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EVALUATE THE CONCENTRATION OF SERUM CFDNA AND INTEGRITY AS A NON-INVASIVE MARKER TO DISTINGUISH BETWEEN MALIGNANT AND BENIGN BREAST LESION

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Abstract

under the curve (AUC) of (0.99), with ALU-247bp exhibited an area under the curve (AUC) of (1.00) and with Integrity area under the curve (AUC) is 0.89 that indicating perfect effect achieve a promising novel noninvasive approach for diagnosis and early detection of malignant breast cancer.Breast cancer is the most common cancer in the women so it important to diagnosis and discriminate between malignant and benign breast lesion by using biomarkers with more sensitivity and specificity than traditional biomarker Therefore, there is an urgent need for novel markers to increase the sensitivity and specificity of diagnostic analysis. The noninvasive method to measure the concentration of circulating cell-free DNA(cfDNA) and cfDNA integrity in serum as a liquid biopsy which could replace tissue biopsy.**The aim** of this study was to assessed the diagnostic values of cfDNA using it's concentration, ALU -247bp, ALU- 115bp and integrity in serum of patients with breast lesion as a noninvasive marker to distinguish between malignant and benign breast tumor .

In order to applicant this study , 90 women in 3 groups were collected. control group, benign breast lesions and malignant breast cancer. The serum sample of all groups were used to measure by ELISA method for determination Cancer Antigen 15.3 (CA15.3). Other part of the serum utilized for extract serum cfDNA in order to measure it's concentration by flourometic method using specific dye and for determined cfDNA integrity through quantitative analysis method real-time PCR , by which cfDNA integrity calculated by the ratio of the concentration of longer cfDNA fragment to shorter cfDNA fragment. The correlations determined cfDNA concentration, ALU-247bp , ALU-115bp, DNA integrity and cancer antigen (CA)15-3 between malignant breast cancer and benign breast lesion group as well as the combined relationship with traditional biomarker CA15-3.

The results showed Serum levels of cfDNA concentration, ALU-247 and cfDNA integrity significantly increased in malignant breast cancer patients compared to benign breast lesion patients (p-value =0.001 for all) but there was less significant increase in ALU-115 (P-value =0.023). Also cfDNA concentration, ALU-247 and cfDNA integrity had the best sensitivity (97%, 100% and 100% respectively) and specificity(93% ,100% and 100% respectively) for discrimination between malignant and benign breast lesion with the value of area under the ROC curve were (0.98 , 1 and 1 respectively).

In conclusion the combined ROC curves increased the ability of CA15-3 to discriminate between

malignant and benign with different noninvasive biomarker. Notably, the combination of CA15-3 with cfDNA concentration, exhibited an area under the curve (AUC) of (0.99), with ALU-247bp exhibited an area under the curve (AUC) of (1.00) and with Integrity area under the curve (AUC) is 0.89 that indicating perfect effect achieve a promising novel noninvasive approach for diagnosis and early detection of malignant breast cancer.

Key Words: Malignant breast cancer, Benign breast lesion , cfDNA concentration , cfDNA integrity, ALU fragments, noninvasive biomarkers.

Introduction

Breast cancer is a type of cancer that develops in the breast tissue. It occurs when abnormal cells in the breast tissue start to grow and divide uncontrollably. Breast cancer can affect both men and women, although it is more common in women (Han et al., 2020)

Early and exact detection of breast cancer is important as it gives a better chance of a successful treatment. can be detected through regular breast self-exams, clinical breast exams, and mammograms (Waks & Winer, 2019). Cancer antigen 15–3 (CA15-3) is the most widely used as serum tumor marker in the clinical field (Henderson et al., 2016) but its specificity is low because elevated levels have been observed in other malignant tumor and in patient with benign breast lesion and liver disease(Desai & Guddati, 2023). Therefore, there is a critical need for novel noninvasive blood markers that either outperform the conventional blood-based biomarkers or are to be used in parallel to them to increase the sensitivity and the specificity of diagnostic tests, The new markers could be examined by noninvasive methods like the measurement of circulating cell-free DNA in serum as a liquid biopsy which could replace tissue biopsy and facilitate the evaluation of focal tumors.

Circulating cell-free DNAs (cfDNA) are double-stranded DNA fragments that can be detected in the non-cellular component of blood (Yuejiao Zhong et al., 2020). Normally cell-free DNA enters the bloodstream after apoptosis but in patients with cancer, cfDNA is shed from both normal and cancer cells. then elevated cfDNA concentrations have been observed in patients . (Stejskal et al., 2023)

Normally, these fragments are cleaned up by macrophages, but in fact the overproduction of cells in cancer leaves more of the cfDNA behind (Kwong, 2020)Evaluation and quantification of cfDNA in serum, termed “liquid biopsy” has become one of the most important clinical analysis for early cancer detection (Alimirzaie, S et al., 2019)

Here, we analyzed the use of a cfDNA concentration and integrity in breast cancer patients based on peripheral blood as a non-invasive biomarker for diagnosis and differentiate between malignant and benign breast lesion.

Cancer Antigen 15-3

Cancer antigen 15-3 also known as CA 15-3, is a protein biomarker that is commonly used in the diagnosis and management of breast cancer. The molecular weight of CA 15-3 is approximately 400-600 kDa (Ribeiro et al., 2018).

CA 15-3 is measured through a simple blood test, elevated levels of CA 15-3 in the bloodstream may be indicative of breast cancer, although it is important to note that other factors can also cause

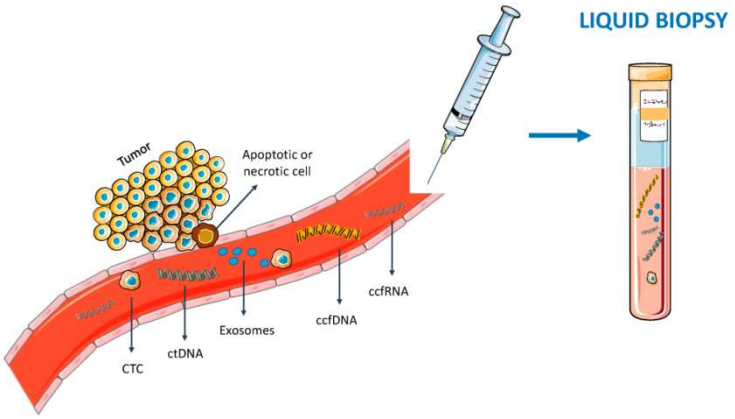
increased levels of this biomarker. As such, CA 15-3 is typically used in combination with other tests to help detect ,monitor and breast cancer screening which has been widely adopted into clinical routine and plays a critical role in breast cancer (.(Coppola et al., 2021) . with imaging, disease history, and clinical course, an aberrant level or increase of CA-15.3 helps to diagnose tumour progression in metastatic breast cancer (Clatot et al., 2020)(Attia et al., 2021).

Despite its relationship with BC cells, an increase in CA 15-3 can also be observed in benign illnesses such as liver disease and benign breast, lung, or ovarian disease; hence, it is not regarded a diagnostically specific biomarker for BC (Coppola et al., 2021)), (Soodabeh H. et al., 2019). Prior to now, there was insufficient evidence supporting the use of CA-15.3 instead of traditional follow-up.

Circulating cell-free DNAs (cfDNA)

(cfDNA) are double-stranded DNA fragments that can be detected in the noncellular component of blood , In healthy individuals, The presence of cfDNA was first reported in 1948 by Mandel and Metais (P. Mandel, P. Metais, 1948);(Hassan et al., 2022)

Cell-free DNA (cfDNA), consists of DNA fragments released after cell death processes from both normal and tumor cells most evidence suggests that the released cfDNA is primarily a consequence of apoptosis that could be released by various pathologic in addition to normal physiologic mechanisms (Zhu et al., 2021). figure(1-6).



Figure(1-6): Sources of cell-free DNA (cfDNA) in body fluids.(Constâncio et al., 2020).

The most widely reported mechanisms for shedding of genomic DNA into the circulation as cfDNA include apoptosis and cell necrosis along with other putative mechanisms such as active secretion and vesicular shedding into the bloodstream(Chan et al., 2021) .

