ACIBADEM MEHMET ALI AYDINLAR UNIVERSITY
INSTITUTE OF HEALTH SCIENCES

ASSOCIATION OF MITOCHONDRIAL DNA COPY NUMBER VARIATIONS IN TRIPLE NEGATIVE BREAST CANCER PATIENTS

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M.Sc. THESIS

DEPARTMENT OF GENOME STUDIES

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DECLARATION

I declare that this thesis work is my own work, I had no unethical behavior at any stage from the planning to the writing of the thesis, I obtained all the information in this thesis in accordance with academic and ethical rules, and I cited all the information and comments that were not obtained with this thesis work, and I provided resources in the list of references. I also declare that there was no violation of any patents and copyrights during the study and writing of this thesis.

25/06/2024

Karin Manto

PREFACE AND ACKNOWLEDGEMENT

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This thesis is dedicated to all those affected by breast cancer, with the hope that our collective efforts will contribute to better outcomes and improved quality of life for patients and their families.

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ABBREVIATIONS AND SYMBOLS

ACR	American College of Radiology
ATP	Adenosine triphosphate
CNV	Copy number variation
COXI	Cytochrome oxidase subunit I
COXII	Cytochrome oxidase subunit II
COXIII	Cytochrome oxidase subunit III
Ct	Threshold cycle number
CYB	Cytochrome B
D-loop	Displacement loop
DNA	Deoxyribonucleic Acid
ER	Estrogen receptor
ESMO	European Society for Medical Oncology
FFPE	Formalin-fixed paraffin-embedded
HER2	Human epidermal growth factor receptor 2
IARC	International Agency for Research on Cancer
IHC	Immunohistochemistry
LAR	Luminal androgen receptor
M	Mesenchymal
MRI	Magnetic resonance imaging
mtDNA	Mitochondrial DNA
N	Corresponding normal tissue
NCCN	National Comprehensive Cancer Network
ND1	NADH dehydrogenase 1
ND2	NADH dehydrogenase 2
ND3	NADH dehydrogenase 3
ND4	NADH dehydrogenase 4
ND5	NADH dehydrogenase 5
ND6	NADH dehydrogenase 6
nDNA	Nuclear DNA
OD	Optical density

pCR	Pathologic complete response
PCR	Polymerase chain reaction
PR	Progesterone receptor
qPCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid
ROS	Reactive oxygen species
T	Tumor tissue
TNBC	Triple negative breast cancer
WHO	World Health Organization

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ABSTRACT

Association of Mitochondrial DNA Copy Number Variations in Triple Negative Breast Cancer Patients

Alterations in mitochondrial DNA (mtDNA) have been associated with various cancer types, impacting cancer risk, metastasis, and prognosis. In this context, mtDNA copy number variations (CNV) are strong candidate biomarkers. Triple negative breast cancer (TNBC) is the most aggressive subtype, therefore it is crucial to choose treatment methods. However, the specific relationship between mtDNA-CNV and TNBC is unknown. Understanding mtDNA's role in diagnosis and treatment approaches can significantly contribute to the prognosis and effective treatment of these patients.

Our aim is to identify mtDNA biomarkers for prognostic purposes by investigating mtDNA CNV in TNBC patients undergoing neoadjuvant treatment. To achieve this, total DNA was isolated from tumors and corresponding normal tissues. Quantification of mtDNA and nuclear DNA (nDNA) was conducted using real-time quantitative PCR with SYBR-green and specific primers. Statistical analyses, including student's t-test, were performed to evaluate the significance of the results ($P<0.05$).

Among 23 samples, 18 exhibited a decrease in mtDNA content in tumor tissue compared to its corresponding normal tissue, significantly. In our study, there was no significant association between age, response to neoadjuvant chemotherapy and mtDNA content in analyzed samples. In conclusion, further research is necessary to fully elucidate the clinical implications of mtDNA CNV to be used as a biomarker for risk assessment.

Keywords: Triple Negative Breast Cancer, mtDNA, copy number variations, biomarker, quantitative PCR

ÖZET

Üçlü Negatif Meme Kanseri Hastalarında Mitokondriyal DNA Kopya Sayısı Değişimlerinin İlişkisi

Araştırmalar mitokondriyal DNA (mtDNA) kopya sayısı değişimlerinin çeşitli kanser türleriyle ilişkili olduğunu ortaya koymaktadır. Bu bağlamda, mtDNA kopya sayısı değişimleri (KSD), kanser için güçlü bir biyobelirteç adayıdır. Üçlü negatif meme kanseri (ÜNMK), meme kanserinin en agresif alt tipi olarak kabul edilmektedir, bu nedenle tedavi yöntemlerinin hastanın gereksinimlerine göre dikkatle seçilmesi önemlidir. Ancak mtDNA KSD ve ÜNMK arasındaki spesifik ilişki bilinmemektir. Tanı ve tedavi yaklaşımlarında mtDNA'nın rolünün anlaşılması bu hastaların prognozuna ve etkin tedavi almalarına önemli katkılar sağlayabilir.

Bu kapsamda amacımız neoadjuvan kemoterapisi gören ÜNMK hastalarında mtDNA KSD'lerini araştırarak prognostik amaçlar için mtDNA biyobelirteci tanımlamaktır. Bu doğrultuda, tümör dokularından ve bu dokulara karşılık gelen sağlıklı dokulardan total DNA izolasyonu gerçekleştirildi. Sonrasında mtDNA KSD'leri SYBR-green'e dayalı kantitatif gerçek zamanlı-PZR ile ölçüldü. Sonuçların anlamlılığını değerlendirmek için Student T testi ile istatistiksel analizler yapıldı, ($P<0.05$).

Yapılan analizler sonucunda 23 örnekten 18'inde tümör dokusundaki mtDNA seviyesi, karşılık gelen normal dokuya kıyasla anlamlı ölçüde daha düşük seviyede tespit edildi. Çalışmamızda analiz edilen örneklerde yaş, neoadjuvan kemoterapiye yanıt durumu ile mtDNA seviyeleri arasında anlamalı bir ilişki bulunamadı. Sonuç olarak, risk değerlendirmesi için biyobelirteç olarak kullanılacak mtDNA KSD'nin klinik etkilerini tam olarak aydınlatmak için daha fazla araştırma yapılması gerekmektedir.

Anahtar Sözcükler: Üçlü Negatif Meme Kanseri, mtDNA, kopya sayısı değişimleri, Biyobelirteç, kantitatif PCR.

1 INTRODUCTION AND AIM

Mitochondria play a crucial role in various biological processes such as energy production, the generation of free oxygen radicals, and apoptosis (1). Human cells contain hundreds to 1,000 mitochondria, with each mitochondrion housing 2-10 copies of the mitochondrial genome (2–4). Unlike the nuclear genome, the mitochondrial genome is more susceptible to damage due to the lack of introns and protective histones, limited DNA repair capacity and a high reactive oxygen species (ROS) rate generated in mitochondria, potentially resulting in mutations or copy number variations (5,6).

The copy number of mitochondrial DNA (mtDNA) represents the quantity of mitochondria in each cell, essential for meeting the cell's energetic requirements (7). Even though mtDNA copy number may differ among cell types, a correlation has been observed among various cell types within the same individual (8). Changes in mtDNA copy number can lead to abnormal mitochondrial function, impacting processes such as apoptosis and cell growth. Therefore, aberrant mtDNA content may alter oxidative phosphorylation and increase ROS production in aerobic metabolism, potentially causing mutations, and facilitating malignant transformation (9). Polymorphisms and mutations in mtDNA have been associated with different cancers, with an increase and decrease in tumor tissue, body fluids, or peripheral white blood cells, suggesting a potential marker for cancer risk, development, and metastasis.

Despite numerous studies on mtDNA content in cancer, clear information is still lacking (10). Various alterations in mtDNA content have been reported in cancerous tissues compared to normal tissues, including an increase in endometrial adenocarcinoma, and a decrease in colorectal and cervical cancers (11).

Regarding breast cancer, several studies have investigated the impact of mtDNA content on drug response, phenotype, and prognosis. When compared to surrounding tissues, breast tumor tissues frequently have lower mtDNA content, with indications that low mtDNA content is associated with a more phenotype that is more aggressive

and altered therapy response (10). However existing studies on breast cancer and mtDNA copy number variations have limitations, such as having a relatively small sample size or lack of information about administered treatments (10,12–14). Notably, there is no dedicated study on mtDNA copy number variations in triple-negative breast cancer (TNBC) patients who received neoadjuvant chemotherapy.

TNBC stands out as the most aggressive breast cancer subtype. It is characterized by notably low levels of three main receptors; estrogen, progesterone receptors and human epidermal growth factor receptor 2 (HER2). These receptors have growth and proliferation effects that can be expressed in breast cancer cells (15). Consequently, hormone therapy cannot be applied in the treatment of TNBC. However, alternative treatments such as chemotherapy or radiotherapy, often coupled with neoadjuvant chemotherapy, can be employed. Additionally, recent advances have brought tumor-specific biomarkers, playing a crucial role in the prognosis and treatment of cancer (16).

It is important to mention that when evaluating TNBC, treatment methods should be carefully chosen to align with the patient's needs as quickly as possible. Understanding the role of mtDNA in the diagnosis and treatment approaches can significantly contribute to these patients' prognosis and allow them to receive good treatment. In cases where neoadjuvant therapy proves unsuccessful, there is a risk of delayed surgery, the development of unresectable disease, or the spread of metastatic tumors.

Investigating mtDNA copy number variations in TNBC patients who received neoadjuvant chemotherapy may uncover specific biomarkers capable of predicting treatment outcomes and refining therapeutic strategies.

Our aim is to identify mtDNA biomarkers for prognostic purposes by comparing mtDNA copy number changes in TNBC patients who have undergone neoadjuvant treatment. To achieve this, total DNA isolation will be performed from deparaffinized tru-cut tumor tissues and corresponding normal tissue. The mtDNA and nuclear DNA

quantities will be measured using real-time quantitative PCR with SYBR-green, and primers specific to nuclear and mitochondrial DNA. The significance of the results will be assessed through statistical tests such as student's t-test calculations. The ultimate goal is to identify an early-stage biomarker that can enhance the treatment process for triple-negative breast cancer patients.

