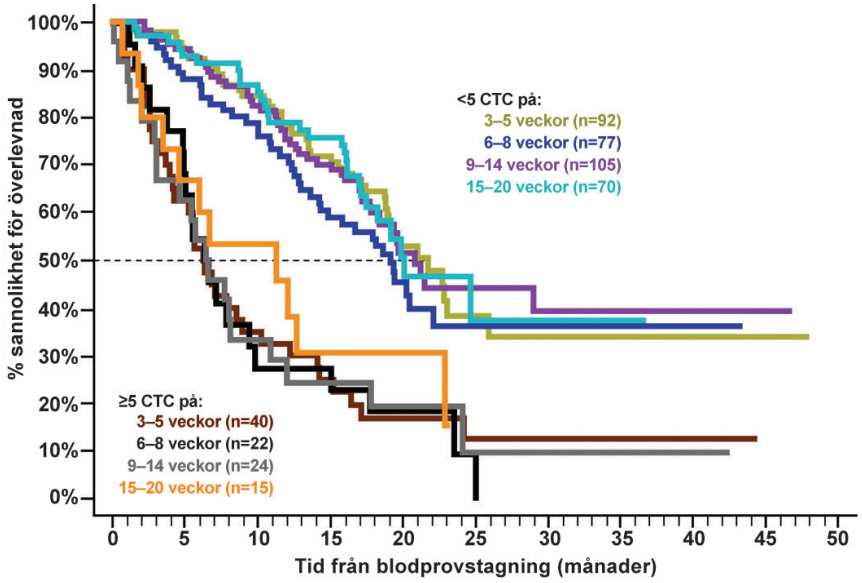


Table 3 summarizes the results of the OS analysis using the CTC levels and a threshold of ≥ 5 CTC/7.5 mL at each of the different blood draw time points.

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 Tx Initiation	**N**	**≥ 5 CTC**	**Median OS in Months (95% CI)**		**Log-rank p-value**
			<5 CTC	**≥ 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

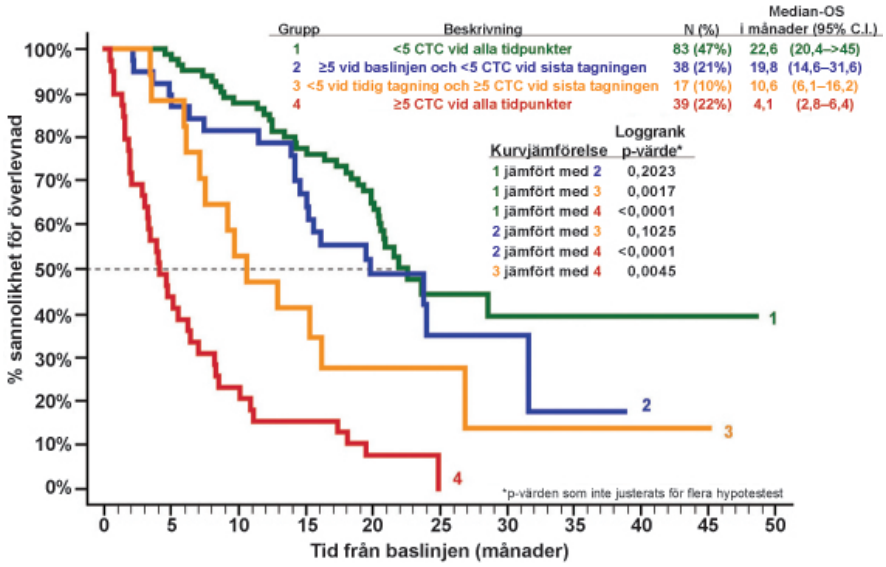
1.3.3 Reduction or Increase in CTC Predicts Improved or Decreased OS

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 **Table 3** in columns 4 & 5, 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.

1.3.4 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		# of MBC Patients	PFS Risk from Baseline		OS 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
Stage at Primary Diagnosis	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	≥ 2nd	1st	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 Immunotherapy alone or Combination of Hormonal and/or Chemo and/or Immunotherapy

1.3.5 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	Hazard Ratio	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	Hazard Ratio	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 (yr)		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

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

1.4.1 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 timepoints and imaging results were compared 1) to the true clinical endpoint overall survival and 2) to each other.

1.4.2 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.

1.4.3 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).

1.4.4 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 7**, **Table 7**). 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 8**, **Table 7**). 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 resulted 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 9**, **Table 7**). 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 ($\geq$5)	34 (25%)	8.3 (5.9 - 15.1)
CTC (±1 Month of Imaging)	134*	
favorable (<5)	105 (78%)	21.9 (19.9 - 31.6)
unfavorable ($\geq$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)

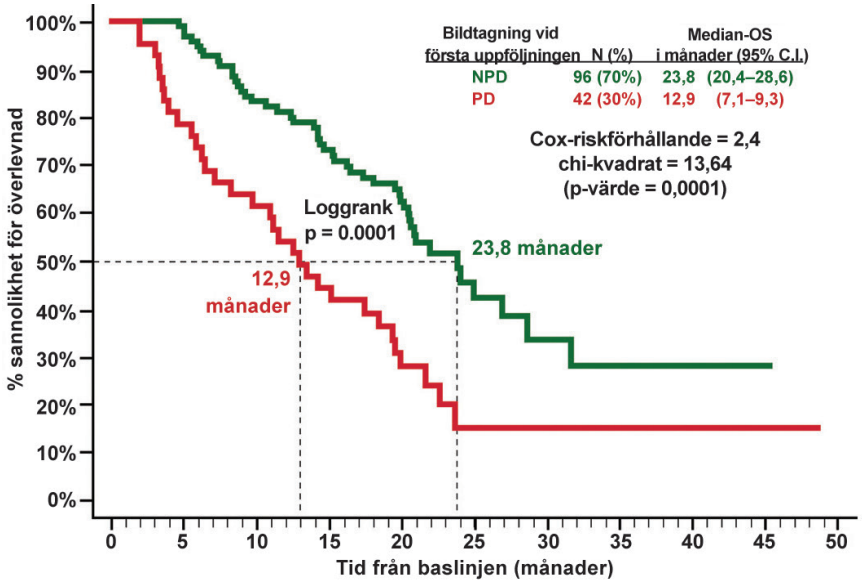


Figure 8: Correlation of Radiological and CTC Assessment with OS: OS of MBC Patients with <5 or ≥5 CTC at 1st Follow-Up after Initiation of Therapy (N=138)

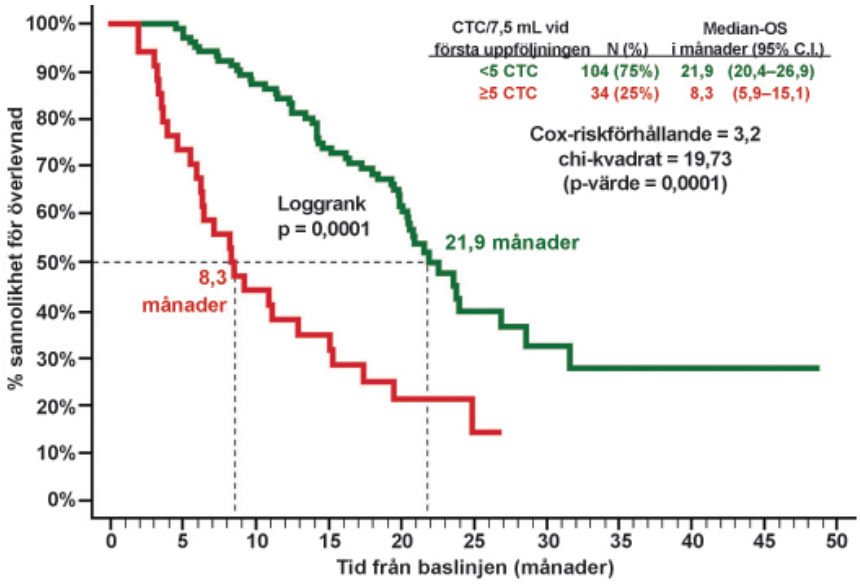
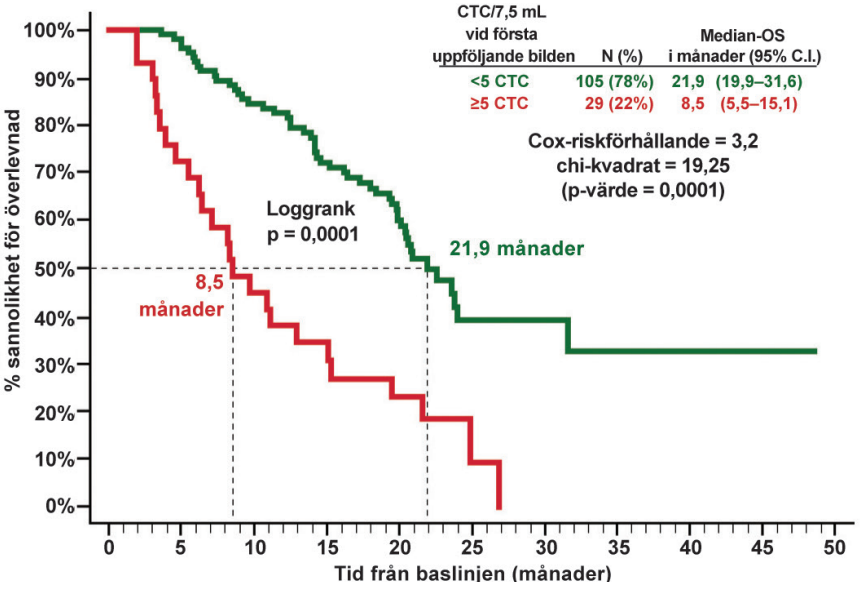


Figure 9: Correlation of Radiological and CTC Assessment with OS: OS of MBC Patients with <5 or ≥5 CTC within ± 1 Month of 1st Follow-Up Imaging Study (N=134)



1.4.5 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
	<5 CTC / 7.5 mL	≥5 CTC / 7.5 mL	
Non-Progressive Disease	85	9	**94**
Progressive Disease	20	20	**40**
Total	**105**	**29**	**134**

Measurement	Estimate	Lower 95% CI	Upper 95% CI
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 / 7.5 mL	≥5 CTC / 7.5 mL	
Non-Progressive Disease	151	16	**167**
Progressive Disease	30	22	**52**
Total	**181**	**38**	**219**

Measurement	Estimate	Lower 95% CI	Upper 95% CI
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 were 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
	<5 CTC / 7.5 mL	≥5 CTC / 7.5 mL	
Non-Progressive Disease	84	12	**96**
Progressive Disease	20	22	**42**
Total	**104**	**34**	**138**

Measurement	Estimate	Lower 95% CI	Upper 95% CI
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

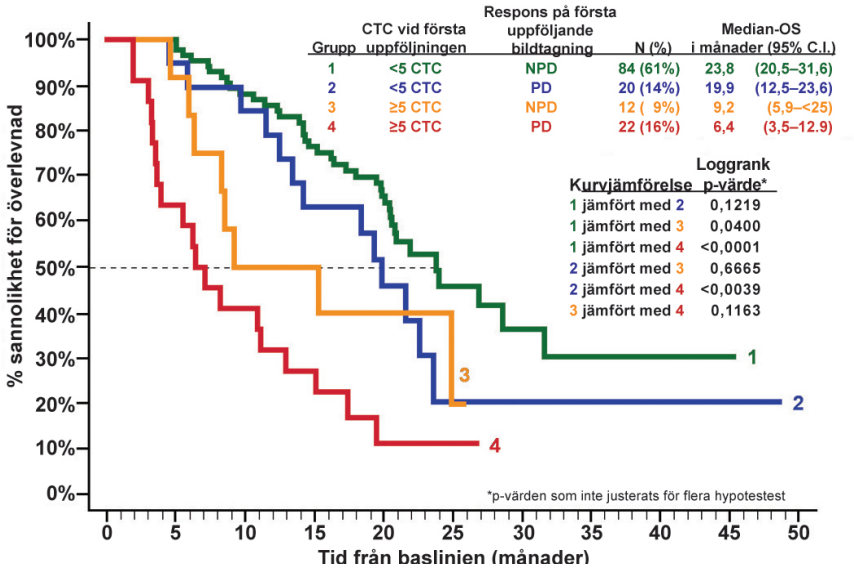
1.4.6 CTC as an Adjunct to Imaging

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



1.5 Variability of CTC and Radiological Assessments

1.5.1 CTC

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.

1.5.2 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.

2 Metastatic Colorectal Cancer (MCRC) Patients

A multi-center prospective, clinical trial was conducted to determine whether the number of CTC predicted disease progression and survival. Metastatic colorectal cancer patients with measurable (N=430) disease starting a new line of therapy were enrolled. Clinical data were analyzed on an intent-to-treat basis. Patient demographic information is presented in **Table 12.**

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 week 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 time of death.

Table 12: MCRC Patient Demographics

Category		N=430 Patients
Age at Baseline (in years)	Mean ± Std. Deviation (Median)	63.0 ± 12.6 (64)
Years to Metastasis	Mean ± Std. Deviation (Median)	0.9 ± 1.4 (0.1)
	Description of Categories	Number of Subjects (% of total)
Gender	Female	192 (45%)
	Male	238 (55%)
Race	White	305 (71%)
	Black	44 (10%)
	Other	12 (3%)
	Unknown	69 (16%)
Baseline ECOG Score	0	196 (46%)
	1	187 (43%)
	2	31 (7%)
	Unknown	16 (4%)
Tumor Type at Primary Diagnosis	Colon	292 (68%)
	Rectal	71 (17%)
	Colorectal	66 (15%)
	Unknown	1 (0%)
Stage at Primary Diagnosis	1	12 (3%)
	2	45 (11%)
	3	118 (27%)
	4	232 (54%)
	Unknown	23 (5%)
Liver metastasis	No	117 (27%)
	Yes	313 (73%)
Line of Therapy	1st line	309 (72%)
	2nd line	95 (22%)
	3rd line	26 (6%)
Type of Therapy	Bevacizumab	243 (56%)
	Irinotecan	103 (24%)
	Oxaliplatin	253 (59%)
	Unknown	25 (6%)

2.1 CTC frequencies

Of the total number of 430 MCRC patients, 9 had a baseline blood draw and no follow-up blood draws. Of these 9 patients, four died before a follow-up blood draw could be obtained, two were taken off their therapy due to treatment related toxicity, one patient had surgery to remove their measurable disease, one patient refused further treatment, and one patient refused any further blood draws. Of the remaining patients, 362, 342, 321, and 211 had follow-up blood draws 1-2 weeks, 3-5 weeks, 6-12 weeks, and 13-20 weeks after the initiation of therapy, respectively. The difference in the number of patients evaluable for PFS and OS at each time point is due to the progression of some patients prior to the blood draw, while the difference in the number of patients at each time point is due to the number of patients with blood draws and evaluable CTC results.

