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Circulating Cell-Free DNA as a Potential Biomarker in Breast Cancer

Israa M. Al-Hawwaz*[a], Nazar S. Haddad[b], Loma AL-Mansouri[c]

[a]Department of Clinical Biochemistry, Basrah Teaching Hospital, Basrah, Iraq

[b]Department of Biochemistry, Basrah Medical College, University of Basrah, Basrah, Iraq

[c]Department of Medicine, Basrah Medical College, University of Basrah, Basrah, Iraq

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ABSTRACT

Background: Breast cancer remains the leading cause of mortality among middle-aged women in the west. Research has shown that cancer patients exhibit significantly elevated levels of circulating cell-free DNA (cfDNA), largely due to the presence of circulating tumor DNA (ctDNA). This study aimed to assess cfDNA concentrations in three groups: newly diagnosed breast cancer patients, individuals with benign breast conditions, and healthy participants. Additionally, it sought to explore cfDNA's potential as a noninvasive biomarker for early breast cancer detection. The study also compared cfDNA's diagnostic sensitivity with that of CA15-3, a traditional tumor marker, to evaluate its effectiveness in breast cancer diagnosis.

Methods: This is a case-control study involving 28 patients with primary breast cancer, 15 patients with benign breast tumors, and 10 healthy individuals. The plasma concentration of circulating cfDNA was measured using real-time PCR.

Results: The study showed a significant difference in the circulating cfDNA levels between the cancer group (mean ± SD = 77.76 ± 152.76 ng/mL) and both benign and control groups (mean ± SD = 0.31 ± 0.87 ng/mL and 1.03 ± 1.46 ng/mL, respectively), with $P < 0.001$ for both. There was a statistically significant association between elevated levels of circulating cfDNA and advanced cancer stages, with $P < 0.001$. Additionally, circulating cfDNA demonstrated 100% sensitivity compared to CA15-3, which had a sensitivity of 25%.

Conclusion: This study found that plasma levels of cfDNA were considerably higher in cancer patients than in the benign and control groups, with cfDNA levels raised significantly when cancer progressed to advanced stages.

INTRODUCTION

Breast cancer is a highly metastatic malignancy that frequently spreads to distant organs, including the liver, bones, lungs, and brain. This aggressive dissemination is a primary reason for its treatment-resistant nature. Early detection of the disease is crucial, as it significantly improves prognosis and enhances patient survival rates. [1,2] Despite its increasing incidence, its mortality has decreased in most developed countries because of screening, early detection and access to adjuvant targeted therapies.[3] Tumor biopsy remains the gold standard for capturing diagnostic, prognostic and predictive information in diagnostic oncology.[4,5] Mammography remains the gold standard for breast cancer screening, with proven efficacy in early detection. However, its accuracy can be compromised in cases where dense fibroglandular tissue obscures malignant

*Address for correspondence:
Israa M. Al-Hawwaz,
Department of Clinical Biochemistry, Basrah Teaching Hospital, Basrah, Iraq
Email: israa.mazin@avicenna.uobasrah.edu.iq

lesions, leading to potential false-negative results. For younger women with dense breast tissue—where mammographic interpretation is challenging—ultrasound serves as a valuable alternative. This non-invasive, radiation-free imaging technique offers a safer diagnostic option for this patient population. Additionally, it excels in differentiating cysts from solid lesions and offers real-time imaging for guiding biopsies. However, it is operator-dependent and not ideal as a screening tool due to its lower sensitivity for some malignancies and higher false-positive rates.[1] Magnetic resonance imaging (MRI) is of increasing interest for breast surgeons because it is useful as a screening tool in high-risk women and in distinguishing scar from recurrence in women with previous conservation therapy for cancer. However, it is of limited use in routine screening because it is expensive, time-consuming, and not recommended for low and average-risk women due to its higher false-positive rates, which may lead to unnecessary biopsies and anxiety.[1] Alongside imaging studies, the serum tumor marker carbohydrate antigen 15-3 (CA15-3) is the most common prognostic biomarker for monitoring patients and predicting their risk of relapse.[8] However, its use as a screening marker is not recommended because of its low sensitivity.[9]

