Correlation between plasma ccfDNA, mtDNA changes, CTCs, and epithelial mesenchymal transition in breast cancer patients undergoing NACT

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Informed consent

The ethical suitability of this study was reviewed and unanimously approved by the Marmara University Faculty of Medicine Clinical Research Ethics Committee (Ethics committee protocol code: 09.2022.246).

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Abstract

Background/aim: Breast cancer is the most prevalent cancer in women, emphasizing need for non-invasive blood biomarkers to aid in treatment selection. Previous studies have demonstrated elevated levels of plasma circulating cell-free DNA (ccfDNA) in breast cancer patients. Both ccfDNA and mitochondrial DNA (mtDNA) are fragments released into the bloodstream. In this study, we investigated effectiveness of ccfDNA and mtDNA as indicators of treatment response and explored their potential as monitoring biomarkers. Additionally, we compared these markers with circulating tumor cell (CTC) data and assessed their relationship with epithelial mesenchymal transition (EMT).

Materials and methods: 36 female breast cancer patients and 21 healthy females were included in the study. Quantitative polymerase chain reaction (qPCR) was performed on plasma samples to measure levels of ND1, ND4, ALU115, ALU247, and GAPDH, and DNA integrity was determined by calculating ratios of ALU247/ALU115 and ND4/ND1.

Results: After treatment, patients had a significant decrease in ccfDNA levels and a significant increase in mtDNA copy number (mtDNAcn). However, there was no significant change in ccfDNA and mtDNA integrity. When comparing all groups, patients exhibited higher levels of ALU115 and ALU247 compared to controls. Moreover, patients demonstrated significantly lower ccfDNA integrity than controls.

Conclusion: This study represents first comprehensive investigation of plasma ccfDNA levels, mtDNAcn, and integrities collectively. Furthermore, it is first study to explore the relationship between these markers and CTCs, cancer stem cell markers, treatment response, and metastatic status. Our findings suggest that plasma ccfDNA and mtDNA may serve as potential biomarkers for assessing chemotherapy response and can be employed alone or in combination with other biomarkers to monitor treatment efficacy in breast cancer patients.

Key words: Breast cancer, ccfDNA, mtDNA, neoadjuvant therapy, EMT

1. Introduction

Breast cancer is the most frequent cancer worldwide, and it is also the most second prevalent cause of cancer-related death in women [1]. Multiple molecular changes that lead to uncontrolled self-renewal, proliferation, transformation and metastasis of normal cells cause cancer [2,3]. Early detection of cancer and accurate identification of metastases have considerably improved the survival rates of women with breast cancer

through enhanced treatment of breast cancer. The monitoring of treatment response is essential to avoid continuation of ineffective treatments, to prevent needless side effects, and to determine the utility of new therapeutics [1,2,4,5]. Accumulating findings over the last couple of years have emphasized the potential use of circulating nucleic acids in peripheral blood, such as DNA, mRNA, and microRNA, in the breast cancer diagnosis, prognosis and monitoring of response to anticancer therapy [6]. Although using of tumor-tissue specimens will remain significant, the utility of biopsy samples is limited since such material may not capture tumor heterogeneity; also, recurrent biopsy is not practical. A considerable alternative method is "liquid biopsy" which enables for sensitive and targeted serial sampling during therapy [5].

Changes in the levels of circulating tumor cells (CTCs), extracellular free DNAs (cfDNAs), and mitochondrial DNA (mtDNA), known as liquid biopsies, have received great attention as cancer biomarkers in plasma and serum. In addition to plasma and serum, it is a minimally invasive tool to detect molecular biomarkers in body fluids, such as peripheral blood, urine, saliva, cerebral spinal fluid, and breast milk [7–13]. This implement provides a perspective for real-time monitoring of tumor dynamics in an individual cancer patient. Although obtaining a tissue biopsy from a cancerous location remains the mainstay of diagnosis, liquid biopsy appears to be an alternative to the restricted solid biopsy approaches due to several advantages; allows sequential sampling for surveillance of tumor progression, response to treatment, metastasis, and disease recurrence [4,9,11].

In 1948, two French researchers, Mandel and Me´tais discovered the presence of cell-free DNA in the blood of healthy and diseased humans [7,10,11,14–19]. While cfDNA may seem petty when it was first discovered in the human circulatory system, its clinical

significance was recognized when researchers observed differences between the properties of cfDNA in healthy individuals and cancer patients [16]. The vast majority of cfDNA is released through apoptosis or necrosis of tumor cells in oncological patients [20,21]. Besides screening healthy and at-risk patient groups for early detection, treatment of cancer, cfDNAs are a biomarker for multiple indications in oncology; including staging and prognosis, tumor localization, initial therapy stratification, monitoring of local or systemic treatment response, identification of acquired resistance mechanisms, monitoring of recurrence [8,16,22].