Table 13 shows the numbers of patients at each time point excluded from the PFS, OS, or PFS & OS analyses and the reasons for their exclusion.

Table 13: Exclusions from Progression Free and Overall Survival Analyses in MCRC Patients

Blood Draw Timing	Reasons for Exclusion of MCRC Patients from Analyses:						Total # of MCRC Patients Evaluable:	
	PFS & OS				PFS Only	OS Only		
	Blood Not Drawn	Blood Drawn 1-7 days after administration of therapy	No Follow-up Beyond Date of Blood Draw	Non-Evaluable CTC Results	Blood drawn after date of disease progression	No Follow-up Beyond Date of Blood Draw	PFS	OS
Baseline	1	11	0	5	0	0	413	413
1-2 Weeks	68	0	0	5	1	0	356	357
3-5 Weeks	88	0	1	8	4	0	329	333
6-12 Weeks	109	0	4	7	26	0	284	310
13-20 Weeks	219	0	9	8	14	1	180	193

The CTC results obtained from the follow-up blood draws at 1-2 weeks, 3-5 weeks, 6-12 weeks, and 13-20 weeks after the initiation of therapy were classified as being favorable (<3 CTC) or unfavorable (≥3 CTC). If more than one CTC result was obtained within any of the designated follow-up timepoints, the CTC result from the blood draw furthest from the baseline blood draw was used.

Table 15 summarizes the total number of MCRC patients and percentage of patients with unfavorable CTC in the clinical trial that differs from the numbers and percentages of patients for Progression Free Survival shown in **Table 14**.

2.2 Progression Free Survival (PFS) Analysis of MCRC Patients

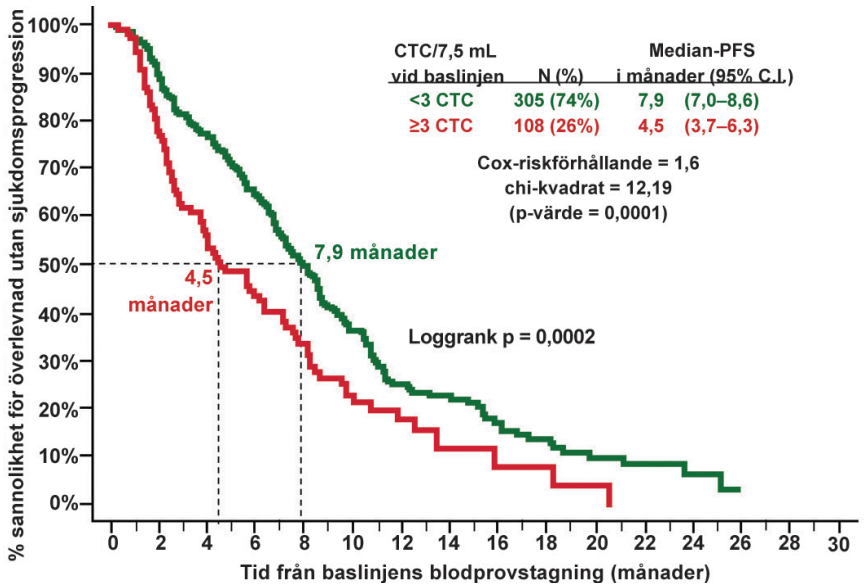
2.2.1 PFS Using Baseline CTC Results

Four hundred and thirteen (413) of the 430 MCRC 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=305), represented in **green**, consisted of patients with <3 CTC.
- The Unfavorable group (N=108), represented in **red**, consisted of patients with ≥3 CTC.

Median PFS was significantly longer in the Favorable group compared to the Unfavorable group (7.9 vs 4.5 months, respectively). These results are illustrated in **Figure 11** and **Table 14**.

Figure 11: PFS of MCRC Patients with <3 or ≥3 CTC at Baseline (N=413).



2.2.2 PFS Using Follow-up CTC Results

For Kaplan-Meier analysis, MCRC 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 12**. 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 12** illustrates the ability of CTC in MCRC patients with <3 and ≥3 CTC 1-2 weeks, 3-5 weeks, 6-12 weeks, and 13-20 weeks after the initiation of therapy to predict PFS.

- The Favorable group, represented in **olive green**, **blue**, **purple**, and **cyan**, consisted of patients with <3 CTC,
- The Unfavorable group, represented in **brown**, **black**, **grey**, and **orange**, consisted of patients with ≥3 CTC.

Figure 12: PFS of MCRC Patients with <3 or ≥3 CTC at different times of Follow-Up

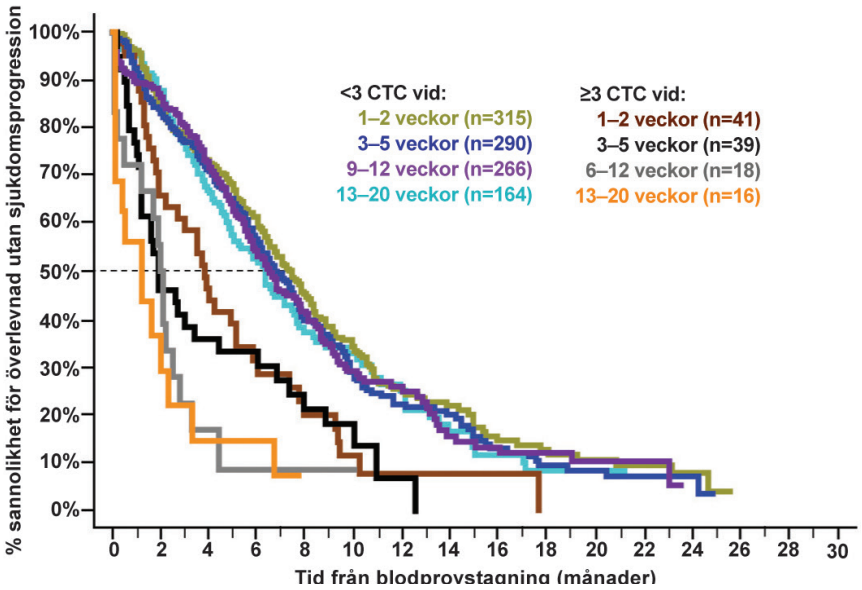


Table 14 summarizes the results of the PFS analysis using the CTC levels and a threshold of ≥3 CTC/7.5 mL at each of the different blood draw time points.

Table 14: Progression Free Survival (PFS) for MCRC patients with <3 or ≥3 CTC at different time points

1	2	3	4	5	6
Sampling Time After Tx Initiation	N	≥3 CTC	Median PFS in Months (95% CI)		Log-rank p-value
			<3 CTC	≥3 CTC	
Baseline	413	108 (26%)	7.9 (7.0 - 8.6)	4.5 (3.7 - 6.3)	0.0002
1-2 Weeks	356	41 (12%)	7.3 (6.5 - 8.1)	3.8 (1.9 - 5.1)	<0.0001
3-5 Weeks	329	39 (12%)	6.8 (6.1 - 7.6)	1.9 (1.2 - 4.4)	<0.0001
6-12 Weeks	284	18 (6%)	6.5 (5.8 - 7.7)	2.0 (0.5 - 2.5)	<0.0001
13-20 Weeks	180	16 (9%)	6.3 (4.9 - 7.4)	1.2 (0.1 - 2.3)	<0.0001

As illustrated in **Figure 12** and **Table 14**, MCRC patients with elevated CTC (≥3 CTC/7.5 mL whole blood) at any of the time points had a much higher likelihood of rapid progression than did those with <3 CTC. **Table 14** column 4 shows the median PFS times for those patients with <3 CTC ranged from 6.3 to 7.9 months and were substantially longer than the median PFS times for those patients with ≥3 CTC, which ranged from 1.2 to 4.5 months (column 5).

2.2.3 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 13**), MCRC patients were segmented into four groups based upon their CTC counts at baseline, 1-2 weeks, 3-5 weeks, 6-12 weeks, and 13-20 weeks after the initiation of therapy:

- Group 1 (**green** curve), 303 (70%) patients with <3 CTC at all time points. Seven (2%) of these patients only had a baseline blood draw while eight (3%) had a single blood draw between their first and last blood draw that had ≥3 CTC;
- Group 2 (**blue** curve), 74 (17%) patients with ≥3 CTC prior to the initiation of therapy but who had decreased to <3 CTC at the time of their last blood draw;
- Group 3 (**orange** curve), 29 (7%) patients with <3 CTC at an early draw (baseline, 1-2 weeks, and/or 3-5 weeks) but who increased to ≥3 CTC at the time of their last blood draw;
- Group 4 (**red** curve), 24 (6%) patients with ≥3 CTC at all time points. Three (13%) of these patients had only a baseline blood draw, one (4%) had only a 3-5 week blood draw, and one (4%) had a single blood draw between their first and last blood draw that had <3 CTC.

Figure 13: A Reduction in CTC Below 3 After the Initiation of Therapy Predicts Longer PFS in MCRC Patients

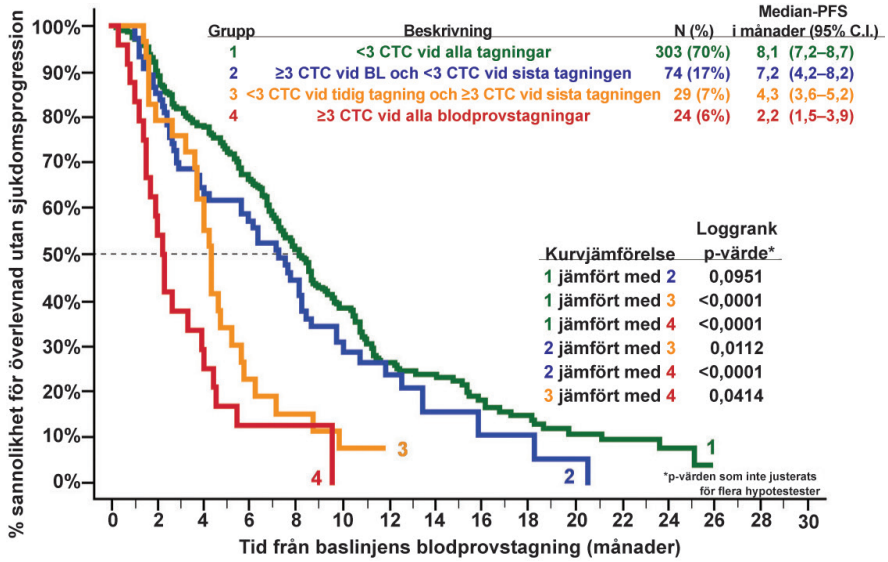


Figure 13 shows that MCRC patients with ≥3 CTC at all time points (**Group 4**) had the shortest median PFS, which was significantly different compared to the median PFS of **Group 3**, **Group 2** and **Group 1**. The difference in the median PFS between those patients who showed a CTC reduction after the initiation of therapy (**Group 2**) was significantly longer compared to those patients who showed a CTC increase (**Group 3**).

2.3 Overall Survival (OS) Analysis of MCRC Patients

2.3.1 OS Analysis Using Baseline CTC Results

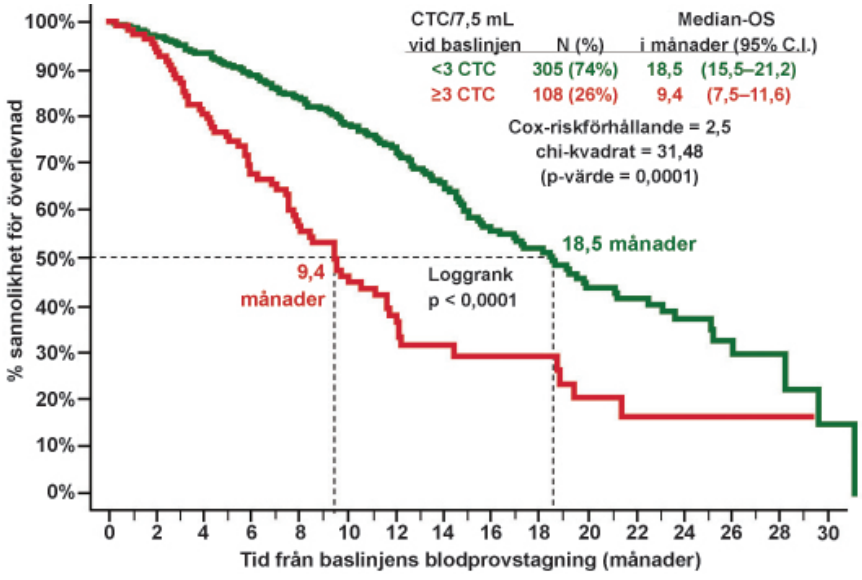
Death occurred in 202 (47%) of the 430 MCRC patients, with a mean follow-up time for the 228 (53%) patients still alive of 12.6 ± 6.5 months (median = 11.0, range = 0.8 to 30.0). At the time of these analyses, 124 (41%) of 305 patients from Favorable group (<3 CTC at baseline) compared to 68 (63%) of 108 from Unfavorable group (≥3 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=305), represented in **green**, consisted of patients with <3 CTC.
- The Unfavorable group (N=108), represented in **red**, consisted of patients with ≥3 CTC.

Median OS was significantly longer in the Favorable group compared to the Unfavorable group (18.5 vs. 9.4 months, respectively). These results are illustrated in **Figure 14**.

Figure 14: OS of MCRC Patients with <3 or ≥3 CTC at Baseline (N=413).



2.3.2 OS Using Follow-up CTC Results

The Kaplan-Meier analyses of both MCRC patient groups at each of the different follow-up blood draw times after initiation of therapy are illustrated in **Figure 15**. This figure illustrates the ability of CTC in MCRC patients with <3 and ≥ 3 CTC 1-2 weeks, 3-5 weeks, 6-12 weeks and 13-20 weeks after the initiation of therapy to predict time to death in 421 patients with metastatic colorectal 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 <3 CTC,
- The Unfavorable group, represented in **brown**, **black**, **grey**, and **orange**, consisted of patients with ≥ 3 CTC.

Figure 15: OS of MCRC Patients with <3 or ≥3 CTC at different times of Follow-Up.