Liquid biopsy has emerged as a promising field of research, offering a potential solution to these diagnostic constraints while enabling a more precise assessment of tumor biology.[10] Liquid biopsy in cancer refers to the isolation and analysis of tumor-derived substances such as DNA, RNA, intact cancer cells, or extracellular vesicles from bodily fluids such as blood, saliva, cerebral spinal fluid, or urine. The non-invasive characteristics of liquid biopsy enable serial monitoring and facilitate early cancer detection, offering significant clinical advantages over conventional diagnostic methods.[11] Overtime, being able to collect multiple blood samples can also help clinicians understand what kind of molecular changes are taking place in the tumor.[10,11] In addition, due to its minimally invasive nature, liquid biopsy has several unique advantages over other methods used for early detection of cancer, screening, and conventional surgical biopsy for cancer diagnosis. The most important advantage of liquid biopsy is its ability to capture information about tumor heterogeneity.[12]

Circulating cell-free DNA (cfDNA) has become particularly important in oncology because of the presence of significantly high plasma levels of cfDNA in cancer patients due to the role of tumor cell-derived DNA (ctDNA) arising from apoptosis, necrosis, and active secretion of cancer cells.[13-17] Multiple research studies have validated that circulating tumor DNA (ctDNA) contains distinct tumor-specific modifications, including point mutations, copy number variations, gene amplifications, and methylation patterns. These molecular signatures accurately reflect the genetic and epigenetic characteristics of the originating malignancy.[5,18,19]

Larger size fragments are usually associated with necrosis. Thus, tumors of small size will have small cfDNA fragments, while large tumors will develop hypoxic central tissues, leading to necrosis and the presence of larger cfDNA fragments in the blood.[12-20] Research indicates that healthy individuals typically exhibit low concentrations of cell-free DNA (cfDNA), ranging from 1.8–35 ng/mL in serum and 3.6–5.0 ng/mL in plasma. In contrast, cancer patients demonstrate significantly elevated cfDNA levels, with concentrations varying from 5 ng/mL to over 1000 ng/mL, representing a marked increase compared to normal physiological ranges.[12] The estimated half-life of cfDNA in blood varies from several minutes to 1–2 hours depending on multiple factors, including its association with molecular complexes that prevent rapid cfDNA degradation, the stage of the tumor, and the treatment modality.[21] In contemporary clinical practice, the application of plasma cfDNA quantification has emerged as a transformative biomarker with significant potential for early cancer detection, prognostic evaluation, and therapeutic monitoring. This innovative approach represents a major advancement in precision oncology.[7]

The aim of this research was to measure the plasma levels of circulating cell-free DNA in newly diagnosed breast cancer patients, patients with benign breast disease, and healthy controls. We also aimed to investigate the potential of cfDNA as a noninvasive biomarker for early breast cancer detection and assess its diagnostic sensitivity by comparative analysis with serum CA15-3 levels.

METHODS

This case-control study was carried out at the Department of Surgery and the Laboratory of Al-Basrah Teaching Hospital and the Oncology Center in Al-Sader Teaching Hospital from July 2020 to May 2021. The total number of patients and controls in the study was 53, including 28 patients with primary breast cancer, 15 patients with benign breast disease, and 10 healthy individuals. All participants, including both cases and controls, were female, aged 15–73 years (mean 38.90 ± 14).

Participant selection criteria

Patients were selected based on the following predefined inclusion and exclusion criteria.

Patients with newly diagnosed, untreated breast lesions (confirmed histologically or radiologically), who had not undergone surgical intervention or received any form of treatment since diagnosis, were included in this study.

Patients with any comorbid illness and pregnant women were excluded. The diagnosis of breast cancer and benign breast disease was confirmed depending on the results of the histological examination of the excisional biopsy. Breast cancer staging was done based on the results of the histological examination, and other imaging examinations such as ultrasound, CT scan, and MRI, employing breast cancer TNM (tumor, node, metastasis) staging. Healthy, non-pregnant women without breast masses or history of concomitant illnesses were recruited as controls.