The cfDNA profile found in a single blood sample contains a mixture of both "wild-type" and genetically and epigenetically modified DNA fragments released by diverse cells from various processes, tissues and organs under environmental factors [23]. All cells seemingly to have the ability to continuously release cell-specific DNA into the extracellular environment. An important point here is that the concentration of cfDNA and the concentration of tumor-derived DNA in tumor microenvironment cells and other healthy cells differ from a considerable extent between individuals [16].

cfDNA presents as ALU (Arthrobacter luteus) sequences. More than 50-65% of the human genome consists of repetitive DNA [18,24]. ALU families belong to the class of retroelements called short interspersed nuclear elements (SINEs) in the more than 10% of the human genome, with a copy number of approximately 1.4 million, are the most abundant ALU [18,25]. They are typically about 300 nucleotides in length [14,26–28]. While the source of cfDNA in healthy individuals is merely by apoptosis, producing evenly sized shorter DNA fragments (ALU 115), on the other hand in cancers, necrosis contributes uneven longer DNA fragments (ALU 247) to the shorter fragments from apoptosis [14,29–31]. Analysis of ccfDNA integrity is a factor independent of the genetic

or epigenetic status of cfDNA and is theoretically representative of all tumors. ccfDNA integrity is calculated as the ratio of the concentration of longer DNA fragments to shorter fragments in plasma or serum [32,33].

Mitochondria is eukaryotic cell organelle that play a central role in energy production, cell proliferation, and apoptosis. It is the main source and target of intracellular reactive oxygen species (ROS), which plays an important role in breast carcinogenesis [34]. Recent advances to increase the diagnostic and prognostic value of cancer patients have also targeted the circulating mitochondrial genome owing to its idiocratical and unique properties. Circulating mitochondrial DNA is known to have short length, relatively simple molecular structure, and high copy number. These properties make it an easily accessible, non-invasive biomarker for the diagnosis of various types of solid tumors together with the function of liquid biopsy [35]. Together with the inconsistent association between peripheral blood mtDNA copy number and breast cancer risk, breast cancer may alter the observed mtDNA levels in peripheral blood and it emphasizes the need of creating forward-looking work designs [36].

There are hotspot locations for deletions along the mtDNA circle, 90% of which are deletions of the nicotinamide adenine dinucleotide dehydrogenase 4 (ND4) sequence, reflecting a population of viable mitochondria but with poor mtDNA integrity [37]. ND4 subunits are often missing in complex I and are a common indicator of mtDNA damage [38]. In contrast, the loss of ND1 subunits has a much more detrimental effect on complex I and the mitochondria itself, making ND1 deletions rare in viable mitochondria. Therefore, the rarely deleted ND1 copy number is a suitable marker for the total mtDNA copy number and the ND4/ND1 ratio can be used to assess the proportion of intact mtDNA [37–40].

Breast cancer treatment typically incorporates a multimodality strategy that includes surgery, radiation, and systemic therapy. Neoadjuvant chemotherapy (NACT) has become in an ideal world treatment for patients with advanced breast cancer. If breast-conserving surgery is not possible, neoadjuvant chemotherapy can be utilized [41]. It has become a standard-of-care for patients who locally advanced breast cancer. NACT provides a unique opportunity for real-time monitoring of tumor response and evaluation of drug efficacy. Secondly, it can reduce the stage of tumors and thus promote the chances of breast-conserving surgery [42–44].

Currently, the need for diagnostic and prognostic biomarkers continues. However, using a combination of blood biomarkers with a non-invasive method is critical for treatment selection. According to the studies examined, ccfDNA and mtDNA play a crucial role in human cancers, especially their important functions on metastasis. Since there is no research to examine the relationship between multiple markers and circulating tumor cells with epidermal-mesenchymal transition, the present study will contribute to clinical studies for disease diagnosis, prognosis and treatment.

In this study, we determined the changes in plasma cfDNA and mtDNA copy number and integrity at before and after NACT in breast cancer patients. By correlating it with CTC molecular analysis data obtained from the same patients in our previous study, it is aimed to examine the relationship of these biomarkers with epidermal-mesenchymal transformation (EMT), provide to predict the development of metastasis in these patients, thereby predicting the clinical course of the patients.

2. Materials and methods

2.1. Study subjects and ethical approval

Breast cancer patients receiving neoadjuvant chemotherapy (n = 36) and healthy individuals as control (n = 21) were included in this retrospective study. Plasma retrieved from the banked specimens at -80 °C. The present study was approved by the Marmara University School of Medicine ethics committee (approval ID 09.2022.246). Informed consent was obtained from all the recruited subjects.