2 BACKGROUND

2.1 Cancer

Cancer is a complex disease characterized by unregulated cell growth which leads to the formation of malignant tumors (17). Tumors may be either malignant or benign. Contrary to malign tumors, benign ones remain in their original location, do not invade nearby normal tissues, and do not spread to the other parts of the body. However, malignant tumors can invade any tissue and spread to other body parts through the circulatory or lymphatic system (18). Various factors, including radiation, chemicals, viruses, and inflammation, can contribute to the development of cancer, thereby prompting the transformation of a tumor into a malignant phenotype (19).

The etiology of cancer has become remarkably clear owing to the modern technology for genome sequencing. The analysis revealed aberrant gene expression profiles and epigenetic alterations in malignancies. Cancer is a hereditary disorder arising from mutations in tumor suppressor genes and oncogenes (20). The majority of mutations are not associated with recurrent cancer subtypes, and most likely arise as the disease progresses, as opposed to initiating or accelerating uncontrolled proliferation. Such mutations are called “passenger” mutations. However, driver mutations contribute to the development or progression of cancer itself (20).

2.1.1 Breast cancer

Breast cancer stands as the second most prevalent cancer type and ranks fourth in terms of mortality worldwide, with over 2.3 million individuals affected, as reported by the World Health Organization (WHO) (21,22). Incidence and mortality rates of breast cancer increased by 50% and 40%, respectively compared to results from around ten years ago (23). By 2040, there will be 3.06 million additional cases of breast cancer, up from the current trend, according to the International Agency for Research on Cancer (IARC) (24). The incidence and mortality rate of breast cancer are rising annually in emerging nations like Turkey. According to 2022 WHO data,

approximately 25.2 thousand new cases were reported in breast cancer, which is the most common type among women in Turkey, and approximately 7,000 cases resulted in death (21).

Metastasis is often the leading cause of mortality in breast cancer cases. Although metastatic breast cancer represents 10-15% of all breast cancer cases, the rate of metastasis is decreasing due to advances in early detection facilitated by mammographic screening and routine clinical examinations (25).

2.1.1.1 Risk factors

Breast cancer is a complex disease, and its development can be affected by genetic, hormonal, environmental and lifestyle factors. While the exact cause is not always clear, various risk factors have been identified that could heighten a person's susceptibility to developing breast cancer. It is crucial to emphasize that having one or more risk factors does not ensure that someone will develop breast cancer. Additionally, many patients diagnosed with breast cancer have no known risk factors that can be listed as below (26,27):

1. Non-modifiable risk factors: These factors are intrinsic, and independent, and do not go through simple alteration in a person's life, such as gender, age, genetic features, ethnicity, family history.
2. Modifiable risk factors: contrary to non-modifiable risk factors, these factors are extrinsic. They can modify the neoplastic course to a certain level. Identified modifiable risk factors may help to develop prevention schemes therefore reducing the occurrence of breast cancer. Modifiable risk factors are lifestyle-related such as the use of birth control pills, breastfeeding, the number of given births, hormone therapy, overweight, physical activity, alcohol use, and smoking.
3. Controversial risk factors: These factors have uncertain, debatable, or unconfirmed effects on the probability of breast cancer development, such as vitamin intake, diet, abortion, and chemicals.

2.1.1.2 Anatomy of breast

The breast is supported and attached to the front of the chest wall on the breastbone by ligaments. Each breast contains 15 to 20 lobes and each of these lobes functions as a milk-producing gland with many small lobules. Lobes and lobules are connected to each other by milk ducts, which are responsible for carrying milk to the nipples (28) (Figure 1). The breast comprises three main parts: skin, subcutaneous, and breast tissues. Breast tissue contains both epithelial and stromal elements. The epithelial components take about 10-15% of the overall breast volume, and the remaining volume consists of the stromal elements (29) .

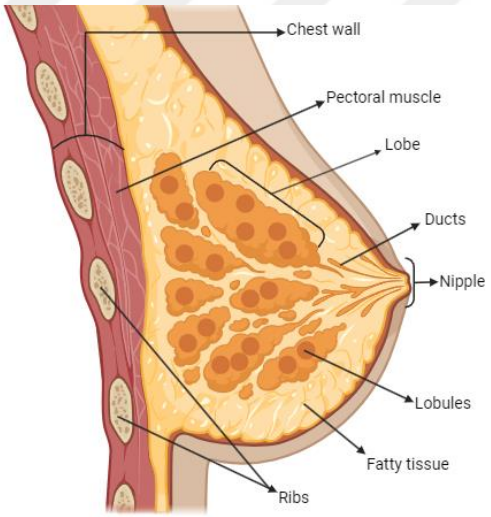

Figure 1 Anatomy of the breast (30)

The breast skin contains hair follicles, sebaceous and sweat glands. Within each breast lies an areola, a pigmented structure, housing a nipple at its center. These structures do not contain any hair follicles, sebaceous and sweat glands (31).

The majority of the breast parenchyma extends from the lower second or third rib down to the lower breast fold, around the sixth or seventh rib level. Laterally, it extends from the edge of the sternum to the anterior axillary line. Apart from the nipple and areola, it is surrounded by fatty tissue from all sides (32).

The breast lacks clearly defined boundaries unlike some other organs, thus two common methods are employed to locate lesions in clinical practice. The first method involves determining positions in a clockwise manner and based on distance from the nipple. Alternatively, lesions can be located according to breast quadrants. These quadrants include the upper inner and outer quadrants, lower inner and outer quadrants. The upper outer quadrant contains the majority of breast volume, making it the primary site for breast tumor occurrence (29).

2.1.1.3 Classification

Breast cancer, like all types of cancer, exhibits distinct histopathological and molecular characteristics and it is also heterogeneous both clinically, and molecularly. This heterogeneity plays a big role in disease classification (33). While histologic appearance forms the basis of classification, various classification methods have been developed.

WHO has identified at least 21 distinct histomorphologic types of breast cancer (34). Following the initial diagnosis with the confirmation of primary breast tumor, further histological classification usually depends on evaluating the type and level of differentiation and histological grade. This assessment involves examining slides stained with hematoxylin and eosin, considering findings supported by special stains, immunohistochemistry (IHC), and other molecular assays (35).

The breast cancer's histopathological classification involves categorizing tumors based on their microscopic characteristics observed in tissue samples. This classification helps in understanding the behavior of the cancer and guides treatment decisions (36). Carcinomas are malignant tumors arising from epithelial tissue, affecting the cells that within lobules and breast milk ducts. They are classified as invasive, in situ and metastatic based on their behavior and spread (37). Ductal carcinoma, originates in breast milk ducts, forming malignant tumors within these structures (37). Invasive ductal carcinoma is one of the most encountered types, accounting for 50-80% of all breast cancers (38). Lobular carcinoma is characterized

by its origin in the lobules of the breast tissue (37). Lastly, metastatic carcinoma, representing advanced breast cancer, refers to the state where tumor cells migrate to distant sites within the body (37). WHO provides additional histological classification of breast cancer, as shown in Table 1 (36,39).

Table 1 Histopathological classification of breast tumors according to the WHO

Histopathological classification of breast tumors according to the World Health Organization
• Adenomas • Adenosis and benign sclerosing lesions • Benign epithelial proliferations and precursors • Ductal carcinoma in situ (DCIS) • Epithelial myoepithelial tumors • Invasive breast carcinoma • Fibroepithelial tumors • Mesenchymal tumors • Neuroendocrine neoplasms • Noninvasive lobular neoplasia • Papillary neoplasms • Rare and salivary gland-type tumors • Tumors of the nipple

Another important classification parameter is defining the stage of breast cancer. The TNM classification staging system is utilized for staging breast cancer, as with all cancer. TNM stands for T: Tumor, N: lymph node, M: metastases, and are staged between 1-3 scores (40). The staging of breast cancer is conducted in two terms: clinical staging (cTNM) which is determined by the patient's physical examination and imaging studies carried out before treatment, and pathologic staging (pTNM) based on findings after surgery. If a patient undergoes surgery after receiving neoadjuvant chemotherapy, it is referred as ypTNM (40). In TNM staging, as the stage increases, the prognosis worsens and 5-year survival decreases (26,40). The TNM classification terms are described in Table 2.

Table 2 TNM classification terms

Classification criterion	Stage	Description	
T (Tumor)	Tx	Primer tumor is not evaluated.	
	T0	There is no sign of tumor	
	Tis	Paget disease or ductal/luminal carcinoma in situ	
	T1	Tumor is 20 mm across or less	
		T1a	1 mm<tumor< 5 mm
		T1b	5 mm<tumor<10 mm
		T1c	10 mm<tumor<20 mm
	T2	20 mm<tumor<50 mm	
	T3	50 mm<tumor	
	T4	T4a	The tumor has extended into the chest wall
		T4b	The tumor invaded the skin and breast, potentially causing swelling
		T4c	The tumor metastasized to the skin and chest wall
		T4d	Inflammatory carcinoma
N (Lymph node)	Nx	It is not possible to evaluate regional lymph node.	
	N0	There is no lymph node metastasis	
	N1	The tumor cells are spread to one or more lymph nodes	
	N2	N2a	Tumor cells that are adhered together or attached to other regions of the breast
		N2b	Tumors are located in lymph nodes behind the breastbone, without any proof of cancer in the lymph nodes of the armpit.
	N3	cN3	Metastasis in the ipsilateral internal mammary lymph nodes may occur independently or alongside level I lymph node involvement. Alternatively, metastasis may extend from the ipsilateral internal mammary lymph nodes to include both level I and II axillary lymph nodes. Another possibility is metastasis to the ipsilateral supraclavicular lymph nodes.