Normally, these fragments are cleaned up by macrophages, but in fact the overproduction of cells in cancer leaves more of the cfDNA behind, so the concentrations of cfDNA were elevated in patients with cancer. these Fragments of cfDNA tend to be short, ranging between approximately 180-200 base pairs in length. (Hu et al., 2021)

The half-life of cfDNA in circulating blood varies from one to two hours and are present at high concentration in both early and late stage disease in many common tumors including non-small cell lung(Michaelidou et al., 2020);(Celec et al., 2018)

The gold standard for breast cancer diagnosis is still tissue biopsy, also Mammography is a sensitive test that is recommended every two years in women older than 50. However, more than20% of patients diagnosed with breast cancer are younger than 50 and there is no specific test for this age group, new methods are needed To resolve that issues, and liquid biopsy is a promising option. For example, cfDNA levels were higher in cancer patients compared to those with benign lesions, (Sant et al., 2022)

Evaluation and quantification of cfDNA in serum, termed "liquid biopsy" has become one of the most important clinical analysis as noninvasive, fast, repeatable, and sensitive biomarkers for molecular detection, prognosis, and treatment follow-up in a variety of cancers including PCa (Zulato et al., 2022)

Extensive research has been performed to utilize blood-based analytics for detection and monitoring of the disease in cancer patients. In many bodily fluids, including blood serum, circulating cell-free DNA(cfDNA) is present (Heitzer, E. et al., 2019)(Gupta et al., 2020)

Advantages and limitations of liquid biopsies

Liquid biopsies have many advantages . ctDNA/cfDNA are both easier to collect serially (and less expensive) than tissue biopsy and, in many cases, can provide critical molecular and response information in real time, especially for patients harboring difficult to biopsy neoplasms .They are non-invasive and less expensive than traditional tissue biopsies. They have the potential to detect material shed from multiple tumor or metastatic sites rather than analyzing a small piece of tissue biopsied; therefore, liquid biopsies have the potential to better detect heterogeneity in the tumor across sites.(Barbirou et al., 2022) (Nikanjam et al., 2022)

Liquid biopsies can be obtained serially to observe changes with therapy. They are an easier means for monitoring therapeutic responses than tissue biopsy, and liquid biopsies have potential in early cancer detection as part of screening, as well as detecting minimal residual disease following therapy. (Nikanjam et al., 2022) (Liang, L. et al., 2023)

There are also several limitations of liquid biopsies . ctDNA/cfDNA can be shed in only small amounts and not all patients can have detectable levels, especially those with low tumor burden. sequencing can be difficult and expensive. Not all detectable cfDNA alterations are cancer-related, Moreover, not all ctDNA/cfDNA is equally shed from the primary tumor and metastases, Shedding of ctDNA can be suppressed by treatment and may be limited at certain disease site. (Adashek, et al., 2021)

The integrity of cfDNA

cfDNA integrity is a method used to evaluate the quality of cfDNA isolated from clinical samples, calculated as the ratio of the concentration of longer DNA fragments to shorter fragments in plasma or serum. (Condappa et al., 2020)

based on The mechanism of cfDNA released into the bloodstream is the primary determinant of fragmentation signatures of serum/plasma DNA. Fundamentally, non-neoplastic cells that undergo apoptotic cell death, shed DNA fragments of about 150 bp in size as a result of enzymatic cleavage of nucleosome units, whereas tumor cells undergo many different death processes, including necrosis and autophagy, and can release longer DNA fragments. During tumor development, the

release of cfDNA increases, and it contains both tumor-derived and normal DNA fragments. This makes the assessment of cfDNA integrity a critical step in liquid biopsy applications. (Gezer et al., 2022)

In order to reliably measure such DNA fragments, researchers have employed the abundant presence in the human genome of DNA ALU repeats - repetitive ~300 bp sequences of retro transposon origin found in genomic introns (Condappa et al., 2020)

Using different primers, fragments of these ALU repeats can be detected of either >200 bp (indicative of necrotic DNA), or of <200bp (detecting both necrotic and apoptotic DNA). Detection of these longer cfDNA fragments and their relative abundance compared to short cfDNA A fragments in serum appears to be a promising tool for diagnosis and prognostic prediction of malignancies (Khani et al., 2019)

Real-time quantitative PCR can used to directly measure serum shorter fragments of 115 bp that were considered as derived from apoptotic normal cells and larger ones of 247 bp as ctDNA, derived from necrosis/autophagy of cancer cells. The cfDI value calculated as the ratio quantity of longer over shorter fragments, ALU247/ALU115 (Vizza et al., 2018)

Therefore, the ratio of short to long DNA fragments can serve as an indirect measure of cfDNA integrity promising biomarker for cancer diagnosis, prognosis, and treatment monitoring.(Gianni et al., 2022) .

Materials and methods

Subjects and Design of the Study:

From 1 April 2022 to 1 June 2023, this case-control study was done on 90 of both apparently healthy , and breast tumor female of both malignant and benign. Women patients were recruited from Al-ZahraaTeaching Hospital, Al-Karama Teaching Hospital, and other private Hospital, Wasit, Iraq. The practical part was conducted in the Research Laboratories of the College of Medicine / Wasit University.

Study Groups

The study included 90 women, age was matched in all groups. The age range was between (25-68) years old These groups consist of the following:

Group1: breast cancer (BCA) consist of 30 women with breast cancer confirmed by histopathology with age range (25-68).

Group 2: benign breast disease (BBD) consist of 30 women with benign breast lesion confirmed by histopathology with age range (25-68).

Group3: (control) 60 apparently healthy women of age matched with group1 and group2 free of breast disease with age range (25-68).

Samples Collection

Blood samples were obtained from the apparently healthy women and patients with breast lesion who examined by a consultant physician and were eligible for surgery , their specimen was sent to the Pathology Department for histopathological examination and diagnosis if the lesion benign or malignant . Five milliliters (5ml) of venous blood were withdrawn using disposable syringes in the sitting position. The blood is discharged slowly into disposable gel test tubes without

anticoagulant (Gel Plus Clot Activator tube) , The blood was allowed to clot at 37˚C for 10minutes, then Serum was separated from whole blood using a two-step, Blood was centrifuged for 10 minutes at4000 rpm at room temperature in a Table centrifuge (Hettich EBA 20, Germany) in order to separate red and white blood cells. The superannuated part of the separated serum was transfer for second centrifuged at 1500 rpm for 10 min. at 4◦C in a cooler centrifuge (Hermle Z216 MK Refrigerated centrifuge ,Germany) . serum samples was aliquot into eppendorf tubes for serum cfDNA extraction and further experimentation and stored at -80°C until analysis. (Sultana et al., 2023)

Methods

Human CA 15-3 Assay :

This assay is based on the sandwich ELISA principle for direct antigen detection including high affinity and specificity antibodies (enzyme labelled and biotinylated) with different and distinct epitope recognition.

Quantitative Determination of cfDNA:

cfDNA extraction was performed using the cfDNA extraction kit based on column-based systems, Quantification of nucleic acids was performed using Quantifluor dsDNA kit (promega–USA) by GloMax multidetection system in which the program of double-strand DNA (ds-DNA) was selected to calculate nucleic acid concentration. fluorescence was used to measure total serum cfDNA concentration

For the purpose of calibrating the fluorometer, both blank and standard samples were generated. In contrast to the Nano Drop TM, the Quants fluorometer is based on the principle of fluorometry, which includes the use of very sensitive and precise fluorescent dyes to measure DNA, RNA, and protein. The UV-induced fluorescence of intercalating dyes is a more accurate and sensitive approach for detecting DNA than spectrophotometers. Since intercalating dye only sticks to double-stranded DNA, it has no effect on proteins or ribonucleic acid molecules that are contaminating the DNA.

Cell Free DNA Integrity

CfDNA integrity was calculated as the ratio of concentrations longer over shorter fragments in each assay:

CfcDNA integrity = ALU 247 bp fragments

ALU 115 bp fragments

has been explored in BC by qPCR by many authors using SYBRGreen fluorescent dye They measured in serum. (Condappa et al., 2020)

GoTaq qPCR Master Mix is a reagent system used for quantitative PCR (qPCR) contains a fluorescent DNA-binding dye (the BRYT Green Dye), that exhibits greater fluorescence enhancement upon binding to double stranded DNA (dsDNA) than SYBR Green .

The Reaction Mix was Assembled to a final volume of 20µl as described in the table 1

Table 1: Reaction mix of real time PCR :

Component	Volume

GoTaq qPCR Master Mix (2X)	10µl
Forward Primer (20X)	0.5µl
Reverse Primer (20X)	0.5µl
cfDNA template	9µl

A set of primers for the 115 base-pair amplicons and 247-bp amplicons were designed to amplify both shorter and long DNA fragments representing the total amount of circulating cell free DNA, A negative control without any template was also performed on each run.