2.2. Isolation of DNA

Blood was collected in an EDTA tube. To obtain plasma, centrifugation was performed at 2000 g for 10 min at 4 °C. DNA was extracted from plasma samples using the QIAmp DNA mini kit (Qiagen, Hilden, Germany, cat. number: 51304) according to the manufacturer's instructions and stored at -20 °C until use.

2.3. RT-PCR of ALU elements and mtDNA copy number

1μL of genomic DNA was used as a template for each real-time polymerase chain reaction (RT PCR) using BlasTaq 2X SYBR Green Master Mix (abm Good) on a real-time PCR device (LightCycler 480, Roche).

The quantitative values from the 115 bp (shorter fragments) primers represent the total level of ccfDNA (ng/μL), while the quantitative values from the 247 bp (longer fragments) primers used for the calculation of the integrity of ccfDNA. The sequences of the ALU 115 and ALU 247 primers were as follows: ALU 115 forward 5′-CCTGAGGTCAGGAGTTCGAG-3′ and reverse 5′- CCCGAGTAGCTGGGATTACA-3′; ALU 247 forward 5′-GTGGCTCACGCCTGTAATC-3′ and reverse 5′-CAGGCTGGAGTGCAGTGG-3′[45]. The absolute amount of ccfDNA in each sample was determined by a standard curve using 10-fold dilutions (10, 1, 0,1, 0,01, 0,001) of genomic DNA obtained from peripheral blood of a healthy donor volunteer. A negative control (without template) was run in each reaction plate.

We analyzed levels of ND1, ND4, and GAPDH for mtDNA copy number in plasma samples. ND1 copy number is a convenient marker for total amount of mtDNA. The sequences of the ND1 and ND4 obtained from NCBI. ND1 forward 5′-ATGGCCAACCTCCTACTCCT-3′ and reverse 5′-GGGCCTTTGCGTAGTTGTAT-3′; ND4 forward 5′-GATGAGGCAACCAGCCAGAA-3′ and reverse 5′-GTAGGGGAAGGGAGCCTACT-3′. After obtaining to Ct values, the mtDNA copy number was calculated using the following formula: $2^{-\Delta Ct}$.

Real-time PCR amplification was performed with the following cycles: Initial holding at 95 °C for 3 min, followed by 50 cycles of 95 °C for 15 s and 60 °C for 60 s. Negative template control was run in each plate. All reactions were conducted in triplicate in 96-well plates.

2.4. DNA Integrity Determination

DNA integrity was calculated as the ratio of ALU 247 to ALU 115 for ccfDNA and the ratio of ND4 to ND1 for ccf-mtDNA. In this study, mtDNA integrity was calculated by proportioning the amounts obtained as a result of the $2^{-\Delta Ct}$ formula of the ND1 and ND4 primer sets while the ratio of longer to shorter fragments (ALU 247 (ng/μL) / ALU 115 (ng/μL)) demonstrates the integrity of ccfDNA in each sample [14,38,45].

2.5. Statistical analysis

In this study, analysis done in patients who received neoadjuvant chemotherapy before treatment, after treatment and control group; SPSS17.0 program was used to examine ccfDNA level, ccfDNA integrity, mtDNA copy number and mtDNA integrity. The results were individually assessed using the Wilcoxon signed rank test and, as a group, using the Mann-Whitney U test. $p < 0.05$ was considered significant unless otherwise stated on statistical analyses.

3. Results

In this study, data were obtained from 34 women before treatment and 30 women after treatment with locally advanced breast cancer. And as a control group, 21 age-matched healthy volunteers were studied. The mean ages of the cases were 50.35 (32 - 80) years. Age range of the control group is < 50. The clinicopathologic characteristics of the patients before and after NACT were given in the previous study and table 1 [41].

3.1. ccfDNA Amount and Integrity Index at Pre- and Post-Treatment

While the mean ALU values increased, the ccfDNA integrity index decreased post-treatment. The ALU values of the healthy controls are lower than the values pre- and post-treatment ($p < 0.01$) (Figure 1).

3.2. mtDNA Amount and Integrity Index at Pre- and Post-Treatment

Analysis of the pre-treatment and post-treatment groups revealed a significant reduction in mtDNA copy number in patients prior to treatment compared to post-treatment group (mean 6956.58 to 1395.16, $p = 0.014$) (Figure 2). Following treatment, an average decrease in mtDNA integrity from 0.58 to 0.50 was observed, although statistical significance was not reached ($p = 0.135$) (Figure 3).

