Doctor of Philosophy

Analysis of serial circulating tumor cell count during neoadjuvant systemic therapy in patients with breast cancer

The Graduate School of the University of Ulsan

Department of Medicine

Sung-chan Gwark

Analysis of serial circulating tumor cell count during neoadjuvant systemic therapy in patients with breast cancer

Supervisor: Prof. Sei Hyun Ahn

A Dissertation

Submitted to

The Graduate School of the University of Ulsan

In partial Fulfillment of the Requirements

For the Degree of

Doctor of Philosophy [in Medicine]

By

Sung-chan Gwark

Department of Medicine

Ulsan, Korea

February 2018

Analysis of serial circulating tumor cell count during neoadjuvant systemic therapy in patients with breast cancer

This certifies that the dissertation of Sung-chan Gwark is approved

Prof. BH. Son

Committee Chair Dr.

Prof. SH. Ahn

Committee Member Dr.

Prof. JW. Lee

Committee Member Dr.

Prof. SB. Kim

Committee Member Dr.

Prof. WS. Lim

Committee Member Dr.

Department of Medicine
Ulsan, Korea
February 2018

PURPOSE: Circulating tumor cells (CTCs) are known to be associated with prognosis and response to therapy. Although most studies have focused on CTCs among metastatic cancers with greater tumor burden, there is insufficient information regarding operable breast cancers. We aimed to evaluate the clinical implication of CTC counts in patients with locally advanced breast cancer undergoing preoperative systemic therapy.

METHODS: In total, 207 patients with locally advanced breast cancer with no evidence of distant metastasis were prospectively enrolled from a single institute (Asan Medical Center, Seoul, Korea) between February 2014 and May 2017. All patients were undergoing neoadjuvant systemic therapy (NST), and CTC counts were calculated from 10 mL of blood drawn at three time points: before, during, and after systemic therapy. CTC isolation was performed using a Smart Biopsy™ System isolation kit; cells were stained for immunofluorescence microscopy with antibodies against EpCAM, cytokeratin, and CD45. Images of stained cells were captured using a fluorescence microscope with a 400× objective. Recurrence-free survival (RFS) and overall survival (OS) were analyzed in relation to CTC counts. Dynamic changes in CTC numbers associated with responses to therapy were also analyzed.

RESULTS: Mean follow-up period was 22.46 months, and mean age was 46.48 years. One or more CTCs were identified in 65.0% (132/203) of patients before NST, in 72.0% (135/186) during NST, and in 60.2% (103/171) after NST. In HER2-overexpressing breast cancers, one or more CTCs were detected in 89.3% (25/28) of patients before NST, 92.0% (23/25) during NST, and 95.7% (22/23) after NST, whereas 87.0% (20/23) of HER2-positive tumors were CTC-positive both before and after NST ($p < 0.05$). Initial tumor burden at diagnosis (clinical stage) did not correlate with CTC positivity, and overall, CTC count did not correlate with response to therapy. Using Response Evaluation Criteria in Solid Tumors (RECIST), 86.5% (179/204) of patients were responders (complete or partial response) and 12.1% (25/204) were non-responders (stable or progressive disease). A pathologic complete response was observed in 14.5% (30/207) of patients; however, no association was found between CTC counts/changes and radiological/pathological response to therapy. CTC count

was also not correlated with overall prognosis. However, in hormone receptor-positive tumors, CTC detection before NST was associated with treatment response according to RECIST (responder vs. non-responder) using different cutoff CTC values of ≥1, ≥2, and ≥5 (p = 0.003, 0.017, and 0.023, respectively). Among patients lacking CTCs before therapy (35.2%, 50/144), appearance of CTCs after one or two cycles of systemic therapy (15.5%, 7/45; five missing values during NST) was associated with worse outcomes (hazard ratio, 16.46; 95% CI, 1.68–161.22; p = 0.016). Similarly, univariate analysis showed that the group displaying two consecutive ≥5 CTCs throughout the period (before, during, and after therapy) exhibited significantly worse RFS (hazard ratio, 22.83; 95% CI, 1.74–298.86; p = 0.017) and OS (98.4% vs 85.7%, $p < 0.05$; 98.4% vs.75.0%, $p < 0.05$) than the group with <5 CTCs at all three time points. This association was only significant among luminal-type breast cancers, whereas no association between CTC count and outcome was found among HER2 and triple-negative subtypes.

CONCLUSIONS: Although CTC count and its dynamic changes are known to reflect tumor burden, prognosis, and response to therapy in metastatic breast cancer, our findings support the limited value of CTC counts for patients with locally advanced breast cancers undergoing NST. Additional studies with a greater number of patients are necessary to conclude the prognostic implication of CTC because the literature reports conflicting results between different studies within this population.

Keywords: Circulating tumor cells, Circulating tumor cell count, Neoadjuvant systemic therapy, Prognosis, Treatment response, RECIST

Index

Table Index

Figure Index

INTRODUCTION

Neoadjuvant systemic therapy (NST) is becoming a treatment of choice for patients with locally advanced breast cancer. Downstaging could result in decreasing the extent of surgery, e.g., increase in breast conservation rate and better cosmetic outcomes [1-3]. Moreover, NST allows to assess *in vivo* drug response and thereby enables prediction of overall prognosis.

Tumors that respond well to a given therapy display better outcomes, and because NST is being used more frequently, extensive clinical/laboratory research has been aimed at more accurately predicting patient response. Despite this, many reports of clinical, radiological, and pathological evaluations have provided discordant findings [4]. Monitoring tumor status via serum markers is a common approach, for example using cancer antigen 15-3 (CA 15-3), a commonly utilized tumor marker for breast cancer. However, because of its low sensitivity and specificity, the clinical utility of CA 15-3 is quite limited [5-7]. Recently, dissecting tumor biology and analyzing disseminated tumor cells (DTC) in patients with breast cancer have been investigated. Evaluation of bone marrow samples after adjuvant therapy to monitor DTCs has shown clinical value in several studies [8, 9]; however, because of its technical difficulty and the risk of complications, repeated bone marrow aspiration is not warranted in routine practice.

Circulating tumor cells (CTCs) are cells that have shed from the primary tumor, invaded blood vessels, and are circulating in the bloodstream. CTCs can be the seeds that act as a fundamental cause of metastasis, and such a hypothesis was first proposed by the Australian pathologist Thomas Ashworth in 1869 [10]. Depending on the "type" of cancer and its burden, CTCs most likely exist at extremely low concentrations, e.g., a single cell in millions of blood cells. Identification of CTCs may offer us more reliable and more direct information that could be used for monitoring response to therapy, selecting appropriate treatment agents and predicting prognosis. These bloodborne tumor cells can be obtained by a simple blood draw, referred to as a "*liquid biopsy*," which proffers a less invasive and simpler assessment when compared to tissue biopsies (core needle biopsy or bone marrow aspiration) [11].

In metastatic breast cancer, CTC counts and their variation during treatment are well-known to be related to poor prognosis [12, 13]. However, conflicting results have been reported in several recent studies analyzing CTCs in non-metastatic patients with different

types of cancer [14-17]. Furthermore, regarding breast cancer treated with NST, only a few studies containing a small number of cases have investigated CTCs and their correlation to treatment response and prognosis [18-20]. Among those latter studies, several were conducted in Korean patients with breast cancer [21, 22].

Here, we evaluate the prognostic impact of CTC counts obtained during the course of NST (before, during, and after) and also their relevance to treatment response.

MATERIALS AND METHODS

From February 2014 to May 2017, 207 patients with breast cancer treated with NST at Asan Medical Center, Seoul, Korea, were enrolled into this study. The NST regimen included either chemotherapy or hormonal therapy [14 patients from two neoadjuvant endocrine clinical trials (NEST and METEOR trials); Table 1] [23, 24]. CTC detection was conducted before, during, and after NST (after two cycles for a total of four cycles of chemotherapy regimen, after four cycles for a total of eight cycles of chemotherapy regimen, and after 3 months of hormonal therapy). Neither patients nor clinicians were informed of the results of CTC analyses. Eligibility criteria for the study were female patients aged >20 years scheduled to undergo NST and with no distant metastasis. This study was approved by the Institutional Review Board of the Asan Medical Center (Seoul, Korea; IRB no. 2013-1048), and written informed consent was obtained from all enrolled patients.

The initial diagnostic and subsequent work-up included mammography, breast ultrasound imaging, magnetic resonance imaging, chest X-ray, blood sampling, and clinical examination. Estrogen receptor and progesterone receptor expressions were evaluated according to the Allred score [25]. HER2 status was confirmed as positive with an immunohistochemistry score of 3+ or scoring of 2+ or 1+ with positive detection of fluorescence or silver in situ hybridization for HER2 amplification [26]. All clinical and histopathological staging was based on the 7th edition of the American Joint Committee on Cancer Manual.

All patients with HER2-positive tumors received adjuvant trastuzumab for 1 year, whereas all patients with hormone receptor-positive tumors received adjuvant tamoxifen or aromatase inhibitors.

CTC detection method

Blood collection and CTC enrichment process

Blood from each patient was collected in acid citrate dextrose tubes (BD Vacutainer®; BD Biosciences, San Jose, CA, USA) and processed within 4 h. CTC isolation was performed using a Smart Biopsy™ System isolation kit (cat no. CIKW10; Cytogen, Inc., Seoul, Korea). Blood samples were incubated for 20 min with an antibody cocktail from the isolation kit against white and red blood cells and then mixed with pre-activation buffer, followed by density gradient centrifugation at 400 × g for 30 min at room temperature. The cell suspension containing CTCs was collected and gradually diluted with dilution buffer (Cytogen, Inc.). Diluted cell suspensions were filtered through an HDM chip (Cytogen, Inc.) as previously described. Cells on the HDM chip were collected and transferred to a microtube. For immunofluorescence staining, isolated cells were fixed on slides in 4% paraformaldehyde for 5 min at room temperature and kept at 4°C until further processing [27].

Immunofluorescence staining

Cells on slides were permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature. Cells were then blocked with 1% bovine serum albumin in PBS for 60 min and incubated with primary antibodies for 60 min, followed by secondary antibody incubation under the same conditions. The primary antibodies used were mouse anti-EpCAM (Cell Signaling Technology Inc., Danvers, MA, USA), mouse anti-cytokeratin (Sigma), and rabbit anti-CD45 (Cell Signaling Technology, Inc.). The secondary antibodies used were goat anti-rabbit Alexa Fluor® 647 (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and goat anti-mouse Alexa Fluor® 546 (Thermo Fisher Scientific, Inc.). Slides were mounted using Fluoroshield™ Mounting Medium with DAPI (ImmunoBioScience Corp, Mukilteo, WA, USA). Stained cells were observed and images were captured using a fluorescence microscope (Eclipse Ti; Nikon Corporation, Tokyo, Japan) and a 400× objective (Figure 2) [27].

NST response and survival analysis

Treatment response assessments were performed by physical examination and imaging modalities, which were used at baseline, after the first phase of treatment, and after the completed course of NST. Tumor responses were determined in accordance with the Response Evaluation Criteria In Solid Tumors (RECIST; Figures 3 and 4) [28]. Physical examinations and laboratory tests were performed at each treatment cycle. All surgical specimens from breast and lymph nodes were evaluated for pathological response. Recurrence-free survival (RFS) and overall survival (OS) were evaluated from a detailed review of EMR data. All patients received standard treatment, and surveillance was likewise performed at the physicians' discretions.

Statistical analysis

The primary goal was to evaluate the association of CTC detection and dynamics (change in CTC count based on the CTC count at enrollment compared with that during/after NST) with treatment response, as determined by the radiological examination performed after NST using RECIST and compared to the pathologic result (pathologic complete response, pCR). The secondary objective was to assess the impact of CTC dynamics on overall prognosis, RFS, and OS by analyzing the whole population as well as different subgroups [luminal, HER2-positive, and triple-negative (TN) subtypes].