ALU115 F 5'CCTGAGGTCAGGAGTTCGAG-3'
R 5'CCCGAGTAGCTGGGATTACA-3'
ALU247 F 5'GTGGCTCACGCCTGTAATC-3'
R 5'CAGGCTGGAGTGCAGTGG-3'

Thermal Cycle

Amplification reactions were carried out by using thermocycler real-time PCR apparatus with program that describe in table 2 below, and Ct value of the RT-qPCR test was indirect indicator of the fragments amount as showed in figure1 and 2:

Table 2: Thermal Cycle Program

Step	Temperature	Time	Cycle
GoTaq Hot Start Polymerase activation	95°C	2 minutes	1
Denaturation	95°C	15 seconds	44
Annealing and extension	60°C	1 minute	

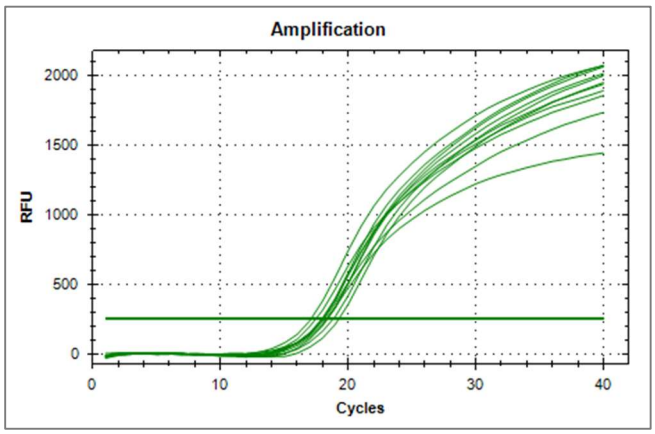


Figure 1: Ct value of RTpcr for ALU 247 bp

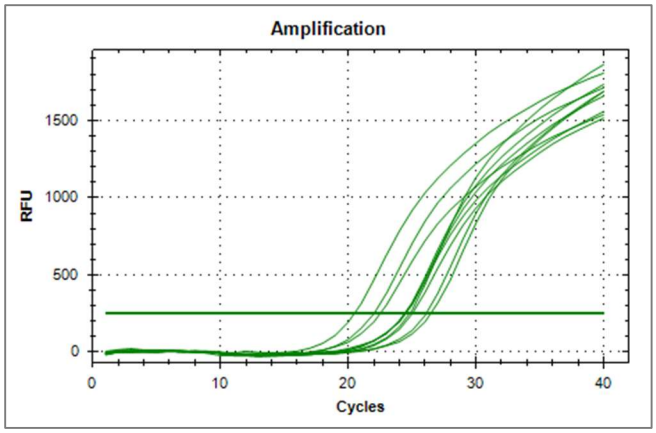


Figure 2: Ct value of RTpcr for ALU 115 bp

the Ct value of each sample was used to determine its absolute concentration of fragments by interpolating it from the DNA standard curve of serial 10X dilutions volunteered female DNA purchased from the Promega Corporation. of known DNA concentrations, water used instead of DNA in the blank sample. figure 3 and 4:

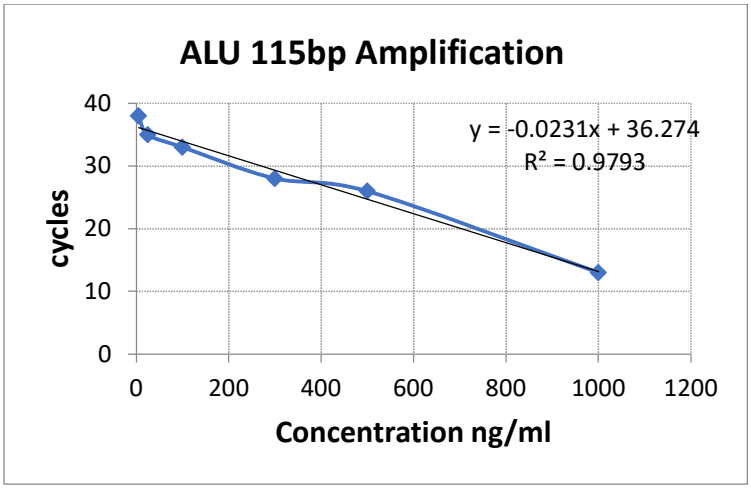


Figure 3: Standard Curve of ALU115bp

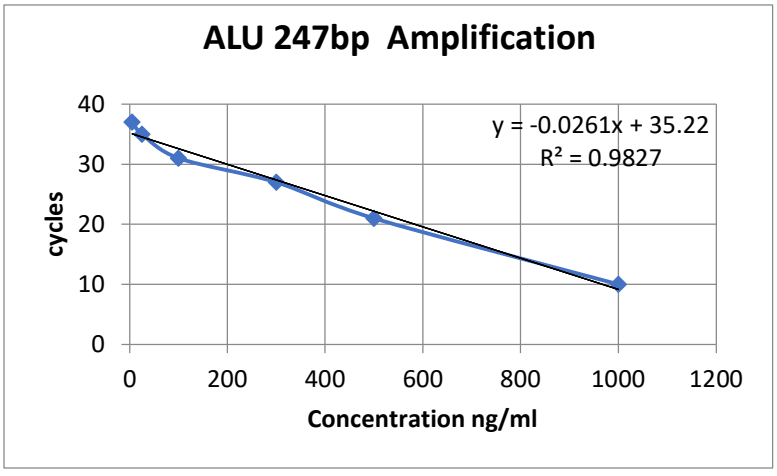


Figure 4: Standard Curve of ALU247bp

From this way , we derived the intercept and slope of the curve , the absolute concentration of each sample was measured from Ct values using the following formula:

{ Absolute concentration = (Ct-intercept)/ slope) }

Finally, The absolute concentration of ALU247 was divided by the absolute concentration of ALU115 in order to obtain the integrity (ALU247/115 ratio) for each sample .

Qualitative Detection of ALU Elements:

Agarose-Gel Electrophoresis is the method that used to detect ALU element qualitatively.

Electrophoresis is the movement of a charged molecules, chiefly proteins and nucleic acids under the influence of an electric field(Brod et al., 2016), Agarose gel electrophoresis is the most effective way and standard method to separate, identify, purify DNA fragments and it is simple, rapid to perform(Bhattacharya & Van Meir, 2019).

Loading dyes used in gel electrophoresis serve three major purposes; First, they add density to the sample, allowing it to sink into the gel. Second, the dyes provide color and simplify the loading process. Finally, the dyes move at standard rates through the gel, allowing for the estimation of the distance that DNA fragments have migrated.(Neoh et al., 2019) After separation, the DNA molecules can be visualized under UV light after staining with an appropriate dye(Dendani Chadi & Arcangioli, 2023)

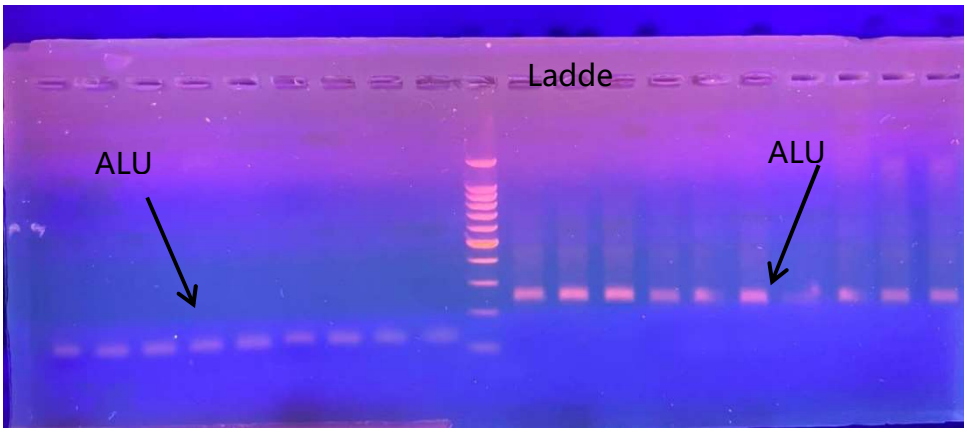


Figure5: cfDNA extracted from serum under UV –light. ladder: DNA marker 100Pb.

Statistical Analysis

The data was analysed using Software Package for Social Science (SPSS-22.0 version). The data was presented as a mean and standard deviation (SD). Continuous variables were tested for normality according to the one way ANOVA and linear regression analysis that have been used to determine the significant difference between the groups.

(ROC) curve analysis was used with its corresponding area under the curve (AUC), accuracy level, sensitivity, specificity and level of significance (P) using using the R statistical programming language within the RStudio integrated development environment (IDE). The 'pROC' package in R facilitated the generation of Receiver Operating Characteristic (ROC) curves to assess the diagnostic performance of individual parameters in cancer detection.

Results

The level of CA15-3 in serum contrasted among study groups are summarized in Table 3 . Where

showed strong significance increased in the serum level of CA15-3, (P<0.001) in malignant patients in comparison to benign group., that was in agreement with (Fu et al., 2017) and (Chao L. et al. 2018)

Table 3: **Descriptive characteristic of cfDNA Among Study Groups**:

Parameters	groups	N (%)	Mean	S.D	Minimum	Maximum	P value
CA153 U/ml	M	30 (50%)	83.9	39.4	29.18	222.7	0.001
	B	30 (50%)	44.2	18.7	7.79	72.3	

as showed in figure 10 below figure the specificity of CA15-3 in our study was 70% and the sensitivity was found 87% (table 4).