The mtDNA copy number pre-treatment was found to be higher than the control group, which was statistically significant ($p < 0.001$) (Figure 2). Analysis of the mtDNA integrity index revealed a value of 0.58 in breast cancer patient's pre-treatment. In contrast, the control group demonstrated a mean integrity index of 0.72. However, statistical analysis showed no significant difference between the two groups ($p = 0.690$) (Figure 3).

When we examine the difference between the post-treatment and control groups; the mtDNA copy number post-treatment was approximately 7.34 times higher than the control group ($p = 0.002$) (Figure 2). However, the mean mtDNA integrity index was 0.72

in the control group, it was 0.50 post- treatment, and this difference was not statistically significant ($p = 0.528$) (Figure 3).

3.3. The Result of Wilcoxon signed-rank Test at Pre- and Post-Treatment

We have pre- and post-treatment data for only 28 of 34 patients. When we look at the pre- and post-treatment results of this patient group, the post-treatment ccfDNA level was lower than the pre-treatment group and it was statistically significant ($p = 0.007$). The ccfDNA integrity index of the post- treatment group was higher than pre-treatment group and these results were not statistically significant ($p = 0.665$) (Figure 4).

It was statistically significant that the mtDNA copy number was higher post-treatment than pre- treatment ($p = 0.031$). The mtDNA integrity index post- treatment was higher than pre- treatment, and these results were not statistically significant ($p = 0.820$) (Figure 5).

3.4. ccfDNA, mtDNA, CTC, EMT ve ALDH1 at Pre- and Post-Treatment

We aimed to examine the relationship between changes in plasma cfDNA and mtDNA copy number and integrity with CTC, EMT and ALDH1 data obtained in a previous study by our team [41]. Detailed results of CTC, EMT and ALDH1 pre- and post- NACT are given in the previous study [41]. All cfDNA and mtDNA results with other biomarker results are given in supplementary data. No correlation was found ccfDNA levels and mtDNA copy number with CTC, EMT and stem cell markers pre- and post- NACT. The results were not statistically significant ($p > 0.05$).

3.5. Relationship of ccfDNA levels and mtDNAcn with Breast Cancer Type, Therapy Response and Metastasis

When we compare the same patient group with previous study data, in only 1 of 6 patients who were both negative for metastasis and showed complete pathological and clinical

response, CTC, EMT and ALDH1 markers pre- and post- NACT are also negative. However, mtDNA copy number decreased while ccfDNA level increased after treatment. But it was not found significant ($p > 0.05$). Patients' ccfDNA and mtDNA levels were analysed according to breast cancer type. No significant results were found between them ($p > 0.05$) (Table 2).

4. Discussion

Breast cancer stands as the leading form of cancer affecting women globally, presenting numerous challenges for effective treatment [1]. Traditional methods such as tissue biopsy, although widely employed, have limitations in terms of comprehensively detecting the disease and monitoring treatment response. Additionally, the site of metastases can pose obstacles to biopsy procedures [9,16]. Therefore, there is a critical clinical need for non-invasive biomarkers that can aid in the diagnosis and follow-up of breast cancer [46].

Breast cancer exhibits a complex molecular landscape, necessitating innovative approaches for its detection and monitoring. The advent of liquid biopsy, which involves the analysis of circulating tumor cells (CTCs), cell-free DNAs (cfDNAs), and mitochondrial DNA (mtDNA) in plasma and serum, has emerged as a promising non-invasive tool in cancer diagnostics [9,16,47]. Cancer cells can enter the bloodstream early in the disease process, even before the detection of a tumor, and can disseminate throughout the body. By capturing and analysing CTCs, cfDNAs, and mtDNA released through apoptosis, necrosis, or active release during tumor growth, liquid biopsy enables the comprehensive assessment of disease dynamics [47].

Plasma ccfDNA and mtDNA were evaluated as blood biomarkers to assess neoadjuvant chemotherapy response in breast cancer patients. Changes in ccfDNA level, mtDNA copy

number, and integrity were detected. The relationship with CTC molecular analysis and EMT was explored, aiming to predict metastasis development and estimate patient outcomes.

