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RESEARCH ARTICLE

Measurement of Total Iron in Breast Tissue Samples by Inductively Coupled Plasma-Mass Spectrometry

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ABSTRACT

Objective: Breast cancer is the commonest and the deadliest malignancy among women. Iron is known as an essential element for cell growth and division. The connection between oxidative stress, antioxidants, and progression, aggressiveness, and recurrence of breast cancer, is widely recognized to involve impaired iron metabolism. The purpose of this study was to investigate the connection between the tumor characteristics and the levels of total iron in breast tissues by employing state of the art technology, inductively-coupled plasma/mass spectrometry (ICP-MS) with our in-house analysis methodology for tissue samples.

Materials and Methods: Iron contents were determined in 25 tissue sets (matched tumor and peritumoral tissues) collected from 25 women diagnosed with invasive ductal breast cancer. In addition, three cancer-free breast tissues, obtained from breast reduction surgery, were analyzed as a normal group. Collected samples were digested and introduced to ICP-MS for iron analysis.

Results: Our method showed a low rate of measurement error (<10%). A highly significant ($p<0.001$) ~3 fold difference of iron concentrations were observed in tumors (24.73±6.15 ppb/mg) as compared to peritumoral tissues (9.10±4.84 ppb/mg). As the grade and stage of the cancer increases, iron levels in tumor tissues were also found to be increased ($p=0.006$, $p=0.022$ respectively).

Conclusion: Our ICP-MS based method can be reliably performed at the established conditions for tissue specimens, and also have potential to be used in clinical practice. Understanding the relationship between tissue iron levels and tumor characteristics is essential in identifying potential prevention and treatment strategies.

Keywords: Iron, Breast cancer, ROS, ICP-MS

INTRODUCTION

Breast cancer is a complex and multifaceted disease that continues to pose significant challenges to public health.[1] Extensive research has been dedicated to unraveling the intricate mechanisms underlying its development and progression. Among the various factors implicated in breast cancer, emerging evidence suggests that tissue iron levels play a crucial role in its pathogenesis.[2]

Animal studies conducted so far provide evidence supporting the notion that iron could potentially contribute to the advancement of breast cancer. These studies demonstrate that diets high in iron or subcutaneously injected iron promote the progression of breast cancer at various stages.[3–6] When examining breast epithelial cells at a cellular level, it is widely acknowledged that the malignant state is marked by an imbalance in cellular iron regulation. This is evident through variations in the expression of several iron-related proteins that correlate with indicators of unfavorable prognosis.[7–11]

Iron has been implicated in the development of breast cancer through several proposed pathways. Experimental evidence suggests that iron-induced oxidative stress may result in damage to DNA, proteins, and organelles. This damage arises from the production of hydroxyl radicals and hydrogen peroxide via chemical reactions like the Haber-Weiss and Fenton reactions.[12–14] Moreover, it is hypothesized that prolonged disturbance in the balance of cellular redox processes can influence specific signaling networks associated with the development of cancer.[15] Such disruption may result in the suppression of tumor-inhibiting factors and the heightened expression of oncogenes.[16,17] Considering the vital role of iron in cellular proliferation, it is logical to assume that iron also significantly

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contributes to the clonal expansion of malignant cells.[18,19] Furthermore, iron provides a discerning advantage and facilitates the increased proliferation of tumor cells. Interestingly, cancer cells often exhibit a phenotype characterized by iron deficiency, which manifests as increased expression of iron importers and decreased expression of iron exporters.[11,20,21] Therefore, the altered iron metabolism in cancer cells contributes to their growth and survival.

Due to the higher metabolic and proliferation rates of breast tumor cells compared to normal breast cells, their iron demand is substantially greater, resulting in elevated oxidative stress. The use of dietary antioxidants as supplements to alleviate this oxidative stress is a common practice.[22,23] However, the precise and optimal dosage of antioxidants administered in clinical trials remains unclear and insufficiently refined. Moreover, not all tumor types rely on reactive oxygen species (ROS) signaling, which means that the benefits of antioxidants may not be universally applicable.[24] Additionally, it is essential to consider endogenous factors such as genetic variability in enzymes, which can limit the efficacy of antioxidants in reducing ROS levels and protecting cells from oxidative stress by preserving the integrity of lipid membranes.