We categorized CTC counts into two groups: (1) positive vs. negative and (2) ≥5 CTCs vs. <5 CTCs. In addition, changes in CTC counts were categorized into two groups according to the patterns of changes during a course of NST (before, during, and after NST): (1) positive to negative and (2) ≥5 CTCs to <5 CTCs. Analyses were performed in the subgroup (luminal, HER2, and TN subtypes) of each category. RFS was defined as the time from the date of study enrollment to the first date of disease recurrence, and OS was defined as the time from the date of study enrollment to the date of death from any cause. The probability of survival was estimated using the Kaplan–Meier method and Cox regression analysis. Multivariate Cox proportional hazards regression analyses were performed with the following clinical parameters: age at diagnosis, clinical tumor stage, lymph node status, and hormone receptor and HER2 positivity. All statistical tests were conducted using IBM SPSS Statistics version 23.0 for Windows (SPSS, Inc., IBM Co.). A p-value of <0.05 was considered to be

statistically significant.

RESULTS

The characteristics of the entire patient cohort are summarized in Table 1. Mean follow-up period was 22.46 months (median, 25.03 months), and mean age of the patients was 46.48 years (range, 28–71 years; median, 46 years). In total, 203 samples were obtained prior to NST, and at least one or more CTCs were detected in 132 patients (detection rate, 65.0%; 95% CI, 58.4–71.6%; mean, 2.7 cells; range, 1–27 cells). CTC detection rate during NST (after the first two or four cycles of regimen) was higher than before NST (p = 0.031). In total, 186 samples were retrieved during NST, and CTCs (at least one cell) were detected in 134 patients (detection rate, 72.0%; 95% CI, 65.7–78.6%; mean, 4.7 cells; range, 1–76 cells). Samples after NST were available for 171 patients, and CTCs were detected in 103 patients (detection rate, 60.2%; 95% CI, 53.0–67.8%; mean, 3.6 cells; range, 1–55 cells; Figure 5). CTC counts using different CTC cutoff values for each time point are shown in Figure 6. While age, tumor size, and lymph node status were not correlated with CTC positivity, CTC detection rate was significantly higher for the HER2-positive subtype compared with HER2-negative subtypes (Table 2).

CTC correlation to responses based on RECIST and pCR status

Patients who exhibited a complete response or partial response (PR) according to RECIST were defined as “responders,” whereas those who demonstrated stable disease or progressive disease were defined as “non-responders.” Patients who were CTC-positive before NST displayed a higher tendency to be responders compared with those who were CTC-negative before NST (90.9% vs.77.7%, p = 0.011; Table 2). Response and CTC counts were associated only at the initial blood draw prior to NST, whereas CTC detection during and after NST was not associated with treatment response (Table 2). Among 207 patients, 30 (14.4%) achieved pCR. However, neither CTC positivity at any time point of NST (before, during, or after) nor changes in CTC count were predictive of pCR (Figure 7). Other clinical variables such as hormone receptor negativity and HER2 positivity were correlated with pCR rate, consistent with known findings ($p < 0.05$, data not shown).

In subgroup analyses, CTC detection before NST (≥1 CTC, ≥2 CTCs, and ≥5 CTCs) was significantly associated with treatment response according to RECIST (responder vs. non-responder) (p = 0.003, p = 0.017, and p = 0.023, respectively; Table 3) among the luminal subtype but not with pCR. CTC detection during or after NST was not associated with treatment response or pCR status (data not shown). Additionally, no association of HER2 and TN subtypes was observed with CTC detection at any time point, with change of CTC number, and with clinical/pathological response to therapy (data not shown).

CTC correlation to survival

Changes in CTC counts during the course of NST in subgroups (as described earlier) showed significant association with RFS and OS in specific groups. The group whose CTC count variation pattern was Negative > Positive > Positive exhibited significantly worse RFS than the group whose CTC count variation pattern was Positive > Positive > Negative (p = 0.036; Figure 10). Among patients with luminal breast cancer in whom CTCs were absent before NST (35.2%, 50/144), appearance of CTCs after one or two cycles of systemic therapy (15.5%, 7/45; five missing values during NST) was associated with worse outcomes (HR, 16.46; 95% CI, 1.68–161.22; p = 0.016; Table 4). Similarly, using univariate analysis, the group displaying two consecutive ≥5 CTCs throughout the period (before, during, and after NST) exhibited significantly worse RFS (HR, 22.83; 95% CI, 1.74–298.86; p = 0.017, figure not shown) and OS (98.4% vs.85.7%, $p < 0.05$; 98.4% vs.75.0%, $p < 0.05$) than the group with <5 CTCs at all three time points (Table 4, Figure 14). These findings were observed only in luminal breast cancer, while no associations of any kind were found among hormone receptor-negative diseases (HER2-positive and/or TNBC; data not shown).

DISCUSSION

Here we analyzed serial CTC counts throughout the course of NST in patients with locally advanced breast cancer, who have been relatively less investigated than those with metastatic breast cancers with evident tumor cells within circulation. Using the Cytogen's staining approach, detection rate of before NST was 65.0%, and it increased to 72.1% during NST after the administration of treatment agent and then decreased to 60.4% after NST. Such an

increase in the detection rate during NST might arise from pre-existing dormant or angiogenesis-suppressed tumor cells exhibiting resistance to initial treatment [29]. According to a study by Komarova et al. [30], drug-resistant subpopulations co-exist within the primary tumor before the initiation of treatment because of the heterogeneous nature of cancer cells. Studies on the association between the detection of CTCs and breast cancer subtype have reported conflicting results. In a study by Fehm et al. [31], CTC positivity rate was the highest in patients with TN subtype followed by those with a luminal subtype, but no CTCs were detected in those with HER2 subtype. In contrast, in our study, HER2 status was correlated with CTC positivity, similar to a study by Lang et al. wherein HER2 was the only factor that correlated with the presence of CTCs, whereas hormone receptor (ER and PR) status was not [32]. Finally, Qi et al. [33] also reported that the HER2 subtype exhibited the highest correlation with the presence of CTCs. These results could possibly stem from the more aggressive features and higher migration potential possessed by the HER2 subtype [34, 35].

Systemic therapy is less effective in patients with luminal subtype than in those with other subtypes. This could be explained by the fact that, in general, the benefits of chemotherapy are most effective in the first few years after treatment, but in this early period, the general risk of recurrence is relatively lower in patients with luminal subtype patients than in those with other subtypes [36, 37]. However, even within the luminal subtype, individual responses to treatment may vary [37]; therefore, predicting the response may still have clinical value. We investigated the predictive impact of CTC detection on tumor response in patients with breast cancer treated with NST. Similar to a study by Ma et al. [38], patients who were CTC-positive before NST exhibited a significantly better response to treatment than those who were CTC-negative before NST. In a subgroup analysis of the luminal subtype, the baseline CTC counts of responders were significantly higher than those of non-responders. Although not reaching statistical significance, the CTC counts of responders after NST were lower than those of non-responders. We did not observe a correlation between CTC detection and pCR rate. This may be because of the nature of NST on evaluating pCR rate as universally accepted pathological response criteria have not been established [39] and supports the finding that CTCs do not predict the treatment sensitivity

of the primary tumor [40, 41].

Even after curative resection of the primary tumor, DTCs and micrometastases can remain in a dormant state for many years [42]. However, DTCs can eventually recirculate through the bloodstream as CTCs and initiate secondary metastases. Studies by Kim et al. [43] and Leung et al. [44] suggest that CTCs can give rise to not only distant metastasis but also to local relapse by so-called "tumor self-seeding." In metastatic breast cancer, various studies have supported the use of CTC detection as a potential prognostic factor [12, 13, 45, 46]. However, in patients with non-metastatic breast cancer treated with NST prior to surgery, the clinical value and prognostic impact of CTCs have not been sufficiently investigated [47, 48]. Studies on metastatic breast cancer by Cristofanilli et al. [12], Nole et al. [49], and Pierga et al. [50] suggested a lower cutoff value for progression-free survival risk as 1 CTC/7.5 mL up to a plateau of 5 CTCs/7.5 mL. Following this suggestion, studies using 1 CTC/7.5 mL as a cutoff value in non-metastatic breast cancer have been reported [17, 48, 51, 52]. Taking into account these previous studies and the mean CTC count presented in this study (mean CTC counts: before NST = 2.7, during NST = 4.7, after NST = 3.6), we applied two thresholds, (1) CTC/7.5 mL and (2) 5 CTCs/7.5 mL, to broaden the span of the analysis. Many studies have shown that not only detection of CTCs themselves but also the dynamics of CTC counts during the course of treatment can provide further information for prognosis [13, 48, 53-56]. Unlike TN and HER2 subtypes, which have a predictive prognostic value for pCR [57, 58], there is a lack of predictive metrics for survival outcomes in the luminal subtype. In this study, patients with luminal subtype whose CTCs were maintained <5 during NST demonstrated significantly better RFS and OS, similar to previous results on metastatic breast cancer [13, 55]. However, because of the low incidence of events and the small sample size in each subgroup, the 95% CI for HR exhibited a wide range and thus the potential for interpreting these results for prognostic purposes is limited.

There are numerous platforms and methods for detecting CTCs, but most CTC isolation technologies are based on the physical or biological properties of CTCs. The CELLSEARCH® system (Veridex LLC, Raritan, NJ, USA), the first and only US Food and Drug Administration-approved system, captures CTCs using antibodies directed against EpCAM and defines CTCs as CK8, CK18 or CK19 positive, and CD45-negative cells [59].

Therefore, this technology may isolate fewer differentiated tumor cells. Rao et al. [60] reported that the expression of EpCAM in carcinoma cells decreases during epithelial-mesenchymal transition. Furthermore, a recent report demonstrated that there are CTCs that do not express EpCAM; therefore, EpCAM-based isolation technology may fail to detect certain CTCs [61]. The technique used in the present study was able to isolate EpCAM-negative CTCs using size-based filtration.

Our study has some limitations, the greatest of which is the lack of validation of the CTC detection method used. Because the validated CELLSEARCH® system is currently not available in Korea, this study was conducted with a unique CTC detection system developed by Cytogen, Inc., Seoul, Korea, because sending samples abroad for analyses was not feasible due to a strict sample processing protocol. For this reason, we were unable to perform a direct head-to-head comparison with other studies conducted using the CELLSEARCH® system. Another limitation of this study is the relatively short follow-up period for the assessment of survival for prognostic purposes. Further analyses with data from longer follow-up periods need to be performed. A final limitation of this study is the heterogeneity of treatment regimens used (Table 1).

In summary, although CTC counts and their dynamic temporal variation are known to reflect tumor burden, prognosis, and response to therapy in metastatic breast cancer, our findings support a limited value of CTC detection among the population of patients with locally advanced breast cancer. In the luminal subtype, changes in CTC counts were neither correlated with tumor burden nor radiological changes, but detection of initial CTCs prior to treatment may be predictive of treatment response, although this observation will need to be fully validated by a study with a larger number of patients.

REFERENCES

1. Fisher B, Brown A, Mamounas E, Wieand S, Robidoux A, Margolese RG, Cruz AB, Jr., Fisher ER, Wickerham DL, Wolmark N *et al*: **Effect of preoperative chemotherapy on local-regional disease in women with operable breast cancer: findings from National Surgical Adjuvant Breast and Bowel Project B-18**. *J Clin Oncol* 1997, **15**(7):2483-2493.

2. van der Hage JA, van de Velde CJ, Julien JP, Tubiana-Hulin M, Vandervelden C, Duchateau L: **Preoperative chemotherapy in primary operable breast cancer: results from the European Organization for Research and Treatment of Cancer trial 10902**. *J Clin Oncol* 2001, **19**(22):4224-4237.
3. Caudle AS BIea: **Use of Sentinel Lymph Node Dissection After Neoadjuvant Chemotherapy in Patients with Node-Positive Breast Cancer at Diagnosis: Practice Patterns of American Society of Breast Surgeons Members**. *Ann Surg Oncol* 2017, **10**:2925-2934.
4. Schott AF, Roubidoux MA, Helvie MA, Hayes DF, Kleer CG, Newman LA, Pierce LJ, Griffith KA, Murray S, Hunt KA *et al*: **Clinical and radiologic assessments to predict breast cancer pathologic complete response to neoadjuvant chemotherapy**. *Breast Cancer Res Treat* 2005, **92**(3):231-238.
5. Safi F, Kohler I, Beger HG, Röttinger E: **The value of the tumor marker CA 15-3 in diagnosing and monitoring breast cancer. A comparative study with carcinoembryonic antigen**. *Cancer* 1991, **68**(3):574-582.
6. Lumachi F, Basso SMM: **Serum tumor markers in patients with breast cancer**. *Expert Review of Anticancer Therapy* 2004, **4**(5):921-931.
7. Bayo J, Castaño M, Rivera F, Navarro F: **Analysis of blood markers for early breast cancer diagnosis**. *Clinical and Translational Oncology* 2017:1-9.
8. Wiedswang G, Borgen E, Karesen R, Qvist H, Janbu J, Kvalheim G, Nesland JM, Naume B: **Isolated tumor cells in bone marrow three years after diagnosis in disease-free breast cancer patients predict unfavorable clinical outcome**. *Clin Cancer Res* 2004, **10**(16):5342-5348.
9. Janni W, Rack B, Schindlbeck C, Strobl B, Rjosk D, Braun S, Sommer H, Pantel K, Gerber B, Friese K: **The persistence of isolated tumor cells in bone marrow from patients with breast carcinoma predicts an increased risk for recurrence**. *Cancer* 2005, **103**(5):884-891.
10. Ashworth T: **A case of cancer in which cells similar to those in tumors were seen in the blood after death**. *Australian Med J* 1869, **14**:146.
11. Alix-Panabieres C, Pantel K: **Circulating tumor cells: liquid biopsy of cancer**. *Clin*

Chem 2013, **59**(1):110-118.

12. Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Matera J, Miller MC, Reuben JM, Doyle GV, Allard WJ, Terstappen LW *et al*: **Circulating tumor cells, disease progression, and survival in metastatic breast cancer**. *N Engl J Med* 2004, **351**(8):781-791.
13. Hayes DF, Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Miller MC, Matera J, Allard WJ, Doyle GV, Terstappen LW: **Circulating tumor cells at each follow-up time point during therapy of metastatic breast cancer patients predict progression-free and overall survival**. *Clin Cancer Res* 2006, **12**(14 Pt 1):4218-4224.
14. Van Dalum G, Van der Stam GJ, Tibbe AG, Franken B, Mastboom WJ, Vermes I, De Groot MR, Terstappen LW: **Circulating tumor cells before and during follow-up after breast cancer surgery**. *International journal of oncology* 2015, **46**(1):407-413.
15. Rack B, Schindlbeck C, Jückstock J, Andergassen U, Hepp P, Zwingers T, Friedl TWP, Lorenz R, Tesch H, Fasching PA *et al*: **Circulating Tumor Cells Predict Survival in Early Average-to-High Risk Breast Cancer Patients**. *JNCI: Journal of the National Cancer Institute* 2014, **106**(5):dju066-dju066.
16. Franken B, de Groot MR, Mastboom WJ, Vermes I, van der Palen J, Tibbe AG, Terstappen LW: **Circulating tumor cells, disease recurrence and survival in newly diagnosed breast cancer**. *Breast Cancer Research* 2012, **14**(5):R133.
17. Bidard FC, Mathiot C, Delaloge S, Brain E, Giachetti S, de Cremoux P, Marty M, Pierga JY: **Single circulating tumor cell detection and overall survival in nonmetastatic breast cancer**. *Annals of Oncology* 2010, **21**(4):729-733.
18. Hall C, Karhade M, Laubacher B, Anderson A, Kuerer H, DeSynder S, Lucci A: **Circulating tumor cells after neoadjuvant chemotherapy in stage I–III triple-negative breast cancer**. *Annals of surgical oncology* 2015, **22**(3):552-558.
19. Fei F, Du Y, Di G, Wu J, Shao Z: **Are changes in circulating tumor cell (CTC) count associated with the response to neoadjuvant chemotherapy in local advanced breast cancer? A meta-analysis**. *Oncology research and treatment* 2014, **37**(5):250-254.
20. Kasimir-Bauer S, Bittner A-K, König L, Reiter K, Keller T, Kimmig R, Hoffmann O: **Does primary neoadjuvant systemic therapy eradicate minimal residual disease?**

Analysis of disseminated and circulating tumor cells before and after therapy. *Breast Cancer Research* 2016, **18**(1):20.

21. Wang H-y, Ahn S, Kim S, Park S, Park S, Han H, Sohn JH, Kim S, Lee H: **Detection of circulating tumor cells in patients with breast cancer using the quantitative RT-PCR assay for monitoring of therapy efficacy**. *Experimental and molecular pathology* 2014, **97**(3):445-452.
22. Wang H-y, Ahn S, Kim S, Park S, Jung D, Park S, Han H, Sohn J, Kim S, Lee H: **Detection of circulating tumor cell-specific markers in breast cancer patients using the quantitative RT-PCR assay**. *International journal of clinical oncology* 2015, **20**(5):878-890.
23. Kim J, Lim W, Kim E-K, Kim M-K, Paik N-S, Jeong S-S, Yoon J-h, Park CH, Ahn SH, Kim LS: **Phase II randomized trial of neoadjuvant metformin plus letrozole versus placebo plus letrozole for estrogen receptor positive postmenopausal breast cancer (METEOR)**. *BMC cancer* 2014, **14**(1):170.
24. Kim hJ, Noh WC, Lee ES, Kim L, Jung Y, Han W, Nam SJ, Ahn S-H: **A phase III, open label, prospective, randomized, multicenter, neoadjuvant study of chemotherapy versus endocrine therapy in premenopausal patient with hormone responsive, HER2 negative, breast cancer (KBCSG 012)**. *Journal of Clinical Oncology* 2017, **35**(15_suppl):517-517.
25. Allred DC, Harvey JM, Berardo M, Clark GM: **Prognostic and predictive factors in breast cancer by immunohistochemical analysis**. *Mod Pathol* 1998, **11**(2):155-168.
26. Wolff AC, Hammond MEH, Hicks DG, Dowsett M, McShane LM, Allison KH, Allred DC, Bartlett JMS, Bilous M, Fitzgibbons P *et al*: **Recommendations for Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer: American Society of Clinical Oncology/College of American Pathologists Clinical Practice Guideline Update**. *Archives of pathology & laboratory medicine* 2014, **138**(2):241-256.
27. Hwang E, Uh JH, Lee HS, Lee CH, Lee SJ, Ahn SH, Son BH, Lee JW, Yu JH, Kwon NJ: **Cancer gene panel analysis of cultured circulating tumor cells and primary tumor tissue from patients with breast cancer**. *Oncology Letters* 2017, **13**(6):4627-4632.

28. Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, Verweij J, Van Glabbeke M, van Oosterom AT, Christian MC: **New guidelines to evaluate the response to treatment in solid tumors**. *Journal of the National Cancer Institute* 2000, **92**(3):205-216.
29. Naumov GN, Townson JL, MacDonald IC, Wilson SM, Bramwell VH, Groom AC, Chambers AF: **Ineffectiveness of doxorubicin treatment on solitary dormant mammary carcinoma cells or late-developing metastases**. *Breast cancer research and treatment* 2003, **82**(3):199-206.
30. Komarova NL, Wodarz D: **Drug resistance in cancer: principles of emergence and prevention**. *Proceedings of the National Academy of Sciences of the United States of America* 2005, **102**(27):9714-9719.
31. Fehm T, Hoffmann O, Aktas B, Becker S, Solomayer EF, Wallwiener D, Kimmig R, Kasimir-Bauer S: **Detection and characterization of circulating tumor cells in blood of primary breast cancer patients by RT-PCR and comparison to status of bone marrow disseminated cells**. *Breast Cancer Research* 2009, **11**(4):R59.
32. Lang JE, Mosalpuria K, Cristofanilli M, Krishnamurthy S, Reuben J, Singh B, Bedrosian I, Meric-Bernstam F, Lucci A: **HER2 status predicts the presence of circulating tumor cells in patients with operable breast cancer**. *Breast cancer research and treatment* 2009, **113**(3):501-507.
33. Qi X, Yang X, Fan L, Zhang Y, Zhang F, Jiang J: **Association between the spread of circulating tumor cells and breast cancer subtypes**. *Breast Cancer Research* 2010, **12**(3):402.
34. Rubin I, Yarden Y: **The basic biology of HER2**. *Annals of Oncology* 2001, **12**(suppl_1):S3-S8.
35. Yarden Y: **Biology of HER2 and its importance in breast cancer**. *Oncology* 2001, **61**(Suppl. 2):1-13.
36. Voduc KD, Cheang MC, Tyldesley S, Gelmon K, Nielsen TO, Kennecke H: **Breast cancer subtypes and the risk of local and regional relapse**. *Journal of Clinical Oncology* 2010, **28**(10):1684-1691.
37. Berry DA, Cirrincione C, Henderson IC, Citron ML, Budman DR, Goldstein LJ,

Martino S, Perez EA, Muss HB, Norton L: **Estrogen-receptor status and outcomes of modern chemotherapy for patients with node-positive breast cancer**. *Jama* 2006, **295**(14):1658-1667.

38. Ma S, Ling F, Gui A, Chen S, Sun Y, Li Z: **Predictive Value of Circulating Tumor Cells for Evaluating Short-and Long-Term Efficacy of Chemotherapy for Breast Cancer**. *Medical Science Monitor* 2017, **23**:4808-4816.
39. Kurosumi M: **Significance and problems in evaluations of pathological responses to neoadjuvant therapy for breast cancer**. *Breast Cancer* 2006, **13**(3):254-259.
40. Becker S, Solomayer E, Becker-Pergola G, Wallwiener D, Fehm T: **Primary systemic therapy does not eradicate disseminated tumor cells in breast cancer patients**. *Breast cancer research and treatment* 2007, **106**(2):239.
41. Fehm T, Becker S, Becker-Pergola G, Sotlar K, Gebauer G, Dürr-Störzer S, Neubauer H, Wallwiener D, Solomayer E-F: **Presence of apoptotic and nonapoptotic disseminated tumor cells reflects the response to neoadjuvant systemic therapy in breast cancer**. *Breast Cancer Research* 2006, **8**(5):R60.
42. Uhr JW, Pantel K: **Controversies in clinical cancer dormancy**. *Proceedings of the National Academy of Sciences* 2011, **108**(30):12396-12400.
43. Kim M-Y, Oskarsson T, Acharyya S, Nguyen DX, Zhang XH-F, Norton L, Massagué J: **Tumor self-seeding by circulating cancer cells**. *Cell* 2009, **139**(7):1315-1326.
44. Leung CT, Brugge JS: **Tumor self-seeding: bidirectional flow of tumor cells**. *Cell* 2009, **139**(7):1226-1228.
45. Budd GT, Cristofanilli M, Ellis MJ, Stopeck A, Borden E, Miller MC, Matera J, Repollet M, Doyle GV, Terstappen LW: **Circulating tumor cells versus imaging—predicting overall survival in metastatic breast cancer**. *Clinical Cancer Research* 2006, **12**(21):6403-6409.
46. Bidard F-C, Vincent-Salomon A, Sigal-Zafrani B, Dieras V, Mathiot C, Mignot L, Thiery J-P, Sastre-Garau X, Pierga J-Y: **Prognosis of women with stage IV breast cancer depends on detection of circulating tumor cells rather than disseminated tumor cells**. *Annals of Oncology* 2008, **19**(3):496-500.
47. Camara O, Rengsberger M, Egbe A, Koch A, Gajda M, Hammer U, Jörke C, Rabenstein

C, Untch M, Pachmann K: **The relevance of circulating epithelial tumor cells (CETC) for therapy monitoring during neoadjuvant (primary systemic) chemotherapy in breast cancer**. *Annals of Oncology* 2007, **18**(9):1484-1492.