Consistent with previous findings, breast cancer patients exhibited higher plasma levels of circulating cell-free DNA (ccfDNA) compared to controls, alongside elevated levels of ALU 115 and ALU 247. This is likely due to increased release of fragmented DNA from apoptotic and necrotic cells in breast cancer patients. Similarly, a study involving breast and prostate cancer patients reported a higher ccfDNA integrity index in prostate cancer patients compared to controls, while breast cancer patients had a lower index. The amount of cfDNA released into circulation is influenced by factors such as cancer stage, tumor mutation load, and DNA clearance rate. The ALU DNA integrity index has been suggested as a more advantageous marker than absolute ccfDNA levels, as it correlates with tumor cell death. However, variations in DNA integrity index among different cancer types and individual cases highlight its heterogeneity and complexity. When we examined all the patients we have, ccfDNA levels increased significantly post-treatment, while the ccfDNA integrity index decreased and was not statistically significant. Severe destruction of cells and different number of pre- and post-treatment data may affect this situation. In studies conducted with various cancer patients differences in the integrity index were observed while ccfDNA levels increased post-treatment [43,45,48,49]. These findings highlight the importance of ALU 115 and ALU 247 levels, as well as ccfDNA integrity, as potential biomarkers for breast cancer.

In 28 patients with both pre- and post-treatment data, a significant decrease in ccfDNA levels and a non-significant increase in ccfDNA integrity were observed post-treatment. These findings align with a study by Adusei et al., who also reported a decline in serum

ALU 247 and ALU 115 levels and an increase in ccfDNA integrity after three cycles of chemotherapy in breast cancer patients [14]. These findings emphasize the dynamic nature of plasma ccfDNA levels in response to treatment in breast cancer patients. The significant decrease in ccfDNA levels following treatment indicates its potential as a monitoring tool for treatment response. Further investigations are warranted to explore the underlying mechanisms driving the alterations in ccfDNA levels and integrity, and to evaluate their clinical implications in breast cancer management.

Chemotherapy treatment leads to the destruction of cancer cells, resulting in the release of cellular DNA into the bloodstream and subsequently increased levels of circulating cell-free DNA (ccfDNA) in the blood [48]. However, post-treatment measurements reveal lower ccfDNA levels, indicating a reduction in cancer cell population and activity, as well as an efficient clearance system for cfDNA. The extensive destruction of cancer cells during treatment may contribute to the release of longer DNA fragments, potentially affecting DNA integrity [14]. Studies in breast and colorectal cancer patients have demonstrated a decrease in ALU 115 levels and an increase in integrity following treatment [31,50,51]. These observations highlight the dynamic nature of ccfDNA and its potential as a valuable biomarker in monitoring chemotherapy response.

Our findings revealed a higher mitochondrial DNA (mtDNA) copy number in breast cancer patients compared to controls, with the highest levels observed pre-treatment. These results were statistically significant. In contrast, mtDNA integrity was higher in the control group, although not statistically significant. Studies have reported conflicting results regarding mtDNA copy number, with some showing higher levels in the patient group and others demonstrating lower levels [52–58]. Consistent with our results, a study in breast cancer patients found the highest mtDNA copy number in late-stage cancer

patients, while healthy individuals and early-stage cancer patients exhibited lower levels [59]. Additionally, elevated ND1 levels have been observed in thyroid and colorectal cancer patients compared to normal individuals, potentially indicating increased replication-induced mtDNA damage and the need for compensatory mtDNA molecules in tumor tissues [38,40,57]. Although the mtDNA integrity index was not statistically significant and lower in patients due to fragmented DNA and increased copy number, further research could explore its potential as a treatment follow-up marker in breast cancer.

In our current study, we also aimed to examine the relevance of the previous findings to our research. However, we did not observe any statistically significant associations between ccfDNA levels, mtDNA copy number (mtDNAcn), and CTCs, EMT, ALDH1, treatment response, or metastasis. To gain a comprehensive understanding of these biomarkers' clinical significance, further investigations involving larger patient cohorts are warranted. Serial monitoring and characterization of these biomarkers at specific time points during treatment are essential to elucidate their potential as clinically meaningful indicators. This study is the first to comprehensively investigate plasma ccfDNA levels, mtDNA copy number, and their integrities simultaneously. It is also the first to explore the relationship between these biomarkers and CTCs, cancer stem cell markers, treatment response, and metastatic status. Differences in biomarker levels observed in our study may stem from variations in factors such as blood collection periods, sample pre-processing, storage, DNA isolation procedures, as well as clinical characteristics including tumor stage, size, and patient age. The findings highlight the potential of ccfDNA and mtDNA as biomarkers for monitoring chemotherapy response in breast cancer. Non-invasive methods for cancer detection and monitoring have gained

significant attention in recent years. These approaches offer the potential for improved patient comfort, reduced invasiveness, and enhanced accessibility. The development of reliable biomarkers that can be detected through non-invasive means is crucial to address these clinical needs. However, due to the limited dataset and lack of pre- and post-treatment results, further confirmation in larger patient cohorts is necessary to validate our findings.