Samples collection

A volume of 8 ml of blood was collected from all patients. The first 4 ml aliquot was collected in a gel separating tube and left for 15 minutes to clot at room temperature. Sera were then separated by centrifugation and used for measurement of renal functions (blood urea and serum creatinine), liver functions (ALT and AST), serum blood glucose, and serum tumor marker CA15-3. The second 2 ml aliquot was transferred to a dipotassium EDTA tube for complete blood count. The third 2 ml aliquot was collected into another EDTA tube and centrifuged at 4000 rpm for 10 minutes. Then, the plasma was transferred into a polypropylene tube and centrifuged again for another 10 minutes at 16000 g force for complete removal of cells from the plasma. DNA was then extracted from the plasma for circulating cell-free DNA (ccfDNA) estimation. Hemolyzed samples were rejected as hemolysis might influence cfDNA levels.

Analytical methods

Complete blood count was done by SYSMEX XT-2000i automated hematology analyzer using SLS SULFOLYZER kit (REF904-1141-4).

Serum CA15-3 was measured by cobas e 411 (Roche Diagnostics GmbH, Mannheim, Germany) using Elecsys CA15-3 II kit (REF03045838 122) which is based on sandwich noncompetitive immunoassay.

Serum glucose was measured using a COBAS INTEGRA 400 plus analyzer (Roche Diagnostics, Germany) with a Glucose HK Gen.3 kit (REF04404483 190), based on the enzymatic reference method with hexokinase.

Urea was measured in serum by COBAS INTEGRA 400 plus analyzer (Roche Diagnostics, Germany) using UREAL kit (REF04460715 190) which is based on kinetic test with urease and glutamate dehydrogenase.

Serum creatinine was measured by COBAS INTEGRA 400 plus (Roche Diagnostic, Germany) using Creatinine Jaffe Gen.2 kit (REF04810716), which is based on kinetic colorimetric assay (Jaffe method).

Serum ALT was measured by COBAS INTEGRA 400 plus analyzer (Roche Diagnostics, Germany) using ALTL kit (REF20764957 322).

Serum AST was measured by COBAS INTEGRA 400 plus analyzer (Roche diagnostics, Germany) using ASTL kit (REF20764949 322).

Estimating Plasma cell-free DNA (cfDNA) levels
DNA extraction method

DNA was extracted from plasma samples using the DNA, RNA, and protein purification NucleoSpin Plasma XS kit provided by MACHEREY-NAGEL GmbH & Co. KG (REF740900.10), specifically designed for the efficient isolation of circulating DNA from human plasma and serum.

DNA fragments as small as 50–1000 bp can be extracted by this kit with a high level of efficiency. This kit is based on the principle of bind-wash-elute procedures. The plasma sample was first mixed with binding buffer, then the mixture is applied to the NucleoSpin Plasma XS Column. Upon loading of the mixture, DNA binds to a silica membrane. After that, two subsequent washing steps efficiently remove the contamination and highly pure DNA is finally eluted with 5–30 µL of a slightly alkaline elution buffer of low ionic strength (5mM Tris-HCl, pH 8.5). The NucleoSpin® Plasma XS columns feature a specialized funnel-shaped design that enables minimal elution volumes (5–30 µL), yielding highly concentrated DNA extracts ideal for downstream applications.[22] Spectrophotometer nanodrop device (Implen, Munich, Germany) was used to measure the concentration of the extracted DNA.

Real-time PCR

Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene in cfDNA was determined by real-time PCR using RotorGene real-time PCR instrument (QIAGEN, Hilden, Germany). The glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene is one of the most common housekeeping genes used to normalize changes in specific gene expression.[32] The gene product is *GAPDH* protein, which is a homo-tetrameric protein predominantly present in the cytoplasm. This protein was identified as a glycolytic enzyme converting glyceraldehyde 3-phosphate (GAP) and inorganic phosphate into 1,3-bisphosphoglycerate (1,3-BPG) in the presence of NAD^+ and serves to break down glucose for energy production. *GAPDH* protein is generally up-regulated in highly glycolytic cancer cells and down-regulated by chemotherapeutic drugs. Cancer cells prefer the production of energy through glycolysis (the Warburg effect) even under aerobic conditions. This is allowed by increased glucose uptake and augmented *GAPDH* gene activity, which determine the production of glycolytic intermediates, stimulating anabolism and abnormal cancer cell proliferation.[33]