Table 2 TNM classification terms (continue)

N (Lymph node)		pN3	Metastases may be present in 10 or more axillary lymph nodes, infraclavicular lymph nodes, or positive ipsilateral internal mammary lymph nodes. Additionally, metastasis may occur in more than 3 axillary lymph nodes, with micrometastases or macrometastases identified by sentinel lymph node biopsy in clinically negative ipsilateral internal mammary lymph nodes or ipsilateral supraclavicular lymph nodes.
M (Metastasis)	MX	Distant metastasis has not yet been investigated	
	M0	No signs of distant metastasis were observed.	
	cM0(i+)	Tumor cells have been detected in the bloodstream, bone marrow, or distant lymph nodes, but there are no indications of the tumor spread to other parts during physical examination or scans.	
	M1	The tumor had spread to another part of the body.	

Recent advancements in technology, such as high-throughput technologies, have provided a new level of understanding the complexity of genetic changes and the presence of intertumoral heterogeneity. Array-based technologies have made it possible to quantify DNA copy number alterations and profile the global expression of thousands of genes in a single experiment. Next-generation sequencing has enabled chromosomal rearrangements, detection of mutations, and copy number variations across the genome, even those confined to minor subclones of tumor cells. These technologies have significantly increased the understanding of the molecular biology of breast cancer, revealing that traditional well-established classification systems are unable to demonstrate the biological and clinical heterogeneity of breast cancer. At present, molecular subtyping of breast cancer is becoming more prominent in its ability to anticipate tumor behavior more accurately, which is essential for oncological decision making and preventing overtreatment with a poor prognosis (41).

The most common molecular subtyping is based on the known gene expression to drive cell growth signals and is typically overexpressed in breast cancer cells, the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) (42). The five primary subtypes of breast cancer identified using this approach are luminal A, luminal B, HER2 positive, basal-like, and normal breast-like (43). The classifications are presented in Table 3.

Table 3 Molecular Classification of Breast Carcinoma

Subtype	Immunohistochemical traits	Other clinical traits
Luminal A	ER^+, $PR^{+/-}$, $HER2^-$	Ki67 is low, endocrine sensitive, often sensitive to chemotherapy
Luminal B	ER^+, $PR^{+/-}$, $HER2^+$	Ki67 is high, often endocrine sensitive, variable to chemotherapy, HER2 trastuzumab sensitive
Basal-like	ER^-, $PR^{-/-}$, $HER2^-$	EGFR + and/or cytokeratin 5/6 +, Ki67 high, does not respond to endocrine, often responds to chemotherapy
HER2	ER^-, PR^-, $HER2^+$	Ki67 is high, sensitive to trastuzumab, sensitive to chemotherapy

Luminal A: Luminal A tumors make up 50-60% of all breast cancer cases. This subtype is defined by several distinct characteristics: it is PR positive, HER2 negative, exhibits low levels of Ki67 expression, demonstrates high ER levels, and shows expression of ER-activated genes and low expression of proliferative genes. Therefore, luminal A is associated with a low histological grade, low cell division and recurrence rates with a good prognosis (43–45).

Luminal B: Like Luminal A, Luminal B has a similar phenotype. Cancers that exhibit more aggressive behavior than Luminal A are referred to as Luminal B in the 2013 St. Gallen Consensus. Luminal B distinguishes itself from Luminal A by having higher Ki67 and HER2 expression. Especially, the Ki67 level is a key index to differentiate it from Luminal A. Luminal B tumors express a higher number of proliferation-related genes while expressing lower levels of ER and ER-related gene expression. Histologically, they tend to exhibit a higher grade and are more likely to

be lymph node positive. Furthermore, Luminal B tumors can be PR-negative and are associated with a worse prognosis than luminal A tumors (41,43). Notably, because luminal B is HER2 positive it exhibits heightened susceptibility to treatment with trastuzumab, an anti-HER2 antibody, and is a candidate for chemotherapy (41,46).

HER2 positive: HER2 positive breast cancer does not express the hormone receptors ER and PR, in contrast to luminal-like breast cancer. This subtype has a poor prognosis, high histological grade, high proliferative index, and early development in life (43). In HER2 positive tumors, overexpression of related genes in the HER2 pathway has been observed, including the *ERBB2* gene located on 17q12 (45).

Basal-like: This subtype is generally called “triple negative breast cancer”, characterized by the lack of three primary breast hormone receptors. It is positive for epidermal growth factor receptor (EGFR) or cytokeratin. As a result of missing three key receptors, it tends to be more aggressive and is often associated with a poor prognosis (43).

2.1.1.4 Diagnosis

Clinically, the screening and evaluation of breast lesions hold significant importance, contributing to the optimal management of the disease. Although breast symptoms are common among women, clinical findings lack specificity. Each symptom is related with an increased risk of malignancy. The most frequent symptom was breast pain followed by a palpable mass. However, most symptoms are defined as benign origin (47).

The diagnosis of breast cancer primarily depends on physical examination and diagnostic imaging, and requires confirmation by pathological evaluation. Therefore, the National Comprehensive Cancer Network (NCCN) has designed specific guidelines for breast cancer imaging and diagnosis, primarily directed towards cisgender females because of the majority of data in this demographic. For transgender

individuals, the NCCN supports the consensus-based guidelines established by the American College of Radiology (ACR) Appropriateness Criteria (47,48).

An accurate prognosis is important for treatment decisions and requires an understanding of the distinction between screening and diagnostic imaging. Hence, the NCCN Clinical Practice Guidelines in Oncology for Breast Cancer Screening and Diagnosis offer clinicians a systematic approach for screening and evaluating a range of clinical breast presentations. While the goal of diagnostic evaluation is to describe clinical findings or potential abnormalities detected during screening, imaging for screening aims to identify cancer in women who do not exhibit any symptoms (47).

Diagnostic evaluation and screening include physical examination and imaging. It also includes diagnostic mammography, ultrasonography, and magnetic resonance imaging (MRI). The decision to proceed with tissue sampling relies on the level of suspicion from imaging and/or clinical examination. In cases where imaging results are negative with suspicious clinical findings, biopsy becomes essential for a more sensitive cancer diagnosis (47).

In addition to screening and diagnostic imaging, obtaining a complete personal and family medical history, particularly regarding breast/ovarian and other cancers as well as menopausal status during physical examination, is important for achieving a definitive diagnosis. Treatment strategies should be personalized based on diagnostic parameters, such as tumor size and location, lymph node involvement, pathology, biomarkers, gene expression, age, menopausal status, and overall health status (50).

Overall, these parameters not only provide a treatment strategy during disease progression but also emphasize the significance of early detection through breast screening. The primary objective of breast screening is the early detection of breast cancer prior to the onset of signs or symptoms, thus providing less aggressive treatments and reducing associated mortality and morbidity. Identifying cancer at its initial stages enables prompt interventions, offering patients a better prognosis and enhancing their quality of life.

2.1.1.5 Triple negative breast cancer (TNBC)

Triple negative breast cancer (TNBC) mirrors the characteristics of normal breast basal epithelial cells and is believed to originate from the outer layer of the breast ducts (51). TNBC accounts for approximately 15% of global breast cancer cases (51,52) and is identified by aggressive behavior, with a high potential for early relapse, metastasis to vital organs such as the liver, lung, and central nervous system, and generally lower survival rates (53). It is a type of breast cancer that is characterized by the immunohistochemical expression absence of the ER, PR, and HER-2 (51–53). The simultaneous absence of these three main breast receptors makes treatment challenging by limiting the availability of targeted molecular therapies (52).

Genomic expression profile assays have been used to molecularly classify TNBC. Typically, TNBC is classified into seven subclasses based on clinical and pathological features: immunomodulatory, mesenchymal (M), mesenchymal stem-like, luminal androgen receptor (LAR), unstable subtype and two basal-like subtypes (BL1 and BL2) (53,54). However, retrospective studies in certain clinical trials have refined this classification into four groups: BL1, BL2, M, and LAR (54).

2.1.1.5.1 Treatment of TNBC

Although TNBC has a poor prognosis, studies indicates that TNBC is more responsive to chemotherapy compared to other subtypes (55). Treatments used for patients with ER-positive and/or HER2-positive breast cancers are unhelpful, therefore, both NCCN and European Society for Medical Oncology (ESMO) guidelines recommend the use of third-generation chemotherapy (55).

The characteristics of TNBC present challenges in treatment; however, neoadjuvant therapy has shown potential for achieving a pathologic complete response (pCR) (56). The standard approach for early stage TNBC involves adjuvant chemotherapy with anthracyclines and taxanes (52). In the advanced stages, immunotherapy has demonstrated promising outcomes, but further evaluation is

required (52). Several studies have shown that TNBC is particularly radiosensitive, and radiotherapy can provide local control and is responsive to DNA-damaging agents, such as PARP inhibitors (51,52).

Treatment for TNBC is determined by recurrence risk factors, such as tumor size and grade, as well as nodal status (51). Moreover, identifying reliable predictive biomarkers such as TP53, Ki-67, and EGFR is crucial for determining the most appropriate therapeutic options (51,52).

2.1.1.6 Neoadjuvant chemotherapy

Neoadjuvant chemotherapy may be beneficial for breast cancer patients who require cytotoxic chemotherapy. Initially, it was used in individuals with locally advanced incurable breast cancers. This approach provides an essential opportunity to evaluate the in vivo responsiveness to chemotherapy. Moreover, it facilitates research on novel treatment targets and examination of tissue imaging predictors of response, thereby contributing to a deeper understanding of treatment efficiency and potential advancements in breast cancer care (57).

Neoadjuvant chemotherapy, when combined with surgery, reduces tumor volume and regional node, allowing for more surgical alternatives (57). Clinical trials have demonstrated the ability of neoadjuvant chemotherapy to downsize tumors, thereby increasing the feasibility of breast-conserving surgery. This therapeutic approach is particularly instrumental for tumors exceeding 2 cm in size, making them operable and facilitating the separation of cancerous tissues from normal structures (58). In addition to making the surgery easier, it can support the postoperative recovery process and reduce the likelihood of long-term surgical recurrence.

Neoadjuvant chemotherapy is the standard approach for early TNBC, aiding in predicting the tumor response and guiding subsequent treatment (59). A pCR to neoadjuvant chemotherapy is related with long-term clinical benefits and improved survival. However, conventional regimens achieve pCR rates of only 35-45%. Efforts

to enhance pCR rates have led to the investigation of alternative neoadjuvant therapy combinations (59).