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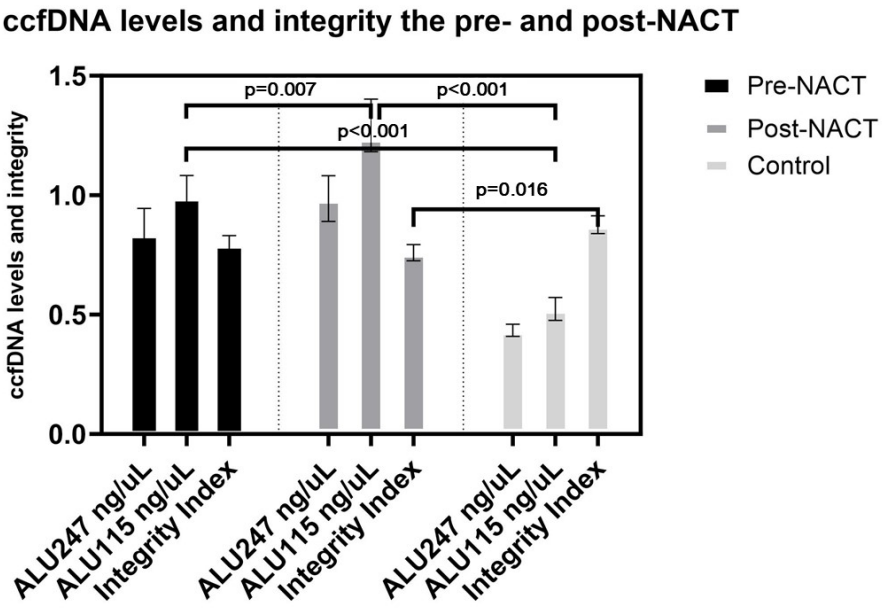
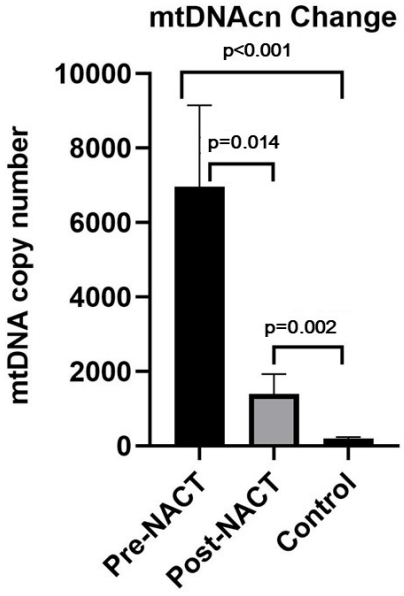