48. Pierga J-Y, Bidard F-C, Mathiot C, Brain E, Delaloge S, Giachetti S, de Cremoux P, Salmon R, Vincent-Salomon A, Marty M: **Circulating tumor cell detection predicts early metastatic relapse after neoadjuvant chemotherapy in large operable and locally advanced breast cancer in a phase II randomized trial**. *Clinical Cancer Research* 2008, **14**(21):7004-7010.
49. Nole F, Munzone E, Zorzino L, Minchella I, Salvatici M, Botteri E, Medici M, Verri E, Adamoli L, Rotmensz N: **Variation of circulating tumor cell levels during treatment of metastatic breast cancer: prognostic and therapeutic implications**. *Annals of Oncology* 2007, **19**(5):891-897.
50. Pierga J-Y, Hajage D, Bachelot T, Delaloge S, Brain E, Campone M, Dieras V, Rolland E, Mignot L, Mathiot C: **High independent prognostic and predictive value of circulating tumor cells compared with serum tumor markers in a large prospective trial in first-line chemotherapy for metastatic breast cancer patients**. *Annals of oncology* 2011, **23**(3):618-624.
51. Lucci A, Hall CS, Lodhi AK, Bhattacharyya A, Anderson AE, Xiao L, Bedrosian I, Kuerer HM, Krishnamurthy S: **Circulating tumour cells in non-metastatic breast cancer: a prospective study**. *The lancet oncology* 2012, **13**(7):688-695.
52. Rack B, Schindlbeck C, Andergassen U, Lorenz R, Zwingers T, Schneeweiss A, Lichtenegger W, Beckmann M, Sommer H, Pantel K: **Abstract S6-5: Prognostic Relevance of Circulating Tumor Cells in the Peripheral Blood of Primary Breast Cancer Patients**. In.: AACR; 2010.
53. Cristofanilli M, Hayes DF, Budd GT, Ellis MJ, Stopeck A, Reuben JM, Doyle GV, Matera J, Allard WJ, Miller MC: **Circulating tumor cells: a novel prognostic factor for newly diagnosed metastatic breast cancer**. *Journal of clinical oncology* 2005, **23**(7):1420-1430.
54. Riethdorf S, Müller V, Zhang L, Rau T, Loibl S, Komor M, Roller M, Huober J, Fehm T, Schrader I: **Detection and HER2 expression of circulating tumor cells: prospective**

monitoring in breast cancer patients treated in the neoadjuvant GeparQuattro trial. *Clinical cancer research* 2010, **16**(9):2634-2645.

55. Iwata H, Masuda N, Yamamoto D, Sagara Y, Sato N, Yamamoto Y, Saito M, Fujita T, Oura S, Watanabe J: **Circulating tumor cells as a prognostic marker for efficacy in the randomized phase III JO21095 trial in Japanese patients with HER2-negative metastatic breast cancer**. *Breast cancer research and treatment* 2017, **162**(3):501-510.
56. Riethdorf S, Müller V, Loibl S, Nekljudova V, Weber K, Huober J, Fehm T, Schrader I, Hilfrich J, Holms F: **Prognostic impact of circulating tumor cells for breast cancer patients treated in the neoadjuvant" Geparquattro" trial**. *Clinical Cancer Research* 2017, **23**(18):5384-5393.
57. Tan MC, Al Mushawah F, Gao F, Aft RL, Gillanders WE, Eberlein TJ, Margenthaler JA: **Predictors of complete pathological response after neoadjuvant systemic therapy for breast cancer**. *The American Journal of Surgery* 2009, **198**(4):520-525.
58. von Minckwitz G, Untch M, Blohmer J-U, Costa SD, Eidtmann H, Fasching PA, Gerber B, Eiermann W, Hilfrich J, Huober J: **Definition and impact of pathologic complete response on prognosis after neoadjuvant chemotherapy in various intrinsic breast cancer subtypes**. *Journal of Clinical Oncology* 2012, **30**(15):1796-1804.
59. Pantel K, Alix-Panabières C, Riethdorf S: **Cancer micrometastases**. *Nature reviews Clinical oncology* 2009, **6**(6):339-351.
60. Rao CG, Chianese D, Doyle GV, Miller MC, Russell T, Sanders RA, Terstappen LW: **Expression of epithelial cell adhesion molecule in carcinoma cells present in blood and primary and metastatic tumors**. *International journal of oncology* 2005, **27**(1):49-57.
61. Sieuwerts AM, Kraan J, Bolt J, van der Spoel P, Elstrodt F, Schutte M, Martens JW, Gratama J-W, Sleijfer S, Foekens JA: **Anti-epithelial cell adhesion molecule antibodies and the detection of circulating normal-like breast tumor cells**. *Journal of the National Cancer Institute* 2009, **101**(1):61-66.

국문요약

목적: 수술 전 전신치료 (Neoadjuvant systemic therapy) 대상 유방암 환자에 있어 순환종양세포(Circulating tumor cell, CTC)의 변화와 예후 및 치료반응과의 상관관계 분석을 통해 치료 반응성 및 예후 예측인자 로서의 CTC 의 의의를 파악하여 임상적 효용성을 재고하기 위함.

방법: 2014 년 2 월부터 2017 년 5 월까지 서울아산병원에서 침윤성 유방암으로 진단받은 환자 중, 20 세 이상 환자 중 본 연구에 동의한 환자 207 명을 대상으로 환자군의 CTC 정보(개수 및 치료에 따른 변화)와 임상병리학적 특성, 영상의학적 정보 및 생존 추적 정보 등을 수집하여 치료 반응성과 예후 분석을 목표로 전향적 연구를 시행함. 순환종양세포 탐지는 (주)싸이토젠사의 SMART BIOPSY™ SYSTEM Isolation kit (cat no. CIKW10; Cytogen, Inc., Seoul, Korea)을 사용하였으며, anti-EpCAM (Cell Signaling Technology), mouse anti-cytokeratin (Sigma), rabbit anti-CD45 (Cell Signaling Technology), goat anti-rabbit Alexa Fluor® 647 (Thermo Fisher Scientific, Inc.), goat anti-mouse Alexa Fluor® 546 (Thermo Fisher Scientific, Inc.)를 이용하여 면역형광염색을 시행, Fluoroshield™ Mounting Medium with DAPI (ImmunoBioScience Corp) 배지에 착상시켜 형광현미경 (Eclipse Ti; Nikon Corporation, Tokyo, Japan) 400 배 배율로 확인함. 이를 바탕으로 순환종양세포개수에 따른 무재발 생존률과 전체 생존률 분석 및 순환종양세포개수 변화에 따른 치료반응성과의 연관성 분석을 시행함.

결과: 평균 추적기간은 22.26개월 (중앙값 25.03개월) 이었으며 평균 나이는 46.48세였음. 전신치료 전 최소 1개 이상의 순환종양세포가 203명중 132명 (65.0%)에서 탐지 되었으며, 전신치료 중에는 186명중 135명 (72.0%), 전신치료 후에는 171명중 103명 (60.2%) 에서 탐지되었음. 본 연구의 전체 환자군에서 순환종양세포의 단순 탐지는 치료반응 및 병리학적 완전 관해 (pathologic complete response) 와 연관성은 없었으나, 전신 치료 전 혹은 치료 후 순환종양세포의 존재는 HER2 상태와 연관성이 있었으며, HER2 양성인 경우 전신치료 전, 후 순환종양세포 탐지율이 HER2

음성의 경우에 비해 통계적으로 유의하게 높았음. 또한 천제 환자군의 경우 순환종양세포와 생존율과의 유의한 연관성은 보이지 않았음. 관내강 아형의 경우 전신치료 전 순환종양세포의 탐지 (≥1 CTC, ≥2 CTCs, ≥5 CTCs)는 RECIST 기준 (반응군;responder vs. 비반응군;non-responder) 치료반응성과 유의한 연관성을 보였으며 (p = 0.003, p = 0.017, p = 0.023, 각각), 전신치료 전 순환종양세포 음성이었으나, 전신치료 중 양성으로 전환된 경우, 음성을 유지한군에 비해 무병생존율이 유의하게 낮았음 (hazard ratio 16.46, 95%CI 1.62-161.22, p = 0.016). 또한 전신치료 전 탐지된 전신치료 과정 중 2번 이상 연속하여 5개 이상의 순환종양세포가 탐지된 경우 5개미만으로 유지된 군에 비해 낮은 무병생존율 (hazard ratio 22.83 95%CI 1.74-298.86, p = 0.017) 과 전체생존율 (98.4% vs. 85.7%, p < 0.05, 98.4% vs. 75.0% p < 0.05)을 보였음. HER2 및 삼중 음성 환자군 에서도 상기와 동일한 분석을 시행하였으나, 생존율과 통계적으로 유의한 연관성은 없었음.

결론: 전이성 유방암에서 순환종양세포는 종양상태와 예후 및 치료반응성을 반영하는 것으로 알려져 있으나, 본 연구 결과에 따르면 수술 전 전신치료를 시행받는 국소 진행 유방암 환자에서 순환종양세포 탐지와 그 특징은 예후를 예측하는 요인으로서의 임상적 효용성은 한계가 있음. 관내강 아형에서 전신치료 전 순환종양세포의 탐지로 치료 반응성을 예측할 수 있을 가능성을 확인하였고, 이에 대한 대규모 환자군에서의 추가 검증이 필요하겠음.

중심단어: 유방암, 순환종양세포, 수술 전 보조항암치료, 예후, 치료반응, RECIST

Table 1. Patient characteristics and CTC status in course of NST and NST regimen.

Variables	No. of patients (%)	
Whole patients N=207		
Age (mean 46.48, range 28-71)		
34≥	10	4.8%
35-50	144	69.6%
51≤	53	25.6%
Tumor subtype (pre NST)		
Luminal	144	69.6%
HER2	17	8.2%
TN	46	22.2%
Clinical T stage		
TX (occult breast ca)	1	0.5%
T1	20	9.7%
T2	132	63.8%
T3	49	23.7%
T4	5	2.4%
Lymph node status		
Negative	56	27.1%
Positive	151	72.9%
Histologic grade		
G1	2	1.0%
G2	152	73.4%
G3	50	24.2%
unknown	3	1.4%
ER status		
Positive	144	69.6%
Negative	63	30.4%
PR status		
Positive	117	56.5%
Negative	90	43.5%
HER2/neu status		
Positive	28	13.5%
Negative	179	86.5%
Baseline CA-15-3		
Normal	195	94.2%
Elevated	7	3.4%
Unknown	5	2.4%
RECIST status (post NST)		
Responder (CR+PR)	179	86.5%
Non-responder (SD+PD)	25	12.1%
unknown	3	1.4%
Pathologic response		
pCR	30	14.5%
Non pCR	177	85.5%

Table 1. (continued)

CTC detection in course of NST	No.of patients (%)	
Pre-NST CTC (N=203) (Mean count=2.7, SD=4.0)		
Negative	71	35.0%
Positive	132	65.0%
<5	160	78.8%
≥5	43	21.2%
Mid-NST CTC (N=186) (Mean count=4.6, SD=9.6)		
Negative	52	28.0%
Positive	134	72.0%
<5	139	74.7%
≥5	47	25.3%
Post-NST CTC (N=171) (Mean count=3.6, SD=6.9)		
Negative	68	39.8%
Positive	103	60.2%
<5	132	77.2%
≥5	39	22.8%
NST(Neoadjuvant systemic therapy) regimen		
Chemotherapy (N=193)(%)	**193**	**93.2%**
AC#4	52	25.1%
AC#4 > D#4	96	46.4%
AC#4 > D+Herceptin#4 (Trastuzumab)	20	9.7%
FEC#4 > D#4	16	7.7%
PEARLY trial (NCT02441933) (AC#4 > D+Carboplatin#4)	2	1.0%
M14-011 trial (NCT02032277) (Veliparib/Placebo+Carboplatin/Placebo+Paclitaxel)	4	1.9%
TCHP#6 (Docetaxel+Carboplatin+Trastuzumab+Pertuzumab)	3	1.4%
Hormonal therapy (N=14)(%)	**14**	**6.8%**
METEOR trial (NCT01589367) (Letrozole +/- Metformin)	4	1.9%
NEST trial (NCT01622361) (GnRHa+Tamoxifen)	10	4.9%

CTC(Circulating tumor cell); NST(Neoadjuvant systemic therapy); RECIST(Response Evaluation Criteria in Solid Tumors); CR(complete response); PR(Partial response); SD(Stable disease); PD(Progressive disease); pCR(Pathologic complete response).

Table 2. Patient characteristics in correlation with CTC status in course of NST.