2.2 Mitochondria

Mitochondria, first described by Richard Altman in 1890, are one of the most important organelles in a cell and are responsible for the generation of ATP by the citric acid cycle and oxidative phosphorylation. Additionally, it plays a role in apoptosis and cell division. Mitochondria are found in almost all eukaryotic cells. While some cells contain singular large mitochondrion, most cells have hundreds or even thousands of mitochondria. The quantity of mitochondria is associated with the cellular level of mitochondrial activity (60).

Unlike other organelles, mitochondria have their own genome. Mitochondria are generally 1-10 μm long with rod-shaped or spherical structures. It is composed of outer and inner membranes that are made of phospholipid bilayers and proteins, cristae generated by inner membrane infoldings, and matrix, that is the space within the inner membrane (61).

2.2.1 Mitochondrial genome

Mitochondria house their own genomes within the mitochondrial matrix. Human cells have 16,569 bp double-stranded circular mitochondrial DNA (mtDNA), with a variable copy number ranging from 100 to 10,000 copies per cell (62,63). The double strands of mtDNA are referred as the light strand and heavy strand. The mitochondrial genome encodes a total of 37 genes, 13 subunits of respiratory chain complexes, a set of 22 transfer RNAs and 2 ribosomal RNAs essential for the synthesis of mitochondrial proteins (64) (Figure 2). The displacement loop (D-loop), approximately 1100 bases long, is responsible for heavy chain replication, does not contain any genes, and shows high polymorphism (65).

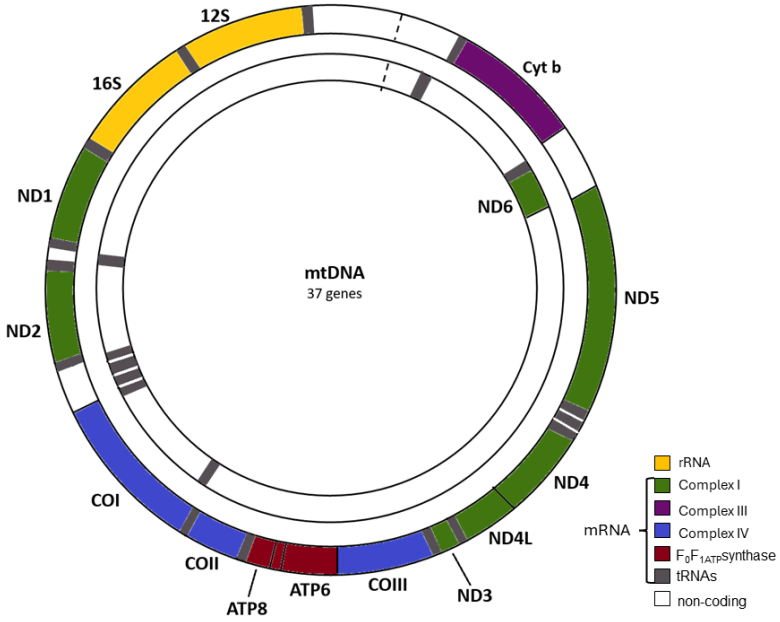

Figure 2 Mitochondrial Genome Structure (66)

Complex I serves as the primary entry point for electrons into the respiratory chain and consists of seven nicotinamide adenine dinucleotide hydride (*NADH*) dehydrogenase subunits, which are NADH dehydrogenase 1 (*ND1*), NADH dehydrogenase 2 (*ND2*), NADH dehydrogenase 3 (*ND3*), NADH dehydrogenase 4 (*ND4*), NADH dehydrogenase 4 (*ND4L*), NADH dehydrogenase 5 (*ND5*), NADH dehydrogenase 6 (*ND6*); complex III contains cytochrome B (*CYB*). Complex IV contains three subunits which are cytochrome oxidase subunit I (*COX I*), cytochrome oxidase subunit II (*COX II*), and cytochrome oxidase subunit III (*COX III*), and complex V includes *ATPase 6* and *ATPase 8* (67). Additionally, other mitochondrial proteins are encoded by the nuclear DNA, which is synthesized in the cytoplasm, and then transported into the mitochondria (65).

Complex I has a vital role in energy metabolism, prompting mitochondria to change their location and number depending on cell type (67). The replication of mtDNA occurs independently of the cell cycle, and it can also occur in post-mitotic cells (68). During mitosis, each daughter cell receives approximately an equivalent number of mitochondria; however, because there is no mechanism for dividing mitochondria evenly, some cells contain more mtDNA than others. Thus, the total

number of mtDNA hinges on mitochondria number, mtDNA molecule size, and the quantity of mtDNA molecules per mitochondrion. Each of these factors varies greatly between cell types (64). Despite this variability, mtDNA copy numbers are partially heritable and indicate a relationship between the cell types (63).

mtDNA lacks introns and histone proteins and does not have a repair mechanism, making it more prone to mutations than the nuclear genome (69). Therefore, mutations only occur on coding sequences of the genome, and in the absence of DNA repair mechanism, mtDNA replicates rapidly which may finally lead to the formation of cancer (63).

2.2.2 mtDNA copy number variations

The peculiarity of mtDNA is that multiple copies can be found in a single mitochondrion as well as multiple mitochondria can be found within a single cell. The quantity of mtDNA molecules per cell varies significantly among tissue types (68,70–72). Generally, the amount of mtDNA depends on the energy consumption of cells. For example, liver cells and skeletal muscle contain thousands of mtDNA, whereas blood lymphocytes contain hundreds (72).

The mitochondrial genome is prone to damage by oxidative stress and mutations. MtDNA mutations are associated with an increased mtDNA copy number to deal with mitochondrial dysfunction (73).

In conclusion, the mtDNA copy number depends on the tissue and cell type. Similarly, mtDNA copy numbers have prognostic value for assessing the individual risk of solid tumors, autoimmune, infectious, and musculoskeletal diseases.

2.2.3 mtDNA and cancer

The relationship between mitochondrial metabolism and cancer was initially described by Otto Warburg in the early 1920s based on his discovery that cancer cells are capable of fulfilling their energy demands through aerobic glycolysis (74).

MtDNA variations, point mutations, deletions, and rearrangements have been identified in different cancers and explored as risk factors or early diagnostic markers. However, the role of variations in disease progression remains unclear (75).

Mutations in mtDNA appear to accumulate in all cancer types; however, it is still not clear whether these mutations are causative factors or simply by-products resulting from replication in fast dividing cancer cells. Studies indicate that mtDNA mutations may accumulate due to the proliferation of pre-existing heteroplasmic polymorphisms or mutations, which passively undergo clonal expansion during tumor cell divisions. There are other arguments that mtDNA mutations have a positive role in cancer development because these mutations cause protein truncations that are counter-selected in many tumors (67).

It has been proposed that mtDNA copy number in cancer cases may vary depending on cellular and environmental factors (70). For example, studies of mitochondrial mutations/variations show that aberrations in the mtDNA D-loop region may contribute to the reduction of mtDNA copy number. Thus, an increase in mtDNA content in cancers may be associated with an increase in oxidative stress (77).

For diagnostic purposes, following the idea that blood mtDNA levels may be a marker for cancer risk, many studies have found a correlation between mtDNA copy number and disease onset. While the literature states that high mtDNA levels in the blood are correlated with an increased risk of developing papillary thyroid, colorectal, ovarian, lung, prostate, head and neck cancers, some studies show that mtDNA content in bone, kidney, liver, stomach, and breast cancer has been reported to decrease in tumor tissues. However, the risk of developing colorectal carcinoma and breast cancer

was associated with both higher and lower amounts of mtDNA in peripheral blood (10,68,70,72,78). Therefore, inconsistencies exist between the data obtained depending on the molecular method, methodology, sample group, and number used in the literature. The main reason for such inconsistencies is the lack of understanding of tissue-specific changes, even in the same individuals, when investigating the effect of mtDNA copy number on mitochondrial function (78). This shows that mtDNA levels are associated with disease severity. In conclusion, it has been suggested that changes in mtDNA copy number are associated with the cancer type.

High copy number and high number of mutations characteristic of mtDNA can be used as a biomarker for cancer detection (79). Owing to this biomarker, it is possible to have information about cancer prognosis, response to treatment, and risk of metastasis. Upregulation of mtDNA is a defense mechanism used by tumors to protect themselves from apoptosis. A low mtDNA number increases the amount of reactive oxygen; as a result of the increased amount of reactive oxygen, tumor cells become sensitive to chemotherapeutic agents and apoptosis is accelerated (80). Therefore, mtDNA copy number changes can be clinically associated with survival outcomes among cancer patients.

There are several studies that considered the effect of mtDNA content and breast cancer in the context of phenotype, drug response, tumor progression, and prognosis (10,81). Most of the studies suggested that breast cancer tumor tissue has lower mtDNA content compared to corresponding normal tissue (12,72,81). Conversely, Lin et al. have reported an increase in mtDNA content in breast cancer (11). This incompatibility suggests more studies are needed to identify a biomarker for breast cancer.

3 MATERIALS AND METHODS

3.1 Material

3.1.1 TNBC patients and tissue samples

Collaborative research was conducted with the Acibadem Health Group, Pathology, and General Surgery Departments at the Maslak Hospital. The study aimed to retrospectively include formalin-fixed paraffin-embedded tru-cut biopsies from pre-neoadjuvant tumors and corresponding normal tissue samples from TNBC patients who had undergone neoadjuvant chemotherapy.

This study adhered to all guidelines and obtained the necessary approvals from the ethics committees of Acibadem University and Acibadem Healthcare Institutions Medical Research Ethics Committee with the decision number 2022-16/01, given in Appendix 1. Patient confidentiality was ensured by anonymizing and coding the biopsy samples. The characteristics of the samples are given in Table 4.

Table 4 Characteristics of the TNBC patients.

Characteristic	**Patients (n=23)**
Age range (years)	31-69
Mean (years)	48.80 ± 10.39
<65 years	21
>65 years	2
T-stage	T0 (12); T1 (3); T2 (4); NA (4)
N-stage	N0 (13); N1 (3); N2 (2); N3(1); NA (4)
Treatment response	Complete (13); Partial (10)
Control (n)*	23

NA: not applicable
*Since control tissues are from the same individual (the corresponding normal tissue), their number and age properties are identical to those of TNBC tissue.