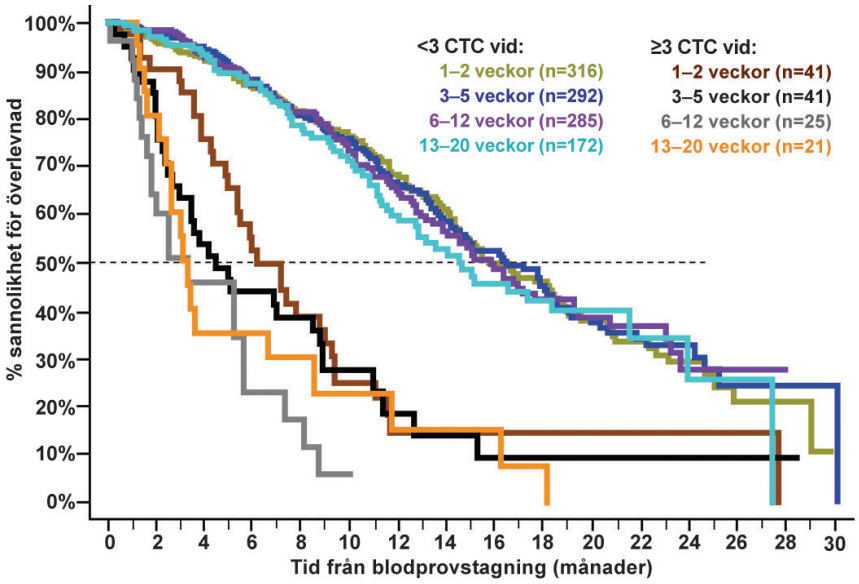


Table 15 summarizes the results of the OS analysis using the CTC levels and a threshold of ≥ 3 CTC/7.5 mL at each of the different blood draw time points.

Table 15: Overall Survival (OS) for MCRC patients with <3 or ≥3 CTC at different time points

1	2	3	4	5	6
Sampling Time After Tx Initiation	N	≥3 CTC	Median OS in Months (95% CI)		Log-rank p-value
			<3 CTC	≥3 CTC	
Baseline	413	108 (26%)	18.5 (15.5 - 21.2)	9.4 (7.5 - 11.6)	<0.0001
1-2 Weeks	357	41 (11%)	15.7 (14.3 - 18.4)	6.1 (4.9 - 8.9)	<0.0001
3-5 Weeks	333	41 (12%)	16.4 (14.1 - 18.3)	4.4 (2.6 - 8.7)	<0.0001
6-12 Weeks	310	25 (8%)	15.8 (13.8 - 19.2)	3.3 (1.8 - 5.6)	<0.0001
13-20 Weeks	193	21 (11%)	14.6 (12.0 - 21.5)	3.3 (2.4 - 8.5)	<0.0001

As illustrated in **Figure 15** and **Table 15** in columns 4 & 5, MCRC patients with ≥ 3 CTC at any of the time points had a much higher likelihood of dying sooner than did those with <3 CTC. The median OS times for those patients with <3 CTC ranged from 14.6 to 18.5 months and were substantially longer than the median OS times for those patients with ≥ 3 CTC, which ranged from 3.3 to 9.4 months.

2.3.3 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 16**), MCRC patients were segmented into four groups based on their CTC counts at baseline, 1-2 weeks, 3-5 weeks, 6-12 weeks, and 13-20 weeks:

- Group 1 (**green** curve), 303 (70%) patients with <3 CTC at all time points. Seven (2%) of these patients only had a baseline blood draw while eight (3%) had a single blood draw between their first and last blood draw that had ≥ 3 CTC;
- Group 2 (**blue** curve), 74 (17%) patients with ≥ 3 CTC prior to the initiation of therapy but who had decreased to <3 CTC at the time of their last blood draw;
- Group 3 (**orange** curve), 29 (7%) patients with <3 CTC at an early draw (baseline, 1-2 weeks, and/or 3-5 weeks) but who increased to ≥ 3 CTC at the time of their last blood draw;
- Group 4 (**red** curve), 24 (6%) patients with ≥ 3 CTC at all draw time points. Three (13%) of these patients had only a baseline blood draw, one (4%) had only a 3-5 week blood draw, and one (4%) had a single blood draw between their first and last blood draw that had <3 CTC.

Figure 16: A Reduction in CTC Below 3 After the Initiation of Therapy Predicts Longer OS whereas an Increase in CTC Count to 3 or above Predicts Shorter OS in MCRC Patients

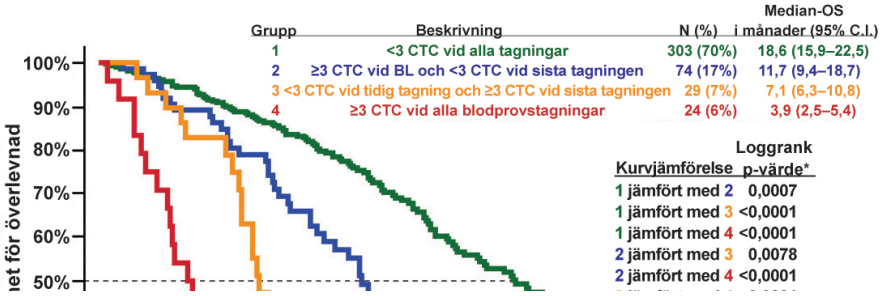


Figure 16 shows that MCRC patients who exceed the threshold of 3 CTC at any point after the initiation of therapy had a much higher likelihood of dying sooner. Patients with ≥3 CTC at all time points (**Group 4**) had the shortest median OS, which was significantly different compared to the median OS of **Group 3**, **Group 2** and **Group 1**. Patients with <3 CTC at all time points (**Group 1**) had the longest median OS, which was significantly different compared to the median OS of **Group 4**, **Group 3** and **Group 2**. Figure 16 also shows that patients who showed a decrease in CTC (**Group 2**) had a significantly lower risk of death compared to those patients with an increase in CTC (**Group 3**).

2.3.4 Univariate Cox Regression Analysis in MCRC Patients

The following parameters were analyzed using Univariate Cox regression analysis to evaluate association with PFS and OS: gender, stage of disease at diagnosis (1-4), time to metastasis (continuous), patient age (≥65 or <65), site of primary disease (colorectal or rectal or colon), ECOG status before initiation of a new line of therapy (0-2), line of therapy (1st or 2nd or 3rd), presence of liver metastasis (yes or no), type of therapy (bevacizumab, irinotecan, and/or oxaliplatin included or not), baseline CTC counts (≥3 or <3 CTC/7.5 mL), and follow-up CTC counts 1-2 weeks, 3-5 weeks, 6-12 weeks and 13-20 weeks after the initiation of therapy (≥3 or <3 CTC/7.5 mL). **Table 16** 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 16: Univariate Cox Regression Analysis in MCRC Patients

Parameter	Categories		# of MCRC Patients	PFS Risk from Baseline		OS Risk from Baseline	
	Positive	Negative		HR	p-value	HR	p-value
Gender	Male (1)	Female (0)	430	1.01	0.944	1.23	0.156
Stage at Primary Diagnosis	4 vs. 3 vs. 2 vs. 1		407	0.98	0.734	1.09	0.330
Time to Metastasis	Time in Years		428	1.00	0.901	0.92	0.121
Age at Baseline Blood Draw	≥65 Years	<65 Years	430	1.65	<0.001	1.82	<0.001
Site of Primary Disease	Colorectal (2) vs. Rectal (1) vs. Colon (0)		429	1.03	0.733	1.02	0.866
Baseline ECOG Status	2 vs. 1 vs. 0		414	1.32	0.002	1.65	<0.001
Line of Therapy	3 vs. 2 vs. 1		430	2.04	<0.001	1.63	<0.001
Liver Metastases	Yes	No	430	0.86	0.225	1.23	0.198
Bevacizumab	Yes	No	405	0.54	<0.001	0.62	0.001
Irinotecan	Yes	No	405	1.51	0.001	1.39	0.029
Oxaliplatin	Yes	No	405	0.53	<0.001	0.69	0.008
Baseline CTC Number	≥3	<3	413	1.59	<0.001	2.48	<0.001
1 - 2 Week CTC Number	≥3	<3	357	2.02	<0.001	3.23	<0.001
3 - 5 Week CTC Number	≥3	<3	334	2.19	<0.001	4.23	<0.001
6 - 12 Week CTC Number	≥3	<3	314	4.59	<0.001	10.88	<0.001
13 - 20 Week CTC Number	≥3	<3	203	5.07	<0.001	4.88	<0.001

2.3.5 Multivariate Cox Regression Analysis in MCRC Patients

Multivariate Cox regression analyses were conducted 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 and OS (**Table 17**).

Table 17: Multivariate Cox Regression Analysis in MCRC Patients

Variable	N	PFS Risk from Baseline		OS Risk from Baseline	
		Hazard Ratio	p-value	Hazard Ratio	p-value
Baseline CTC (<3 vs. ≥3)	373	1.76	<0.001	2.46	<0.001
Age at Baseline (<65 vs. ≥65)		1.47	0.002	1.84	<0.001
Baseline ECOG Status (0 vs. 1 vs. 2)		1.16	0.107	1.48	0.001
Line of Therapy (1st vs. 2nd vs. 3rd)		1.59	<0.001	1.41	0.009
Bevacizumab (No vs. Yes)		0.65	0.001	0.68	0.021
Irinotecan (No vs. Yes)		0.76	0.156	1.25	0.363
Oxaliplatin (No vs. Yes)		0.57	0.002	1.00	0.984
1-2 Week CTC (<3 vs. ≥3)	321	1.76	0.003	2.77	<0.001
Age at Baseline (<65 vs. ≥65)		1.53	0.001	1.85	<0.001
Baseline ECOG Status (0 vs. 1 vs. 2)		1.26	0.025	1.54	0.001
Line of Therapy (1st vs. 2nd vs. 3rd)		1.76	<0.001	1.62	0.001
Bevacizumab (No vs. Yes)		0.66	0.003	0.77	0.156
Irinotecan (No vs. Yes)		0.67	0.066	1.25	0.402
Oxaliplatin (No vs. Yes)		0.53	0.002	0.97	0.904
3-5 Week CTC (<3 vs. ≥3)	302	2.35	<0.001	4.54	<0.001
Age at Baseline (<65 vs. ≥65)		1.58	0.001	2.06	<0.001
Baseline ECOG Status (0 vs. 1 vs. 2)		1.16	0.149	1.33	0.032
Line of Therapy (1st vs. 2nd vs. 3rd)		1.74	<0.001	1.65	0.001
Bevacizumab (No vs. Yes)		0.68	0.007	0.86	0.410
Irinotecan (No vs. Yes)		0.58	0.012	0.99	0.966
Oxaliplatin (No vs. Yes)		0.47	<0.001	0.88	0.594
6-12 Week CTC (<3 vs. ≥3)	279	3.04	<0.001	9.43	<0.001
Age at Baseline (<65 vs. ≥65)		1.43	0.013	1.73	0.005
Baseline ECOG Status (0 vs. 1 vs. 2)		1.30	0.027	1.53	0.004
Line of Therapy (1st vs. 2nd vs. 3rd)		1.73	<0.001	1.20	0.282
Bevacizumab (No vs. Yes)		0.61	0.001	0.82	0.337
Irinotecan (No vs. Yes)		0.78	0.258	1.47	0.181
Oxaliplatin (No vs. Yes)		0.62	0.020	1.35	0.278
13-20 Week CTC (<3 vs. ≥3)	186	4.50	<0.001	4.97	<0.001
Age at Baseline (<65 vs. ≥65)		1.26	0.218	1.55	0.061
Baseline ECOG Status (0 vs. 1 vs. 2)		1.13	0.417	1.13	0.526
Line of Therapy (1st vs. 2nd vs. 3rd)		1.68	0.004	1.12	0.628
Bevacizumab (No vs. Yes)		0.68	0.058	0.89	0.655
Irinotecan (No vs. Yes)		0.73	0.311	1.20	0.636
Oxaliplatin (No vs. Yes)		0.65	0.135	1.31	0.477

2.4 Use of CTC to Monitor Clinical Status of Metastatic Colorectal Cancer

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

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

2.4.2 CTC

Previous data has shown that metastatic colorectal cancer patients with ≥ 3 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 <3 CTC / 7.5 mL of blood. The CTC results obtained 3-5 weeks after the initiation of therapy as well as the CTC results obtained within ± one month of the imaging study were classified as Favorable (<3 CTC) and Unfavorable (≥3 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.

2.4.3 Imaging

Each MCRC patient had to have measurable disease, i.e. a minimum of one 2cm lesion up to and including a maximum of 10 such lesions. The method of imaging for each patient was determined by the treating oncologist in keeping with the current standard of care. Either CT or MRI of the chest, abdomen and pelvis were performed with the requirement that all lesions seen at baseline were followed using the same method for all subsequent imaging studies. Image interpretation was performed by a certified radiologist at the participating site using RECIST uni-dimensional criteria to classify each follow-up disease assessment as complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD).

Each patient was imaged at a minimum of two time points up to 8 different time points. These studies included a baseline image, imaging at subsequent intervals of 2-3 months (6-12 weeks), and a final image study when the patient went off study. Copies of all patients' imaging studies were forwarded to the study coordinator at each clinical site for filing with the patient clinical data.

Out of the total of 430 evaluable MCRC patients enrolled into the study, 28 (7%) did not have a follow-up imaging study performed, 18 (4%) died before a follow-up imaging study could be performed, and 384 (89%) had one or more follow-up imaging studies performed that were assessed using RECIST criteria. At the time of the 1st follow-up in the 384 patients with a follow-up imaging study, 4 (1%) showed a complete response, 117 (31%) showed a partial response, 186 (48%) had stable disease, and 77 (20%) showed progressive disease. For the purposes of these analyses, patients who died before a follow-up imaging study were considered to have progressive disease.

For response to therapy at the first follow-up disease assessment, the Favorable group was defined as those having stable disease (S), partial response (PR) or a complete response (CR) by RECIST criteria (non-progressive disease, NPD) and the Unfavorable group as those with progressive disease or death (PD).

2.4.4 Relationship between survival to imaging and CTC

Separate Kaplan-Meier analyses were performed to compare the overall survival of MCRC patients in the Favorable (<3 CTC) and Unfavorable (≥3 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 9.1 ± 2.9 weeks (median = 8.6 weeks) after initiation of therapy (i.e. the baseline blood draw), the median survival of the 307 (76%) patients determined by imaging to have NPD was 19.1 months (95% CI = 17.0 to 23.1) (**Figure 17, Table 18**). For the 95 (24%) patients determined by imaging to have PD, the median survival was 5.8 months (95% CI= 4.4 to 7.7).

A total of 320 MCRC patients had imaging studies performed before and after initiation of therapy or they died prior to a follow-up imaging study being performed and they had CTC assessed 3-5 weeks after initiation of therapy (average = 3.8 ± 0.7 weeks from the time of the baseline blood draw, median = 4.0 weeks). The median survival of 282 (88%) patients with Favorable CTC results (<3 CTC) was 17.3 months (95% CI = 15.0 to 19.5 months) (**Figure 18, Table 18**). The 38 patients (12%) with Unfavorable CTC results (≥3 CTC) had a median survival of 5.4 months (95% CI = 3.6 to 9.4 months).

To determine if CTC assessments performed closer to the time of the imaging resulted in similar survival prospects compared to CTC assessments performed 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 were analyzed (**Figure 19, Table 18**). Three hundred and sixty-four (364) of the 402 patients (91%) had CTC assessments within one month of the first follow-up imaging study, which was performed 9.0 ± 2.9 weeks (median = 8.5 weeks) after the initiation of therapy. The median survival of 335 (92%) patients with Favorable CTC results was 17.2 months (95% CI = 15.0 to 19.2 months). For the 29 (8%) patients with Unfavorable CTC results, the median survival was 5.4 months (95% CI = 3.2 to 7.5 months). These data showed that CTC assessments at both time points provided similar results to imaging conducted approximately nine weeks after the initiation of therapy.