Variables	Initial CTCs (pre NST, N=203)		*p*	Mid CTCs (During NST, N=186)		*p*	Post NST CTCs (N=171)		*p*
	Negative N=71 (%)	Positive N=132 (%)		Negative N=52 (%)	Positive N=134 (%)		Negative N=68 (%)	Positive N=103 (%)	
Age (mean 46.48) (range 28-71)			0.340			0.620			0.075
34≥	5 (7.0%)	5 (3.8%)		2 3.8%	8 6.0%		1 1.5%	6 5.8%	
35-50	51 (71.8%)	89 (67.4%)		39 75.0%	92 67.9%		54 79.4%	66 64.1%	
51≤	15 (21.1%)	38 (28.8%)		11 21.2%	35 26.1%		13 19.1%	31 30.1%	
Tumor subtype (pre NST)			0.031			0.102			0.002
Luminal	50 (70.4%)	92 (69.7%)		37 71.2%	93 69.5%		49 72.1%	75 71.8%	
HER2	2 (2.8%)	15 (11.4%)		2 3.8%	14 10.4%		1 1.5%	13 12.6%	
TN	19 (26.8%)	25 (18.9%)		13 25.0%	27 20.1%		18 26.5%	16 15.6%	
Clinical T stage			0.310			0.541			0.360
TX (occult breast ca)	0 (0.0%)	1 (0.8%)		0 0.0%	0 0.0%		0 0.0%	0 0.0%	
T1	8 (11.3%)	11 (8.3%)		3 5.8%	17 12.7%		6 8.8%	12 11.7%	
T2	49 (69.0%)	81 (61.4%)		34 65.4%	83 61.9%		47 69.1%	61 59.2%	
T3	14 (19.7%)	34 (25.8%)		13 25.0%	31 23.1%		15 22.1%	27 26.2%	
T4	0 (0.0%)	5 (3.8%)		2 3.8%	3 2.3%		0 0.0%	3 2.9%	
Lymph node status			0.601			0.408			0.649
Negative	18 (25.4%)	38 (28.8%)		12 23.1%	39 29.1%		17 25.0%	29 28.2%	
Positive	53 (74.6%)	94 (71.2%)		40 76.9%	95 70.9%		51 75.0%	74 71.8%	
Histologic grade			0.194			0.774			0.969
G1	2 (2.8%)	0 (0.0%)		0 0.0%	2 1.5%		1 1.5%	1 1.0%	
G2	55 (76.4%)	95 (72.0%)		40 76.9%	97 72.4%		51 75.0%	75 72.8%	
G3	14 (19.4%)	35 (26.5%)		11 21.2%	33 24.6%		15 22.1%	25 24.3%	
unknown	1 (1.4%)	2 (1.5%)		1 1.9%	2 1.5%		1 1.5%	2 1.9%	
ER status			0.914			0.815			0.976
Positive	50 (70.4%)	92 (69.7%)		37 71.2%	93 69.4%		49 72.1%	74 71.8%	
Negative	21 (29.6%)	40 (30.3%)		15 28.8%	41 30.6%		19 27.9%	29 28.2%	
PR status			0.948			0.435			0.223
Positive	40 (56.3%)	75 (56.8%)		32 61.5%	74 55.2%		44 64.7%	57 55.3%	
Negative	31 (43.7%)	57 (43.2%)		20 38.5%	60 44.8%		24 35.3%	46 44.7%	
HER2/neu status			0.003			0.016			0.000
Positive	3 (4.2%)	25 (18.9%)		2 3.8%	23 17.2%		1 1.5%	22 21.4%	
Negative	68 (95.8%)	107 (81.1%)		50 96.2%	111 82.8%		67 98.5%	81 78.6%	
Baseline CA-15-3			0.711			0.443			0.077
Normal	66 (93.0%)	126 (95.4%)		48 92.3%	127 94.8%		64 94.1%	96 93.2%	
Elevated	3 (4.2%)	3 (2.3%)		3 5.8%	3 2.2%		4 5.9%	2 1.9%	
Unknown	2 (2.8%)	3 (2.3%)		1 1.9%	4 3.0%		0 0.0%	5 4.9%	
RECIST status (post NST)			0.011			0.926			0.842
Responder (CR+PR)	56 (77.7%)	120 (90.9%)		46 88.5%	117 86.7%		60 88.2%	91 89.2%	
Non-responder (SD+PD)	14 (19.4%)	10 (7.5%)		6 11.5%	16 11.9%		8 11.8%	11 10.8%	
unknown	2 (2.9%)	2 (1.6%)		0 0.0%	2 1.4%		0 0.0%	1 0.9%	
Pathologic response			0.532			0.996			0.150
pCR	12 (16.9%)	18 (13.6%)		7 13.5%	18 13.4%		6 8.8%	17 16.5%	
Non pCR	59 (83.1%)	114 (86.4%)		45 86.5%	116 86.6%		62 91.2%	86 83.5%	

CTC(Circulating tumor cell); NST(Neoadjuvant systemic therapy); RECIST(Response Evaluation Criteria in Solid Tumors); CR(complete response); PR(Partial response); SD(Stable disease); PD(Progressive disease); pCR(Pathologic complete response).

Table 3. Treatment response and pCR status in relation to Initial CTCs (pre NST) in the Luminal subtype.

	RECIST response			pCR status		
Initial CTC (pre NST)	**Responder**	**Non-responder**	***p***	**pCR**	**Non pCR**	***p***
Negative	39 (78.0%)	11 (22.0%)	0.003	4 (8.0%)	46 (92.0%)	1.000
Positive	85 (94.4%)	5 (5.6%)		8 (8.7%)	84 (91.3%)	
CTCs <5	94 (85.5%)	16 (14.5%)	0.023	8 (7.1%)	104 (92.9%)	0.280
CTCs≥5	30 (100.0%)	0 (0.0%)		4 (13.3%)	26 (86.7%)	

CTC(circulating tumor cell); NST(Neoadjuvant systemic therapy); pCR(Pathologic complete responde); RECIST(Response Evaluation Criteria In Solid Tumor).

Table 4. Univariate and Multivariate Cox analyses of RFS and OS for CTC detection and changes during treatment.

	RFS			OS		
	Univariate *p*[1]	**Multivariate HR (95 CI)[2]**	***P***	**Univariate *p*[1]**	**Multivariate HR (95 CI)[2]**	***P***
Whole patient cohort						
CTC detection						
Initial						
<5		1			1	
≥5	0.375			0.884		
Mid						
<5		1			1	
≥5	***0.015***	2.72 (1.05-7.01)	***0.038***	0.106		
Post						
<5		1			1	
≥5	0.268			0.455		
Luminal subgroup						
CTC detection						
Initial		1			1	
<5						
≥5	0.600			0.468		
Mid						
<5		1			1	
≥5	***0.002***	6.84 (1.95-23.71)	***0.003***	***0.000***	30.09 (1.67-539.39)	***0.021***
Post						
<5		1			1	
≥5	0.233			0.378		
CTC changes						
Pre > Mid						
Neg > Neg		1				
Neg > Pos	***0.038***	16.46 (1.62-161.22)	***0.016***	0.446		
Pre > Mid > Post NST						
<5 - <5 - <5		1			1	
<5 - ≥5- ≥5	***0.000***	319.662 (9.05-11283.48)	***0.002***	***0.000***	N/A	
≥5 - ≥5 - <5	***0.000***	553.90 (11.13-27559.15)	***0.002***	***0.000***	N/A	

CTC(circulating tumor cell); NST(Neoadjuvant systemic therapy); RFS(Relapse-free survival) ;OS(Overall survival); HR(Hazard ration); CI(Confidence interval); Neg(Negative); Pos(Positive); [1]Log-rank test; [2]Cox model.

Figure 1. Time points of blood sampling. 1.Initial diagnosis 2.During NST 3. After NST (prior to operation). CTC(Circulating tumor cell); NST(Neoadjuvant systemic therapy).

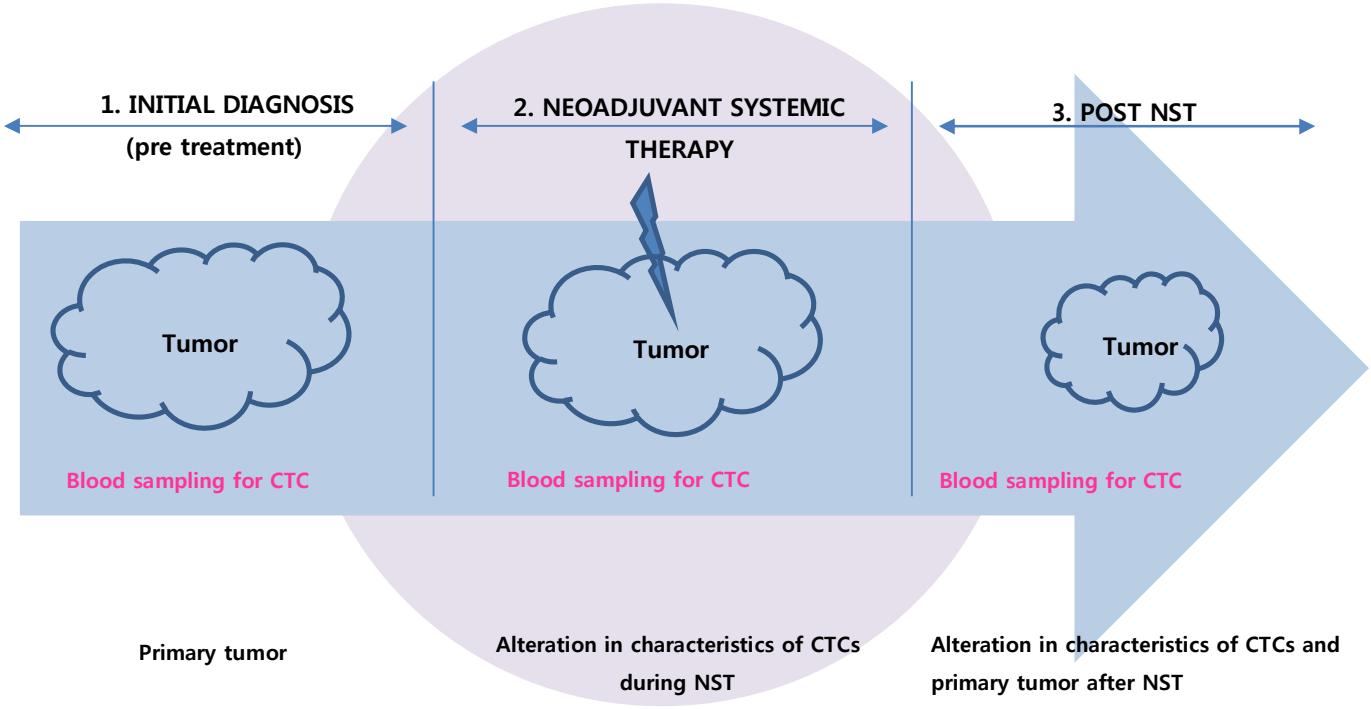


Figure 2. Immunofluorescence staining of CTCs for DAPI, EpCAM, CK, CD45 staining and merged image. Magnification, x400. CTCs(Circulating tumor cells); DAPI(4',6-diamidine-2'-phenylindole dihydrochloride); EpCAM(Epithelial cell adhesion molecule); CK(Cytokeratin); CD(Cluster of differentiation).

Immunophenotyping findings (magnified x400 image)

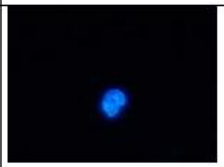
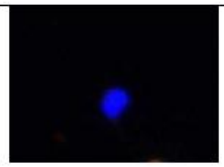
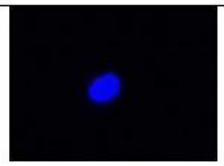
CTC count	DAPI stain	EpCAM stain	CK stain	CD 45 stain	Merged finding
WBC					
EpCAM (+) control					
CD45 (+) control					

Figure 3. Breast ultrasound. RECIST PR(Partial response). A. Pretreatment: mass in left breast (blue arrow). B. Post-treatment: decreased mass in the left breast (yellow arrow), with partial tumor reduction.