The forward primer 5′-GGAAGGTGAAGGT CGGAGTC-3′, the reverse primer 5′-GAAGAT GGTGATGGGATTTC-3′, and RealMOD TM Green SF 2X qPCR mix were used to amplify the sequence of the *GAPDH* gene. The forward and the reverse primers were provided by Macrogen company [Macrogen, Seoul, South Korea].[23] The master mix was RealMOD Green W² 2x qPCR mix, which was provided by LilifTM Diagnostics from South Korea (REF25353), which is an optimized ready-to-use solution for real-time quantitative PCR assays, incorporating SYBR Green I dye.[23] The master mix comprised Taq DNA Polymerase, Ultrapure dNTPs, MgCl2, and SYBR Green I dye. The real-time PCR mixture was composed of Master Mix: 10 µL, Forward primer: 1 µL, Reverse primer: 1 µL, Extracted DNA: 10 µL, and 1 µL nuclease-free water (Table 1).

Table 1. Cycling Conditions of the Real-Time Polymerase Chain Reaction

Step	Time	Temperature
Hold1	2 min	50 °C
Hold2	15 min	95 °C
Denaturation	15 sec	94 °C
Combined annealing and extension	60 sec	59 °C

Standard curve

Genomic DNA extracted from volunteer blood (13 ng/µL) was serially diluted 10-fold to generate a standard curve ranging from 13 ng/µL to 0.0013 ng/µL. Each dilution (10 µL) was analyzed in triplicate qPCR reactions using identical *GAPDH* primers and cycling conditions as those for the experimental samples. The same primers were used to amplify the *GAPDH* gene, and the same qPCR conditions were applied (Figures 1 and 2).

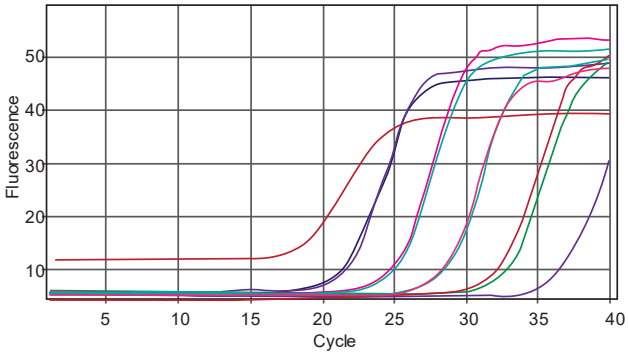

Figure 1. Quantitation Data for Cycling A. Green from 10-fold serial dilutions of genomic DNA (13 to 0.0013 ng/µL) using *GAPDH*-specific primers in qPCR. cf-DNA, cell-free DNA.

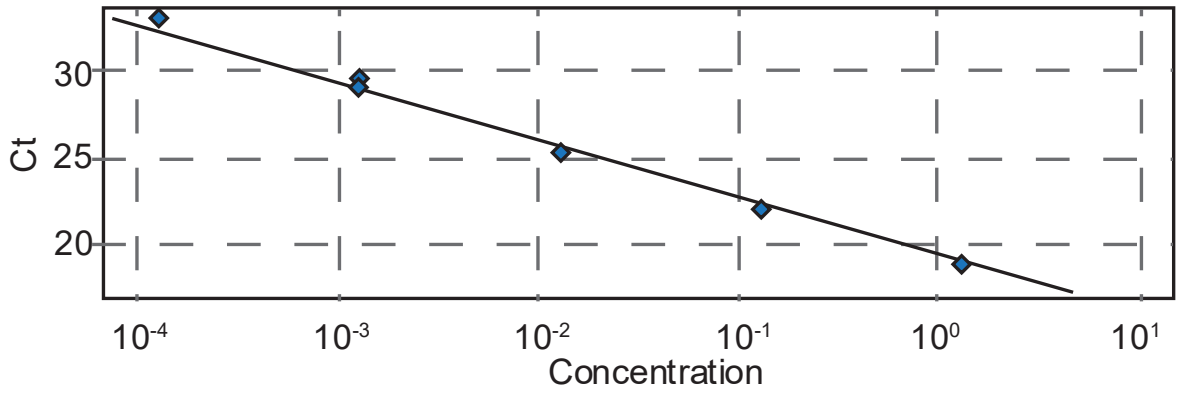

Figure 2. Standard Calibration Curve for Circulating Cell-Free DNA. A standard curve was generated using 10-fold serial dilutions of genomic DNA ranging from 13 ng/µL to 0.0013 ng/µL, with 10 µL of each dilution used per qPCR reaction. The resulting Ct values were plotted against the logarithm of DNA concentration to assess amplification efficiency using *GAPDH*-specific primers under consistent qPCR conditions.