Increased ROS levels are known to impede cancer cell survival during the initial stages of tumor development and progression. Consequently, dietary supplementation with antioxidants during these stages may inadvertently support cancer cell survival and hasten tumor growth. In fact, studies have revealed that the intake of dietary antioxidants can negatively impact breast cancer prognosis in postmenopausal women and accelerate metastasis while reducing survival rates in mouse models of cancer.[25–29] Although clinical trials have not convincingly demonstrated the effectiveness of antioxidants as standalone therapies, these compounds are gradually being incorporated as supplements or adjuncts to conventional treatments.[30–32]

In addition to its well-known function as a crucial element in cellular growth, division and co-carcinogenesis, iron can contribute to breast carcinogenesis through an alternative pathway. Current scientific understanding suggests a synergistic interaction between iron and estrogen.[33] The connection between estrogen and iron has been suggested as a notable factor in controlling the aggressiveness of breast cancer and the variations in recurrence rates observed in pre- and postmenopausal women.[7] Estrogen and iron have the ability to activate pathways of oxidative stress, leading to the production of ROS. This oxidative stress plays a pivotal role in inducing and sustaining the oncogenic phenotype of cancer cells. Consequently, the combined effects of estrogen and iron may result in elevated ROS production and the occurrence of site-specific or random DNA damage.[34]

In our study, we utilized inductively coupled plasma mass spectrometry (ICP-MS) to quantify tissue iron levels. ICP-MS is a specialized elemental analysis technique, specifically designed for the measurement of elements rather than molecules and compounds, which are typically analyzed using liquid chromatography-mass spectrometry and gas chromatography–mass spectrometry methods. Unlike the qualitative and less sensitive colorimetric approaches used in the past, which relied on the formation of insoluble metal sulfides, ICP-MS provides precise, sensitive, and highly specific quantification of heavy metals, including iron. This technique is applicable to liquid samples or those that can be dissolved or undergo acid digestion to obtain a liquid sample. The versatility of ICP-MS has resulted in its widespread adoption in various industries, including routine environmental monitoring, consumer product testing, food and pharmaceutical safety evaluations, as well as research in life sciences and clinical settings.[35] The popularity of ICP-MS can be attributed to its remarkable capability to achieve remarkably low detection limits for nearly all detectable elements. It can detect numerous elements at levels below 0.1 part per trillion (ppt), surpassing the capabilities of other techniques in terms of broad coverage of elements, low detection limits, and extensive measurement range.[36]

Our study aimed to examine the relationships between various tumor characteristics and the levels of iron in breast tissue using an in-house method using ICP-MS for analyzing iron in breast tissue samples. This method has the potential to be implemented in clinical laboratories for future use.

MATERIALS AND METHODS

Sample Collection

Fresh tumor and peritumoral tissues samples were collected via the Department of Pathology at Istanbul University-Cerrahpasa between June 2018 and December 2020. 50 tissue samples were gathered, comprising 25 pairs of tumor and peritumoral tissues, obtained from 25 women who underwent surgical procedures (mastectomy or breast conservation surgery) due to breast cancer. These women did not receive any additional treatments prior to surgery. Peritumoral tissues were taken at least 1 cm from the tumor boundary. Furthermore, three control samples were procured from premenopausal women who had no previous diagnosis of breast cancer and underwent reduction mammoplasty surgery. Histopathological evaluation was performed independently by two pathologists. The collected samples were rapidly frozen using liquid nitrogen and kept at a temperature of -80°C until they were utilized for iron measurement analysis. Tumor characteristics such as grade, stage, lymphatic invasion status, Ki-67 status and molecular type information were also recorded. The research protocol received approval from the Ethical Committee of Acibadem University (no: 2018-5/9). Prior to their participation, each patient provided informed written consent regarding the study.

Instrumentation

Wet digestion procedure was carried out with an incubator (Nuve EN 400, Turkey) and magnetic stirrer with ceramic heating plate (IKA C-MAG HS 7, China). ICP-MS measurements were performed with a 7700 Series x (Agilent Technologies, Germany) equipped with a third generation Octopole Reaction System (ORS^3) using helium gas. Low matrix was selected as plasma mode. Each measurement was performed with three repetitions. Data acquisition and analysis was performed with MassHunter 4.4 Workstation Software (Agilent Technologies, Germany).