Table 4: **Receiver operator characteristics (ROC) curve parameters for serum markers between malignant and benign breast mass.**

Parameters	AUC*	Cutoff	Sensitivity %	Specificity %	p-value
CA153	0.88	53.7	87%	70%	.000
CEA	0.85	14.13	97%	60%	.000

***AUC** measures the performance of the predictor in distinguishing between 'malignant' and 'benign' cases The **cutoff value** is the threshold used by the predictor to classify between 'malignant' and 'benign' cases. For example, if the predictor value is above the cutoff, it might be classified as 'malignant', and vice versa, **Sensitivity** is the true positive rate and **Specificity** is the true negative rate

Evaluation of Cell free DNA concentration within study groups

The mean of circulating cell-free DNA concentration in serum samples of malignant breast cancer patients was 641.4±147.2, while the patients with benign breast lesions was 281± 94 and in healthy individuals was 73.7± 30 ,the descriptive characteristic showed in (Table 5). The significantly higher in patients with malignant and benign breast lesions than in healthy controls at (p=<0.01) table 6 ,figure 6

Table 5 Descriptive characteristic of cfDNA Among Study Groups:

Descriptive						
Parameters		N(%)	Mean	SD.	Minimum	Maximum
cfDNA concentration (ng/ml)	M	30(33.3%)	641.4	147.2	342	894
	B	30(33.3%)	281.0	94.7	136	496
	H	30(33.3%)	73.7	30.7	17	129

n: *denotes the number of cases****; %:*** *denotes to percentage of distribution* ***SD****: is the standard deviation****; M:*** *denotes malignant breast carcinoma****; B*** *benign breast lesion H: healthy group.,*

Table 6:Multiple Comparisons of circulating cell free DNA Among Study Groups

Parameter	Groups		p-value
cfDNA concentration (ng/ml)	M	B	0.001 *
	M	H	0.001 *
	B	H	0.001 *

M: *denotes malignant breast carcinoma****; B*** *benign breast lesion H: healthy group.,*

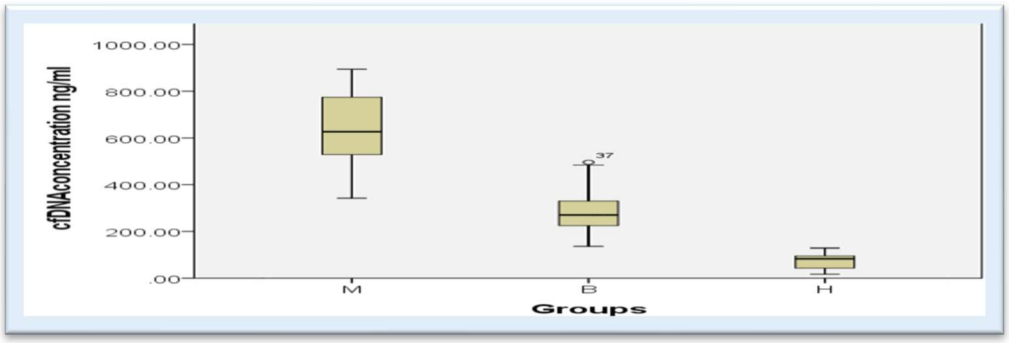


Figure6: cfDNA concentration for all study groups .

Determine the ALU fragments in cfDNA and compared it between study groups

DNA integrity is determined by evaluation the ratio of ALU long fragment 247bp to ALU short fragment 115bp concentrations , mean±SD. of ALU 115BP for malignant, benign and healthy women were 265.2±91.6, 223.9±74.7 and 41.9±19.4 respectively, ALU 247bp mean± SD. for malignant, benign and healthy were 559.8 ±122.0 , 80.8±20.7 and 24.4±11.04 respectively while the integrity were 2.3±0.8 , 0.40±0.1 and 0.61±0.1 respectively (table 7)

Table 7 : Descriptive characteristic of ALU 115 , ALU247fragments between study groups

Descriptive						
Parameters	Groups	N	Mean	SD.	Minimum	Maximum
ALU115	M	30	265.2	91.6	129	485
	B	30	223.9	74.7	119	353
	H	30	41.9	19.4	17	97
ALU247	M	30	559.8	122	355	785
	B	30	80.8	20.7	23	114
	H	30	24.4	11.04	13	56
Integrity	M	30	2.3	0.8	1.27	5.91
	B	30	0.4	0.1	0.13	0.80
	H	30	0.61	0.1	0.34	1.00

This study demonstrated significant differences between patients of malignant breast cancer and benign breast lesion in the concentration of long fragment DNA ALU247bp at P value <0.001 and

cfDNA integrity pvalue <0.001 ,but there is less significant difference in the concentration of short DNA fragment ALU115bp at p value 0.025, the result showed in table8

Table 8 :Multiple Comparisons of ALU 115BP, ALU 247BP and cfDNA integrity between study groups

Parameters	Groups		p-value
ALU115	M	B	0.023 *
	M	H	0.001 *
	B	H	0.001 *
ALU247	M	B	0.001 *
	M	H	0.001 *
	B	H	0.003 *
Integrity	M	B	0.001 *
	M	H	0.001 *
	B	H	0.121 N.S.

*The mean difference is significant at the 0.05 level

LSD *multiple comparison test **: denotes significant at p ≤ 0.01* ***N.S*** *denotes not significant;* ***; M:*** *denotes malignant breast carcinoma****; B*** *benign breast lesion* ***H****: healthy group.,*

The study show high significant difference in ALU 247 bp biomarker to differentiate between malignant and benign than ALU115bp or integrity as cleared in the (figures 7,8,9)

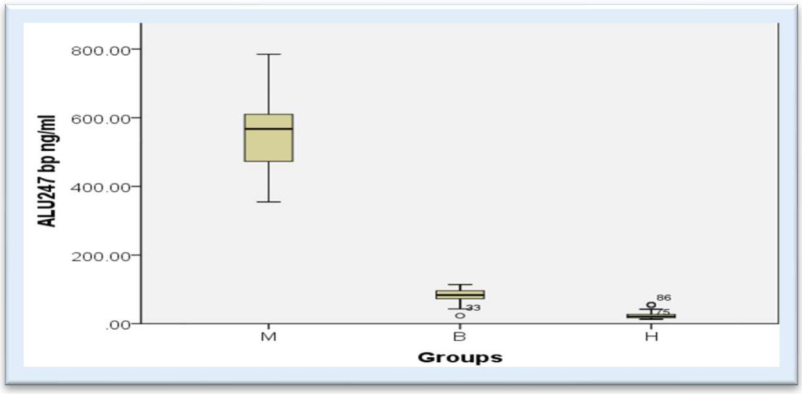


Figure 7:comparison of ALU247bp concentration among study group

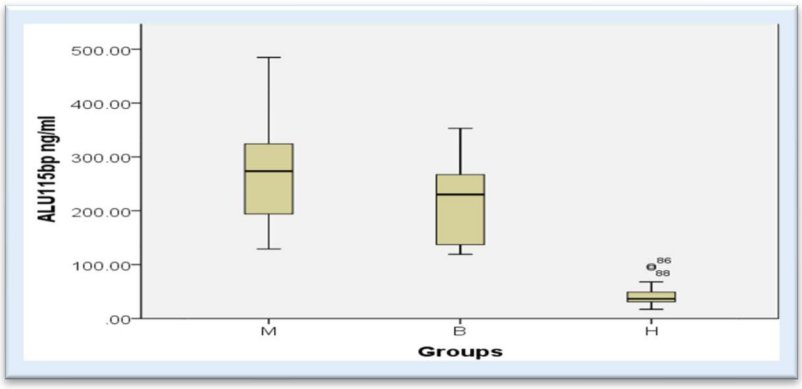


Figure 8: comparison of ALU115bp concentration among study group

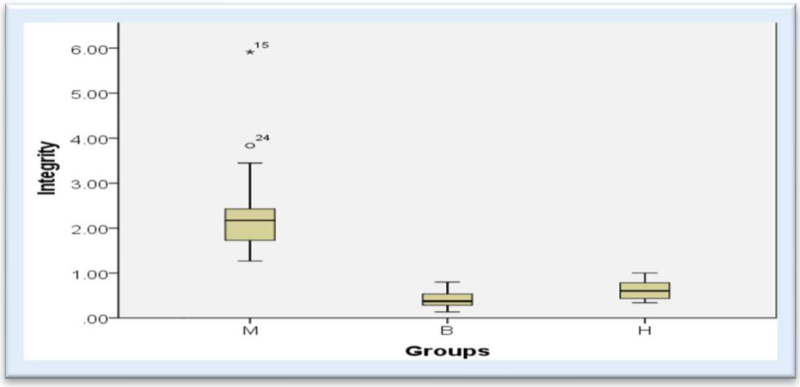


Figure 9 :comparison of cfDNA integrity among study group

The diagnostic potential of serum markers in detecting breast carcinoma

The results obtained from A receiver operator characteristics curve analysis ROC Curve analysis include Sensitivity, Specificity, AUC (Area Under the Curve), and Cutoff values for serum markers to each predictor variable in distinguishing between 'malignant' and 'benign' cases the result showed in figure 10 and table 9

serum ALU-247, integrity and cfDNA were excellent predictors, but serum ALU-115 was not good predictors since the area under curve (AUC) was 0.61 .