In this study, applying multivariate Cox regression analysis to adjust for imaging indicated that both CTC and imaging at 6-12 weeks are independently associated with overall survival but CTC [adjusted hazard ratio: 7.9 (4.6-13.6)] are a stronger predictor than imaging [adjusted hazard ratio: 3.1 (2.1-4.6)].

Table 18: OS of MCRC 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) in Months
A. Imaging	402	
Favorable (NPD)	307 (76%)	19.1 (17.0 – 23.1)
Unfavorable (PD)	95 (24%)	5.8 (4.4 – 7.7)
B. 3-5 week CTC	320	
Favorable (<3 CTC)	282 (88%)	17.3 (15.0 - 19.5)
Unfavorable ($\geq$3 CTC)	38 (12%)	5.4 (3.6 - 9.4)
C. CTC (±1 month of Imaging)	364	
Favorable (<3 CTC)	335 (92%)	17.2 (15.0 - 19.2)
Unfavorable ($\geq$3 CTC)	29 (8%)	5.4 (3.2 - 7.5)

Figure 17: Correlation of Radiological and CTC Assessment with OS: OS of MCRC Patients with NPD or PD at 1st Follow-Up Imaging Study (N=402)

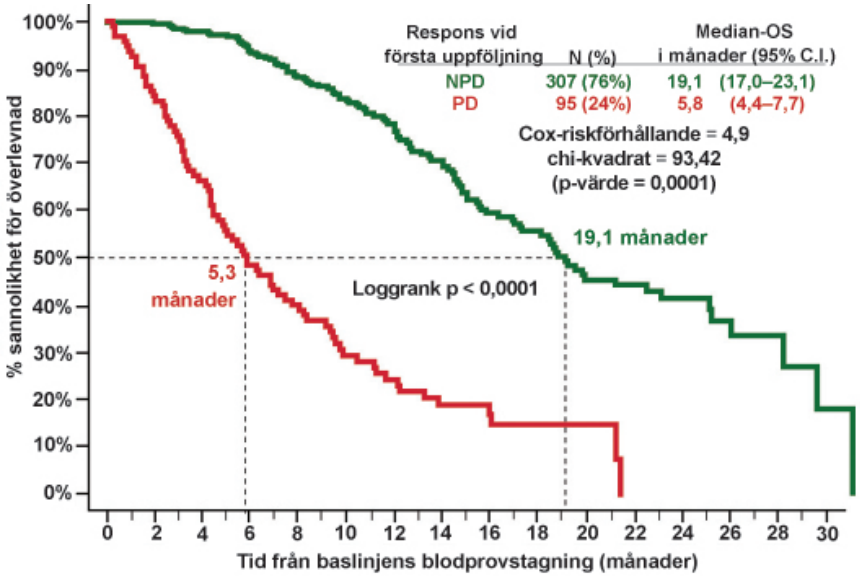


Figure 18: Correlation of Radiological and CTC Assessment with OS: OS of MCRC Patients with <3 or $\geq$3 CTC at 1st Follow-Up after Initiation of Therapy (N=320)

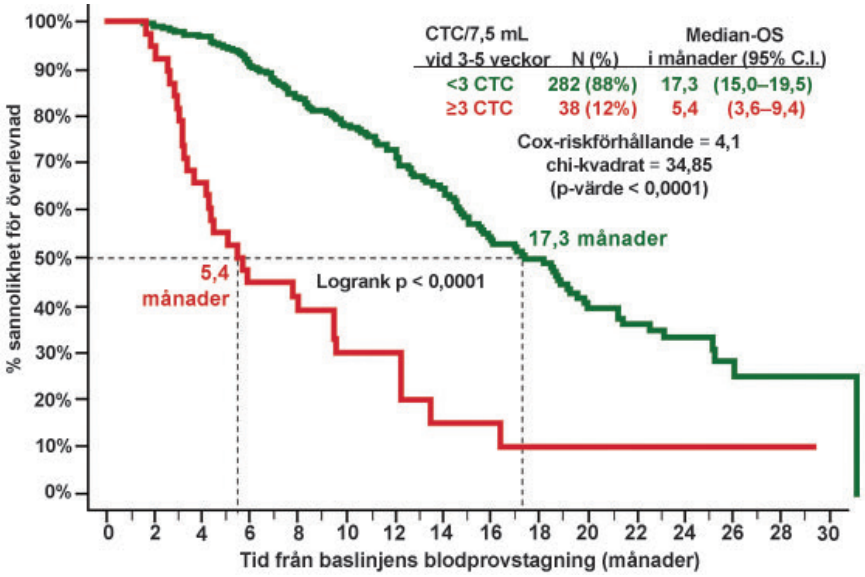
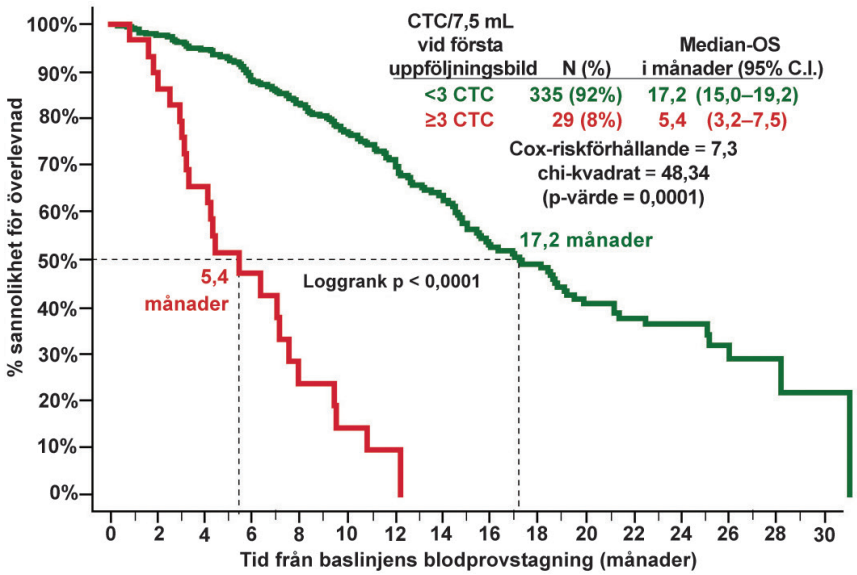


Figure 19: Correlation of Radiological and CTC Assessment with OS: OS of MCRC Patients with <3 or ≥3 CTC within ±1 Month of 1st Follow-Up Imaging Study or Death (N=364)



2.4.5 Concordances between CTC and Radiological Monitoring in MCRC Patients

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 colorectal cancer setting. To further support the effectiveness of CTC in making these clinical assessments, two-by-two tabulations of concordant and discordant observations between CTC and radiological imaging were constructed.

For response to therapy, the Favorable group was defined as those having stable disease (S), partial response (PR) or a complete response (CR) by RECIST criteria (non-progressive disease, NPD) and the Unfavorable group as those with progressive disease (PD). Out of the 18 patients who died prior to a follow-up imaging study, 10 had a follow-up blood draw within 30 days of death and these 10 patients were classified as having progressive disease (PD) for the purposes of these comparisons.

The CTC results obtained within ± one month of the imaging study were classified as Favorable (<3 CTC) and Unfavorable (≥3 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. This analysis used all evaluable blood draws from the patients to match up CTC with the imaging studies, not just the ones that were selected for the designated time points as described in 2.1 above.

A total of 366 MCRC patients had CTC results within one month of the imaging study or death. The result of this "patient-wise" comparison between CTC and imaging (or death) is shown in **Table 19**.

Table 19: MCRC Patient-Wise Comparison of CTC and Imaging

Response at 1st Follow-Up Imaging Study	CTC within ± 1 Month of Imaging Study or Death		Total
	<3 CTC / 7.5 mL	≥3 CTC / 7.5 mL	
Non-Progressive Disease	272	13	**285**
Progressive Disease	65	16	**81**
Total	**337**	**29**	**366**

Measurement	Estimate	Lower 95% CI	Upper 95% CI
Positive % Agreement	20%	12%	30%
Negative % Agreement	95%	92%	98%
Positive Predictive Value	55%	36%	74%
Negative Predictive Value	81%	76%	85%
Overall Agreement	79%	74%	83%
Odds Ratio	5.2	2.4	11.2

Of the 384 MCRC patients with one or more follow-up imaging studies, a total of 911 imaging studies that rendered a useable radiological response were performed. A total of 805 of the 911 (88%) imaging studies had CTC results obtained within ± one month of the imaging study. Of the 18 patients who died prior to a follow-up imaging study, 10 had a follow-up blood draw within 30 days of death and these 10 patients were classified as having progressive disease (PD) for the purposes of these comparisons. The result of this "observation-wise" comparison between CTC and imaging (or death) in the 815 observations is shown in **Table 20**.

Table 20: MCRC Observation-Wise Comparison of CTC and Imaging

Response at All Follow-Up Imaging Studies	CTC within ± 1 Month of Imaging Study or Death		Total
	<3 CTC / 7.5 mL	≥3 CTC / 7.5 mL	
Non-Progressive Disease	597	33	**630**
Progressive Disease	147	38	**185**
Total	**744**	**71**	**815**

Measurement	Estimate	Lower 95% CI	Upper 95% CI
Positive % Agreement	21%	15%	27%
Negative % Agreement	95%	93%	96%
Positive Predictive Value	54%	41%	65%
Negative Predictive Value	80%	77%	83%
Overall Agreement	78%	75%	81%
Odds Ratio	4.7	2.8	7.7

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 <3 and ≥3 CTC / 7.5 mL.

Because the prognostic value of the CTC results at an earlier time-point were equivalent to that of the CTC results at the time of imaging (**Figure 18** & **Figure 19**), 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 approximately 4 weeks after initiation of therapy was constructed. A total of 320 (80%) of the 402 patients had CTC results 3-5 weeks after the initiation of therapy. The result of this "patient-wise" comparison between CTC at an earlier time point and imaging (or death) is shown in **Table 21**.

Table 21: MCRC Patient-Wise Comparison of CTC and Imaging

Response at 1st Follow-Up Imaging Study	CTC 3-5 Weeks After Initiation of Therapy		Total
	<3 CTC / 7.5 mL	≥3 CTC / 7.5 mL	
Non-Progressive Disease	228	18	**246**
Progressive Disease	54	20	**74**
Total	**282**	**38**	**320**

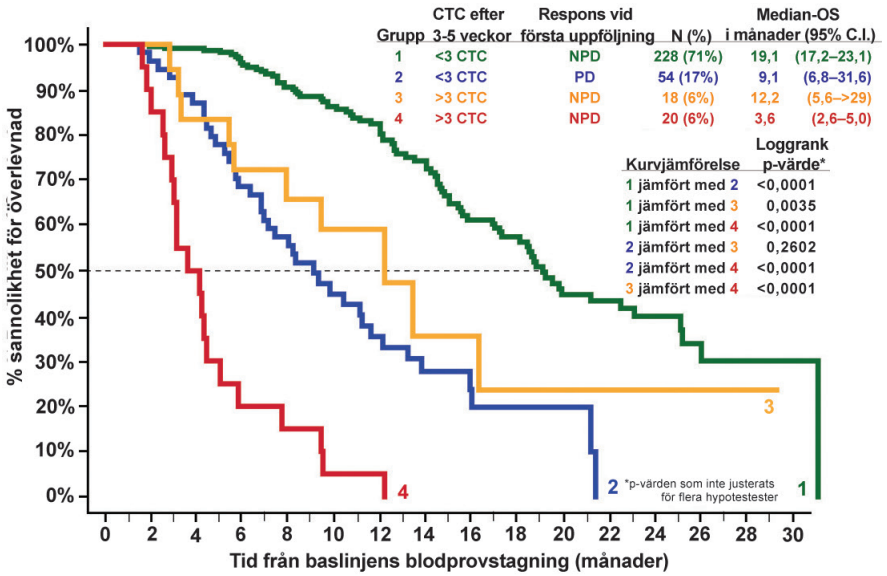
Measurement	Estimate	Lower 95% CI	Upper 95% CI
Positive % Agreement	27%	17%	39%
Negative % Agreement	93%	89%	96%
Positive Predictive Value	53%	36%	69%
Negative Predictive Value	81%	76%	85%
Overall Agreement	78%	73%	82%
Odds Ratio	4.7	2.3	9.5

2.4.6 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 MCRC patients. As the information from CTC assessments is intended to be used in conjunction with other diagnostic modalities to make treatment decisions, CTC assessment 3-5 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 20**):

- Group 1 (**green** curve), 228 (71%) patients with <3 CTC at 3-5 weeks and NPD;
- Group 2 (**blue** curve), 54 (17%) patients with <3 CTC at 3-5 weeks and PD;
- Group 3 (**orange** curve), 18 (6%) patients with ≥3 CTC at 3-5 weeks and NPD;
- Group 4 (**red** curve), 20 (6%) patients with ≥3 CTC at 3-5 weeks and PD.

Figure 20: OS of MCRC Patients in Groups 1, 2, 3 and 4 using CTC 3-5 Weeks after Initiation of Therapy (n=320) and the Disease Status Determined at the 1st Follow-Up Imaging Study



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.

3 Metastatic Prostate Cancer (MPC) Patients

A multi-center prospective, clinical trial was conducted to determine whether the number of CTC predicted disease progression and survival. Metastatic prostate cancer patients in this study were defined as having two consecutive increases in the serum marker prostate-specific antigen (PSA) above a reference level, despite standard hormonal management. These patients are commonly described as having androgen-independent, hormone-resistant, or castration-resistant prostate cancer. A total of 231 metastatic prostate cancer patients with evidence of PSA progression despite standard hormonal therapy and starting a new line or type of chemotherapy were enrolled. Clinical data were analyzed on an intent-to-treat basis. Patient demographic information is presented in **Table 22**.