3.1.2 Equipment, kits chemicals

The equipment, kits and chemicals that were used during the experiments are listed in Table 5.

Table 5 Equipment, kits and chemicals

Chemicals, Kits
• QIAamp FFPE DNA kit, (QIAGEN, Germany) • 99% ethanol, (Isolab, Germany) • Xylene • GM SYBR qPCR kit (w/o Rox), (GeneMark) • Distilled and filtered water, (ddH_2O) (Milli-Q, Merck Millipore, Turkey)
Consumables
• 0.2 ml 96-well plate for qPCR (Appleton Woods LTD, UK) • 1.5 ml microcentrifuge tubes (Isolab, Germany)
Lab Devices
• Refrigerator and Freezers, (-20°C, Arctiko, Denmark; +4°) • Vortex, (Neuation, India) • Micropipette set, (Thermo Fisher, USA) • Pipette gun, (Thermo Fisher, USA) • Microcentrifuge, (Thermo Scientific, USA) • Real Time PCR device, (Bio-Rad CFX Real-Time device, USA) • Spectrophotometer, (Nanodrop) (Thermo Fisher, USA) • Heat Block, (Witeg, Germany) • Microarray Miniplate Centrifuge, (Arrayit Corporation, USA) • Mini Centrifuge/Vortex, (Combi-spin, FVL-2400N, Biosan, Latvia)

3.2 Method

3.2.1 Formalin-fixed paraffin-embedded (FFPE) DNA isolation

Total DNA was isolated using the QIAamp DNA FFPE Tissue Kit (QIAGEN, Germany) after deparaffinization with xylene. The protocol for deparaffinization and DNA isolation according to Manufacturer's Protocol with some adjustments and optimizations* is given below:

1. As starting material, 3 sections of 5 μm thick FFPE breast tissue were sliced using a microtome and then placed into a 1.5 ml microcentrifuge tube.

2. 1000 µl of xylene was added, and vortexed for 10 sec. The mixture was centrifuged at 14000 rpm for 2 min at room temperature (15-25 °C).
3. The supernatant was carefully removed using a pipette, without damaging the pellet.
4. 1000 µl ethanol (96-100%) was added and vortexed. Then, the tubes were centrifuged at 14000 rpm for 2 min at room temperature (15-25 °C).
5. The supernatant was then removed without disturbing the pellet. The cap of the tube was left open at room temperature for 10 min to allow residual ethanol to evaporate. Any remaining ethanol residue was removed using a pipette.
6. The pellet was resuspended by adding 180 µl of ATL buffer. Subsequently, 20 µl of proteinase K was added and vortexed.
7. The sample was placed in a pre-adjusted heat block at 56 °C for 3 h, with gentle shaking every 15 min.
8. Another heat-block was adjusted to 90 °C. After the initial 3 h of incubation, the sample was transferred to a 90 °C heat block for an additional 1 h.
9. The tube was spun down briefly to remove the remaining drops from the lid.
10. 200 µl Buffer AL was added to the samples and vortexed. Then, 200 µl ethanol (96-100%) was added and vortexed.
11. The tube was briefly centrifuged in order to remove the remaining drops from the lid.
12. The lysate was transferred to the QIAamp MinElute column (in a 2 ml collection tube) avoiding the wetting of the rim. Then the tube was centrifuged at 8000 rpm for 1 min. The column was then put into a new 2 ml collection tube, and the old one containing the flow-through was discarded.
13. 500 µl of Buffer AW1** was added to the column avoiding the wetting of the rim. Then the tube was centrifuged at 8000 rpm for 1 min. The column was placed into a new 2 ml collection tube, and the old one containing the flow-through was discarded.
14. 500 µl of Buffer AW2** was added to the column avoiding the wetting of the rim. The tube was centrifuged at 8000 rpm for 1 min. Then the column was placed into a clean 2 ml collection tube, and the old collection tube containing the flow-through was discarded.

15. To dry the membrane, the lid of the tube was opened and left at room temperature for 2 min, followed by centrifugation at 14000 rpm for 3 min.
16. The column was transferred to a new 1.5 ml microcentrifuge tube, and the collection tube was discarded.
17. Lids of the columns for all samples were carefully opened, and 20 µl of distilled and autoclaved water (ddH_2O) was applied to the center of the membrane.
18. The lids of all tubes were opened, left at room temperature for 5 min, following centrifugation at 14000 rpm for 1 min. This process was repeated in steps 17 and 18, until the total volume reached 100 µl.
19. The QIAamp MinElute columns were discarded, and DNA concentrations were measured using a nanodrop.

* The proteinase K treatment duration was originally indicated as 1 h in the Manufacturer's protocol. However, in a study by Frazer Z et al., it was found that the optimal time for treatment was extended to 3h rather than 1 h (82). Subsequently, in our laboratory, DNA yield was optimized by substituting the original elution buffer with ddH_2O. Additionally, instead of a one-time application of 100 µl of elution, 20 µl of ddH_2O was applied five times until the total volume reached 100 µl.

** AW1 and AW2 buffers were prepared following the Manufacturer's protocol. For AW1,19 ml buffer was mixed with 25 ml 96-100% ethanol. For AW2, 13 ml buffer was mixed with 30 ml 96-100% ethanol.

3.2.2 Spectrophotometric measurement of concentration and purity of total DNA

The purity and concentration of total DNA were measured by spectrophotometric measurement via NanoDrop ScientificTM 1000 (Thermo Fisher Delaware, USA). Among these measurement standards, DNA concentration was determined from its optical density (OD) at 260 nm; purity was determined from the OD ratio at wavelengths 260 nm/280 nm ratio of 1.70-2.00, generally. However, for

DNA isolation from FFPE tissue, this ratio can be a minimum of 1.60 or even 1.30 (83–85).

3.2.3 qPCR for measurement of nuclear and mitochondrial DNA content

Quantitative real-time polymerase chain reaction (qPCR) allows real-time detection of DNA amplification by quantifying the amount of DNA in the reaction tube. In this method, the fluorescent dye binds to the DNA samples in proportion to its quantity. The amount of the product produced through qPCR is evaluated by measuring the signal generated by fluorescent dyes and probes or primers which increases according to the amount of product synthesized during the reaction.

Among the dyes utilized in qPCR, SYBR-green binds to the minor groove of double-stranded DNA, thereby enhancing fluorescence emission upon attachment (86). When the fluorescent signal reaches a certain level, it is captured by the signaling instrument, generating an amplification curve. The threshold value line is the level or point at which the fluorescence measurement detection exceeds the background fluorescence radiation (87). This value is placed in the exponential phase of the amplification. The number of cycles required for samples to reach this threshold is called the Threshold Cycle (Ct), which can be used to determine the DNA content for relative quantification.

To assess the specificity of the qPCR assay, a melting curve analysis is used. This technique uses an interacting dye as a reporter that is associated with the amplicon (87). A singular peak in the resulting plot indicates high assay specificity, confirming the accuracy of the detected DNA targets.

To determine variations in mtDNA copy number, qPCR was performed. This technique makes it possible to estimate relative changes in mtDNA copy number variation. qPCR was performed on a Bio-Rad CFX Real-Time device. Three sets of primers were used to detect both mtDNA and nuclear DNA (nDNA), with 18S rRNA

primers for nDNA, and ND1 and ND5 primers for mtDNA. The primer sets used are listed in Table 6.

Table 6 Primer sequences for nDNA and mtDNA

Primer	Sequence 5'-->3'
18S rRNA forward	TAGAGGGACAAGTGGCGTTC
18S rRNA reverse	CGCTGAGCCAGTCAGTGT
ND1 forward	CCCTAAAACCCGCCACATCT
ND1 reverse	GAGCGATGGTGAGAGCTAAGGT
ND5 forward	CCGGAAGCCTATTCGCAGGA
ND5 reverse	ACAGCGAGGGCTGTGAGTTT

2x GM SYBR Green (QPSY02, GeneMark) was used for qPCR for real-time detection. For each sample, 50 ng of DNA was used, and three distinct tube mixtures were prepared for nDNA and mtDNA as given in Table 7.

Table 7 qPCR reaction mixtures

Component	Volume	Final concentration
2x GM SYBR Green qPCR Kit (W/o ROX)	10 µl	1X
Forward Primer (10 µM)	0.4 µl	0.2 µM
Reverse Primer (10 µM)	0.4 µl	0.2 µM
Template DNA	Variable	50 ng
Water, nuclease-free	to 20 µl	-

The program for qPCR was set according to Table 8. For the melting curve, the default setting of BioRadCFXManager was used.

Table 8 Parameters for qPCR

Step	Temperature	Time	Cycle
Initial Denaturation	95 °C	3 min	Holding Stage
Denaturation	95 °C	10 sec	Cycling Stage 40 Cycles
Annealing	59 °C	10 sec	
Extension	72 °C	20 sec	
Melting Curve Analysis	65 to 95 °C, increment 0.50 °C, for 0:05		

3.2.3.1 Determination of mtDNA content

mtDNA content was estimated using the PCR threshold cycle number (Ct) for each corresponding normal and tumor tissue sample using the formula $2 \times 2^{\Delta Ct}$ (ΔCt=CtnDNA-CtmtDNA) (88). ΔCt represents the difference in Ct values between nuclear and mitochondrial DNA. For every sample, qPCR was performed in duplicate, and average Ct values were determined.

3.2.4 Statistical analysis

GraphPad Prism 10.2.2 software was used to perform statistical analyses. Differences in mtDNA content between TNBC FFPE and the corresponding normal tissue from the same individual were calculated using the Wilcoxon matched-pairs signed-rank test. For the correlation analysis, both Pearson's and Spearman's correlation tests were performed. The Pearson correlation coefficient (parametric) evaluates the strength of the linear relationship between the normally distributed variables. Conversely, the Spearman nonparametric correlation test makes calculations based on ranks rather than actual values, making no assumptions about the value distribution. A significance threshold of $P < 0.05$ was considered statistically significant. All statistical tests were two-sided.