Table9: Receiver operator characteristics (ROC) curve parameters for serum markers between malignant and benign breast mass.

Parameters	AUC*	Cutoff	Sensitivity %	Specificity %	p-value
CA153	0.88	53.7	87%	70%	.000
CEA	0.85	14.13	97%	60%	.000
cfDNA conc.	0.98	445	97%	93%	.000
ALU115	0.61	269.5	53%	77%	0.132
ALU247	1	234.5	100%	100%	.000
Integrity	1	1.035	100%	100%	.000

***AUC** measures the performance of the predictor in distinguishing between 'malignant' and 'benign' cases The **cutoff value** is the threshold used by the predictor to classify between 'malignant' and 'benign' cases. For example, if the predictor value is above the cutoff, it might be classified as 'malignant', and vice versa, **Sensitivity** is the true positive rate and **Specificity** is the true negative rate

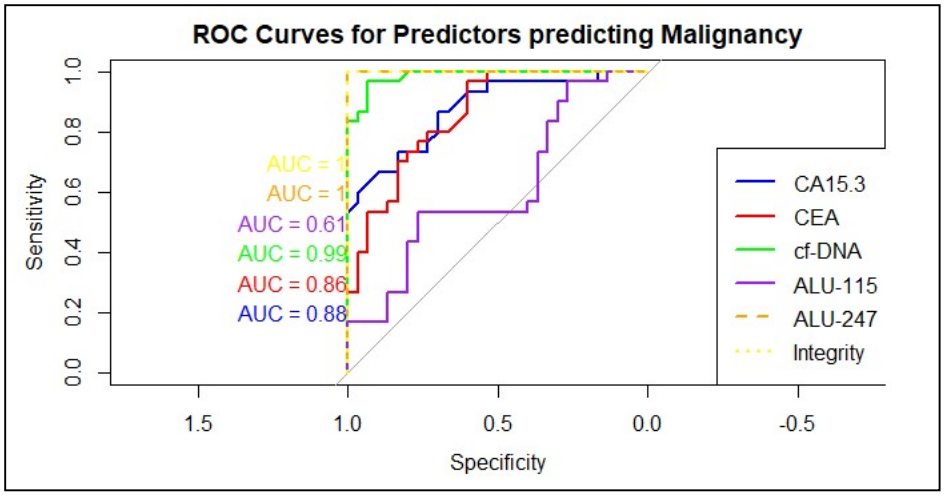


Figure 10 :A receiver operator characteristic (ROC) curve to find the optimal cutoff value for serum markers to distinguish between benign and malignant breast mass.

CA15.3 area under the ROC curve is (0.88) indicates a reasonably good discriminatory ability. It suggests that CA15.3 is fairly effective in distinguishing between malignant and benign cases.

CEA area under the ROC curve is (0.86) that exhibits a slightly lower than CA15.3, indicating relatively good discrimination but slightly less effective compared to CA15.3.

cfDNA area under the ROC curve is (0.99) presents the highest AUC, suggesting exceptional discriminatory ability between malignant and benign cases.

ALU-115 area under the ROC curve is (0.61) displays a relatively lower AUC compared to the other predictors, indicating weaker discrimination between the classes.

ALU-247 and Integrity area under the ROC curve is (1.00) for Both ALU-247 and Integrity demonstrate perfect AUCs, indicating ideal discriminatory ability.

Combined ROC curve:

ROC curves were constructed for three combinations: CA15-3 with cfDNA, ALU-247, and Integrity. Sensitivity, specificity, and optimal cutoff values were derived to assess the discriminatory ability of these combined biomarkers.

The combined ROC curves revealed That the ability of CA15-3 to discriminate between malignant and benign increased at varying performance among different combinations. Notably, the combination of CA15-3 with cf-DNA exhibited an area under the curve (AUC) of 0.99, reflecting high discriminatory power. The CA15-3 with ALU-247 showed an AUC of 1, indicating perfect discriminatory ability. However, CA15-3 with Integrity combination displayed an AUC of 0.89, suggesting moderate discriminatory performance. as showed in table 10 and figure 11

Table 10: Analysis of Combined ROC Curves between CA15-3 and other novel parameters

Parameters	AUC	Optimal Cutoff	Sensitivity%	Specificity %

CA15-3 & cf-DNA	0.99	532.9	93%	100%
CA15-3 & ALU-247	1	302.07	100%	100%
CA15-3 & Integrity	0.89	72.4	65%	98.5%

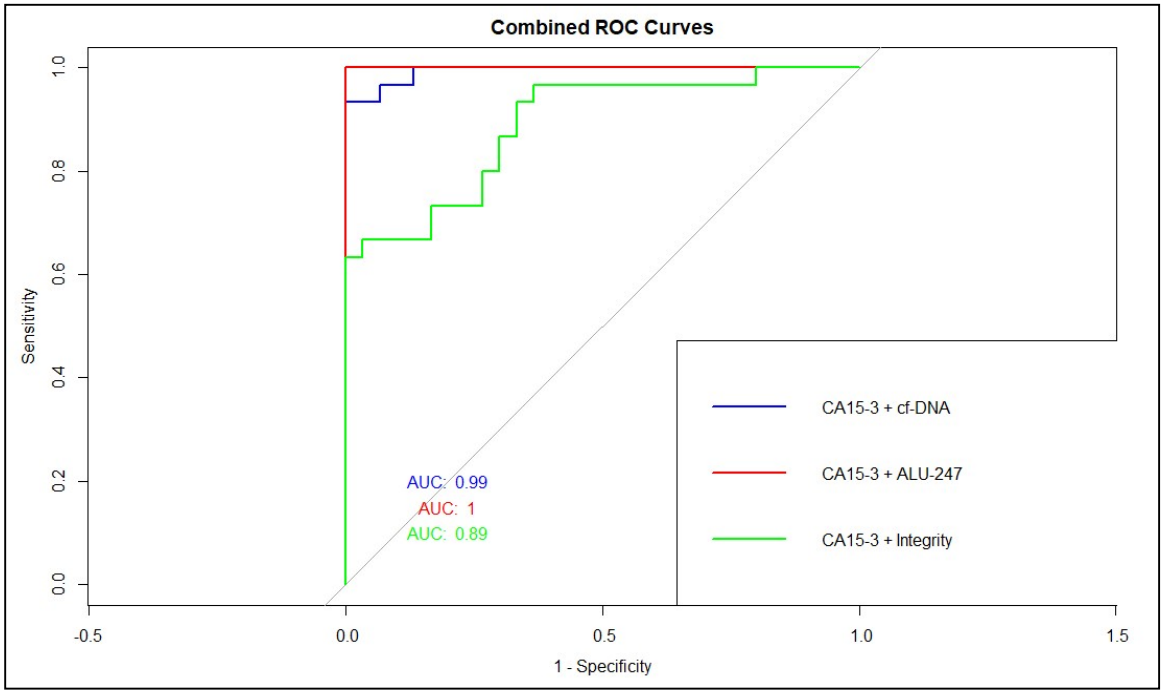


Figure11: The receiver operating characteristic (ROC) curves were generated to assess the predictive performance of various parameters in distinguishing between benign and malignant cases.

Discussion

Circulating Carcinoma Glycoproteins CA15-3

The data of this study showed significant differences in the estimation of serum biomarkers CA15-3 level of breast cancer patients, benign and healthy groups and this results agreement with (Khadhum et al., 2022) that referred a raised amount of CA15-3 in breast cancer patients compared to healthy control group, and the level of CA15-3 much higher in malignant patient than benign group at significant differences that was in agreement with (Fu et al., 2017) and (Chao L. et al. 2018)

This results may be due to the glycoprotein CA15-3 is naturally produced in small amounts by breast cells. but is highly expressed in breast cancer cells. Therefore, CA15-3 levels are often elevated in breast cancer patients and are related to the malignancy and stage of the tumor. However, the serum concentration of this protein are significantly higher in cancer cells compared to healthy people or people with benign tumors. many studies were consistent with our findings

such as (Jia et al., 2022) , other studies had proved the importance of this antigen in following up used as prognostic biomarker in patients with breast cancer, as it was noted that the high levels of CA 15-3 return to decline after treatment, (Sottotetti et al., 2022)

Because of the specificity of CA15-3 in our study was 70% the elevation peripheral blood CA153 levels may indicate that some tumor has appeared. on the other hand the sensitivity was found 87% , not accurate enough for early diagnosis, However, CA15-3 elevation its performance might benefit from additional features or complementary markers to enhance its predictive power. (Sun et al., 2023)

Carcinoembryonic antigen (CEA)

CEA level was elevated at significant differences in breast cancer patients and benign groups these results agreement with (Fazli Khalaf et al., 2023), the results showed that increased CEA level are more commonly with malignant condition than in benign .