Table 22: MPC Patient Demographics

Category	Mean ± Std. Deviation (Median)	Number of Subjects
Age at Baseline (in years)	70 ± 9 (70)	**231**
Pre-Therapy:		
PSA (ng/mL)	547 ± 1616 (144)	**231**
Hemoglobin (g/dL)	12.3 ± 1.6 (12.4)	**221**
Alkaline Phosphatase(AlkPhos) (IU/mL)	235 ± 271 (144)	**223**
Lactate dehydrogenase(LDH) (IU/mL)	293 ± 228 (224)	**219**
Albumin (g/dL)	3.9 ± 2.6 (3.8)	**214**
	Description of Categories	**Number of Subjects (% of total)**
Race	White	209 (90%)
	Black	17 (7%)
	Other	5 (3%)
Baseline ECOG Score	0	101 (44%)
	1	100 (43%)
	2	21 (9%)
	Unknown	9 (4%)
Gleason Score	≥5	18 (8%)
	6	28 (12%)
	7	63 (27%)
	8	45 (20%)
	≥9	54 (23%)
	Unknown	23 (10%)
Stage at Primary Diagnosis	1	14 (6%)
	2	30 (13%)
	3	58 (25%)
	4	19 (8%)
	Unknown	110 (48%)
Line of Therapy	1st	154 (67%)
	2nd	38 (16%)
	≥3rd	39 (17%)
Taxotere in Current Therapy Line?	No	67 (29%)
	Yes	162 (70%)
	Unknown	2 (1%)
Bone Metastasis	Negative	20 (8%)
	Positive	207 (90%)
	Unknown	4 (2%)
Measurable Disease	No	142 (62%)
	Yes	88 (38%)
	Unknown	1 (0%)
Visceral Metastasis	No	141 (61%)
	Yes	89 (39%)
	Unknown	1 (0%)

Baseline CTC count was determined prior to initiation of a new line of chemotherapy. The following timeframes were chosen for evaluation: baseline (prior to the initiation of therapy), 2-5 weeks (14 - 41 days from baseline), 6-8 weeks (42 - 62 days from baseline), 9-12 weeks (63 - 90 days from baseline), and 13-20 weeks (91 - 146 days from baseline) after the initiation of therapy. If more than one blood draw fell within the designated timeframes, the blood draw furthest from the baseline blood draw was used as the result for each timeframe.

3.1 CTC frequencies

All 231 evaluable MPC patients had a baseline blood draw. Two hundred and twenty-one (221) of these MPC patients had one or more follow-up blood draws after the initiation of therapy. Of the ten MPC patients with only a baseline blood draw, three died before a follow-up blood draw could be obtained, one progressed and was sent to hospice, one stopped their chemotherapy due to a broken hip, one patient moved, three refused any further blood draws, and one withdrew their consent for the study. There were a total of 214, 171, 158, and 149 MPC patients with follow-up blood draws 2-5 weeks, 6-8 weeks, 9-12 weeks, and 13-20 weeks after the initiation of therapy, respectively.

In metastatic prostate cancer, disease progression is primarily determined using changes in PSA. For this study, disease progression was determined by the sites using PSA, imaging, and/or clinical signs and symptoms. For the baseline analyses, progression free survival (PFS) was determined from the time of the baseline blood draw to the determination of progression or death, and overall survival (OS) was determined from the time of the baseline blood draw to the date of death or the date of last contact with the patient. For the follow-up analyses, PFS was determined from the time of the follow-up blood draw to diagnosis of progression or death, and OS was determined from the time of the follow-up blood draw to the date of death or the date of last contact with the patient. Patients with

progression prior to the date of the blood draw being evaluated were excluded from the PFS analyses of that time point and all subsequent follow-up blood draws. Patients with no additional survival follow-up beyond the date of the blood draw being evaluated were excluded from the PFS & OS analyses of that time point. **Table 23** shows the numbers of patients at each time point excluded from the PFS or PFS & OS analyses and the reasons for their exclusion.

Table 23: Exclusions from PFS and OS Analyses in MPC Patients

Blood Draw Timing	MPC Patients Not Evaluable:					Total # of MPC Patients Evaluable	
	PFS & OS				PFS Only		
	Blood Not Drawn	Blood Drawn 1-7 days after therapy administration	No Follow-up Beyond Date of Blood Draw	No or Non-Evaluable CTC Results	Blood drawn after date of disease progression	PFS	OS
Baseline	0	6	0	6	0	219	219
2-5 Weeks	17	0	0	11	4	199	203
6-8 Weeks	60	0	0	8	22	141	163
9-12 Weeks	73	1	0	8	15	134	149
13-20 Weeks	82	0	1	5	27	116	143

The CTC results obtained from the baseline and follow-up blood draws at 2-5 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). The PSA, Alkaline Phosphatase, and LDH levels summarized in the demographics table and used in the analyses were all measured at a central laboratory in serum samples collected at the same time as the blood samples used for CTC evaluation. The hemoglobin and albumin levels summarized in the tables and used in the analyses were values provided by the sites and verified from the patient's medical records that were determined within ± 30 days of the baseline CTC evaluation.

3.2 Progression Free Survival (PFS) Analysis of MPC Patients

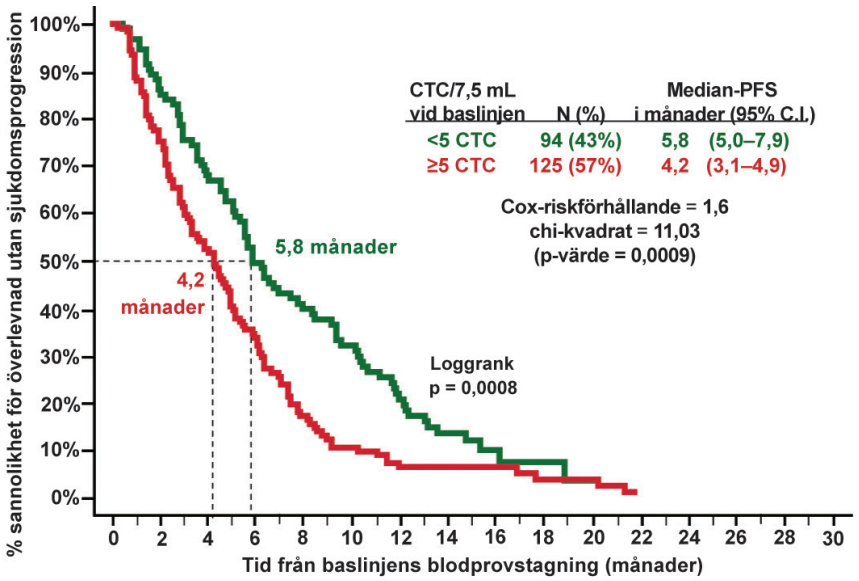
3.2.1 PFS Using Baseline CTC Results

Two hundred and nineteen (219) of the 231 evaluable 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=94), represented in **green**, consisted of patients with <5 CTC.
- The Unfavorable group (N=125), represented in **red**, consisted of patients with ≥5 CTC.

Median PFS was longer in the Favorable group compared to the Unfavorable group (5.8 vs. 4.2 months, respectively.) These results are illustrated in **Figure 21** and **Table 24**.

Figure 21: PFS of MPC Patients with <5 or ≥5 CTC at Baseline (N = 219).



3.2.2 PFS Using Follow-up CTC Results

For Kaplan-Meier analysis, MPC 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 draws after initiation of therapy for PFS are illustrated in **Figure 22**. This figure illustrates the ability of CTC in MPC patients with <5 and ≥5 CTC to predict time to clinical progression or death at 2-5 weeks (n=199), 6-8 weeks (n=141), 9-12 weeks (n=134) and 13-20 weeks (n=116) after the initiation of therapy.

- The Favorable group represented in **olive green**, **blue**, **purple**, and **cyan** consisted of patients with <5 CTC at 2-5, 6-8, 9-12, and 13-20 weeks after the initiation of therapy, respectively.
- The Unfavorable group, represented in **brown**, **black**, **grey**, and **orange** consisted of patients with ≥5 CTC at 2-5, 6-8, 9-12, and 13-20 weeks after the initiation of therapy, respectively.

Figure 22: PFS of MPC Patients with <5 or ≥5 CTC at different times of Follow-Up

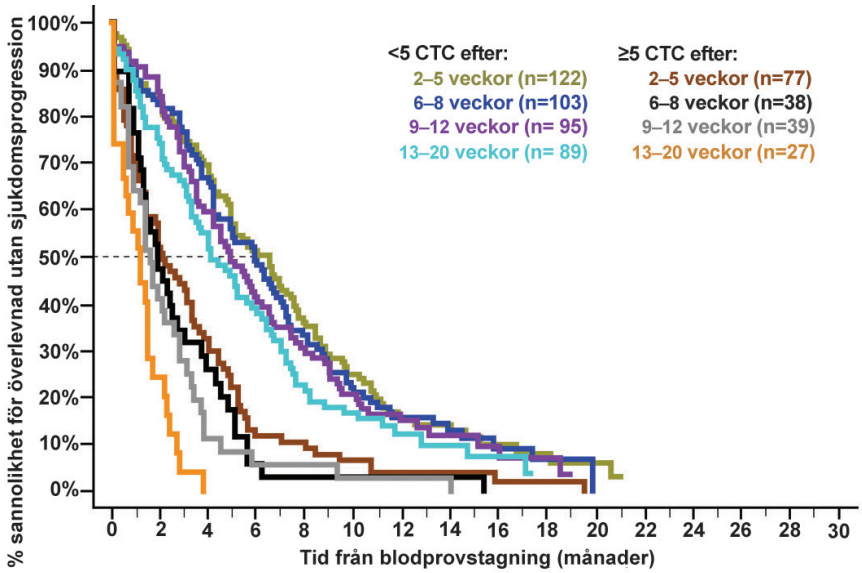


Table 24 summarizes the results of the PFS analysis using the CTC levels and a threshold of ≥5 CTC/7.5 mL at each of the different blood draw time points.

Table 24: Progression Free Survival (PFS) for MPC patients with <5 or ≥5 CTC at different time points

1	2	3	4	5	6
Sampling Time After Tx Initiation	**N**	**≥5 CTC**	**Median PFS in Months (95% CI)**		**Log-rank p-value**
			<5 CTC	**≥5 CTC**	
Baseline	219	125 (57%)	5.8 (5.0 – 7.9)	4.2 (3.1 – 4.9)	0.0008
2-5 Weeks	199	77 (39%)	6.5 (4.9 – 7.4)	2.1 (1.4 – 3.3)	<0.0001
6-8 Weeks	141	38 (27%)	5.9 (4.2 – 7.0)	1.9 (1.3 – 2.7)	<0.0001
9-12 Weeks	134	39 (24%)	4.9 (3.8 – 6.2)	1.6 (0.9 – 2.6)	<0.0001
13-20 Weeks	116	27 (23%)	4.1 (3.3 – 5.8)	1.2 (0.5 – 1.5)	<0.0001

As illustrated in **Figure 22** and **Table 24**, MPC 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 24** column 4 shows the median PFS times for those patients with <5 CTC ranged from 4.1 to 6.5 months and were substantially longer than the median PFS times for those patients with ≥5 CTC, which ranged from 1.2 to 4.2 months (column 5).

3.2.3 Reduction or Increase in CTC Predicts Improved or Decreased PFS

Elapsed PFS times were calculated from the baseline blood draw. For the Kaplan-Meier analysis shown in **Figure 23**, MPC patients were segmented into four groups based upon their CTC counts at baseline, 2-5 weeks, 6-8 weeks, 9-12 weeks, and 13-20 weeks after the initiation of therapy:

- Group 1 (**green** curve), 88 (38%) patients with <5 CTC at all time points. Five (6%) of these patients only had a baseline blood draw while seven (8%) had a single blood draw between their first and last blood draw that had ≥5 CTC;
- Group 2 (**blue** curve), 45 (20%) 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), 26 (11%) patients with <5 CTC at an early draw (baseline, 2-5 weeks, and/or 6-8 weeks) but who increased to ≥5 CTC at the time of their last blood draw;
- Group 4 (**red** curve), 71 (31%) patients with ≥5 CTC at all draw time points. Eight (11%) of these patients had only a baseline blood draw and two (3%) had a single blood draw between their first and last blood draw that had <5 CTC.

Figure 23: A Reduction in CTC Below 5 After the Initiation of Therapy Predicts Longer PFS in MPC Patients

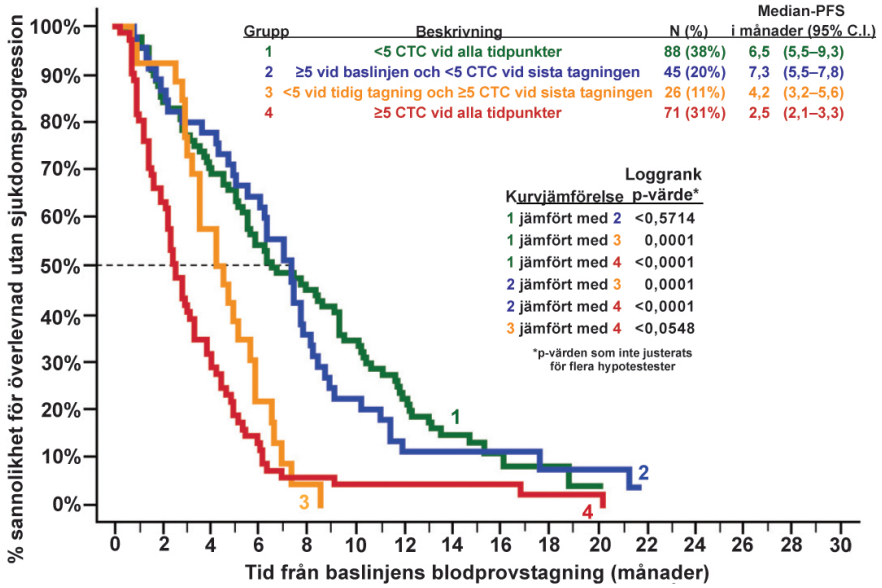


Figure 23 shows that MPC patients with≥5 CTC at all time points (**Group 4**) had the shortest median PFS, which was significantly different compared to the median PFS of **Group 3**, **Group 2** and **Group 1**. The difference in the median PFS between those patients who showed a CTC reduction after the initiation of therapy (**Group 2**) was significantly longer compared to those patients who showed a CTC increase (**Group 3**).

3.3 Overall Survival (OS) Analysis of MPC Patients

3.3.1 OS Analysis Using Baseline CTC Results

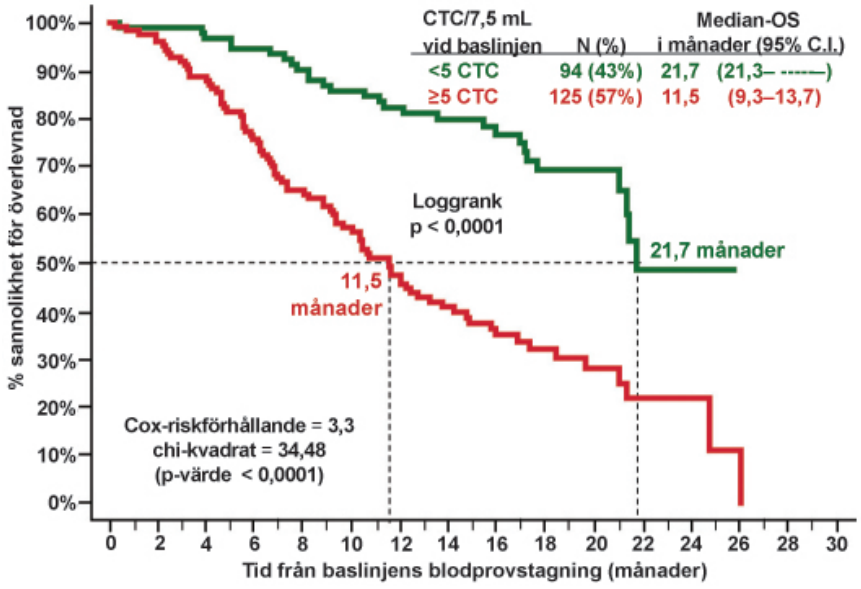
Death occurred in 119 (52%) of the 231 MPC patients, with a mean follow-up time for the 112 (48%) patients still alive of 16.1 ± 4.9 months (median = 16.5 months, range = 1.9 to 25.7 months). At the time of these analyses, 28 (30%) of 94 patients from the Favorable group (<5 CTC at baseline) compared to 83 (66%) of 125 from the Unfavorable group ≥5 CTC at baseline) had died.