Statistical analysis

Data analysis was performed using IBM SPSS Statistics (version 22), with continuous variables expressed as mean ± standard deviation (normally distributed) or median (interquartile range) (non-normally distributed), and categorical variables as percentages; group comparisons utilized One-way ANOVA (normally distributed variables across ≥3 groups) with post-hoc testing, Kruskal-Wallis test (non-normal distributions across ≥3 groups), independent t-tests (normal distributions between two groups), or Mann-Whitney U tests (non-normal distributions between two groups).

Diagnostic performance of circulating cell-free DNA and CA15-3 was evaluated via Receiver Operating Characteristic (ROC) curve analysis to determine sensitivity (true positive rate), specificity (true negative rate), and optimal cutoff values, with positive (PPV = true positives/[true + false positives]) and negative (NPV = true negatives/[true + false negatives]) predictive values calculated from 2×2 contingency tables; all tests were two-tailed with $P < 0.05$ considered statistically significant.

RESULTS

In this study, all patients and controls were females aged 15–73 years (mean 38.90 ± 14.00) (Table 2).

Table 2. Clinical Characteristics of the Study Population

Total number	53
Breast cancer cases, n (%)	28 (52.8%)
Benign cases, n (%)	15 (28.3%)
Controls, n (%)	10 (18.8%)
Gender, %	100% females
Age, y (mean ± SD)	38.90 ± 14.00

The analysis shows that, regarding serum levels of CA15-3, there is a statistically significant difference between the breast cancer and benign breast lesions groups ($P = 0.02$, mean serum level of CA15-3 = 24.16 IU/mL for the breast cancer group and 13.23 IU/mL for benign breast lesions group).

Table 3. Statistical Comparison of Circulating Cell-Free DNA Plasma Levels and CA15-3 Serum Levels Between Study Groups

Variables	Breast cancer (n=28)	Benign breast lesions (n=15)	Controls (n=10)	Test of significance (k)	P	Mann-Whitney test
CA15-3 IU/mL Mean ± SD	24.16 ± 21.72	13.23 ± 4.02	14.89 ± 4.39	5.88	0.053	P1 = 0.02 P2 = 0.22 P3 = 0.34
cfDNA ng/mL Mean ± SD	77.76 ± 152.76	0.31 ± 0.87	1.03 ± 1.46	39.79	<0.001	P1 < 0.001 P2 < 0.001 P3 = 0.46

Statistical comparisons were performed using the Kruskal-Wallis test (K) for overall group differences, followed by Mann-Whitney U tests for pairwise comparisons between: breast cancer vs. benign lesions (P1), breast cancer vs. controls (P2), and benign lesions vs. controls (P3); $P < 0.05$ was considered statistically significant.

Hence, there is no statistically significant difference either between the breast cancer group and controls or between controls and the benign breast lesions group ($P = 0.22$ and $P = 0.34$, respectively) (Table 3).

Plasma cfDNA levels showed significant elevation in breast cancer patients (77.76 ± 152.76 ng/mL) compared to both benign breast lesions (0.31 ± 0.87 ng/mL; $P < 0.001$) and healthy controls (1.03 ± 1.46 ng/mL; $P < 0.001$). There is a statistically significant difference in the mean levels of plasma cfDNA between the breast cancer patients and benign breast lesions and controls ($P < 0.001$ for both). There is no statistically significant difference between benign breast lesions and controls ($P = 0.46$) (Table 3). Also, there is a statistically significant difference between elevated levels of plasma cfDNA and cancer stages with $P < 0.001$ (Table 4).