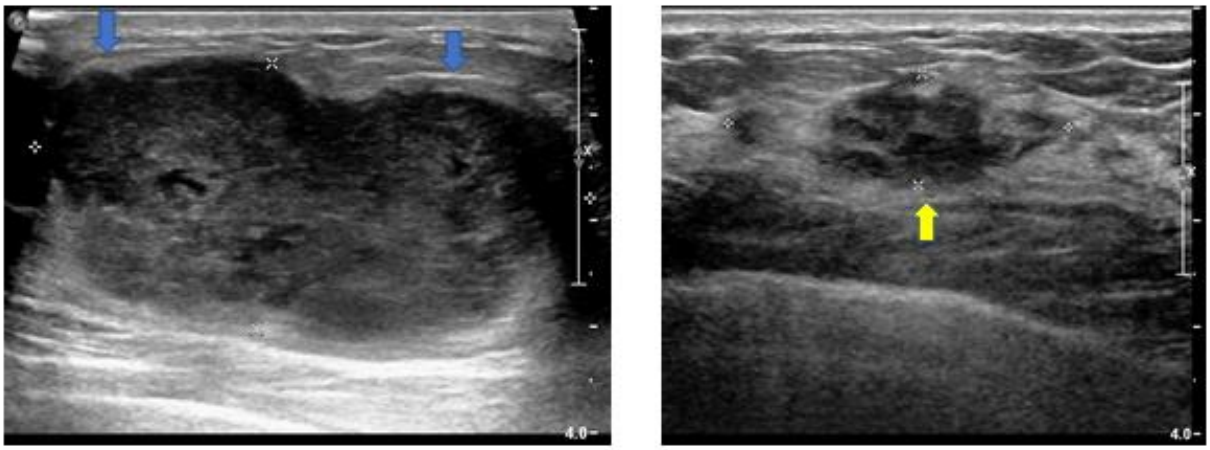


Figure 4. Breast MRI(Magnetic resonance imaging). RECIST PR(Partial response). A. Pretreatment: single large mass in left breast (blue arrow). B. Post-treatment: single mass in the left breast (yellow arrow), with partial tumor reduction.

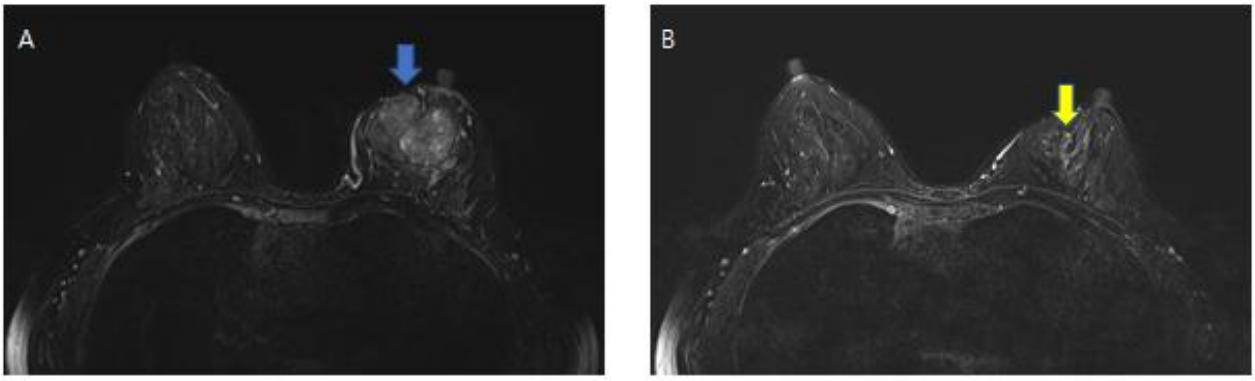


Figure 5. Circulating tumor cell detection in course of NST(Neoadjuvant systemic therapy).

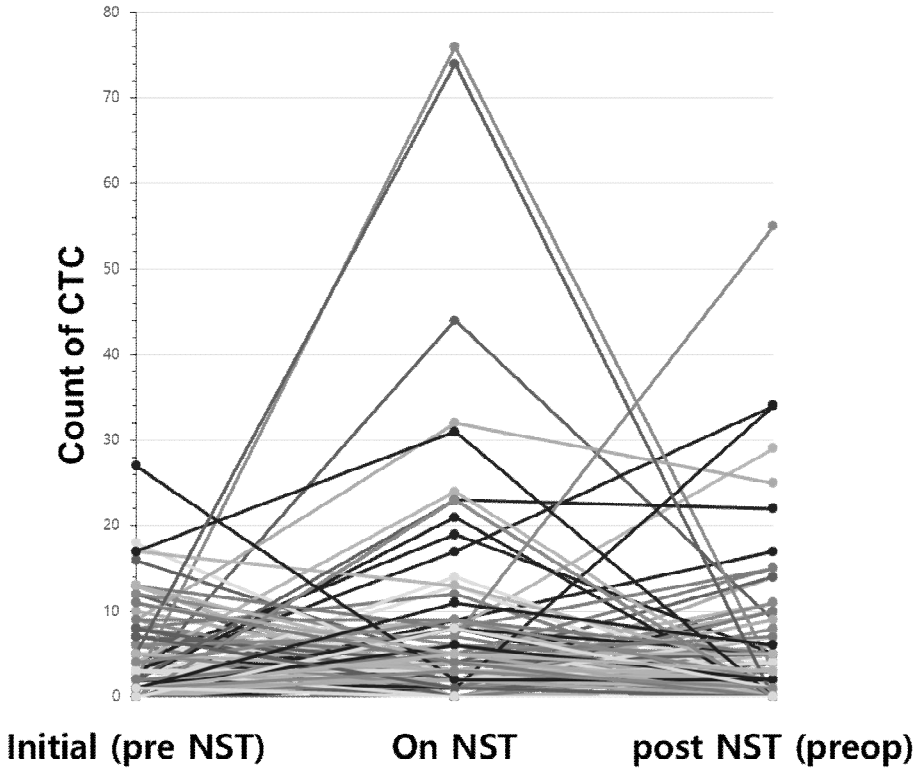


Figure 6. Frequency of CTCs detected according to NST. A. CTC count composition at each time point., B. Proportional change of each CTC category according to each time point. CTC(circulating tumor cell); NST(Neoadjuvant systemic therapy).

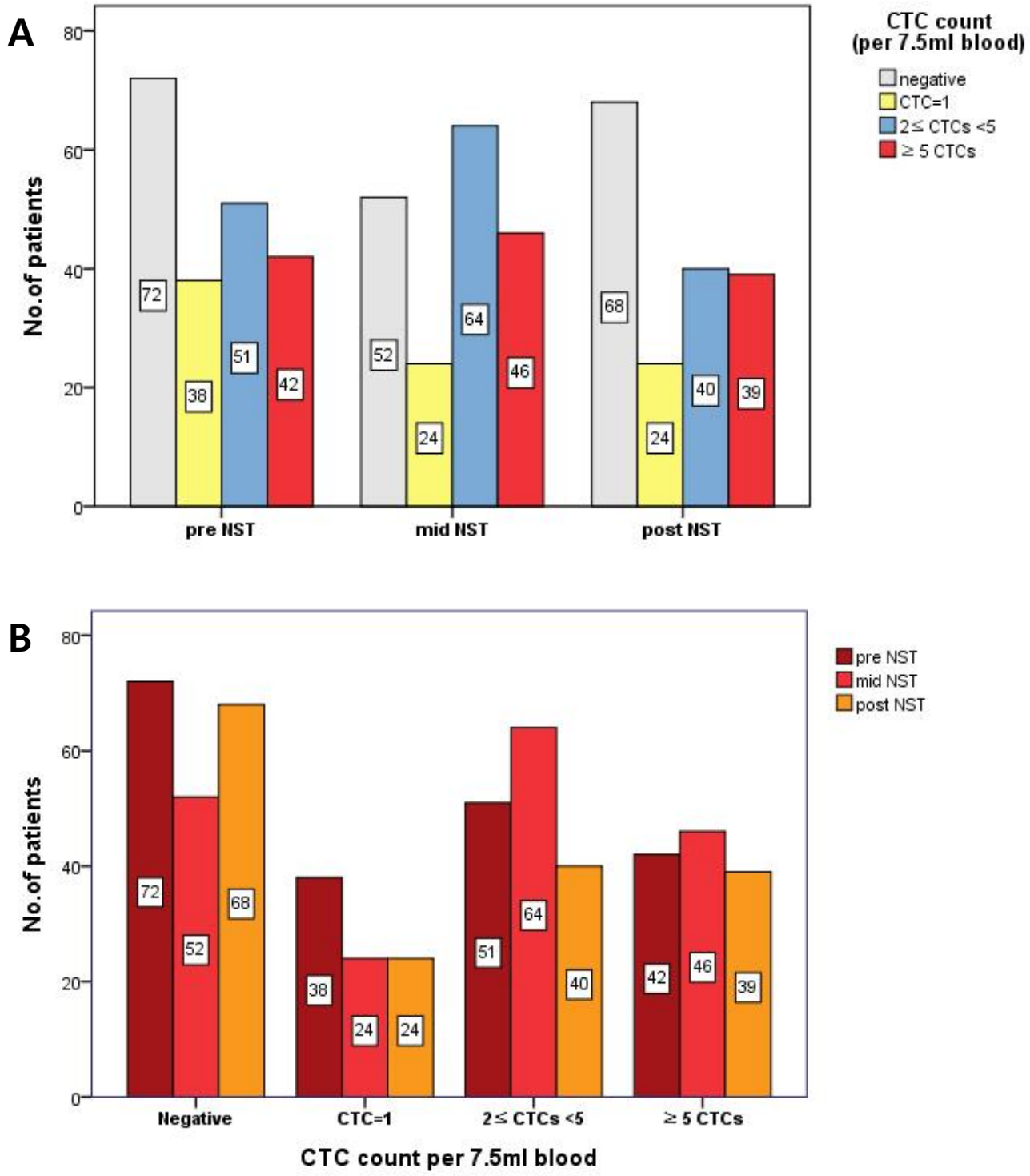


Figure 7. Treatment response in relation to CTC detection before, during and after NST. CTC(Circulating tumor cell; NST(Neoadjuvant systemic therapy); pCR(Pathologic complete response).

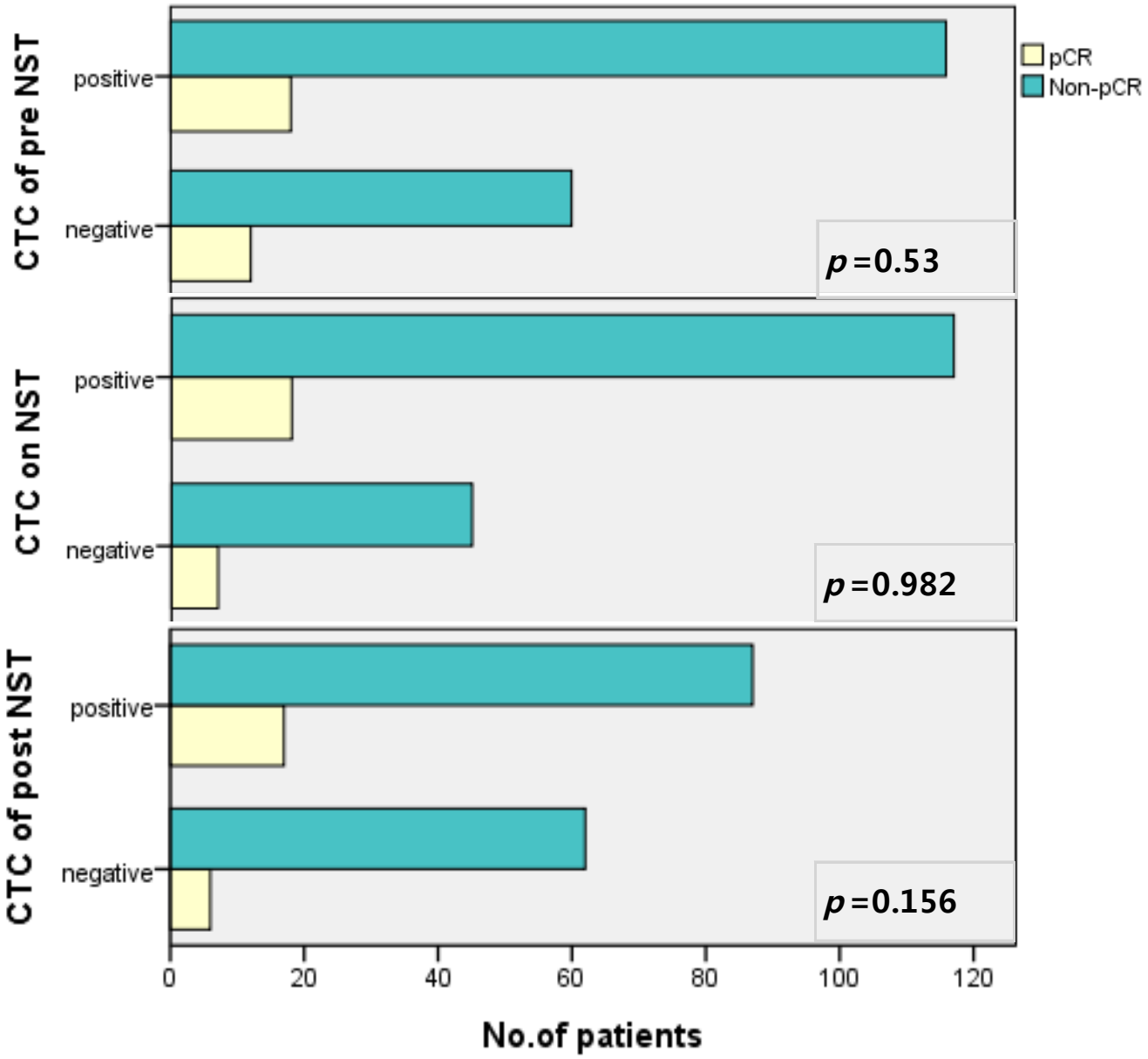


Figure 8. Kaplan–Meier plots for RFS(A,C,E) and OS(B,D,F) according to CTC detection. A,B=Pre NST, C,D=During NST, E,F=Post NST. CTC(circulating tumor cell); NST(Neoadjuvant systemic therapy); RFS(Relapse-free survival); OS(Overall survival).