1

2 Figure 1: ccfDNA levels and integrity the pre- and post-NACT



3

4 Figure 2: mtDNAcn the pre- and post-NACT

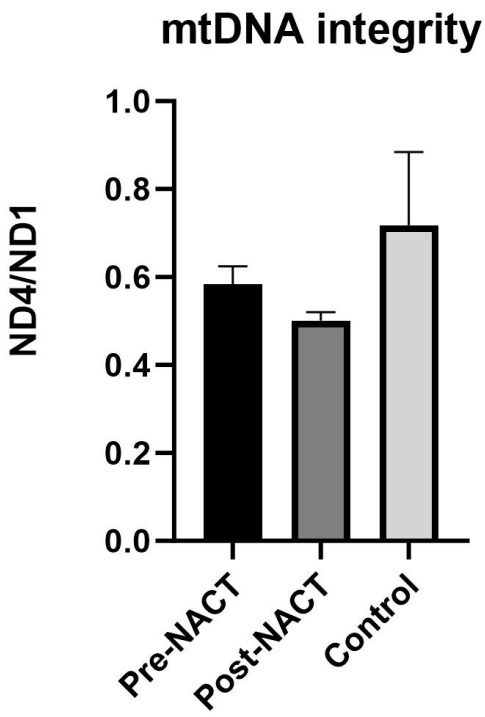


Figure 3: mtDNA integrity the pre- and post-NACT

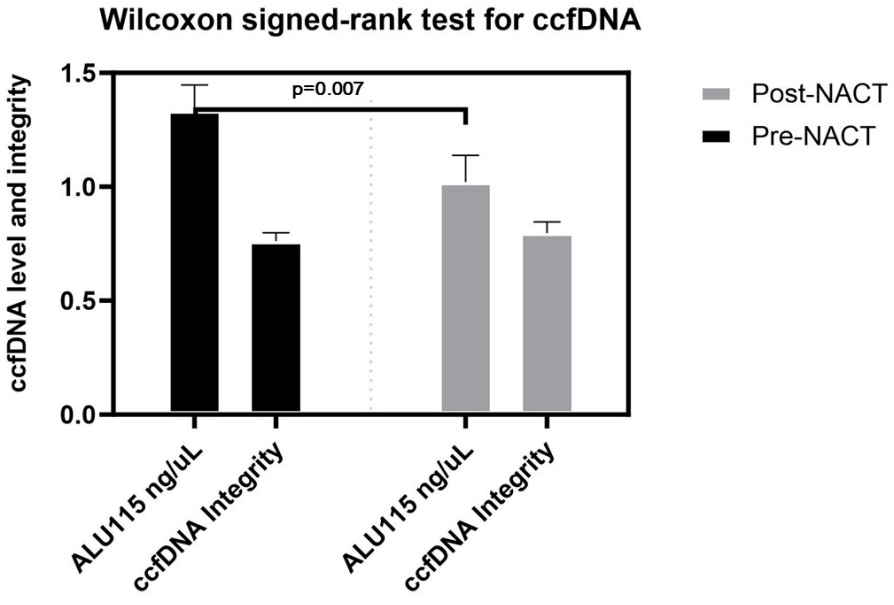


Figure 2: Wilcoxon signed-rank test for ccfDNA

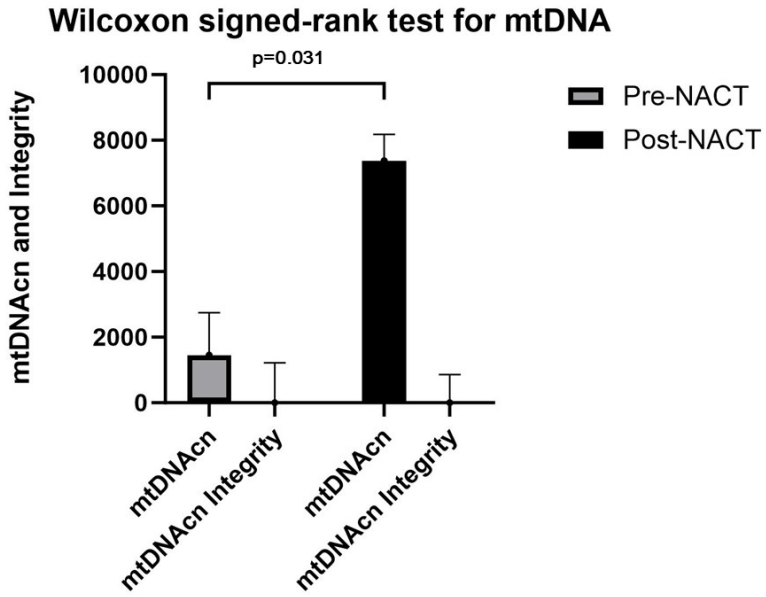


Figure 5: Wilcoxon signed-rank test for mtDNA

Table 1: Clinicopathologic Features of Patients

Parameters	Patients	Parameters	Patients
Age		**Node**	
>50	17	Pozitif	28
<50	16	Negatif	5
Menopouse		**ER**	
Pre	17	ER(+)	23
Post	16	ER(-)	10
Grade			
G0	22	**PR**	
G1	2	PR(+)	13
G2	6	PR(-)	20
G3	3		
Molecular Subtype			
Luminal A	7	**HER2**	
Luminal B	16	HER2(+)	12
TNBC	5	HER2(-)	21
HER2 Enriched	5		
Histologic Subtype			
Invasive Ductal Carcinoma	26		
İnvasive Lobular Carcinoma	2	**Lymphatic Invasion**	
Invasive Breast Carcinoma	2	Positive	13
Invasive Mucinous Carcinoma	1	Negative	6
Medullary Carcinoma	2		
Tumor size			
T1	4	**Masculine Invasion**	
T2	19	Positive	1
T3	5	Negative	18
T4	4		

Table 2: Pre- and post-NACT ccfDNA, mtDNAcn, CTC, EMT, ALDH1 and Pathologic and Clinical Response

			ccfDNA	mtDNA cn	CTC		EMT		ALDH1		Theraphy Response		Metastasis During
Patient No	**Molecular Subgroup**	**Neoadjuvant Chemotherapy Regime**	**Pre-NACT**	**Post-NACT**	**Pre-NACT**	**Post-NACT**	**Pre-NACT**	**Post-NACT**	**Pre-NACT**	**Post-NACT**	**Pathologic**	**Clinical**	
P1	Lum A	4 × EC + 12 × Paclitaxel	-	-	N		N		N		PR	CR	N
P2	Lum B	-	-	-	P	N	N	P	P	N	PR	PR	N
P3	Lum B, HER2^{+}	-	-	-	N	P	N	N	N	N	PR	CR	N
P4	Lum B, HER2^{+}	4 × EC + 12 × Paclitaxel + Herceptin	↑	↑	N	N	N	N	N	N	PR	PR	N
P5	Lum B, HER2^{+}	12 × Paclitaxel + 4 × Herceptin + 4 × EC	↑	↑	N	N	N	N	N	N	CR	PR	N