Reagents and Other Materials

Analytical-grade reagents were used throughout the study. Digestion buffer (DB) was prepared via mixing 65% nitric acid (HNO_3, Merck, Germany) solution and 30% hydrogen peroxide (H_2O_2, Merck, Germany) solution (1:1, v/v). Standard iron (56 Fe) solutions were prepared from a 1 g/L stock solution (TraceCERT Merck, Germany). 5, 10, 25, 50 and 100 ppb standard solutions were prepared in 1% HNO_3 solution. Ultra-purified water (Milli-Q Plus® – Millipore, USA) was used for preparation of all solutions. Mineral oil (Sigma-Aldrich, Germany) was used for heating the samples.

Glassware, sample vials, and pipette tips were decontaminated by immersing them in a 10% HNO_3 solution for a duration of 4 hours. They were then thoroughly rinsed with water and placed on a plastic tray in an oven at 60°C for drying. Following the drying process, all items were stored in sterilized plastic bags to maintain their decontamination until they were ready for use.

Wet Digestion

The wet digestion method was modified from the work of Badran et al.[37] Approximately 50 mg of breast tissue samples in flat bottom glass sample vials were dried overnight at 37°C, and constant weight was obtained for each sample. 1 mL of DB was added to dry tissue samples and it was allowed to soak thoroughly. Then sample vials were placed into the digestion set up (Figure 1) under the fume hood. The temperature was slowly ramped up until reaching 100°C. 1 mL of DB was added to each vial up to 5 times until a clear solution was obtained. Samples were evaporated to 100 mL and cooled to room temperature. 1% HNO_3 solution was added to complete each sample to 1 mL.

Statistical Analysis

All calculations were performed using the SPSS Statistical Program version 24.0 (SPSS Inc. Chicago, IL, USA). The significance of differences in Fe levels was determined by the Wilcoxon signed-rank test (WSR), Mann-Whitney U (MU) or Kruskal Wallis H (KW) test as needed. All reported p values are from two-sided tests, and a value less than 0.05 was considered statistically significant.

RESULTS

Optimization of ICP-MS Analysis

The collision gas utilized in the experiment was helium. Modifying the flow rate of helium gas introduced into the cell resulted in alterations in the production of molecular ions derived from argon gas, specifically ArO and ArN, along with changes in the intensity of the iron signal. To evaluate these alterations, the fluctuations in ArN, ArO and ^{56}Fe signals were quantified at various volume flow rates of helium gas. During each measurement, the instrument settings, such as the ion lens voltage, were optimized to reduce the detection of molecular ions and enhance the detection of iron ions. Optimal instrumental parameters were shown in Table 1.

The instrument was calibrated using aqueous standards of 5, 10, 25, 50 and 100 ppb for Fe. There was a good linear relation between measurements and standard concentrations. Linearity was evaluated by calculating the R-square value, which was 0.999 (Figure 2).

In order to evaluate the % error rates of the method a series of Fe standard samples (300, 600, 800 and 1600 ppb) were digested in triplicate under the optimized conditions and analyzed. All samples were diluted with 1% HNO_3 (1:16) before being introduced to the system. The % error of the measured standard concentrations compared to the true value of the standards were calculated by the following equation: % error = [(standard concentration value-calculated value)/ standard concentration value]*100. Our results showed that our method has less than 10% error rate in 300-1600 ppb ranges (Table 2).

Breast Tissue Iron Levels and Alterations within Specific Groups

Tumor, peritumoral and normal breast tissue samples were taken into consideration in order to compare iron concentrations. The levels in tumor (T) tissues were compared to peritumoral (P) tissues as well as to normal (N) tissues (Figure 3). Overall, the lowest iron levels were in N (n=3, 3.72±0.69 ppb/mg) and the highest were in T (n=25, 24.73±6.15 ppb/mg). The difference between T and P (9.10 ±4.84 ppb/mg) tissues was found to be highly significant (WSR, $p < 0.001$), approximately 3 fold higher in favor of tumor samples. The highest difference between tissues (> 4 fold) was observed in stage II patients' tissues, however, it did not reach statistical significance.