Carcinoembryonic antigen (CEA) is a protein that is produced in the developing fetus but usually disappears or is present at very low levels in adults. Elevated levels of CEA in the blood can be a marker for various medical conditions, most notably certain types of cancer, especially colon cancer, liver cancer rather than breast cancer these result was in agreement with (Hall et al., 2019).

In healthy adults, CEA levels in the blood are typically very low as present in our study result that in Table (3.6). Elevated CEA levels may indicate the presence of an underlying medical condition , this elevation can be utilized in monitoring treatment in cancer patients to track the progress of treatment. A decrease in CEA levels may suggest that the treatment is effective, while an increase may indicate disease progression or recurrence (Uygur & Gümüş, 2021)

Carcinoembryonic Antigen (CEA), not specific biomarker for breast cancer at specificity 60% in this study, and it commonly linked to colorectal cancer it show reasonable discrimination. However, its performance require supplementary markers or clinical data to enhance its predictive accuracy further. Different studies represent that it can also be elevated in other types of cancer, such as pancreatic(van Manen et al., 2020).lung, (Cheng, C et al., 2022)and ovarian cancer (Cheng, C et al., 2022)

It's important to note that elevated CEA levels are not exclusive to cancer. Other noncancerous conditions, such as certain inflammatory bowel diseases (Baj et al., 2022) and smoking, can also cause elevated CEA levels (Fang et al., 2022)Therefore, CEA results are usually interpreted in conjunction with other clinical and diagnostic information.

CA15-3 and CEA are routine biomarkers commonly used in the diagnosis . However, as no appearance to have a great ability ,sensitivity and specificity to predict breast cancer , so we tried to compare these biomarkers with the newer non-invasive biomarkers available such as (cfDNA and its integrity).

Cell free DNA Concentration and Integrity

cfDNA levels showed an individually predictive marker in patients with malignant breast cancer from healthy individuals with significant increased , these result in agreement with (Fernandez-Garcia et al., 2019) which indicate that total cfDNA is good diagnostic indicator in patients during the treatment period reflective of disease response and indicators of overall survival

and the preoperative period high concentrations of cfDNA were observed consistent with the presence of tumor burden in breast cancer patients with subsequent drop in cfDNA levels following surgery.

The results of this study also in agreement with (Jongbloed et al., 2021) and(Panagopoulou et al., 2019) that showed that cfDNA levels were higher in patient with metastatic group in relation to healthy individuals, This elevation was because cfDNA consists of DNA fragments released from both normal and tumor cells after cell death, the concentrations of cfDNA were elevated in patients with cancer due to overproduction of cancer cells which leaves more of the cfDNA behind, this result agreement with (Foda et al., 2023).

The main goal of this study was to prove that women with positive biopsy for malignant breast cancer have higher level of cfDNA than benign breast tumor . this study found high significant differences between malignant and benign at (p-value<0.001) and that was in agreement with (Abd El Hafeez et al. 2023).

On the other hand, other studies found that cfDNA levels can be low in cancer patients due to low cell death rates and a low half life time of cfDNA in the plasma as a result of high DNA clearance. (Peled et al., 2020).

Circulating cfDNA concentration and integrity may be suitable for monitoring of breast cancer progression.(Zijian Hu et al., 2021).

our study revealed that the serum level of both ALU-115 and ALU-247 as well as integrity were statistically significantly higher in breast cancer patients as compared to control group, These results agreed with study done by (Elhelaly et al., 2022) , these fragments ALU-247 and ALU-115 are used to distinguish between necrotic cell death as well as cells. The longer ALU-247 is considered a necrotic product, whereas the shorter ALU-115 corresponded to the total amount of DNA. Since necrotic cell death is mainly related to tumor progressive process, so the presence of longer DNA fragments in serum is taken as a sign of enhanced necrosis taking place in the body and is thought to be indicative of cancer, and is planned to be a promising marker for cancer. (Gezer et al. 2022) (de Miranda et al., 2021)

DNA integrity showed significant higher concentration in breast cancer patients in comparison to healthy (p<0.001) with no significant difference between benign and healthy groups (p=0.121) , DNA integrity was lower in healthy individuals, possibly due to decreased necrotic activity in body tissues, that way lowered the concentration of longer DNA fragments ALU247bp in the blood stream than shorter DNA fragments ALU115bp (Adusei et al., 2021)

In this study, we found that patients with newly breast cancer had statistically significant higher serum cfDNA level combined with ALU-247 levels and integrity in comparison to the benign lesions group, (p<0.001). that was in agreement with (Abd El Hafeez et al. 2023).that was because the released DNA from tumor cells into the circulation which is elevated by lymph vascular invasion not local inflammation ,because blood or direct lymphatic flow through the tumor cells allows spreading of viable tumor cells and increases diffusion of DNA released from dead tumor cells into the circulation. Consequently, long fragment ALU increased in the circulating.

Receiver Operating Characteristic Curve (ROC curve)

The receiver operating characteristic curve used in assessment of diagnostic and prognostic data of diverse markers. The values of serum cf-DNA, ALU115, ALU-247, and integrity in diagnosis of breast cancer were evaluated by the ROC curve analysis. Area under the ROC curve (AUC) was used To explanation the validity of a specific marker in the early detection of breast cancer and to distinguished from benign breast lesion.

cfDNA presents the highest AUC 0.986, suggesting exceptional discriminatory ability between malignant and benign cases. demonstrates remarkable performance, likely due to its sensitivity in capturing genetic alterations associated with cancer. It's highly promising as a standalone or complementary marker for cancer detection or monitoring.

ALU-115 displays a relatively lower AUC compared to the other predictors AUC (61%) , indicating weaker discrimination between malignant and benign. with specificity and sensitivity (77% and 53%), respectively. This lower performance might imply that this specific sequence released from necrotic and apoptotic cells and might not hold significant discriminatory power for malignancy prediction on its own.. While both ALU-247 and Integrity demonstrate perfect AUCs (1.00), indicating ideal discriminatory ability, this perfect AUCs suggest excellent discrimination between malignant and benign patients. (Abd El Hafeez et al., 2023)

Combined ROC Curve

ROC curves were constructed for three combinations: CA15-3 with cfDNA, ALU-247, and Integrity. The discriminatory ability of these combined biomarkers was used to assess.Sensitivity, specificity, and optimal cutoff values .

Combined ROC curves revealed varying performance among different combinations. Notably, the combination of CA15-3 with cfDNA exhibited an area under the curve (AUC) of 0.99, reflecting high discriminatory power. The CA15-3 with ALU-247 showed an AUC of 1, indicating perfect discriminatory ability. However, the CA15-3 with Integrity combination displayed an AUC of 0.89, suggesting moderate discriminatory performance.

These result can improve the sensitivity of combined marker CA15-3 with cfDNA ,and CA15-3 with ALU-247 (93% and 100% respectively) and reduce missed diagnosis rate of traditional marker , which is of great significance to the early diagnosis and postoperative follow-up monitoring of breast cancer(Sun et al., 2023)..

With the continuous development of immune marker technology, it believes that more tumor markers will be detected in serum, which will play a critical role in the early diagnosis of cancerous tumor from benign lesion, evaluation of treatment effects, and judgment of prognosis, providing clinicians with more options for treatment options.

Conclusion

The result of this study shows that serum cfDNA is non-invasive biomarker for discriminate between malignant and benign

1. The study has revealed that CA15-3 and CEA increased significantly in malignant breast cancer as compared with benign breast lesion at low specificity (70% and 60% respectively).

2. Result shows there are significant deferences in concentration of cfDNA between study groups (malignant ,benign and healthy)at pvalue <0.001for each comparison.
3. Depending on real time PCR , the present study shows ALU247bp and integrity have excellent ability to distinguish between malignant and benign breast lesion at significant differences value p< 0.001, with sensitivity100% , specificity 100% and AUC = 1.
4. Combined ROC curves increased the ability of CA15-3 to discriminate between malignant and benign with different noninvasive biomarker. Notably, the combination of CA15-3 with cfDNA concentration, ALU-247bp and Integrity, exhibited an area under the curve (AUC) of (0.99, 1.00 and 0.89 respectively) indicating perfect discriminatory ability and would be a promising novel noninvasive approach for diagnosis and early detection of malignant breast cancer.

References

Abd El Hafeez, H., Abd El Rahman, M., Kamel, T., Rezk, K., Mohamed, F., & Abdel-Hameed, Z. (2023). The role of circulating cell-free DNA and its integrity as a biomarker for diagnosis of breast cancer using ALU (247/115) bP sequences. *Egyptian Journal of Immunology*, *30*(03), 44–55.

Adashek, J. J., Janku, F., & Kurzrock, R. (2021). Signed in blood: Circulating tumor dna in cancer diagnosis, treatment and screening. *Cancers*, *13*(14), 1–16.