For Kaplan-Meier analysis, the 219 of the 231 evaluable patients that had baseline results were segmented into two groups based upon their CTC count at baseline:

- The Favorable group (N=94), represented in **green**, consisted of patients with <5 CTC.
- The Unfavorable group (N=125), represented in **red**, consisted of patients with ≥5 CTC.

Median OS was significantly longer in the Favorable group compared to the Unfavorable group (21.7 vs. 11.5 months, respectively). These results are illustrated in **Figure 24**.

Figure 24: OS of MPC Patients with <5 or ≥5 CTC at Baseline (N = 219).



3.3.2 OS Using Follow-up CTC Results

The Kaplan-Meier analyses of both MPC patient groups at each of the different follow-up blood draw times after initiation of therapy are illustrated in **Figure 25**. This figure illustrates the ability of CTC in MPC patients with <5 and ≥5 CTC 2-5 weeks (n=203), 6-8 weeks (n=163), 9-12 weeks (n=149) and 13-20 weeks (n=143) after the initiation of therapy to predict time to death. 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 25: OS of MPC Patients with <5 or ≥5 CTC at different times of Follow-Up.

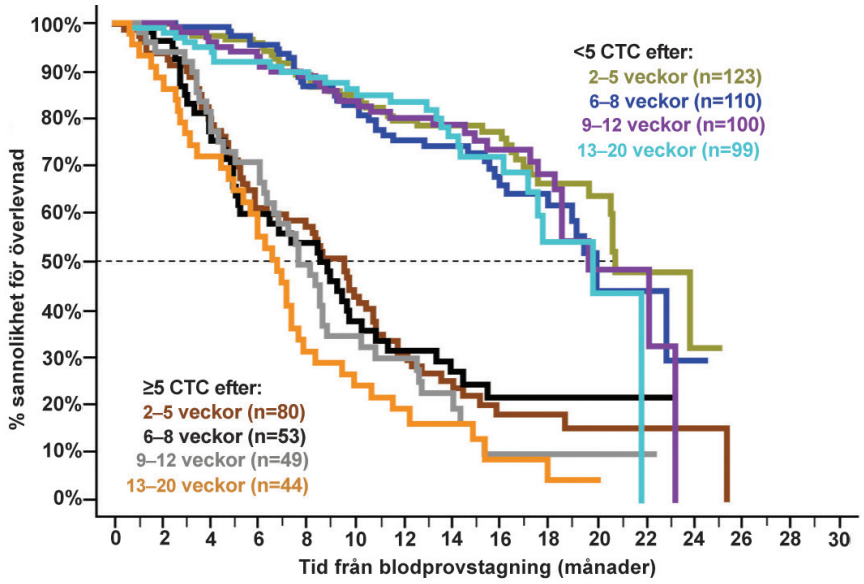


Table 25 summarizes the results of the OS analysis using the CTC levels and a threshold of ≥5 CTC/7.5 mL at each of the different blood draw time points.

Table 25: Overall Survival (OS) for MPC patients with <5 or≥ 5 CTC at different time points

1	2	3	4	5	6
Sampling Time After Tx Initiation	N	≥5 CTC	Median OS in Months (95% CI)		Log-rank p-value
			<5 CTC	≥5 CTC	
Baseline	219	125 (57%)	21.7 (21.3 - NR)	11.5 (9.3 - 13.7)	<0.0001
2-5 Weeks	203	80 (39%)	20.7 (20.5 - NR)	9.5 (5.8 - 10.7)	<0.0001
6-8 Weeks	163	53 (33%)	19.9 (17.9 - NR)	8.5 (5.0 - 10.2)	<0.0001
9-12 Weeks	149	49 (33%)	19.6 (18.5 - NR)	7.6 (6.2 - 8.6)	<0.0001
13-20 Weeks	143	44 (31%)	19.8 (17.1 - NR)	6.7 (4.9 - 7.6)	<0.0001

As illustrated in **Figure 25** and **Table 25** in columns 4 & 5, MPC patients with ≥5 CTC at any of the time points had a much higher likelihood of dying sooner than did those with <5 CTC. The median OS times for those patients with <5 CTC ranged from 19.6 to 21.7 months and were substantially longer than the median OS times for those patients with ≥5 CTC, which ranged from 6.7 to 11.5 months.

3.3.3 Reduction or Increase of CTC Predicts Improved or Decreased OS

Elapsed OS times were calculated from the baseline blood draw. For Kaplan-Meier analysis (**Figure 26**), patients were segmented into four groups based upon their CTC counts at baseline, 2-5 weeks, 6-8 weeks, 9-12 weeks, and 13-20 weeks after the initiation of therapy:

- Group 1 (**green** curve), 88 (38%) patients with <5 CTC at all time points. Five (6%) of these patients had only a baseline blood draw while seven (8%) had a single blood draw between their first and last blood draw that had ≥5 CTC;
- Group 2 (**blue** curve), 45 (20%) 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), 26 (11%) patients with <5 CTC at an early draw but who increased to ≥5 CTC at the time of their last blood draw;
- Group 4 (**red** curve), 71 (31%) patients with ≥5 CTC at all draw time points. Eight (11%) of these patients had only a baseline blood draw and two (3%) had a single blood draw between their first and last blood draw that had <5 CTC.

Figure 26: 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 MPC Patients

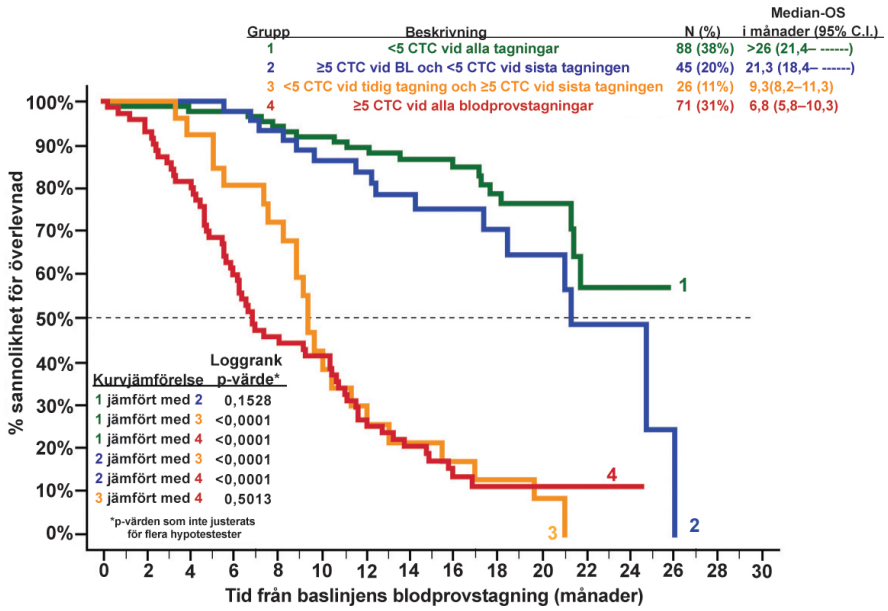


Figure 26 shows that those patients with ≥5 CTC at any point after the initiation of therapy had a much higher likelihood of dying sooner. Patients with ≥5 CTC at all time points (**Group 4**) had the shortest median OS, which was significantly different compared to the median OS of **Group 2**, and **Group 1** but not **Group 3**. Patients with <5 CTC at all time points (**Group 1**) had the longest median OS, which was significantly different compared to the median OS of **Group 4** and **Group 3**, but not **Group 2**. **Figure 26** also demonstrated that patients who showed a decrease in CTC (**Group 2**) improve their survival chances and had a median OS similar to those patients with favorable CTC at all draws (**Group 1**). The figure also shows that unfavorable CTC levels after the initiation of therapy significantly decreased overall survival (**Group 3** and **Group 4**).

3.3.4 Univariate Cox Regression Analysis in MPC Patients

Univariate Cox proportional hazards regression analysis was used to evaluate the association of the following pre-treatment parameters with PFS and OS: stage of disease at diagnosis (1-4), patient age (≥70 or <70 years), ECOG status before initiation of a new line of therapy (0-2), Gleason score (2-10), hemoglobin level within ± 30 days of baseline draw (g/dL, continuous), albumin level within ± 30 days of baseline draw (g/dL, continuous), testosterone level at the time of the baseline draw (ng/mL, continuous), LDH level at the time of the baseline draw (IU/mL, continuous), alkaline phosphatase level at the time of the baseline draw (IU/mL, continuous), PSA level at the time of the baseline draw (ng/mL, continuous), pre-treatment PSA doubling time (months, continuous), pre-treatment PSA velocity (ng/mL/month, continuous), line of therapy (1st, 2nd, 3rd, 4th, 5th, or 6th), type of therapy (taxotere included or not), presence of measurable disease (yes or no), presence of bone metastasis (yes or no), presence of visceral metastasis (yes or no), and baseline CTC level (≥5 CTC/7.5 mL or <5 CTC/7.5 mL) and follow up CTC counts at 2-5, 6-8, 9-12 and 13-20 weeks.

For these analyses, the elapsed times for both PFS and OS were calculated from the time of the baseline blood draw. The Cox regression results (i.e. the hazards ratio and associated 95% confidence interval, chi-square value, and associated p-values) for the ability of the parameters to independently predict PFS and OS are provided in **Table 26** as well as the number of patients in each evaluation.

Table 26: Univariate Cox Regression Analysis in MPC Patients

Parameter	Categories		# of MPC Patients	PFS Risk from Baseline		OS Risk from Baseline	
	Positive	Negative		HR	p-value[2]	HR	p-value[2]
Stage at Primary Diagnosis	4 vs. 3 vs. 2 vs. 1		121	0.88	0.206	0.83	0.174
Age at Baseline Blood Draw	≥70	<70	231	0.96	0.764	1.28	0.178
ECOG Status at Study Entry	2 vs. 1 vs. 0		222	1.34	0.011	2.36	<0.001
Gleason Score	10 to 2		208	1.01	0.919	1.02	0.717
Pre-treatment PSA Doubling Time (Months)	Continuous		230	0.97	0.542	0.97	0.664
Pre-treatment PSA Velocity (ng/mL/Month)	Continuous		230	1.00	0.200	1.00	0.544
Baseline Hemoglobin (g/dL)	Continuous		221	0.87	0.002	0.71	<0.001
Baseline Albumin (g/dL)	Continuous		214	0.99	0.748	1.02	0.557
Baseline Testosterone (ng/mL)[1]	Continuous		223	1.07	0.900	2.71	0.060
Baseline LDH (IU/mL)[1]	Continuous		219	1.001	<0.001	1.002	<0.001
Baseline Alkaline Phosphatase (IU/mL)[1]	Continuous		223	1.00	0.158	1.0008	0.001
Line of Therapy	Continuous (1 - 6)		231	1.23	0.003	1.28	0.003
Type of Therapy (Taxotere: Yes/No)	Yes	No	229	0.57	<0.001	0.59	0.006
Measurable Disease?	Yes	No	230	1.00	0.993	1.28	0.181
Bone Metastasis?	Yes	No	227	1.02	0.933	2.22	0.057
Visceral Metastasis?	Yes	No	230	1.01	0.918	1.26	0.216
Baseline PSA (ng/mL)[1]	Continuous		231	1.00	0.746	1.00	0.907
2 - 5 Week PSA (ng/mL)[1]	Continuous		207	1.00	0.819	1.00	0.794
6 - 8 Week PSA (ng/mL)[1]	Continuous		167	1.00	0.426	1.00	0.654
9 - 12 Week PSA (ng/mL)[1]	Continuous		155	1.00	0.684	1.00	0.324
13 - 20 Week PSA (ng/mL)[1]	Continuous		143	1.00	0.639	1.00	0.205
2 - 5 Week PSA Reduction from BL (%)[1]	<30%	≥30%	207	1.56	0.006	1.24	0.318
6 - 8 Week PSA Reduction from BL (%) [1]	<30%	≥30%	167	2.21	<0.001	2.27	0.001
9 - 12 Week PSA Reduction from BL (%)[1]	<30%	≥30%	155	2.76	<0.001	2.30	<0.001
13 - 20 Week PSA Reduction from BL (%)[1]	<30%	≥30%	143	2.69	<0.001	3.19	<0.001
Baseline CTC Number	≥5	<5	219	1.62	0.001	3.33	<0.001
2 - 5 Week CTC Number	≥5	<5	203	2.34	<0.001	4.46	<0.001
6 - 8 Week CTC Number	≥5	<5	163	3.29	<0.001	3.66	<0.001
9 - 12 Week CTC Number	≥5	<5	149	3.23	<0.001	5.82	<0.001
13 - 20 Week CTC Number	≥5	<5	144	4.82	<0.001	7.18	<0.001

[1] Determined from Serum Drawn on the Same Date as the Blood Drawn for CTC
[2] p-value from Wald test of Z statistic

3.3.5 Multivariate Cox Regression Analysis in MPC Patients

Multivariate Cox regression analyses were conducted 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 strongest predictor at most time points of PFS and OS (**Table 27**).