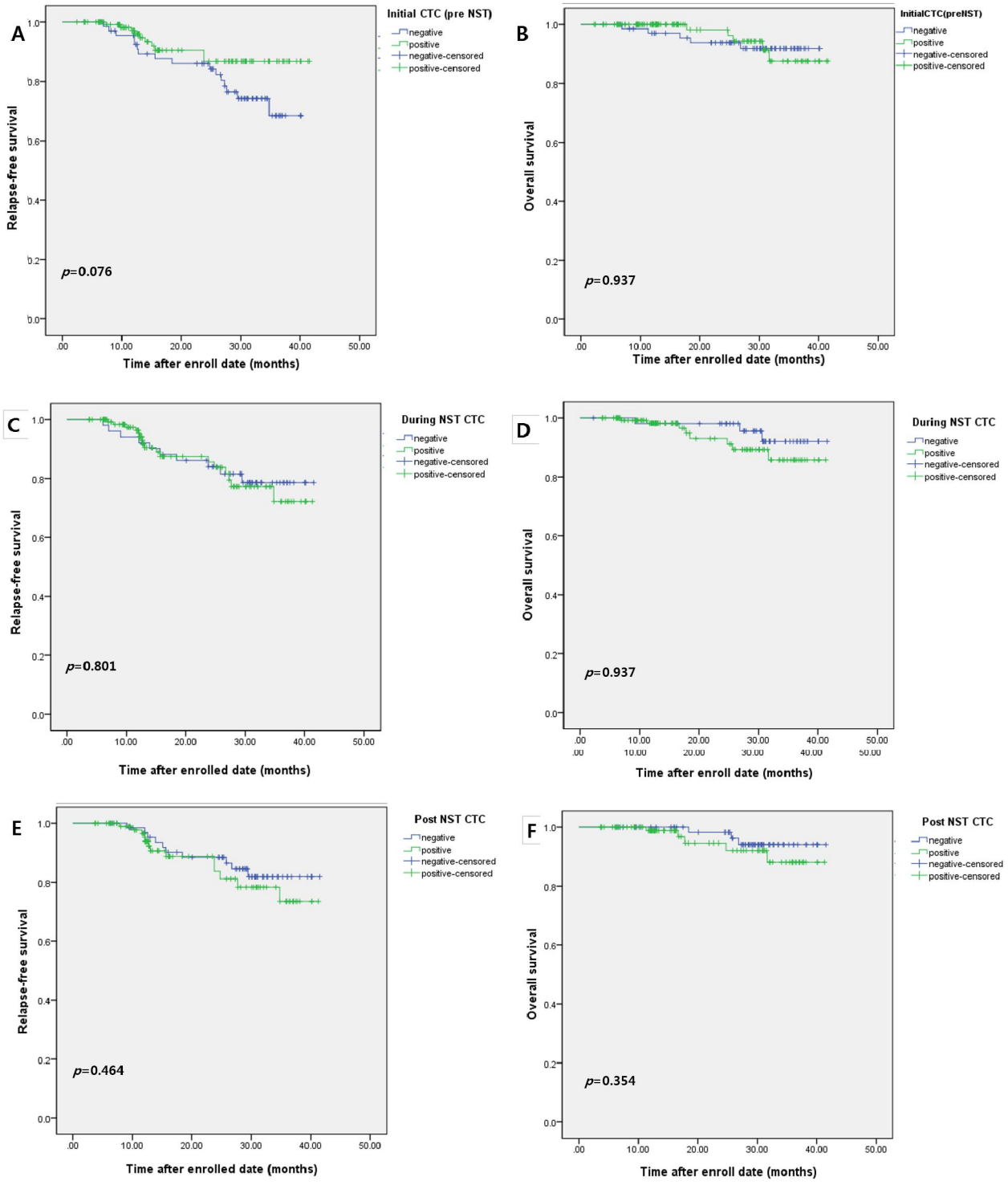


Figure 9. Kaplan–Meier plots for RFS(A,C,E) and OS(B,D,F) according to CTC detection (cut-off ≥5) A,B=Pre NST, C,E=During NST, E,F=Post NST. CTC(circulating tumor cell); NST(Neoadjuvant systemic therapy); RFS(Relapse-free survival); OS(Overall survival).

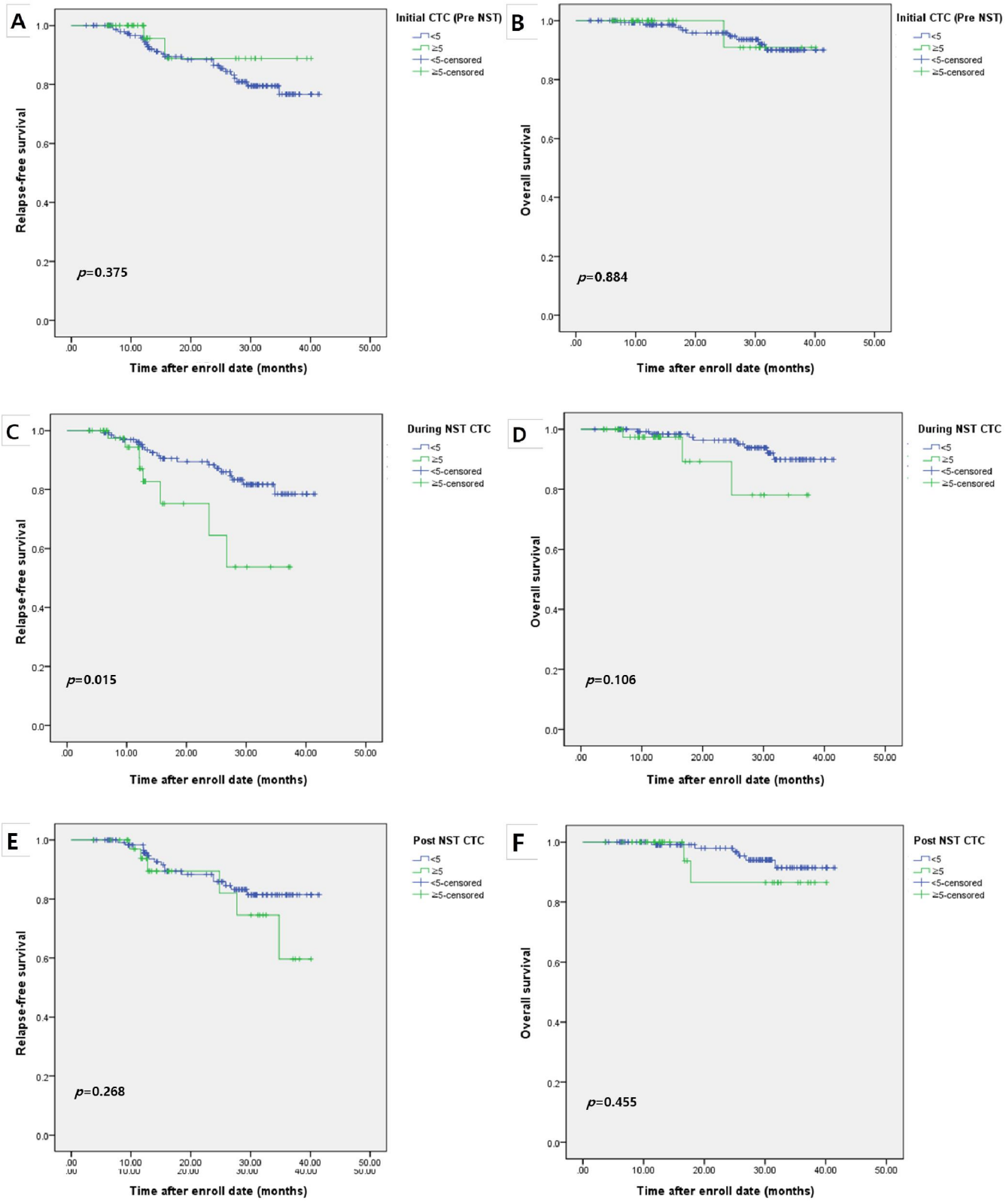
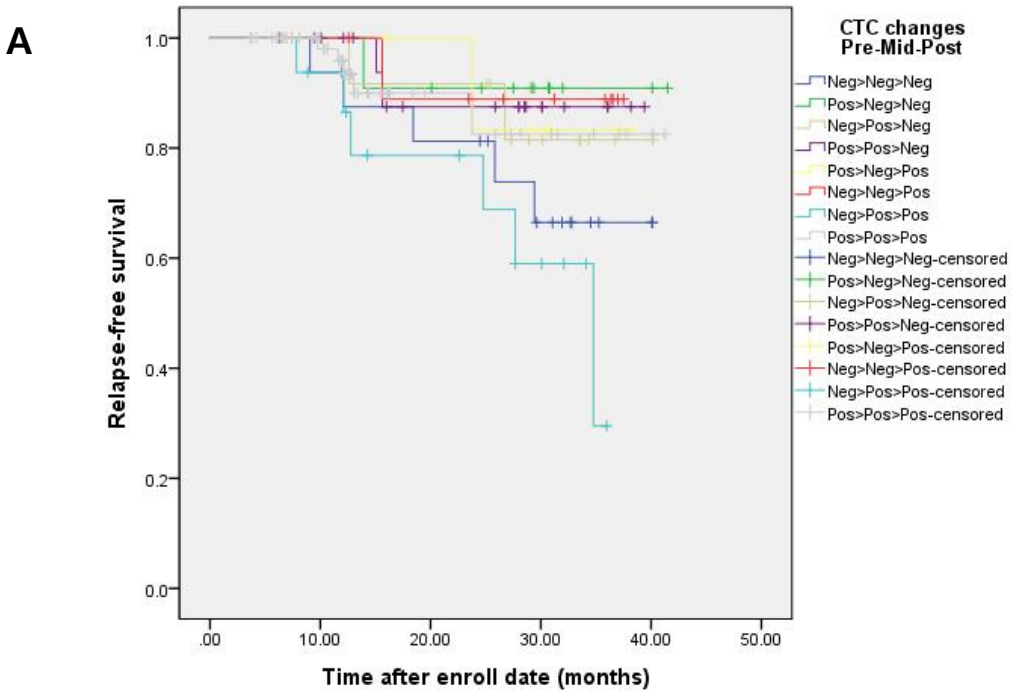


Figure 10. Kaplan–Meier plots for RFS(A) and OS(B) according to changes in CTC counts (negative-positive variations) (in the course of NST). CTC(circulating tumor cell); NST(Neoadjuvant systemic therapy); RFS(Relapse-free survival); OS(Overall survival); Neg(Negative); Pos(Positive).