Adusei, E., Ahenkorah, J., Adu-Aryee, N. A., Adutwum-Ofosu, K. K., Tagoe, E. A., Koney, N. K. K., Nkansah, E., Aryee, N. A., Blay, R. M., Hottor, B. A., Clegg-Lamptey, J. N., & Arko-Boham, B. (2021). Reduced Serum Circulation of Cell-Free DNA Following Chemotherapy in Breast Cancer Patients. *Medical Sciences (Basel, Switzerland)*, *9*(2).

Alimirzaie, S ; Bagherzadeh, M ; Akbari, M. (2019). Liquid biopsy in breast cancer: A comprehensive review. *CLINICAL GENETICS*, *95*(6), 643–660.

Attia, M. S., Youssef, A. O., Goma, M., & Ibrahim, T. (2021). Terbium bipyridyl complex as a photo probe for the determination of carbohydrate antigen CA15.3 in different breast cancer patient samples. *Egyptian Journal of Chemistry*, *64*(7), 3541–3546.

Baj, J., Bryliński, Ł., Woliński, F., Granat, M., Kostelecka, K., Duda, P., Flieger, J., Teresiński, G., Buszewicz, G., Furtak-Niczyporuk, M., & Portincasa, P. (2022). Biomarkers and Genetic Markers of Hepatocellular Carcinoma and Cholangiocarcinoma—What Do We Already Know. *Cancers*, *14*(6), 1–22.

Barbirou, M., Miller, A. A., Gafni, E., Mezlini, A., Zidi, A., Boley, N., & Tonellato, P. J. (2022). Evaluation of cfDNA as an early detection assay for dense tissue breast cancer. *Scientific Reports*, *12*(1), 1–14.

Bhattacharya, D., & Van Meir, E. G. (2019). A simple genotyping method to detect small CRISPR-Cas9 induced indels by agarose gel electrophoresis. *Scientific Reports*, *9*(1), 1–7.

Brod, E., Ben-Yosef, V. S., Bandhakavi, S., & Sivan, U. (2016). Charge-based separation of proteins and peptides by electrically induced dynamic pH profiles. *Journal of Chromatography A*, *1431*, 166–175.

Celec, P., Vlková, B., Lauková, L., Bábíčková, J., & Boor, P. (2018). Cell-free DNA: the role in

pathophysiology and as a biomarker in kidney diseases. *Expert Reviews in Molecular Medicine*, *20*, 1–14.
Chan, R. H., Lin, P. C., Chen, S. H., Lin, S. C., Chen, P. C., Lin, B. W., Shen, M. R., & Yeh, Y. M. (2021). Clinical Utility of a Cell-Free DNA Assay in Patients With Colorectal Cancer. *Frontiers in Oncology*, *11*(March), 1–9.
Chao Liu, Bing Sun, Bin Xu, Xiangying Meng, Lan Li, Yang Cong, Jiannan Liu, Qian Wang, Liang Xuan, Qibin Song &Shikai WuChao Liu, Bing Sun, Bin Xu, Xiangying Meng, Lan Li, Yang Cong, Jiannan Liu, Qian Wang, Liang Xuan, Q. S. &Shikai W. (2018). A panel containing PD-1, IL-2Rα, IL-10, and CA15-3 as a biomarker to discriminate breast cancer from benign breast disease. *Cancer Management and Research*, *10*, 1749–1761.
Cheng, C., Yang, Y., Yang, W., Wang, D., & Yao, C. (2022). The diagnostic value of CEA for lung cancer-related malignant pleural effusion in China: a meta-analysis. *Expert Review of Respiratory Medicine*, *16*(1), 99–108.
Clatot, F., Perdrix, A., Beaussire, L., Lequesne, J., Lévy, C., Emile, G., Bubenheim, M., Lacaille, S., Calbrix, C., Augusto, L., Guillemet, C., Alexandru, C., Fontanilles, M., Sefrioui, D., Burel, L., Guénot, S., Richard, D., Sarafan-Vasseur, N., & Di Fiore, F. (2020). Risk of early progression according to circulating ESR1 mutation, CA-15.3 and cfDNA increases under first-line anti-aromatase treatment in metastatic breast cancer. *Breast Cancer Research*, *22*(1), 1–12.
Condappa, A., McGrowder, D., Aiken, W., McLaughlin, W., & Gossell-Williams, M. (2020). Evaluation of Plasma Circulating Cell Free DNA Concentration and Integrity in Patients with Prostate Cancer in Jamaica: A Preliminary Study. *Diseases*, *8*(3), 34.
Constâncio, V., Nunes, S. P., Henrique, R., & Jerónimo, C. (2020). DNA Methylation-Based Testing in Liquid Biopsies as Detection and Prognostic Biomarkers for the Four Major Cancer Types. *Cells*, *9*(3).
Coppola, L., Cianflone, A., Pane, K., Franzese, M., Mirabelli, P., & Salvatore, M. (2021). The impact of different preanalytical methods related to CA 15-3 determination in frozen human blood samples: a systematic review. *Systematic Reviews*, *10*(1), 1–11.
de Miranda, F. S., Barauna, V. G., Dos Santos, L., Costa, G., Vassallo, P. F., & Campos, L. C. G. (2021). Properties and application of cell-free DNA as a clinical biomarker. *International Journal of Molecular Sciences*, *22*(17).
Dendani Chadi, Z., & Arcangioli, M. A. (2023). Pulsed-Field Gel Electrophoresis Analysis of Bovine Associated Staphylococcus aureus: A Review. *Pathogens*, *12*(7), 1–20.
Desai, S., & Guddati, A. K. (2023). Carcinoembryonic Antigen, Carbohydrate Antigen 19-9, Cancer Antigen 125, Prostate-Specific Antigen and Other Cancer Markers: A Primer on Commonly Used Cancer Markers. *World Journal of Oncology*, *14*(1), 4–14.
Elhelaly, R., Effat, N., Hegazy, M. A. E. F., Abdelwahab, K., Hamdy, O., Hashem, E. M. A., & Elzehery, R. R. (2022). Circulating Cell Free DNA and DNA Integrity Index as Discriminating Tools between Breast Cancer and Benign Breast Disease. *Asian Pacific Journal of Cancer Prevention*, *23*(2), 545–552.
Fang, Y. J., Lee, L. J. H., Luo, K. H., Fang, P. S., Yang, C. C., & Chuang, H. Y. (2022). The

Association of Carcinoembryonic Antigen (CEA) and Air Pollutants—A Population-Based Study. *Atmosphere*, *13*(3), 1–11.
Fazli Khalaf, F., Asadi Gharabaghi, M., Balibegloo, M., Davari, H., Afshar, S., & Jahanbin, B. (2023). Pleural CEA, CA-15-3, CYFRA 21-1, CA-19-9, CA-125 discriminating malignant from benign pleural effusions: Diagnostic cancer biomarkers. *International Journal of Biological Markers*, *38*(2), 81–88.
Fernandez-Garcia, D., Hills, A., Page, K., Hastings, R. K., Toghill, B., Goddard, K. S., Ion, C., Ogle, O., Boydell, A. R., Gleason, K., Rutherford, M., Lim, A., Guttery, D. S., Coombes, R. C., & Shaw, J. A. (2019). Plasma cell-free DNA (cfDNA) as a predictive and prognostic marker in patients with metastatic breast cancer. *Breast Cancer Research*, *21*(1), 1–13.
Foda, Z. H., Annapragada, A. V., Boyapati, K., Bruhm, D. C., Vulpescu, N. A., Medina, J. E., Mathios, D., Cristiano, S., Niknafs, N., Luu, H. T., Goggins, M. G., Anders, R. A., Sun, J., Meta, S. H., Thomas, D. L., Kirk, G. D., Adleff, V., Phallen, J., Scharpf, R. B., … Velculescu, V. E. (2023). Detecting Liver Cancer Using Cell-Free DNA Fragmentomes. *Cancer Discovery*, *13*(3), 616–631.
Fu, S., Yun, Z. Y., Cui, M. M., Meng, H., Qian, C., Liu, T., Liu, Z. P., Wang, R. T., & Yu, K. J. (2017). Cancer antigen 15-3, platelet distribution width, and fibrinogen in combination to distinguish breast cancer from benign breast disease in non-conclusive mammography patients. *Oncotarget*, *8*(40), 67829–67836.
Gezer, U., Bronkhorst, A. J., & Holdenrieder, S. (2022). The Utility of Repetitive Cell-Free DNA in Cancer Liquid Biopsies. *Diagnostics*, *12*(6), 1–17.
Gianni, C., Palleschi, M., Merloni, F., Di Menna, G., Sirico, M., Sarti, S., Virga, A., Ulivi, P., Cecconetto, L., Mariotti, M., & De Giorgi, U. (2022). Cell-Free DNA Fragmentomics: A Promising Biomarker for Diagnosis, Prognosis and Prediction of Response in Breast Cancer. *International Journal of Molecular Sciences*, *23*(22).
Gupta, R., Othman, T., Chen, C., Sandhu, J., Ouyang, C., & Fakih, M. (2020). Guardant360 Circulating Tumor DNA Assay Is Concordant with FoundationOne Next-Generation Sequencing in Detecting Actionable Driver Mutations in Anti-EGFR Naive Metastatic Colorectal Cancer. *The Oncologist*, *25*(3), 235–243.
Hall, C., Clarke, L., Pal, A., Buchwald, P., Eglinton, T., Wakeman, C., & Frizelle, F. (2019). A review of the role of carcinoembryonic antigen in clinical practice. *Annals of Coloproctology*, *35*(6), 294–305.
Han, Y. L., Pegoraro, A. F., Li, H., Li, K., Yuan, Y., Xu, G., Gu, Z., Sun, J., Hao, Y., Gupta, S. K., Li, Y., Tang, W., Kang, H., Teng, L., Fredberg, J. J., & Guo, M. (2020). Cell swelling, softening and invasion in a three-dimensional breast cancer model. *Nature Physics*, *16*(1), 101–108.
Hassan, F., Wang, J. H., Cullinane, C., Ita, M., Corrigan, M., O'Leary, D. P., & Redmond, H. P. (2022). Assessment of cell-free DNA (cfDNA) concentrations in the perioperative period can predict risk of recurrence in patients with non-metastatic breast cancer. *Surgical Oncology*, *42*(April), 101753.
Heitzer, E.; Haque, I.S.Roberts, C.E.S.; Speicher, M. R. (2019). Current and future perspectives