When we investigated the iron levels in the same tissue types

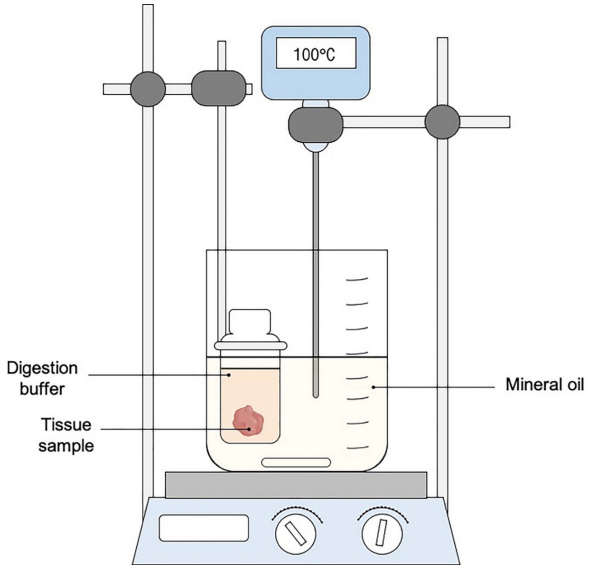

Figure 1. Wet digestion set up for breast tissue samples.

Table 1. Operating conditions of the ICP-MS

RF Power (W)	1400
Cooling gas flow rate (L min^{-1})	15
Carrier gas (He) (mL min^{-1})	12.0
Sampling depth (mm)	10
Isotope	^{56} Fe
Replicates	3
Sweeps	100
Acquisition time (s)	55.5

Table 2. Measurement results of iron in standard samples.

Std. Conc.(ppb)	Observed (ppb)	Calculated (ppb)	% Error
300	17.03	272.43	9.19
600	36.25	579.93	3.35
800	48.54	776.57	2.93
1600	94.46	1511.33	5.54

regarding the tumors' characteristics, T tissues that were diagnosed with higher grade had a significant correlation with high tissue iron levels (p = 0.006). On the contrary, P tissues of grade-3 tumors tend to have lower iron levels compared to grade-2 tumors' P tissues (p = 0.054). Iron level difference was also found to be significant between T samples of stage II and III patient group (p = 0.022). There was no statistically significant association between factors such as molecular types, lymphatic invasion and Ki-67 and tissue iron level differences (Table 3).

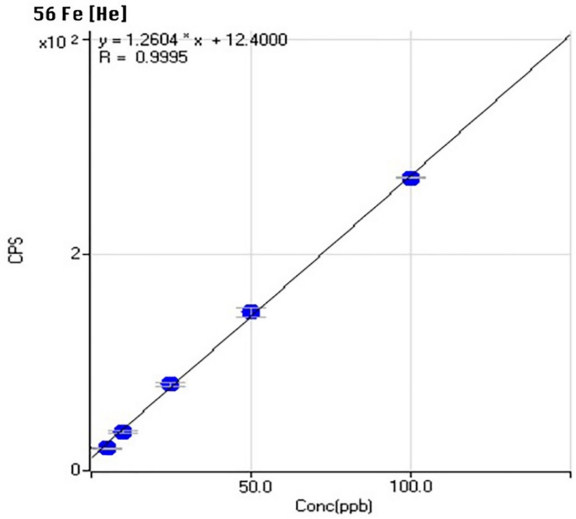

Figure 2. Calibration graph for iron. Blue dots represents 5, 10, 25, 50 and 100 ppb standard solutions measurements respectively. All measurements were triplicates.