To evaluate the diagnostic power of the plasma level of cfDNA, the ROC curve analysis was performed on both plasma levels of cfDNA and serum levels of CA15-3 between breast cancer cases and nonmalignant cases, as can be seen in Figure 3 and Table 5.

Table 4. Relationship Between Plasma Levels of Circulating Cell-Free DNA and Stages of Breast Cancer Using TNM Staging for Breast Cancer

Cancer stage	Number	Mean ± SD (ng/mL)	Test of significance (ANOVA)	P value
Stage 2	14	18.33 ± 15.60[ab*]		
Stage 3	11	65.01 ± 30.72[c*]	9.36	<0.001
Stage 4	3	401.88 ± 354.31		
Total	28	77.76 ± 152.76		

* Tukey's test: [a] $P > 0.05$ (stage 2 vs stage 3), [b] $P < 0.001$ (stage 2 vs stage 4); [c] $P < 0.001$ (stage 3 vs stage 4)
ANOVA, analysis of variance; SD, standard deviation.

Regarding serum levels of CA15-3, ROC curve analysis shows a sensitivity of 25% and a specificity of 100% for diagnosis of breast cancer (area under the curve [AUC] = 0.68, cutoff point = 26.4 IU/mL, P = 0.023, positive predictive value [PPV] = 100%, and negative predictive value [NPV] = 54%). Regarding plasma levels of cfDNA, ROC curve analysis shows a sensitivity of 100% and a specificity of 88% for diagnosis of breast cancer (AUC = 1.00, cutoff point = 3 ng/mL, P < 0.001, positive predictive value [PPV] = 90%, and negative predictive value [NPV] = 100%).

DISCUSSION

While mammography remains the sole screening modality with demonstrated efficacy in both early breast cancer detection and mortality reduction, it is not without limitations. The integration of reliable noninvasive biomarkers with mammographic screening could enhance diagnostic sensitivity, potentially reducing unnecessary invasive procedures (biopsies, surgeries) and overtreatment. Such a combined approach may also mitigate the associated psychological distress for patients, optimizing the risk-benefit ratio of breast cancer screening programs.[6,24]

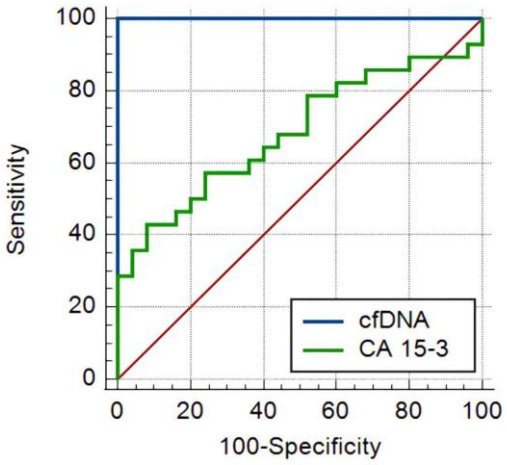

Figure 3. Receiver Operating Characteristics Curve Comparing the Diagnostic Performance of Cell-Free DNA (CfDNA) Concentration and CA15-3 Levels in Distinguishing Malignant from Nonmalignant Cases

Table 5. Receiver Operating Characteristics Curve Values for Circulating Cell Free DNA Plasma Levels and CA15-3 Serum Levels to Differentiate Between Malignant and Nonmalignant Cases

Markers	Cutoff point	Sensitivity	Specificity	PPV	NPV
Circulating cell-free DNA	3 ng/mL	100%	88%	90%	100%
CA15-3	26.4 IU/mL	25%	100%	100%	54%

NPV, negative predictive value; PPV, positive predictive value.