4 RESULTS

4.1 Total DNA Measurements

The concentration of total DNA was quantified using nanodrop. The concentration and purity results are given in Table 9.

Table 9 Total DNA concentration and purity of TNBC samples

	Corresponding Normal Tissue		Tumor Tissue	
Sample	Nanodrop concentration (ng/µl)	260/280	Nanodrop concentration (ng/µl)	260/280
1	30.00	1.58	118.45	1.81
2	57.40	1.63	273.15	1.95
3	17.65	1.56	31.20	1.67
4	22.00	1.37	9.40	1.29
5	17.75	1.54	108.95	1.90
6	20.50	1.45	40.65	1.70
7	17.45	1.69	47.05	1.77
8	18.95	1.97	64.35	1.72
9	21.90	1.61	49.10	1.78
10	328.50	2.02	34.85	1.79
11	15.60	1.45	36.60	1.63
12	89.25	2.03	53.35	1.78
13	11.55	2.08	67.75	1.77
14	23.45	1.70	544.15	2.06
15	42.45	1.32	20.15	1.37
16	18.45	1.85	53.3	1.74
17	39.40	1.90	135.85	1.89
18	22.30	1.70	96.90	1.85
19	24.80	1.96	10.25	1.28
20	13.60	1.92	44.30	1.95
21	15.40	1.49	34.70	1.68
22	29.45	1.66	45.25	1.86
23	22.55	2.12	108.30	1.84

4.2 mtDNA Copy Number Changes

4.2.1 qPCR results

qPCR analyses were used to determine the mtDNA content in both the corresponding normal and tumor tissues of the 23 TNBC samples. For each reaction, 50 ng of DNA was used based on the measurements given in Table 9. MtDNA content was evaluated using ND1 and ND5 primers. To demonstrate the absence of nonspecific bindings during qPCR amplification, the amplification and melting curves

of samples 13-20 are presented in Figure 3, and the findings are presented in Table 10 and Table 11, respectively.

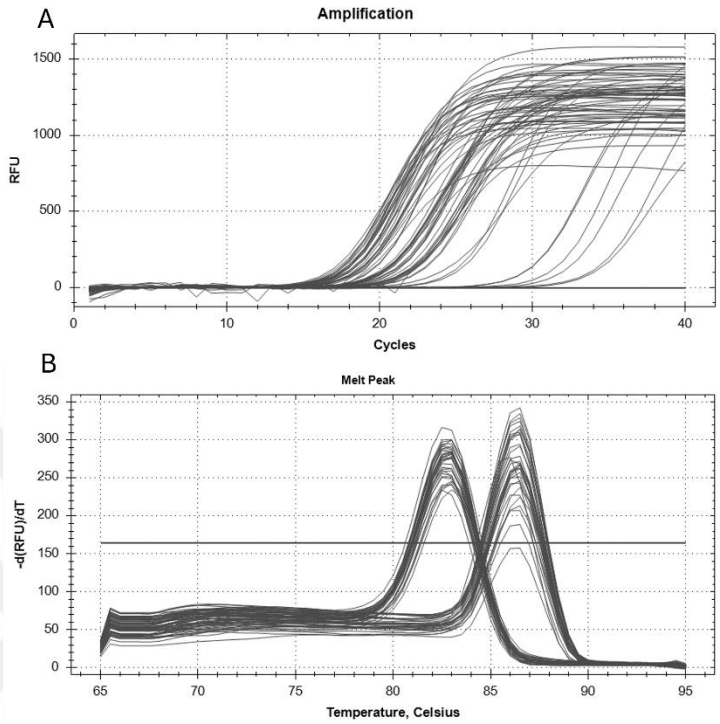

Figure 3 Amplification and melting curves of samples 13-20
A: Amplification curves of sample 13-20; B: Melting curves of sample 13-20. The curves on the right represent 18s rRNA amplification, and the left curves represent ND1 amplification.

The mtDNA content of ND1 is presented in Table 10. Across the 23 samples, the average mtDNA content in the tumor tissues was lower than that in the corresponding normal tissues in 18 cases. The average mtDNA content ranged from 8.65 to 159.71, 3.10 to 53.14, in the corresponding normal and tumor tissues, respectively.

Similarly, in Table 11 the mtDNA content of ND5 is presented. Consistent with the findings from ND1 analysis, tumor tissues exhibited decreased mtDNA content compared to corresponding normal tissues in 18 of the 23 samples. The average mtDNA content ranged from 2.70 to 50.35, 0.82 to 25.16, in corresponding normal and tumor tissues, respectively.

Table 10 mtDNA content of ND1

	Corresponding Normal Tissue			Tumor Tissue		
Sample	18S rRNA	ND1	2 x $2^{\Delta Ct}$	18S rRNA	ND1	2 x $2^{\Delta Ct}$
1	23.38 ± 0.26	17.71 ± 0.07	101.61 ± 0.17	26.18 ± 0.06	21.72 ± 0.12	43.98 ± 0.09
2	20.29 ± 0.18	18.17 ± 0.13	8.65 ± 0.15	18.67 ± 1.05	17.46 ± 0.03	4.62 ± 0.54
3	24.60 ± 0.39	21.07 ± 0.15	23.03 ± 0.27	24.51 ± 0.03	21.48 ± 0.17	16.33 ± 0.10
4	26.01 ± 0.08	21.65 ± 0.23	41.15 ± 0.16	27.34 ± 0.36	26.71 ± 0.18	3.10 ± 0.27
5	23.76 ± 0.25	20.30 ± 0.05	21.92 ± 0.15	21.12 ± 0.18	18.66 ± 0.03	11.03 ± 0.10
6	23.12 ± 1.32	16.99 ± 0.20	59.45 ± 0.76	21.40 ± 0.43	16.81 ± 0.10	42.59 ± 0.27
7	22.83 ± 0.01	19.26 ± 0.08	23.79 ± 0.05	20.21 ± 0.22	18.80 ± 0.10	5.34 ± 0.16
8	20.03 ± 0.14	17.32 ± 0.30	13.02 ± 0.22	18.69 ± 0.10	16.75 ± 0.12	7.67 ± 0.11
9	22.05 ± 0.04	18.54 ± 0.06	22.91 ± 0.05	22.71 ± 0.09	19.20 ± 0.15	22.76 ± 0.12
10	21.04 ± 0.03	16.99 ± 0.38	33.18 ± 0.20	18.53 ± 0.39	16.67 ± 0.03	7.27 ± 0.21
11	21.83 ± 0.01	18.16 ± 0.09	25.49 ± 0.05	19.71 ± 0.16	17.30 ± 0.08	10.60 ± 0.12
12	20.66 ± 2.89	16.85 ± 0.01	28.09 ± 1.45	19.97 ± 0.14	18.52 ± 0.26	5.47 ± 0.20
13	20.79 ± 0.36	17.32 ± 0.20	22.24 ± 0.28	19.80 ± 0.11	18.16 ± 0.06	6.23 ± 0.08
14	21.42 ± 0.10	17.15 ± 0.04	38.65 ± 0.07	20.21 ± 0.07	16.64 ± 0.13	23.69 ± 0.10
15	23.97 ± 0.07	20.29 ± 0.11	25.6 ± 0.09	23.95 ± 0.33	20.35 ± 0.20	24.21 ± 0.26
16	22.93 ± 0.05	18.98 ± 0.18	30.83 ± 0.11	19.29 ± 0.04	17.17 ± 0.05	8.70 ± 0.04
17	22.17 ± 0.03	17.75 ± 0.05	42.97 ± 0.04	19.43 ± 0.55	16.33 ± 0.08	17.24 ± 0.31
18	24.94 ± 0.43	18.62 ± 0.17	159.71 ± 0.30	20.22 ± 0.17	17.31 ± 0.10	15.06 ± 0.13
19	20.41 ± 0.04	16.73 ± 0.20	25.70 ± 0.12	30.11 ± 0.03	25.38 ± 0.05	53.14 ± 0.04
20	20.09 ± 0.04	16.96 ± 0.12	17.49 ± 0.08	21.43 ± 0.12	17.27 ± 0.10	35.84 ± 0.11
21	22.20 ± 0.10	19.12 ± 0.05	16.91 ± 0.07	21.58 ± 0.27	18.46 ± 0.07	17.34 ± 0.17
22	20.72 ± 0.01	18.21 ± 0.12	11.34 ± 0.06	20.37 ± 0.01	17.69 ± 0.13	12.85 ± 0.07
23	20.68 ± 0.13	17.05 ± 0.02	24.88 ± 0.07	20.40 ± 0.25	16.34 ± 0.05	33.32 ± 0.15