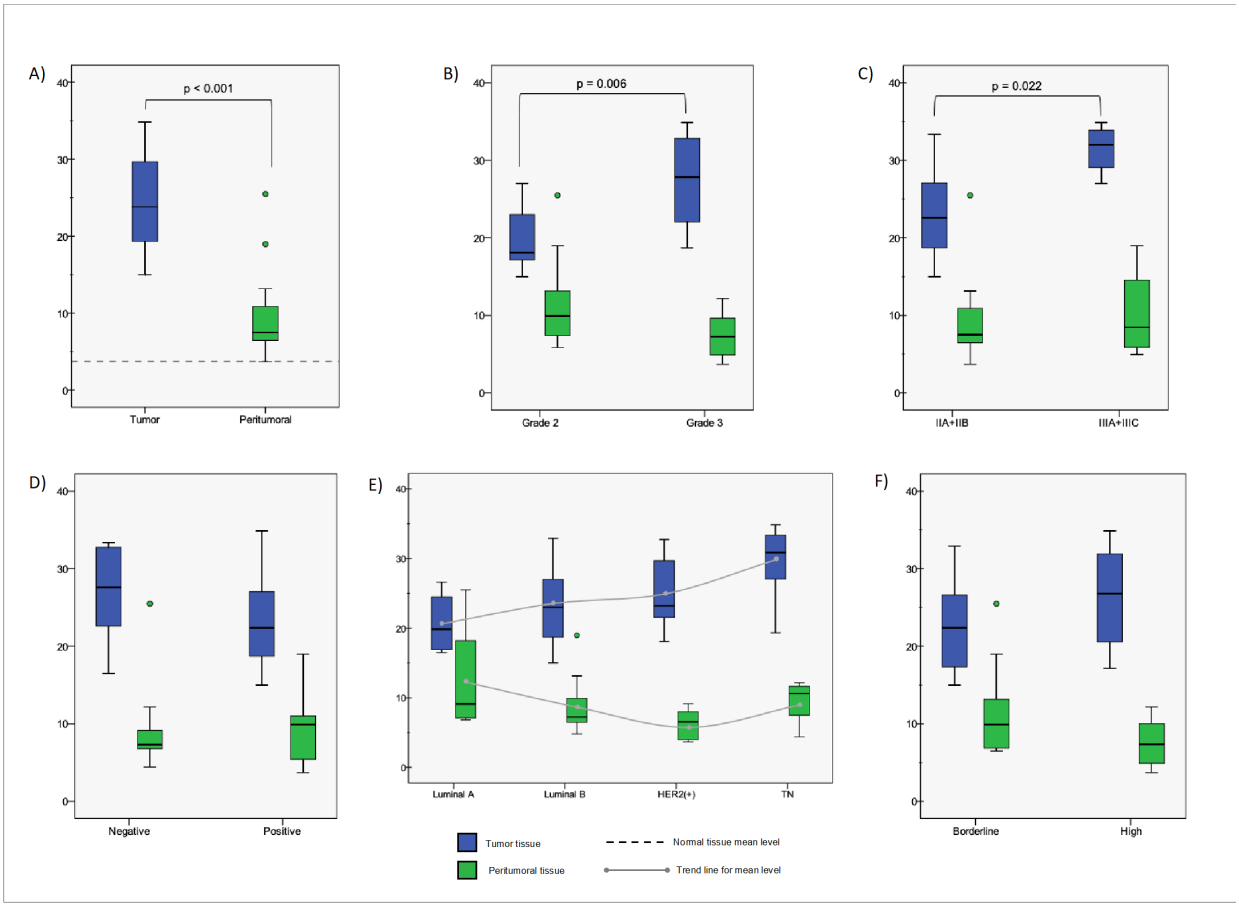

Figure 3. Comparison of iron concentrations among different breast tissue types together with tumor characteristics. Plots represent the difference between A) overall tissue types, B) histological grades, C) stages, D) lymph node status, E) molecular types and F) Ki-67 expression levels respectively. All concentration measurements are presented as ppb per mg tissue.

Table 3. Distribution of iron levels regarding tissue types and tumors' characteristics