Circulating cell-free DNA has attracted considerable attention in the last few years as a promising component of the liquid biopsy. Circulating cell-free DNA concentration and integrity vary with the pathological state. Furthermore, circulating tumor DNA (ctDNA) carries tumor-specific genetic and epigenetic signatures, making it a promising multi-purpose biomarker. Its applications include: (1) early cancer detection, (2) real-time monitoring of therapeutic response, and (3) assessment of tumor aggressiveness and disease progression.[21–24]

In our study, elevated serum levels of CA15-3 were identified in 7 patients with breast cancer (25%). Also, higher levels were found in patients with larger tumor sizes and advanced TNM stages. These findings are in agreement with those obtained by Shao *et al.* and JS *et al.,* who found that CA15-3 levels were elevated in 12.3% and 10.5% of breast cancer patients, respectively.[25,26] A possible explanation is that there is an association between tumor burden and elevated levels of serum CA15-3, and that the preoperative serum levels of CA15-3 are associated with the tumor size and lymph node metastasis, which represent tumor burden. As a result, significantly higher levels of CA15-3 were seen in patients with advanced disease than in those with locoregional breast cancer.[26,27] These results demonstrate the clinical relevance of cfDNA as a complementary biomarker to CA15-3 in breast cancer management. While CA15-3 exhibits limited sensitivity for early detection, its elevated baseline levels may serve as a prognostic indicator for adverse outcomes. Importantly, our data reveal significantly higher plasma cfDNA concentrations in breast cancer patients compared to both benign cases (P < 0.001) and healthy controls (P < 0.001). Notably, no statistically significant difference was observed between benign and control groups (P = 0.46), underscoring cfDNA's potential specificity for malignant disease.

These findings are in agreement with those obtained by Gamaal *et al.,* who found that there is a significant elevation in plasma levels of cfDNA in breast cancer patients in comparison with nonmalignant cases (P = 0.001), and no significant

difference between patients with benign breast lesions and controls ($P = 0.34$).[13] Khurram *et al.* also found elevated cfDNA levels in patients with breast cancer compared to healthy controls, indicating its relevance for early detection and disease monitoring.[31] A possible explanation is that the high levels of cfDNA in breast cancer patients come from apoptosis, necrosis, in addition to the active secretion by cancer cells, which overloads the clearance system.[17-21] Another possible explanation is that, during the onset and the progression of the cancer, nucleic acids from the cancer cells are released into the blood circulation in small particles (microparticles) coated by the cell membrane, protecting cfDNA from DNAase.[28]

The results of this study also show that there is a significant statistical association between elevated plasma levels of cfDNA and cancer staging. Plasma cfDNA concentrations showed significant stage-dependent elevation, with advanced-stage breast cancer patients demonstrating markedly higher levels compared to early-stage cases ($P < 0.001$). These findings are in line with those obtained by Hashad *et al.* and Tangvarasittichai *et al.,* who found that elevated levels of cfDNA were significantly associated with clinical stage ($P < 0.001$ and 0.046, respectively).[29-32] This increase likely reflects greater tumor cell turnover and necrosis in advanced-stage disease. To evaluate the diagnostic power of the plasma level of cfDNA, the ROC curve analysis was performed on plasma levels of cfDNA between breast cancer and non-malignant groups. ROC curve analysis shows a sensitivity of 100% and a specificity of 88% for the diagnosis of breast cancer (AUC = 1.00). These findings are in agreement with those obtained by Yu *et al.,* who found that cfDNA concentrations in samples from breast cancer patients collected before surgery revealed a pooled sensitivity and specificity of 90% and 88%, respectively, with an AUC of 0.95.[7]

CONCLUSION

This study revealed that plasma levels of cfDNA were significantly increased in patients with breast cancer compared with controls and patients with benign breast lesions. Also, plasma levels of cfDNA were found to increase as breast cancer progressed to advanced stages. For this reason, quantitative identification of plasma cfDNA may possess a noninvasive diagnostic value for early detection of breast cancer.

Therefore, we recommend further investigation into plasma cfDNA as a potential noninvasive screening tool for early breast cancer detection, complementary to mammography. Large-scale studies are needed to validate these findings and establish population-based cutoff values. Future research should focus on improving sensitivity for very early-stage cancer detection, where cfDNA levels may be minimal, and explore combining cfDNA quantification with genetic mutation or epigenetic modification analysis to enhance diagnostic accuracy.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest related to this publication.

DATA AVAILABILITY

The data supporting the findings of this study are available from the corresponding author upon request.

ETHICAL CONSIDERATIONS

This study was approved by the research ethical committee at Al-Basrah Medical College. Informed consent was taken from the patients and controls before collecting blood samples (No. 145 in 01/03/2020).

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