Table 11 mtDNA content of ND5

	Corresponding Normal Tissue			Tumor Tissue		
Sample	18S rRNA	ND5	2 x $2^{\Delta Ct}$	18S rRNA	ND5	2 x $2^{\Delta Ct}$
1	23.38 ± 0.26	21.15 ± 0.93	9.37 ± 0.60	26.18 ± 0.06	26.07 ± 0.19	2.15 ±0.13
2	20.29 ± 0.18	19.85 ± 1.15	2.70 ± 0.66	18.67 ± 1.05	18.59 ± 0.09	2.11 ± 0.57
3	24.60 ± 0.39	22.67 ± 0.02	7.58 ± 0.20	24.51 ± 0.03	25.56 ± 0.01	0.97 ± 0.02
4	26.01 ± 0.08	24.13 ± 0.27	7.39 ± 0.18	27.34 ± 0.36	28.62 ± 0.10	0.82 ± 0.23
5	23.76 ± 0.25	20.30 ± 0.05	21.92 ± 0.15	21.12 ± 0.18	18.66 ± 0.03	11.03 ± 0.10
6	23.12 ± 1.32	19.77 ± 0.24	20.48 ± 0.78	21.40 ± 0.43	20.00 ± 2.85	5.25 ± 1.64
7	22.83 ± 0.01	20.59 ± 0.16	9.44 ± 0.08	20.21 ± 0.22	20.13 ± 0.05	2.12 ± 0.14
8	20.03 ± 0.14	18.00 ± 0.04	8.17 ± 0.09	18.69 ± 0.10	17.30 ± 0.16	5.24 ± 0.13
9	22.05 ± 0.04	20.37 ± 0.06	6.42 ± 0.05	22.71 ± 0.09	21.84 ± 0.74	3.67 ± 0.41
10	21.04 ± 0.03	18.53 ± 0.70	11.37 ± 0.37	18.53 ± 0.39	18.52 ±0.48	2.02 ± 0.43
11	23.20 ± 0.19	20.65 ± 0.16	11.76 ± 0.18	20.49 ± 0.03	19.37 ± 0.08	4.35 ± 0.06
12	19.27 ± 0.01	17.10 ± 0.04	8.98 ± 0.02	20.87 ± 0.07	21.20 ± 0.09	1.59 ± 0.08
13	22.02 ± 0.47	20.27 ± 0.16	6.71 ± 0.32	20.92 ± 0.26	19.87 ± 0.09	4.12 ± 0.17
14	21.42 ± 0.10	18.57 ± 0.10	14.39 ± 0.10	20.21 ± 0.07	18.17 ± 0.12	8.24 ± 0.10
15	23.97 ± 0.07	22.35 ± 0.09	6.13 ± 0.08	23.95 ± 0.33	22.55 ± 0.01	5.25 ± 0.17
16	22.93 ± 0.05	20.19 ± 0.14	13.34 ± 0.10	19.29 ± 0.04	19.90 ± 0.17	1.31 ± 0.10
17	22.17 ± 0.03	20.15 ± 0.45	8.10 ± 0.24	19.43 ± 0.55	18.25 ± 0.17	4.55 ± 0.36
18	24.94 ± 0.43	20.29 ± 0.04	50.35 ± 0.23	20.22 ± 0.17	18.88 ± 0.21	5.08 ± 0.19
19	20.41 ± 0.04	18.56 ± 0.13	7.23 ± 0.08	30.11 ± 0.03	28.02 ± 0.82	8.55 ± 0.43
20	20.88 ± 0.08	20.17 ± 0.09	3.26 ± 0.08	22.19 ± 0.21	19.52 ± 0.06	12.75 ± 0.14
21	23.23 ± 0.16	22.80 ± 0.003	2.70 ± 0.08	22.89 ± 0.07	22.28 ± 0.15	3.04 ± 0.11
22	21.68 ± 0.19	20.02 ± 0.17	6.33 ± 0.18	22.35 ± 0.27	19.73 ± 0.19	12.25 ± 0.23
23	20.68 ± 0.13	18.44 ± 0.26	9.49 ± 0.20	20.40 ± 0.25	16.75 ± 0.11	25.16 ± 0.18

Figure 4 illustrates the relative mtDNA copy number content in TNBC samples for both ND1 and ND5 primers. The bar graph represents the mtDNA content in both corresponding normal and tumor tissues for each sample, emphasizing the decreased mtDNA content observed in tumor tissues compared with corresponding normal tissues in the majority of samples.

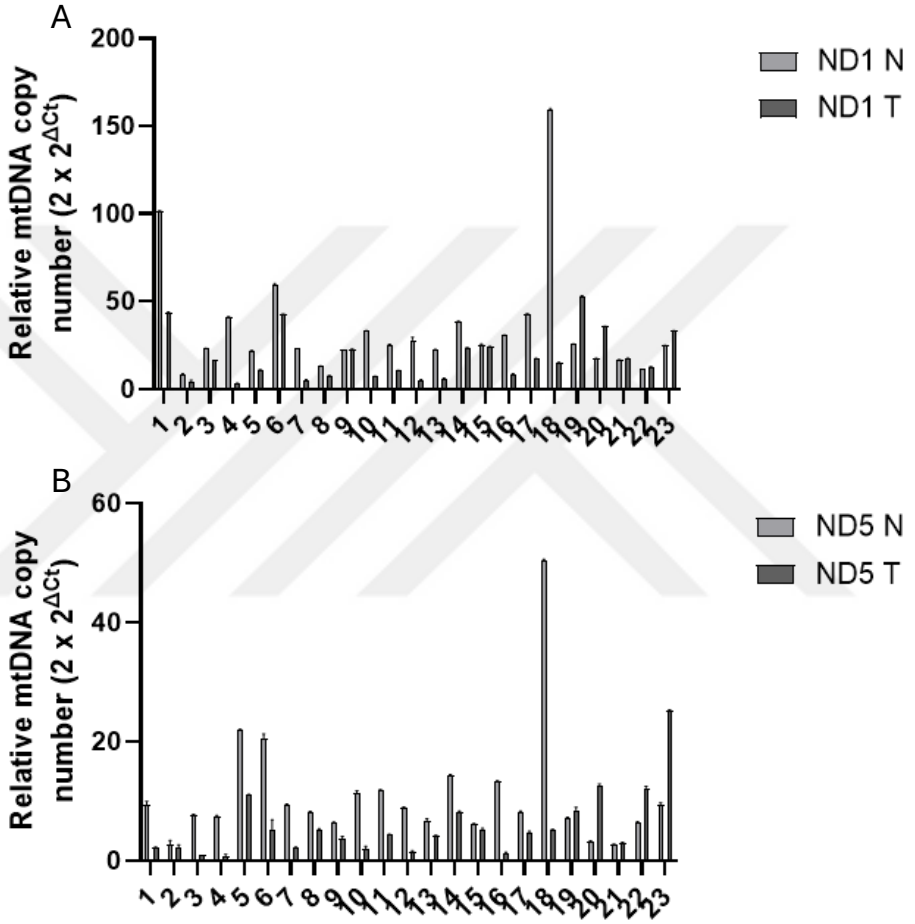

Figure 4 Relative mtDNA copy number content in TNBC samples for ND1&ND5
A: ND1 based mtDNA copy number variations; B: ND5 based mtDNA copy number variations. The bar graph represents the content of mtDNA in both corresponding normal (N) and tumor (T) tissues of each sample.

4.2.2 Statistical analysis

Statistical analysis of the qPCR results was performed using the Wilcoxon matched-pair signed-rank test. Of the 23 pairs of samples evaluated, 18 showed

significantly higher mtDNA levels in corresponding normal tissues than in tumor tissues (*P<0.05*) (Figure 5).

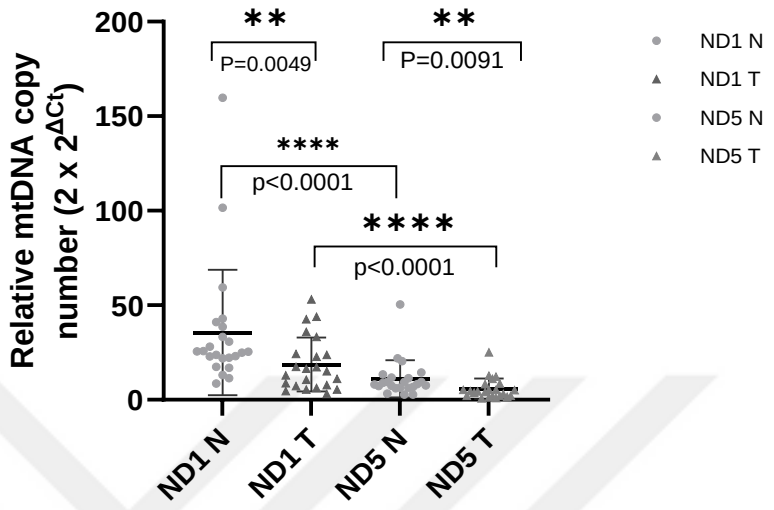

Figure 5 Relative mtDNA copy number variations in samples
Relative mtDNA copy number was determined by the 2 x $2^{\Delta Ct}$ method and compared between values (Mean ± SD) of the patient corresponding normal tissue and tumor tissue. Student's t-test (unpaired, two-tailed) was performed to analyze the significance. **(P<0.05)*.

The correlation between mtDNA content measured using the ND1 and ND5 primers in both corresponding normal and tumor tissues was determined using Pearson's and Spearman's correlation coefficients (Table 12). The results demonstrated a significant correlation between the ND1 and ND5 mtDNA content in both tissue types, suggesting a consistent association between the two primers in all samples. Additionally, all samples were analyzed for changes in mtDNA content, including both decreases and increases. More significant results were observed only in samples with a decreasing mtDNA content, whereas no statistical significance was observed in samples with an increasing mtDNA content (Table 12).

Pearson's and Spearman's correlation coefficients were used to calculate the association between mtDNA content and age, as well as response to neoadjuvant chemotherapy. The findings of these analyses are presented in Table 13 and Table 14, respectively. No correlation was found between mtDNA content and age (Table 13) or response to neoadjuvant chemotherapy (Table 14).

Table 12 Correlation of mtDNA content in corresponding normal and tumor tissue between ND1 and ND5

		Pearson		Spearman	
		r*	*P-value***	r*	*P-value***
All samples (n=23)	ND1N vs ND5N	0.81	<0.0001	0.59	0.003
	ND1T vs ND5T	0.40	0.06	0.60	0.003
Decreasing mtDNA content (n=18)	ND1N vs ND5N	0.91	<0.0001	0.43	0.072
	ND1T vs ND5T	0.67	0.002	0.62	0.006
Increasing mtDNA content (n=5)	ND1N vs ND5N	0.55	0.33	0.60	0.35
	ND1T vs ND5T	0.15	0.81	0.10	0.95

*r is the correlation coefficient. ** *P* is a statistical significance of association, (*p<0.05*).

Table 13 Correlation of mtDNA content with response to age (n=23)

	Pearson		Spearman	
	r*	*P-value***	r*	*P-value***
Age vs ND1N	0.12	0.59	-0.05	0.83
Age vs ND1T	-0.08	0.71	-0.20	0.35
Age vs ND5N	0.05	0.83	0.08	0.70
Age vs ND5T	-0.05	0.83	0.01	0.94

*r is the correlation coefficient. ** *P* is a statistical significance of association, (*P<0.05*).