Variable (%)	**Fe (ppb/mg)**		
	T (Mean,SD)	P (Mean,SD)	FC (T/P)
Histological Grade			
Grade 2 (36)	20.33 ±4.50	12.04 ±6.40	1.7
Grade 3 (64)	27.21 ±5.62	7.45 ±2.75	3.7
P value	0.006 (MU)	0.054 (MU)	
Stage			
IIA+IIB (92)	23.46 ±5.74	8.89 ±4.69	4.1
IIIA+IIIC (8)	31.46 ±3.35	10.21 ±6.22	3.1
P value	0.022 (MU)	0.824 (MU)	
Lymphatic invasion			
Negative (40)	26.81 ±5.93	9.43 ±5.98	2.8
Positive (60)	23.35 ±6.10	8.89 ±4.13	2.6
P value	0.222 (MU)	0.868 (MU)	
Ki-67 status			
Borderline (36)	22.16 ±5.89	11.76 ±6.54	1.9
High (64)	26.18 ±5.98	7.60 ±2.83	3.4
P value	0.101 (MU)	0.141 (MU)	
Molecular Type			
Luminal A (16)	20.71-4.72	12.65-8.74	1.6
Luminal B (36)	23.44-6.31	9.13-4.56	2.6
Her2 (+) (24)	24.74-5.44	6.31-2.20	3.9
Triple Negative (24)	29.38-5.76	9.49-2.99	3.1
P value	0.090 (KW)	0.205 (KW)	

T: Tumor tissue P: Peritumoral tissue FC: Fold change, MU: Mann-Whitney Utest, KW: Kruskal Wallis H test

DISCUSSION

The pathways mentioned in the introduction part, which generate ROS and contribute to the development and progression of breast cancer by disrupting iron metabolism, can potentially be restored through the incorporation of antioxidants into patient treatment regimens. This has been proposed as a promising therapeutic strategy to mitigate tissue damage caused by oxidative stress. Importantly, numerous bioactive compounds derived from plants have demonstrated the ability to regulate both iron metabolism and the cellular redox state, possibly through interactive mechanisms.[38] Before considering such treatments, it is essential to measure and assess breast tissue iron levels using a specific method.

Iron is a well-known trace element that plays a significant role in the human body.[39] The study of trace elements in biology and medicine has garnered considerable research attention due to their crucial involvement in the metabolism and growth of living organisms.[40–42] Various methods are currently employed for trace element analysis, including optical spectrometry, atomic mass spectrometry, X-ray Fluorescence Spectrometry, ICP/MS, chromatography, neutron activation, and photon activation techniques.[43–45] Among these methods, ICP-MS is widely recognized as the most accurate technique for determining the content of trace elements in biological samples.[46] Through the optimization of our method, we were able to achieve error rates below 10%. However, the utilization of ICP-MS for trace element analysis necessitates the conversion of solid biological samples, such as breast tissues, into a liquid state through digestion procedures.[47] Therefore, sample digestion, particularly acid digestion, is a crucial and essential process for accurately analyzing the trace element content in biological samples. The elemental analysis of biological samples involves the destruction of organic matter, the primary component of such samples, followed by their dissolution using acid digestion. Various acids and acid mixtures containing nitric, sulfuric, or perchloric acids have been employed for the acid digestion of biological samples to analyze trace elements using ICP-MS.[48–51] Additionally, wet oxidation procedures involving the combination of oxidizing agents such as hydrogen peroxide with nitric, sulfuric, and perchloric acids have been utilized. While most studies investigating breast cancer and iron have been conducted using serum samples, our samples, being derived from breast tissues, possess a more complex matrix. Consequently, we optimized the ratio of nitric acid and hydrogen peroxide in the digestion solution, as well as the temperature, to achieve the most effective dissolution of iron in breast tissue samples.

The causative factors of human breast cancer remain a topic of debate, although various factors such as hormonal influences, toxic substances, oxidative stress, and lipid peroxidation have been proposed as potential contributors to breast cancer development. In biological systems, the concentration of redox-active transition metals, which have the ability to catalyze or generate free radicals like superoxide, hydrogen peroxide, and hydroxyl radical, is typically low. However, in certain pathological conditions such as breast cancer, these transition metals and their transport proteins can accumulate in different target organs, leading to cellular lipid peroxidation and DNA damage. Excessive iron, in particular, is known to facilitate the production of hydroxyl radicals, impair cellular immune functions, and promote tumor growth, as supported by substantial evidence.[52–55]

Several studies have documented alterations in protein expression that may provide insights into the accumulation of iron in breast tumor cells. These investigations have not only focused on ferritin, but also on various other proteins involved in the molecular mechanisms of iron absorption, storage, utilization, and elimination. However, only a limited number of studies have directly measured iron levels in breast tissue. These studies have consistently observed a significant accumulation of iron in breast tumor samples, particularly in postmenopausal women, indicating a correlation between iron accumulation, advancing age, and the cessation of menstrual periods.[56,57] Our analysis aligns with these findings, revealing an overall elevation of iron concentrations in tumor tissues compared to adjacent peritumoral tissues. Furthermore, we observed that iron levels in normal breast tissue were notably lower compared to other tissues.