Table 27: Multivariate Cox Regression Analysis in MPC Patients

Parameter	# of Patients	PFS Risk from Baseline		OS Risk from Baseline	
		HR	p-value[2]	HR	p-value[2]
Baseline CTC (<5 vs. ≥5)	188	1.14	0.455	1.92	0.009
Baseline ECOG Status (0 vs. 1 vs. 2)		1.00	0.982	1.46	0.032
Baseline Hemoglobin (g/dL)[3]		0.88	0.027	0.81	0.007
Baseline LDH (IU/mL)[1, 3]		1.0007	0.018	1.002	<0.001
Baseline Alkaline Phosphatase (IU/mL)[1, 3]		---	---	1.00	0.410
Line of Therapy (1st through 6th)		1.14	0.145	1.07	0.547
Type of Therapy (Taxotere: Yes/No)		0.63	0.009	0.70	0.139
2 - 5 Week CTC (<5 vs. ≥5)	173	1.48	0.041	2.91	<0.001
2 - 5 Week PSA Reduction from Baseline (≥30% vs. <30%)[1]		1.40	0.077	1.13	0.637
Baseline ECOG Status (0 vs. 1 vs. 2)		0.97	0.836	1.46	0.054
Baseline Hemoglobin (g/dL)[3]		0.93	0.246	0.89	0.141
Baseline LDH (IU/mL)[1, 3]		1.002	0.002	1.003	<0.001
Baseline Alkaline Phosphatase (IU/mL)[1, 3]		---	---	1.00	0.622
Line of Therapy (1st through 6th)		1.11	0.274	1.11	0.399
Type of Therapy (Taxotere: Yes/No)		0.75	0.133	0.80	0.397
6 - 8 Week CTC (<5 vs. ≥5)	139	2.14	<0.001	2.13	0.009
6 - 8 Week PSA Reduction from Baseline (≥30% vs. <30%)[1]		1.88	0.002	2.38	0.007
Baseline ECOG Status (0 vs. 1 vs. 2)		1.04	0.810	1.52	0.088
Baseline Hemoglobin (g/dL)[3]		0.97	0.695	0.79	0.013
Baseline LDH (IU/mL)[1, 3]		1.002	0.003	1.004	<0.001
Baseline Alkaline Phosphatase (IU/mL)[1, 3]		---	---	1.00	0.780
Line of Therapy (1st through 6th)		1.37	0.001	1.35	0.035
Type of Therapy (Taxotere: Yes/No)		0.80	0.278	1.45	0.276
9 - 12 Week CTC (<5 vs. ≥5)	125	1.74	0.015	3.94	<0.001
9 - 12 Week PSA Reduction from Baseline (≥30% vs.<30%)[1]		2.23	<0.001	1.46	0.221
Baseline ECOG Status (0 vs. 1 vs. 2)		1.21	0.307	1.89	0.004
Baseline Hemoglobin (g/dL)[3]		0.93	0.322	0.97	0.758
Baseline LDH (IU/mL)[1, 3]		1.00	0.190	1.003	<0.001
Baseline Alkaline Phosphatase (IU/mL)[1, 3]		---	---	1.00	0.989
Line of Therapy (1st through 6th)		1.25	0.052	1.11	0.499
Type of Therapy (Taxotere: Yes/No)		0.97	0.903	1.26	0.486
13 - 20 Week CTC (<5 vs. ≥5)	123	2.95	<0.001	3.75	0.001
13-20 Week PSA Reduction from Baseline (≥30% vs.<30%)[1]		1.97	0.002	1.52	0.275
Baseline ECOG Status (0 vs. 1 vs. 2)		0.98	0.919	1.98	0.002
Baseline Hemoglobin (g/dL)[3]		1.03	0.723	0.87	0.232
Baseline LDH (IU/mL)[1, 3]		1.00	0.380	1.003	<0.001
Baseline Alkaline Phosphatase (IU/mL)[1, 3]		---	---	1.00	0.078
Line of Therapy (1st through 6th)		1.25	0.050	1.06	0.751
Type of Therapy (Taxotere: Yes/No)		1.04	0.882	0.90	0.770

[1] Determined from Serum Drawn on the Same Date as the Blood Drawn for CTC
[2] p-value from Wald test of Z statistic
[3] Assessed as a continuous parameter

3.4 Use of CTC to Monitor Clinical Status of Metastatic Prostate Cancer Patients

3.4.1 Relationship between survival, CTCs and disease assessment by PSA

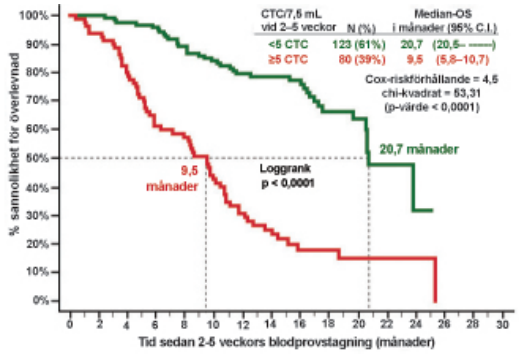
At present, a reduction in PSA is one of the primary means to determine response to therapy in MPC patients. To establish the relationship of clinical status as determined by a PSA to CTC, reduction of ≥30% or ≥50% PSA and CTC were measured 2-5 weeks, 6-8 weeks, 9-12 weeks and 13-20 weeks after initiation of therapy and compared to overall survival.

For the Kaplan-Meier analysis the elapsed OS times were calculated from the time of blood draw. Patients were segmented into Favorable groups based upon a CTC of <5 at the time of evaluation and a ≥30% reduction of PSA from baseline to the time of evaluation. Patients were segmented into Unfavorable groups based upon a CTC of ≥5 and <30% reduction of PSA from baseline to the time of evaluation.

Figure 27 shows the results of the analysis 2-5 weeks after initiation of therapy, Figure 28 the analysis 6-8 weeks after initiation of therapy, Figure 29 the analysis 9-12 weeks after initiation of therapy and Figure 30 the analysis 13-20 weeks after initiation of therapy.

Figure 27: OS of MPC Patients 2-5 weeks after the Initiation of Therapy

A. 2-5 Week CTC



B. ≥30% PSA Reduction at 2-5 Weeks

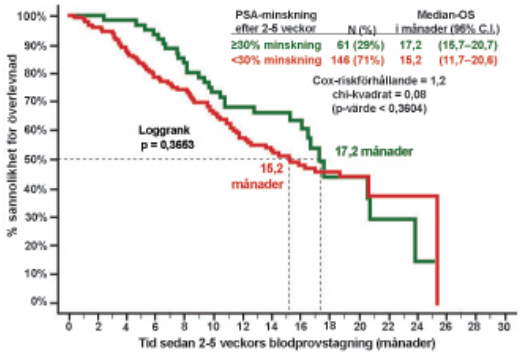
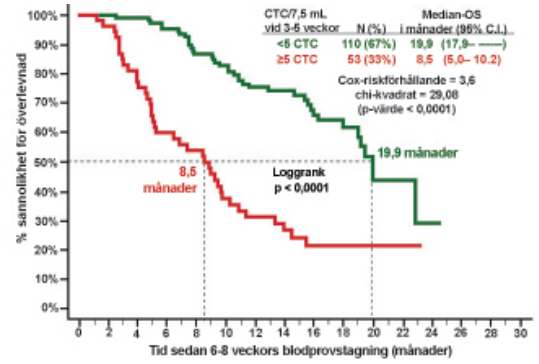


Figure 28: OS of MPC Patients 6-8 weeks after the Initiation of Therapy

A. 6-8 Week CTC



B. ≥30% PSA Reduction at 6-8 Weeks

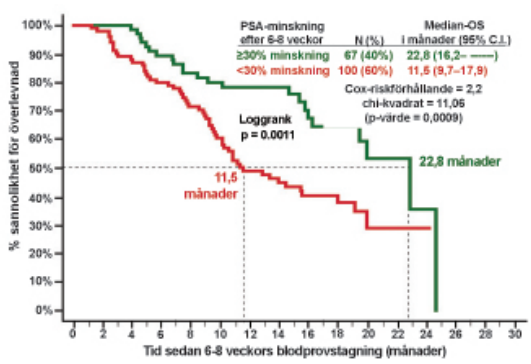
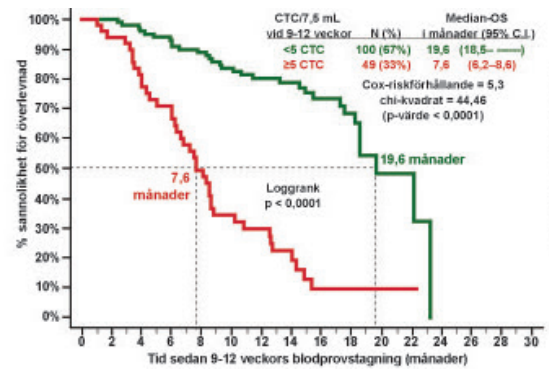


Figure 29: OS of MPC Patients 9-12 weeks after the Initiation of Therapy

A. 9-12 Week CTC



B. ≥30% PSA Reduction at 9-12 Weeks

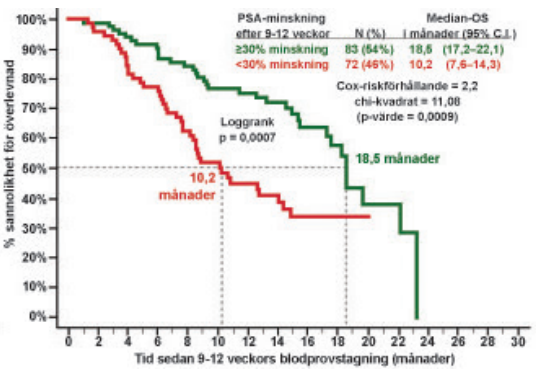
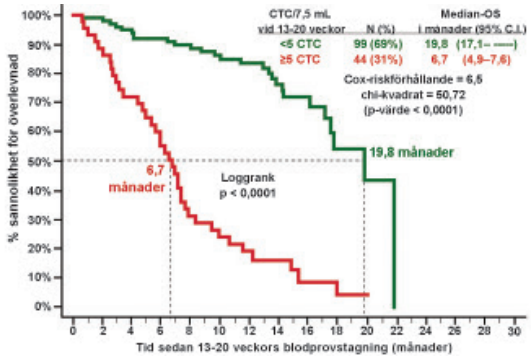


Figure 30: OS of MPC Patients 13-20 weeks after the Initiation of Therapy

A. 13-20 Week CTC



B. ≥30% PSA Reduction at 13-20 Weeks

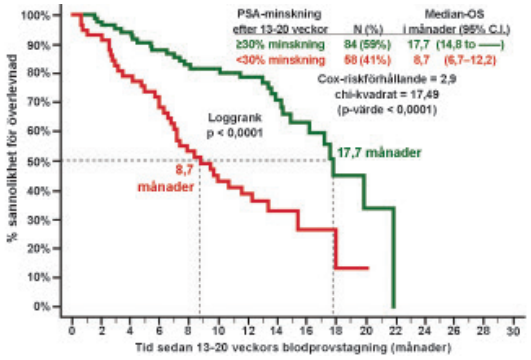


Table 28 illustrates the comparison of median overall survival at different time points after therapy with CTC, a 30% PSA reduction and a 50% PSA reduction.

Table 28: Comparison of Median OS between Favorable and Unfavorable CTC and PSA Reduction Groups.

Time Point	CTC / 7.5 mL						30% PSA Reduction from Baseline						50% PSA Reduction from Baseline					
	N	≥5 (%)	Median OS		logrank p-value	HR	N	<30 (%)	Median OS		logrank p-value	HR	N	<50 (%)	Median OS		logrank p-value	HR
			<5	≥5					≥30%	<30%					≥50%	<50%		
2-5 Weeks	203	39%	20.7	9.5	<0.0001	4.5	207	71%	17.2	15.2	0.3653	1.2	207	83%	17.5	16.2	0.5599	1.2
6-8 Weeks	163	33%	19.9	8.5	<0.0001	3.6	167	60%	22.8	11.5	0.0011	2.2	167	75%	22.8	14.4	0.0117	2.1
9-12 Weeks	149	33%	19.6	7.6	<0.0001	5.3	155	46%	18.5	10.2	0.0007	2.2	155	59%	19.6	10.8	0.0006	2.3
13-20 Weeks	143	31%	19.8	6.7	<0.0001	6.5	142	41%	17.7	8.7	<0.0001	2.9	142	46%	17.7	9.9	0.0001	2.6

The data in **Figure 27** through **Figure 30** and in **Table 28**, illustrate a highly significant difference in overall survival between patients with Unfavorable CTC and Favorable CTC at all time points tested, whereas PSA evaluations were not significant until 6-8 weeks after the initiation of therapy. Although the differences in median OS between the Favorable (≥30% or ≥50% PSA reduction from baseline) and Unfavorable (<30% or <50% PSA reduction from baseline) PSA reduction groups were significant, the separation between the Favorable (<5 CTC) and Unfavorable (≥5 CTC) CTC groups appeared greater and was significant at all time points after the initiation of therapy.

3.4.2 Concordances between CTC and PSA Changes in MPC Patients

At present, either a ≥30% or ≥50% reduction in PSA is commonly used to evaluate disease progression in metastatic prostate cancer patients. Therefore, to establish the relationship between CTC and changes in PSA two by two tabulations of concordant and discordant observations between CTC and PSA changes for each time point after the initiation of therapy were constructed. Although comparisons of CTC to PSA change at both magnitudes were calculated, only data from the CTC vs. ≥30% PSA change are reported. This decision was based on a recent publication (*J Nat Ca Inst*. 98 (8):p.516-521, 2006) demonstrating that a 3-month 30% PSA decline showed a stronger association with decrease in risk of death than did a 50% decrease in PSA. Furthermore, a comparison of patient-wise and observation-wise results from the 30% and 50% PSA decline vs. CTC analyses did not demonstrate substantial differences in the Positive % Agreement, Negative % Agreement and Overall Agreement at any of the observed time points.

A total of 197, 159, 146, and 138 patients had serum samples analyzed by the central laboratory and had evaluable CTC results 2-5 weeks, 6-8 weeks, 9-12 weeks, and 13-20 weeks after the initiation of therapy, respectively. To determine a patient's response to therapy, the percent change in PSA from the baseline value was calculated for each of the time points after the initiation of therapy. For PSA changes at each time point, the Favorable group was defined as patients with a ≥30% reduction in PSA and the Unfavorable group was defined as patients with a <30% reduction in PSA. For CTC at each time point, the Favorable group was defined as patients having <5 CTC per 7.5 mL of blood and the Unfavorable group was defined as patients having ≥5 CTC.

Because CTC vs. PSA results of the patient-wise comparisons between CTC and a ≥30% PSA reduction at 2-5 weeks and 13-20 weeks after the initiation of therapy showed the most significant discordance and concordance, respectively, these data are presented in **Table 29** and **Table 30**, respectively.

Table 29: MPC Patient-Wise Comparison of CTC and 30% PSA Reduction at 2-5 Weeks

% Reduction in PSA from Baseline at 2-5 Weeks After Initiation of Therapy	CTCs 2-5 Weeks after the Initiation of Therapy		Total
	<5 CTCs/7.5 mL	≥5 CTCs/ 7.5 mL	
≥30% Reduction in PSA	50	11	**61**
<30% Reduction in PSA	69	67	**136**
Total	**119**	**78**	**197**

Measurement	Estimate	Lower 95% CI	Upper 95% CI
Positive % Agreement	49%	41%	58%
Negative % Agreement	82%	70%	91%
Positive Predictive Value	86%	76%	93%
Negative Predictive Value	42%	33%	51%
Overall Agreement	59%	52%	66%
Odds Ratio	4.4	2.1	9.2

Table 30: MPC Patient-Wise Comparison of CTC and 30% PSA Reduction at 13-20 Weeks.