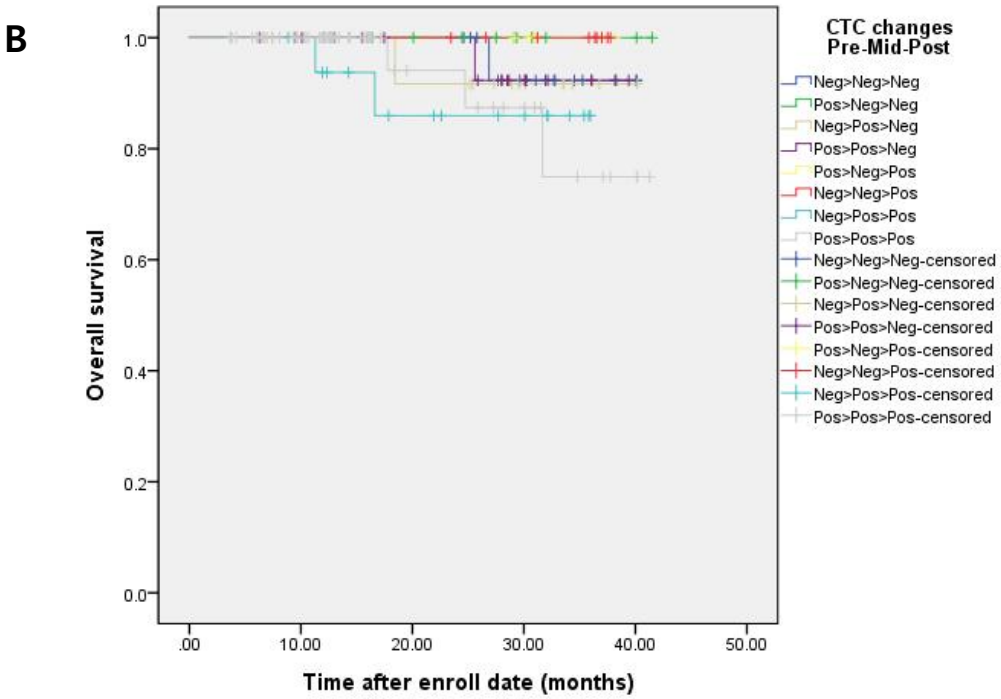


Figure 11. Kaplan–Meier plots for RFS(A) and OS(B) according to changes in CTC counts (cut-off ≥5 Increase-Decrease variations) (in the course of NST). CTC(circulating tumor cell); NST(Neoadjuvant systemic therapy); RFS(Relapse free survival); OS(Overall survival).

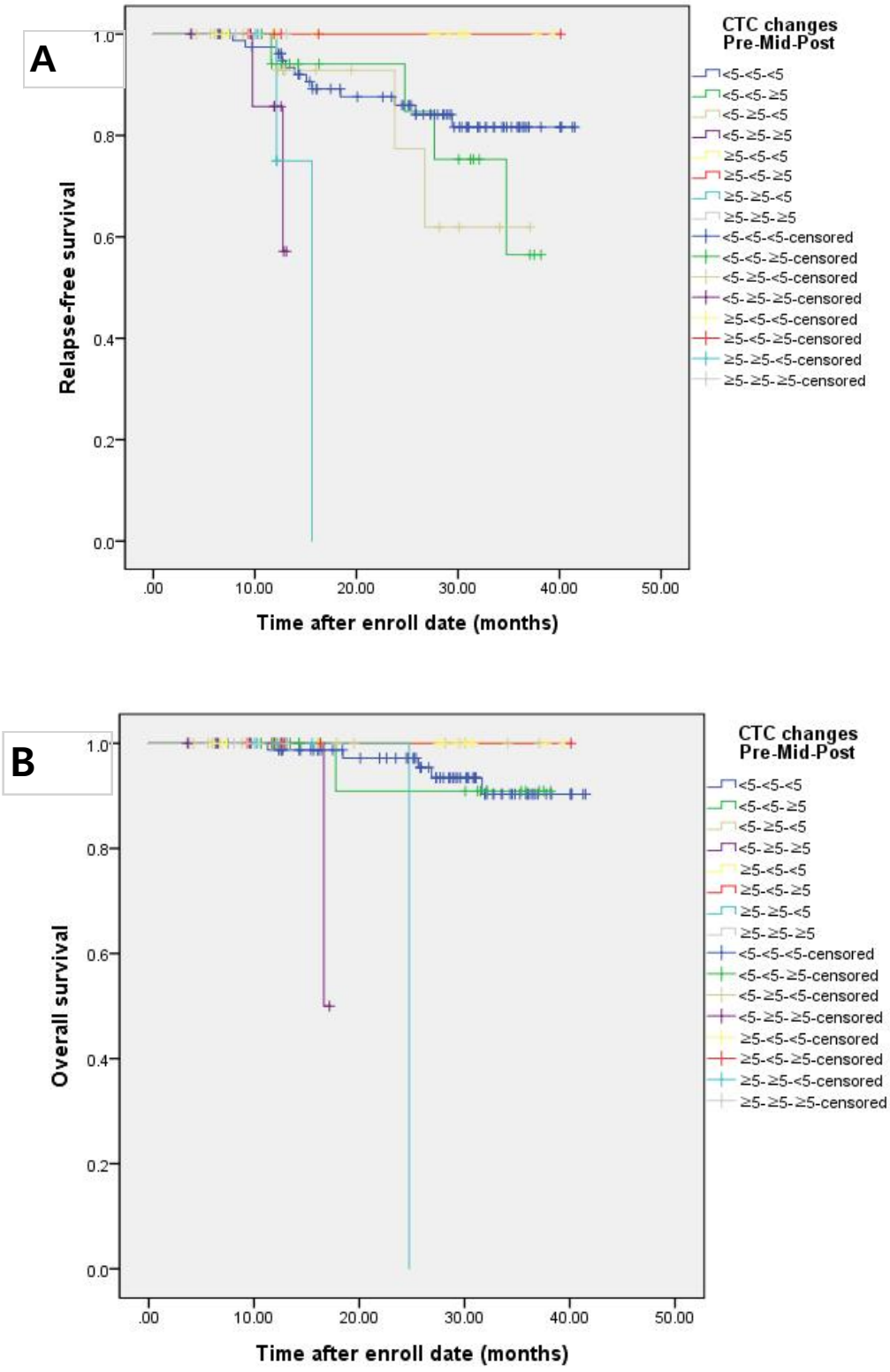
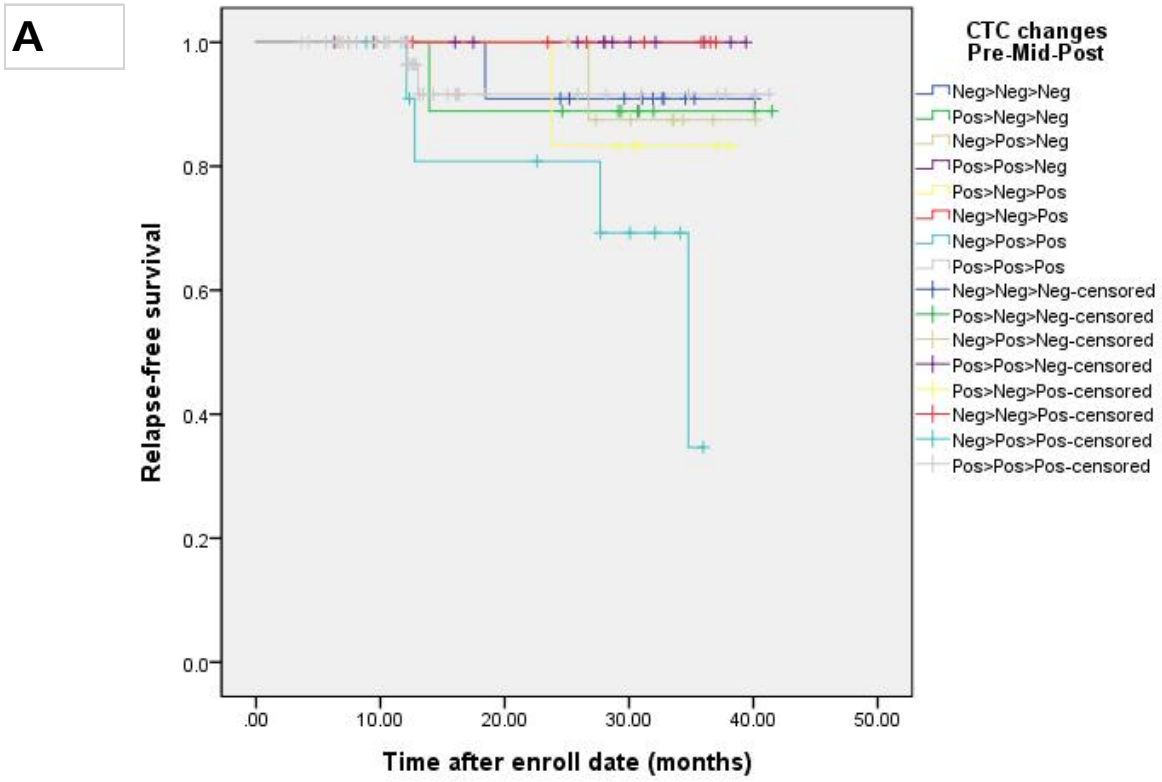


Figure 12. Kaplan–Meier plots for RFS(A) and OS(B) according to changes in CTC counts (negative-positive variations) of the **Luminal** subtype (in the course of NST). CTC(circulating tumor cell); NST(Neoadjuvant systemic therapy); RFS(Relapse-free survival); OS(Overall survival); Neg=Negative; Pos=Positive.



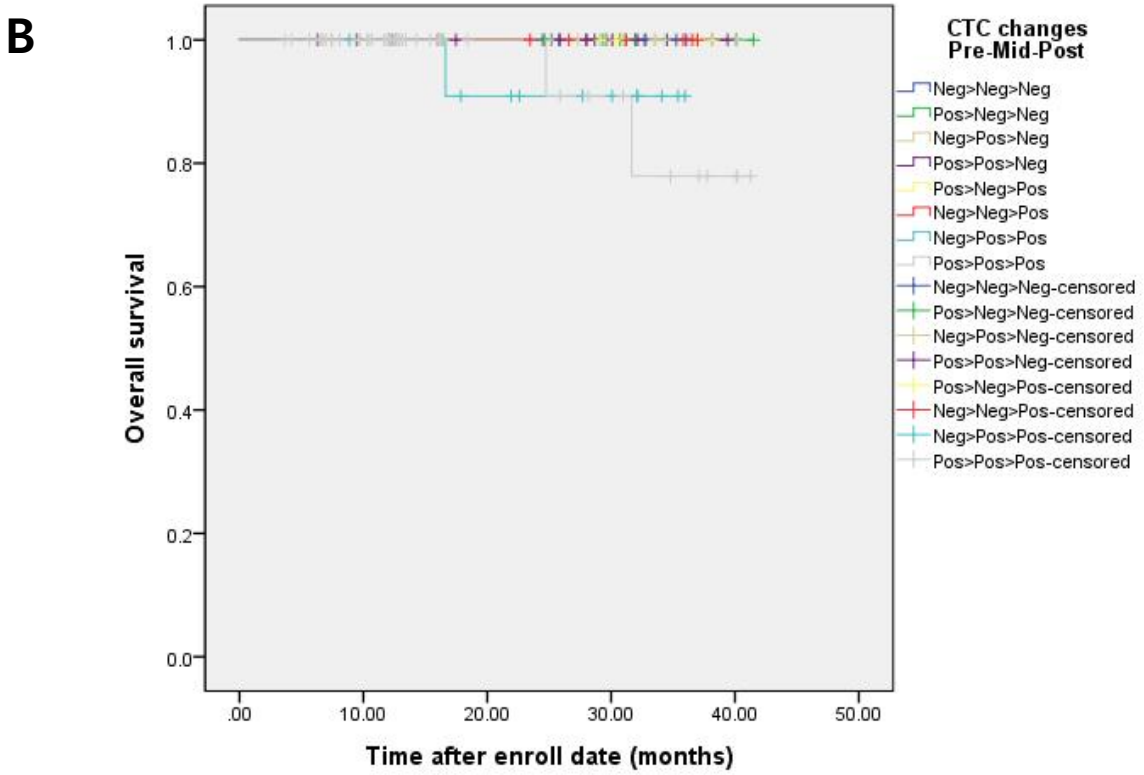


Figure 13. Kaplan–Meier plots for RFS(A) and OS(B) according to changes in CTC counts (cut-off ≥5 CTCs, Increase-Decrease variations) of the **Luminal** subtype (in the course of NST). CTC(circulating tumor cell); NST(Neoadjuvant systemic therapy); RFS(Relapse-free survival); OS(Overall survival).