P6	Lum B, $HER2^+$	-	-	-	N		N		P		CR	PR	N
P7	Lum B	4 × EC + 12 × Paclitaxel	↑	↓	N	N	N	P	P	N	CR	CR	N
P8	Lum B	4 × EC + 12 × Paclitaxel	↑	↓	N	N	N	N	N	N	PR	PR	N
P9	Lum B	4 × EC + 12 × Paclitaxell	↑	↑	N	N	N	N	N	N	CR	CR	N
P10	Lum A	4 × EC + 12 × Paclitaxel	↓	↓	N	N	N	N	P	N	PR	PR	N
P11	TN	4 × EC + 9 × Paclitaxel	↓	↓	N	N	N	P	N	N	PR	PR	N
P12	Lum A	-	↑	↓	P	N	P	P	P	N	PR	PD	P
P13	Lum A	Anastrozole	↑	↑	N	N	N	N	N	N	PR	PR	N
P14	HER2 enriched		-	-	N	N	P	N	N	N	PR	PD	N
P15	Lum B, $HER2^+$	4 × EC + 12 × Paclitaxel + 4 × Herceptin	↑	↓	N	N	N	N	N	N	PR	CR	N
P16	Lum B, $HER2^+$	4 × EC + 12 × Paclitaxel + 4 × Herceptin	↑	↓	N	N	N	N	N	N	PR	PR	N
P17	Lum A	12 × Paclitaxel + 4 × EC	↑	↓	P	N	P	N	P	N	PR	PR	P
P18	Lum B	12 × Paclitaxel + 4 × AC	↑	↓	N	N	N	N	N	N	PR	PR	N
P19	TN	4 × AC + 10 × Paclitaxel	↓	↓	N	N	N	N	N	N	PR	PD	P
P20	Lum B, $HER2^+$	-	-	-	N	N	N	N	N	P	PR	PR	N
P21	Lum A	4 × EC + 12 × Paclitaxel	↑	↓	N	N	N	N	N	N	PR	PR	N
P22	Lum B	4 × EC + 9 × Paclitaxel	↑	↓	N	N	N	N	N	N	CR	CR	N
P23	TN	4 × EC + 12 × Paclitaxel	↓	↑	N	N	N	N	N	N	CR	CR	N
P24	Lum B	4 × EC	↓	↓	N	N	N	P	N	P	PR	PR	N
P25	Lum B	4 × EC + 11 × Paclitaxel	↑	↓	N	N	N	N	N	N	PR	PR	N
P26	HER2 enriched	12 × Paclitaxel + 4 × Herceptin + 4 × AC	↑	↓	N	N	N	N	P	N	CR	CR	N
P27	Lum B	4 × AC	↑	↑	N	P	N	N	P	N	PR	PR	N
P28	TN	4 × EC + 12 × Paclitaxel	↑	↓	P	P	N	N	N	N	PR	PR	N
P29	HER2 enriched	12 × Paclitaxel + 6 × Herceptin + 4 × AC	-	-	N	N	N	N	N	P	CR	CR	P
P30	Lum B	4 × EC + 12 × Paclitaxel	↑	↓	P	N	N	N	P	N	PR	PR	P
P31	Lum B, $HER2^+$	12 × Paclitaxel + 4 × Herceptin + 4 × AC	↑	↓	N	P	N	N	N	N	PR	PR	N
P32	Lum A	4 × AC + 9 × Paclitaxel	↓	↓	N	N	N	N	N	N	PR	PD	N
P33	HER2 enriched	10 × Paclitaxel + 7 × Herceptin + 4 × AC	↑	↑	N	P	N	N	N	N	CR	PR	N
P34	TN	4 × AC + 12 × Paclitaxel	↑	↑	N	P	N	N	N	N	PR	PR	P
P35	HER2 enriched	4 × AC + 12 × Paclitaxel + 4 × Herceptin	↑	-	N	N	N	N	N	N	CR	CR	N
P36	Lum B, $HER2^+$	12 × Paclitaxel + 4 × Herceptin + 4 × AC	-	-	P		N		N		-	-	N

Abbreviations: ALDH1: tumor stem cells marker; CR: complete response; CTC: circulating tumor cell; EMT: epithelialemesenchymal transition; HER2: Human epidermal growth factor receptor 2; Lum A: luminal A; Lum B; luminal B; NACT: neoadjuvant chemotherapy; N: negative; P: positive; PD: progressive response; PR: partial response; TN: triple negative