% Reduction in PSA from Baseline at 13-20 Weeks After Initiation of Therapy	CTC 13-20 Weeks after the Initiation of Therapy		Total
	<5 CTC	≥5 CTC	
≥30% Reduction in PSA	72	9	**81**
<30% Reduction in PSA	23	34	**57**
Total	**95**	**43**	**138**

Measurement	Estimate	Lower 95% CI	Upper 95% CI
Positive % Agreement	60%	46%	72%
Negative % Agreement	89%	80%	95%
Positive Predictive Value	79%	64%	90%
Negative Predictive Value	76%	66%	84%
Overall Agreement	77%	69%	84%
Odds Ratio	11.8	4.9	28.3

The results of an "observation-wise" comparison of CTC and PSA changes using a ≥30% reduction threshold at 2-5 weeks, 6-8 weeks, 9-12 weeks, and 13-20 weeks after the initiation of therapy combined are shown in **Table 31**.

Table 31: MPC Observation-Wise Comparison of CTC and 30% PSA Reduction.

% Reduction in PSA from Baseline After Initiation of Therapy	CTC after the Initiation of Therapy		Total
	<5 CTC	≥5 CTC	
≥30% Reduction in PSA	243	41	**284**
<30% Reduction in PSA	175	181	**356**
Total	**418**	**222**	**640**

Measurement	Estimate	Lower 95% CI	Upper 95% CI
Positive % Agreement	51%	46%	56%
Negative % Agreement	86%	81%	89%
Positive Predictive Value	82%	76%	86%
Negative Predictive Value	58%	53%	63%
Overall Agreement	66%	62%	70%
Odds Ratio	6.1	4.1	9.1

The overall concordance between CTC and PSA changes at the various time points after the initiation of therapy ranged from 59% to 77% when comparing to a ≥30% PSA reduction and from 52% to 75% when comparing to a ≥50% PSA reduction, showing that there was discordance between CTC and PSA changes in ~25% to 40% of the patients.

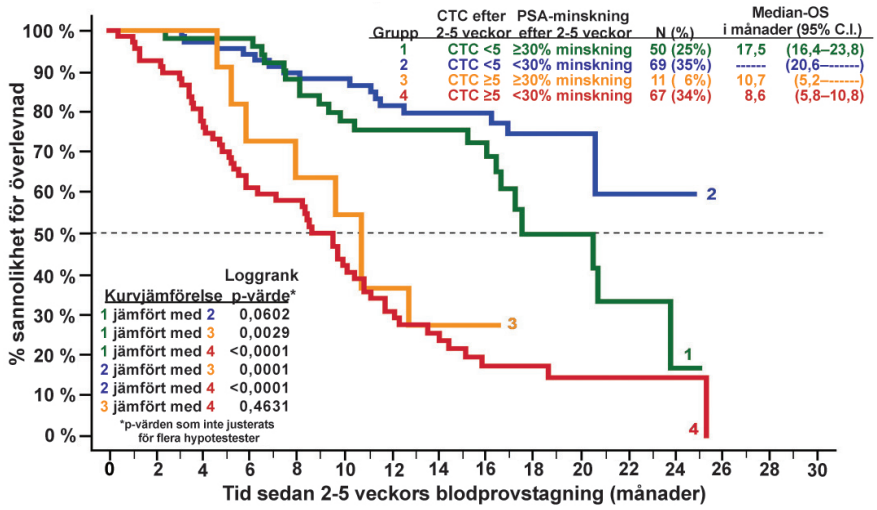
3.4.3 CTC Levels and PSA Reduction Combined to Predict OS in MPC Patients

To determine which of the discordant results better reflected the prognosis of the patient, CTC assessment and changes in PSA 2-5 weeks, 6-8 weeks, 9-12 weeks and 13-20 weeks after initiation of therapy were compared to overall survival. Elapsed OS times were calculated from the blood draw being evaluated. For the Kaplan-Meier analysis **Figure 31** (**Panels A, B, C and D**) patients were segmented into four groups based upon their CTC counts and PSA reduction at 2-5 weeks, 6-8 weeks, 9-12 weeks, and 13-20 weeks after the initiation of therapy, respectively:

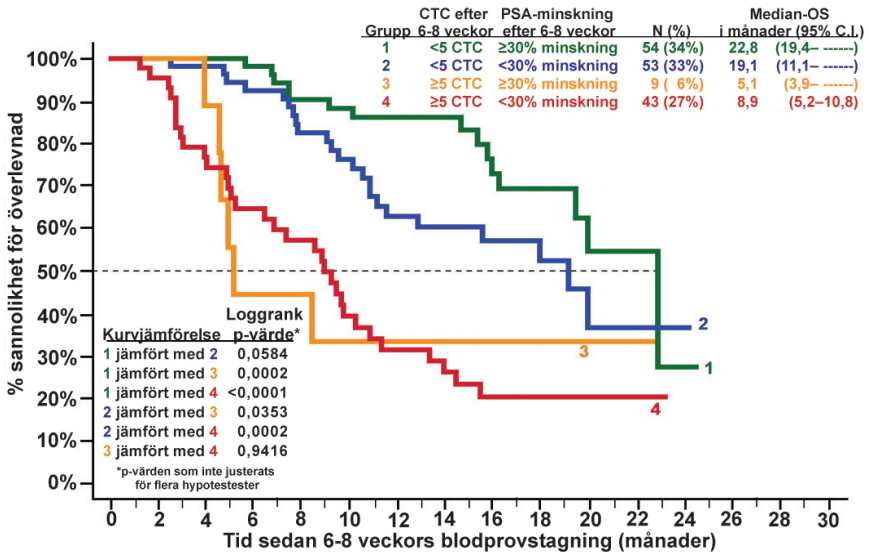
- Group 1 (**green** curve), patients with <5 CTC at the time of evaluation and a ≥30% reduction of PSA from baseline to the time of evaluation;
- Group 2 (**blue** curve), patients with <5 CTC at the time of evaluation and a <30% reduction of PSA from baseline to the time of evaluation;
- Group 3 (**orange** curve), patients with ≥5 CTC at the time of evaluation and a ≥30% reduction of PSA from baseline to the time of evaluation
- Group 4 (**red** curve), patients with ≥5 CTC at the time of evaluation and a <30% reduction of PSA from baseline to the time of evaluation.

Figure 31: CTC Levels and PSA Changes Combined to Predict OS 2-5 Weeks (Panel A), 6-8 Weeks (Panel B), 9-12 Weeks (Panel C), and 13-20 Weeks (Panel D) After the Initiation of Therapy

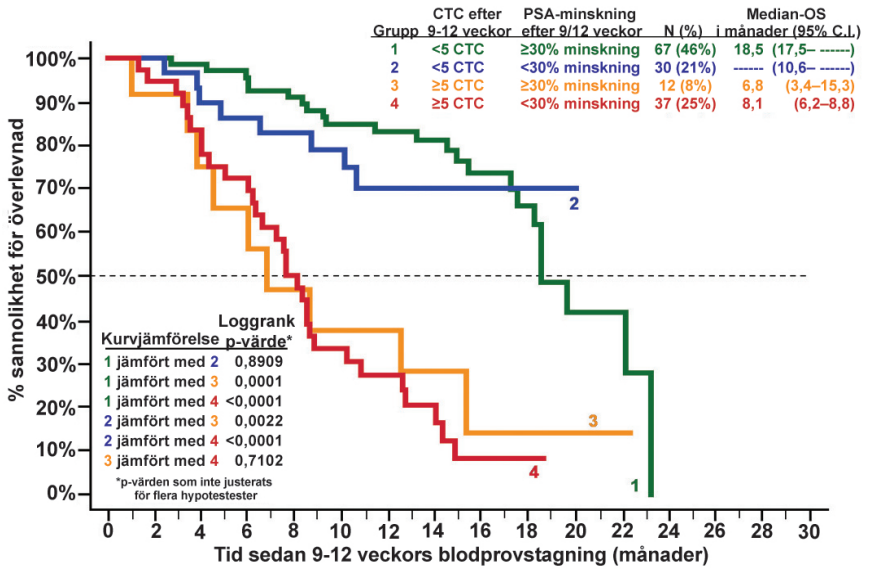
A. 2-5 Weeks



B. 6-8 Weeks



C. 9-12 Weeks



D. 13-20 Weeks

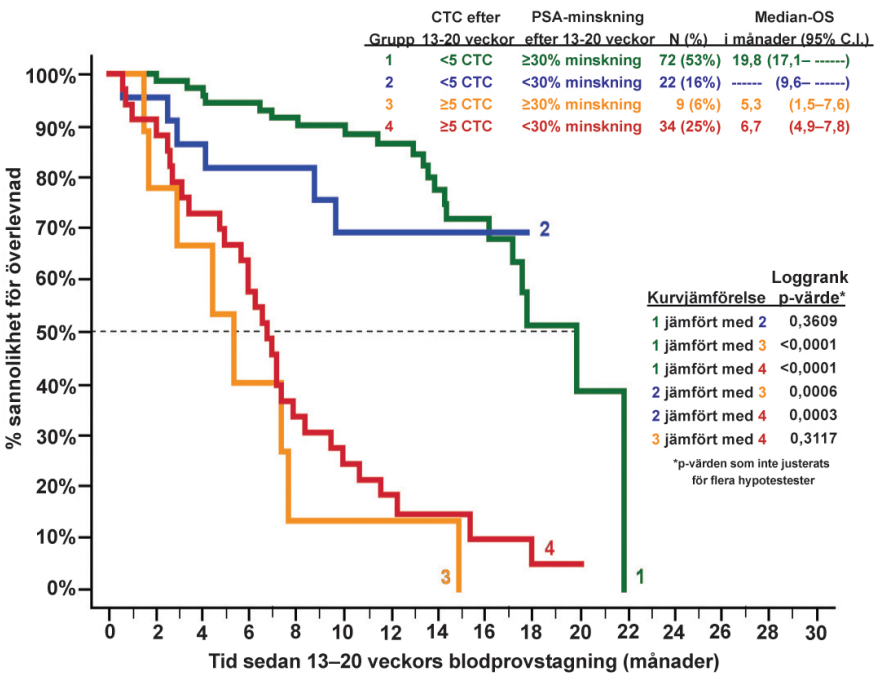


Figure 31 shows that patients with ≥5 CTC at any point after the initiation of therapy had a much higher likelihood of dying sooner, irrespective of the changes in PSA levels from baseline. Patients with ≥5 CTC at all time points (**Group 3** and **Group 4**) had the shortest median overall survivals, which were not significantly different. However, the median OS of these two groups was significantly different compared to the median OS of the patients with <5 CTC at all time points (**Group 1** and **Group 2**). These two groups (**Group 1** and **Group 2**) had the longest median overall survivals, which were not significantly different. The important finding illustrated in **Figure 31** is that although a reduction of PSA at some points after initiation of therapy may reach significance for prediction of survival, Favorable CTC at any time point were more accurate than the PSA evaluation. The practical implication is the use of CTC analysis for the evaluation of the probability of survival of MPC patients. In cases where CTC and PSA change were discordant, CTC provided the most accurate assessment of prognosis.

REVISION HISTORY

Date of Revision	Component Code	Description of Technical Changes
2017-07-06	e631600005_EN	• Added LBL-0018 under part number • Rolled tailcode to e631600005_EN • Updated 'JANSSEN DIAGNOSTICS a division of JANSSEN PHARMACEUTICA NV' company name to 'Menarini Silicon Biosystems Inc.' • Updated to Menarini Silicon Biosystems business attributes, including: – Menarini Silicon Biosystems logo – EC/REP address – Manufacture address • Updated Revision Date
2017-04-01	e631600004-EN	• Rolled tailcode to e631600004_EN • Updated patent information • Updated Revision Date
2016-04-20	e631600003_EN	• Rolled tailcode to e631600003_EN • Removed all instances of MAGNEST trademark registration • Updated 'LIMITATIONS' section: – Added U. S. Federal law sales restrictions caution • Updated 'KEY TO SYMBOLS' section: – Removed languages other than English • Updated BVBA company name to 'JANSSEN DIAGNOSTICS a division of JANSSEN PHARMACEUTICA NV'
2015-05-22	e631600002_EN	• Added LBL-0018 under part number • Rolled tailcode to e631600002_EN • Updated WARNINGS AND PRECAUTIONS section: – Updated step 7 sodium azide from 'Warning' to 'Caution' – Removed Risk and Safety phrases R22 and S28 • Added 'HAZARDS and PRECAUTIONS' section and statements: – Removed ProClin® 300 symptoms of overexposure statement • 'KEY TO SYMBOLS' section: – Replaced Harmful pictogram with Hazard Warning pictogram – Updated the Biological Risk pictogram • Updated address
2013-08-29	e631600001_EN	Technically equivalent to e631500023_EN with the following changes: • Assigned a new part number • Updated to Janssen business attributes, including: – Janssen logo – Manufacture address – EC/REP address – Phone numbers – Website • Eliminated all instances of CELLSPOTTER® Analyzer • Updated all instances of CELLSEARCH® Conical Tube wording to CELLSEARCH® Conical Centrifuge Tubes (15 mL) • Updated all instances of CELLSEARCH® Kit to CELLSEARCH® CTC Kit • In **Interpretation of Results** Section: – Updated cell carryover information to '5000 or greater' • In **Janssen Technical & Customer Support** Section: – Updated all instances of Veridex, LLC to Janssen Diagnostics, LLC – US Patent Statement updated • In **Key to Symbols** Section: – Added Date of Manufacture symbol and text 'Date of Manufacture' • Updated Revision Date

Menarini Silicon Biosystems Inc.
10355 Science Center Drive, Suite 210
San Diego, CA 92121
USA
documents.cellsearchctc.com
Phone: 1-877-837-4339
00 8000 8374339

CELLSEARCH®, CELLTRACKS®, CELLTRACKS ANALYZER II®, and AUTOPREP® are registered trademarks of Menarini Silicon Biosystems Inc. This technology, including products and/or associated components thereof, and procedures and instrument systems described herein, are protected by United States patents and corresponding international patents and pending patent applications, including one or more of the following: US Patent Numbers 6,136,182; 6,551,843; 6,623,982; 6,790,366; 7,011,794 and 7,332,288.

KEY TO SYMBOLS

The following symbols may have been used in this instruction for use or in the associated labeling.

Use by
YYYY-MM-DD or YYYY-MM

Batch code

Serial number

Caution, consult accompanying documents

Date of Manufacture

Manufacturer

Contains sufficient for < n > tests

Warning

Catalog number

Authorized representative in the European Community

Temperature limitation

Biological risks

Consult instructions for use

In vitro diagnostic medical device

EC REP

Menarini Silicon Biosystems SpA
Via Giuseppe Di Vittorio 21B/3
40013 Castel Maggiore (Bologna)
Italy

CE

Menarini Silicon Biosystems Inc.
10355 Science Center Drive, Suite 210
San Diego, CA 92121
USA
documents.cellsearchctc.com
Phone: 1-877-837-4339
00 8000 8374339 (EU)

MENARINI silicon biosystems