Table 14 Correlation of mtDNA content with response to neoadjuvant chemotherapy response (n=23)

	Pearson		Spearman	
	r*	*P-value***	r*	*P-value***
Response vs ND1N	0.09	0.67	0.06	0.76
Response vs ND1T	-0.09	0.68	0.01	0.95
Response vs ND5N	0.29	0.18	0.17	0.43
Response vs ND5T	0.25	0.25	0.26	0.22

*r is the correlation coefficient. ** *P* is a statistical significance of association, (*P<0.05*).

5 DISCUSSION

Triple-negative breast cancer accounts for approximately 15% of all breast cancer cases (50,51) and is characterized by the absence of ER, PR, and HER-2 simultaneously. Compared to other subtypes, this aggressive form of breast cancer is associated with a worse prognosis and limited treatment options (50–52). Mitochondrial DNA copy number variation has been identified as a promising biomarker candidate for future cancer therapeutics and patient care (87). Although advancements in diagnostic and treatment strategies have decreased the mortality rate in Western countries, their impact remains limited in developing nations (76). Understanding the function of mtDNA copy number variations in TNBC shows potential for the development of novel biomarkers and targeted therapies to address the unique challenges associated with this aggressive subtype.

The relationship between mtDNA variations and cancer risk has long been studied. However, mtDNA copy number changes are highly diverse and influenced by various factors, including mitochondrial activity, in different cancer types (9,76). Numerous studies have examined mtDNA copy number changes in breast cancer using both paired tissue and blood samples. However, these investigations have yielded contrasting findings, underscoring the complexity of the relationship between mtDNA copy number changes and breast cancer (76). Furthermore, while previous studies have focused on the general association between mtDNA content and breast cancer, there remains a notable lack of literature concerning the specific relationship between mtDNA copy number variations in patients with TNBC who received neoadjuvant chemotherapy.

In our study, total DNA was isolated from FFPE tumor tissues and corresponding normal tissues of 23 patients with TNBC prior to neoadjuvant chemotherapy. Isolated total DNA was used to determine mtDNA copy number variation using SYBR-green based qPCR. Despite the use of FFPE preservatives to maintain the tissue architecture and cellular components, challenges arise because of prolonged formalin fixation. This process can lead to protein-nucleic acid crosslinking and random nucleotide sequence

breakages (83,88). In addition, there is no gold standard method for tissue sample fixation; therefore, even a small change in the fixation protocol may result in large differences in the quality and quantity of extracted DNA (83). Therefore, to achieve the best yield of DNA, we improved the QIAamp DNA FFPE Tissue Kit Manufacturer's Protocol, as mentioned in section 3.2.1 FFPE DNA isolation. Although our results generally meet satisfactory standards for FFPE samples, inherent heterogeneity and potential tumor-normal tissue contamination from tru-cut biopsy specimens remain significant considerations. While single-source samples offer enhanced accuracy, the possibility of tumor tissue infiltration into normal tissue cannot be overlooked, necessitating meticulous validation protocols.

This study revealed that the mtDNA content was significantly lower in 18 of the 23 tissue samples than in the corresponding normal tissues. However, there was no significant correlation between mtDNA copy number and age or response to neoadjuvant chemotherapy. Although these results are mostly compatible with those in the literature, there are also incompatibilities, as mentioned below.

A study analyzed the mtDNA copy number in 59 cases of invasive breast cancer tumors and paired noncancerous tissues, and the results showed that the level of mtDNA was significantly decreased in tumor tissues (*P=0.001*). In addition, reduced mtDNA was associated with older age (≥50 years old, *P=0.035*) as well as a higher histological grade (12). Another study conducted by Cheng Fan A.X. et al. on 51 breast cancer patients showed that mtDNA content was significantly decreased in 82% of cancerous breast tissues compared to that in corresponding normal tissues, and there was no association between clinical parameters such as age and mtDNA content (89). However, they also found an association between mtDNA content and hormone receptor status (89). Additionally, there are studies that showed the mtDNA content in breast cancer tissues is lower than in the corresponding normal tissues (14,90).

Few studies have indicated an association between a high mtDNA copy number and an increased risk of developing breast cancer. A pilot study of 103 breast cancer patients revealed that a high mtDNA copy number was associated with a significantly

increased risk of breast cancer compared to a low copy number (8). Furthermore, mtDNA copy number has a significantly negative association with several curical endogenous oxidants and antioxidants present in the blood (8). Lemnrau A. et al., and Thyagarajan B. et al. consistently showed that a higher mtDNA copy number was significantly associated with increased breast cancer risk (91,92).

Another study focused on the relationship between mtDNA and drug response conducted by Hsue et al., which suggested that low mtDNA content was associated with increased drug sensitivity and a higher rate of ROS production during doxorubicin treatment (79). In addition, decreased mtDNA content can result in decreased oxidative phosphorylation capacity under hypoxic conditions during cancer development and progression (89). When oxidative stress reaches a certain threshold, it triggers an apoptotic program that kills cancer cells (79). Consequently, biochemical characteristics associated with low mtDNA content may serve as potential biomarkers for predicting patient outcomes after ROS-generating chemotherapy (79).

In other cancer types, the mtDNA copy number shows different characteristics. In colorectal cancer, cervical cancer, osteosarcoma, and lung cancer, the mtDNA copy number was found to be lower in cancerous tissues (9,93–97). Conversely, in endometrial adenocarcinoma, acute lymphoblastic leukemia the mtDNA copy number was increased (9,97,98).

Our study focused on the *ND1* and *ND5* genes of mtDNA; however, some studies have used different primers and methods to amplify different regions of mtDNA using the D-loop (12), cytochrome c oxidase subunit I (90), and *MTATP 8* gene of mtDNA (89). Yu M. et al demonstrated that tumors carrying mutations in the D-loop exhibit lower mtDNA content compared to those without D-loop alterations (12). Somatic mutations within the D-loop region are significant contributing factors to decreased mtDNA levels in breast tumors (12). Further studies that can be conducted by evaluating different regions, especially D-loop, may shed light on our results.

Overall, studies have explored the relationship between mtDNA variation and cancer risk, drug response, and progression across different subtypes. Studies have investigated mtDNA copy number changes in breast cancer using both tumor tissue and blood samples. However, these findings are often contradictory, highlighting the complexity of mtDNA copy number changes and breast cancer pathogenesis.

It is important to acknowledge that several studies have presented conflicting results, yet our findings are in line with the body of existing literature. While our study demonstrates a consistent decrease in mtDNA copy number in cancerous tissue compared to corresponding normal tissue, certain articles have reported higher mtDNA content in cancerous tissues, highlighting the complexity of mtDNA dynamics in cancer (8,91,92). Additionally, the inconsistencies in clinical parameters emphasize the importance of further investigations, and our study showed that there was no significant association between age and mtDNA content, suggesting that age may not be a determining factor in mtDNA alterations, as in research conducted by Cheng Fan A.X. et al. (89). Similarly, our results revealed no correlation between the response to neoadjuvant chemotherapy and mtDNA content, indicating that mtDNA dynamics may not directly influence treatment response in patients with TNBC. However, the lack of post-neoadjuvant chemotherapy samples, owing to limited material resources, limits our analysis. Comparing mtDNA content before and after treatment could provide insights into the treatment response.

Similar to all other studies, our study also has several limitations. We only performed an association analysis between mtDNA copy number, age, and neoadjuvant chemotherapy response, yet understanding the underlying mechanisms of mtDNA copy number variation in TNBC development requires further investigation, such as the functional analysis of aging and oxidative stress. Additionally, future correlation analyses of mtDNA copy number variation in tissues other than FFPE samples, such as fresh tissues, leukocytes, and blood, would enhance our understanding of the relationship between the mitochondria and carcinogenesis.

In the present study, mtDNA copy number variation was determined using SYBR-green based qPCR. SYBR-green is considered a less specific dye because it binds nonspecifically to any double-stranded DNA, potentially leading to amplification of nonspecific products or primer dimers (84). However, our results, as presented in Figure 3**,** demonstrate no evidence of nonspecific binding. Moreover, we assessed the relative variations in mtDNA copy number. Relative mtDNA content provides information on the relative abundance of mtDNA compared to nDNA within a given cell or tissue (99). For a comprehensive biomarker assessment, it is essential to consider the absolute mtDNA content, which quantifies the actual quantity of mtDNA molecules in a sample. Digital PCR is a preferred method for determining absolute mtDNA copy number variations, as it eliminates the need for a reference gene and offers advanced sensitivity, enabling the detection of subtle mtDNA levels (100).

It is also important to note that our study, conducted with a sample size of 23 patients, represents a preliminary exploration of the role of mtDNA copy number variation in TNBC. Although larger studies and advancements are required to validate our findings and establish mtDNA as a robust biomarker, our study contributes valuable insights to the literature. In conclusion, our results suggest that mtDNA may serve as a potential biomarker of TNBC.

6 CONCLUSION

The management of triple negative breast cancer is a significant challenge in oncology because of its aggressive behavior and limited available treatment options. Our study, examining mitochondrial DNA copy number variation in TNBC patients who received neoadjuvant chemotherapy, contributes to a growing body of research investigating biomarkers to improve patient care.

Consistent with previous findings, our study demonstrated a significant decrease in mtDNA content in TNBC tumor tissues compared to that in the corresponding normal tissues. However, the lack of a significant correlation between mtDNA copy number and age or response to neoadjuvant chemotherapy suggests that mtDNA dynamics may not directly influence these clinical parameters in TNBC patients.

Although our results align with the existing literature, it is important to acknowledge the presence of conflicting evidence in certain studies. Some studies suggest higher mtDNA content in cancerous tissues, emphasizing the complexity of mtDNA dynamics in cancer. Moreover, inconsistencies in clinical parameters highlight the need for further investigation to clarify the role of mtDNA in TNBC development and treatment response.

Despite the modest sample size of our study, it offers valuable insights into the mtDNA as a potential TNBC biomarker. Larger studies are necessary to validate our findings and establish that mtDNA is a good indicator of prognosis and therapeutic strategies in patients with TNBC. In conclusion, our results indicate that mtDNA still shows promise as a potential biomarker for TNBC, which could lead to personalized medicine and enhanced clinical outcomes in this challenging breast cancer subtype.

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8 APPENDIX

9 CURRICULUM VITAE