Although not statistically significant, we observed a 2.5-fold difference between peritumoral and normal breast tissues in terms of iron levels. This finding sheds light on the potential involvement of peritumoral tissue, which encompasses the cells of the tumor microenvironment, in the dysregulation of iron metabolism associated with breast cancer progression. Previous research by Marquez et al. has also identified iron accumulation in stromal inflammatory cells associated with breast cancer, suggesting that these cells could serve as a significant reservoir of iron for the tumor core.[58] Furthermore, their discovery of disrupted iron-related protein expression in infiltrating lymphocytes and macrophages provides further evidence supporting the notion that stromal cell responses within the breast microenvironment play a critical role in tumor progression.[59,60]

In our study, we also explored the potential links between tissue iron levels and factors influencing the prognosis and treatment of breast cancer. The analysis results revealed that the accumulation of iron in tumor tissues was associated with well-known indicators of poor prognosis, such as high histological grade and advanced stage. Although not statistically significant, we also observed an increase in the extent of iron accumulation in tumor tissues compared to peritumoral tissues as the disease exhibited more aggressive characteristics, became resistant to treatment, and showed decreased survival rates (as indicated by Ki-67, molecular type, and lymph node status). Another study, which included the analysis of tumors from 251 breast cancer

patients, explained the relationship between these unfavorable prognostic tumor characteristics and impaired iron metabolism through low expression levels of ferroportin.[61] Overall, it is plausible that the disruption of mechanisms crucial for maintaining a controlled iron balance may contribute to tumor development, increased aggressiveness, metastasis, and higher rates of disease recurrence.[7,62]

Except for normal tissues, all the samples used in our study were obtained from individuals diagnosed with invasive ductal breast cancer. Previous research, including our own, has provided evidence of local estradiol production within breast tissues, particularly in hormone-positive tumors.[63–65] The elevated levels of estrogen in breast tissue disrupt intracellular iron metabolism, causing an excess accumulation of iron. This imbalance in iron levels can trigger the production of superoxide anions and the conversion of Fe^{3+} bound to ferritin to Fe^{2+}, resulting in estrogen-induced oxidative stress on nucleic acids. This oxidative stress can subsequently contribute to the development of cancer.[66–68]

CONCLUSION

In conclusion, our findings indicate a strong correlation between elevated iron levels in breast tumor tissue and the progression and prognosis of the disease. Additionally, we have developed a practical methodology utilizing ICP-MS for accurately measuring iron concentration in breast tissue samples. Iron, as a redox-active metal, possesses the potential to ROS, which can contribute to the development of breast cancer. While cancer cells maintain similar iron metabolism pathways to normal cells, alterations in the expression of proteins and enzyme activities suggest a reprogramming of iron metabolism crucial for tumor cell survival. The upregulation of iron-dependent proteins in cancer cells, accompanied by the avoidance of iron overload-induced damage, leads to an "adjusted iron homeostasis" aligned with tumor metabolism. Furthermore, excess iron and the disruption of intracellular iron metabolism by estrogen play significant roles in breast cancer development and progression. Iron depletion and the use of various antioxidants have the potential to inhibit cancer cell growth and reduce inflammation by suppressing ROS generation. Consequently, antioxidant supplements may complement other therapeutic approaches in managing breast cancer. However, preclinical research, both in vitro and in vivo, is required to elucidate the role of antioxidants in breast cancer initiation and progression, their dual role (antioxidant or oxidant) depending on concentration, and their interaction with other compounds and therapies.

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Ethics Committee Approval: This study was approved by the Ethical Committee of Acibadem University (no: 2018-5/9).

Informed Consent: Written consent was obtained from the participants.

Peer Review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- M.B.T.; Data Acquisition- M.B.T.; Data Analysis/Interpretation- M.B.T., B.T.; Drafting Manuscript- M.B.T, B.T.; Critical Revision of Manuscript- M.B.T., B.T.; Final Approval and Accountability- M.B.T.,B.T.

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