of liquid biopsies in genomics-driven oncology. *Nature Reviews Genetics*, *20*, 71–88.
Henderson, M. C., Hollingsworth, A. B., Gordon, K., Silver, M., Mulpuri, R., Letsios, E., & Reese, D. E. (2016). Integration of serum protein biomarker and tumor associated autoantibody expression data increases the ability of a blood-based proteomic assay to identify breast cancer. *PLoS ONE*, *11*(8), e0157692.
Hu, X. M., Li, Z. X., Lin, R. H., Shan, J. Q., Yu, Q. W., Wang, R. X., Liao, L. S., Yan, W. T., Wang, Z., Shang, L., Huang, Y., Zhang, Q., & Xiong, K. (2021). Guidelines for Regulated Cell Death Assays: A Systematic Summary, A Categorical Comparison, A Prospective. *Frontiers in Cell and Developmental Biology*, *9*(March), 1–28.
Hu Z, Chen H, Long Y, et al. (2021). The main sources of circulating cell-free DNA: apoptosis, necrosis and active secretion. *Critical Reviews in Oncology*, *157*(4), 103-166.
Jia, L., Li, G., Ma, N., Zhang, A., Zhou, Y., Ren, L., & Dong, D. (2022). Soluble POSTN is a novel biomarker complementing CA153 and CEA for breast cancer diagnosis and metastasis prediction. *BMC Cancer*, *22*(1), 1–10.
Jongbloed, E. M., Deger, T., Sleijfer, S., Martens, J. W. M., Jager, A., & Wilting, S. M. (2021). A systematic review of the use of circulating cell-free DNA dynamics to monitor response to treatment in metastatic breast cancer patients. *Cancers*, *13*(8).
Khadhum, H. S., Ameen, A. A., & Thuwaini, M. M. (2022). *Assessment of CA 15-3 and P53 biomarkers in diagnosis of breast cancer* (Vol. 140, Issue 01).
Khani, M., Hosseini, J., Mirfakhraie, R., Habibi, M., Azargashb, E., & Pouresmaeili, F. (2019). The value of the plasma circulating cell-free DNA concentration and integrity index as a clinical tool for prostate cancer diagnosis: A prospective case-control cohort study in an Iranian population. *Cancer Management and Research*, *11*, 4549–4556.
Kwong, G. A. (2020). Macrophage Sensors for Early Cancer Detection. *Clinical Chemistry*, *66*(2), 268–270.
Liang, L., Cao, C., Ji, L. et al. (2023). Complementary Alu sequences mediate enhancer–promoter selectivity. *Nature*, *619*, 868–875.
Michaelidou, K., Koutoulaki, C., Mavridis, K., Vorrias, E., Papadaki, M. A., Koutsopoulos, A. V., Mavroudis, D., & Agelaki, S. (2020). Detection of KRAS G12/G13 Mutations in Cell Free-DNA by Droplet Digital PCR, Offers Prognostic Information for Patients with Advanced Non-Small Cell Lung Cancer. *Cells*, *9*(11), 1–18.
Neoh, H. min, Tan, X. E., Sapri, H. F., & Tan, T. L. (2019). Pulsed-field gel electrophoresis (PFGE): A review of the "gold standard" for bacteria typing and current alternatives. In *Infection, Genetics and Evolution* (Vol. 74, p. 103935). Elsevier.
Nikanjam, M. ., Kato, S., & Kurzrock, R. (2022). Liquid biopsy: current technology and clinical applications. *Journal of Hematology and Oncology*, *15*(1), 1–14.
P. Mandel, P. Metais. (1948). Blood plasma nucleic acids in humans. *C R Acad Sci Paris*, 142 241.
Panagopoulou, M., Karaglani, M., Balgkouranidou, I., Biziota, E., Koukaki, T., Karamitrousis, E., Nena, E., Tsamardinos, I., Kolios, G., Lianidou, E., Kakolyris, S., & Chatzaki, E. (2019). Circulating cell-free DNA in breast cancer: size profiling, levels, and methylation patterns lead to

prognostic and predictive classifiers. *Oncogene*, *38*(18), 3387–3401.
Peled, M., Agassi, R., Czeiger, D., Ariad, S., Riff, R., Rosenthal, M., Lazarev, I., Novack, V., Yarza, S., Mizrakli, Y., & Douvdevani, A. (2020). Cell-free DNA concentration in patients with clinical or mammographic suspicion of breast cancer. *Scientific Reports*, *10*(1), 1–9.
Ribeiro, J. A., Pereira, C. M., Silva, A. F., & Sales, M. G. F. (2018). Disposable electrochemical detection of breast cancer tumour marker CA 15-3 using poly(Toluidine Blue) as imprinted polymer receptor. *Biosensors and Bioelectronics*, *109*(February), 246–254.
Sant, M., Bernat-Peguera, A., Felip, E., & Margelí, M. (2022). Role of ctDNA in Breast Cancer. *Cancers*, *14*(2), 1–14.
Soodabeh Hassanpour , Mohammad Hasanzadeh , Arezoo Saadati , Nasrin Shadjou , Jafar Soleymani, A. J. (2019). A novel paper based immunoassay of breast cancer specific carbohydrate (CA 15.3) using silver nanoparticles-reduced graphene oxide nano-ink technology: A new platform to construction of microfluidic paper-based analytical devices (μPADs) towards biomedica. *Microchemical Journal, 146*, 345–358.
Sottotetti, F., Ferraris, E., Tagliaferri, B., Palumbo, R., Quaquarini, E., Teragni, C., Balletti, E., Leli, C., Premoli, A., Mollica, L., Puglisi, S., Sardi, S., Malovini, A., Pedrazzoli, P., & Bernardo, A. (2022). The prognostic role of variations in tumour markers (CEA, CA15.3) in patients with metastatic breast cancer treated with CDK4/6 inhibitors. *Drugs in Context*, *11*, 1–10.
Stejskal, P., Goodarzi, H., Srovnal, J., Hajdúch, M., van 't Veer, L. J., & Magbanua, M. J. M. (2023). Circulating tumor nucleic acids: biology, release mechanisms, and clinical relevance. *Molecular Cancer*, *22*(1), 1–21.
Sultana, G. N. N., Akter, F., Israfil, S. M. H., Ray, U. C., Jahan, R. A., Ali, M. S., Din, S. Al, Rahman, S., Halim, R., & Alam, M. S. (2023). Quantitative analysis of serum cell-free DNA as a predictive and prognostic marker in breast cancer patients. *Frontiers in Oncology*, *13*(June), 1–9.
Sun, J., Ma, Q., Chen, H., Zhou, X., & Zhang, J. (2023). Detection and Clinical Significance of Peripheral Blood CTC, CA125, CA153, and CEA Levels in Patients with Ductal Carcinoma in situ. *Proceedings of Anticancer Research*, *7*(5), 89–94.
Uygur, M. M., & Gümüş, M. (2021). The utility of serum tumor markers CEA and CA 15–3 for breast cancer prognosis and their association with clinicopathological parameters. *Cancer Treatment and Research Communications*, *28*.
van Manen, L., Groen, J. V., Putter, H., Vahrmeijer, A. L., Swijnenburg, R. J., Bonsing, B. A., & Mieog, J. S. D. (2020). Elevated CEA and CA19-9 serum levels independently predict advanced pancreatic cancer at diagnosis. *Biomarkers*, *25*(2), 186–193.
Vizza, E., Corrado, G., De Angeli, M., Carosi, M., Mancini, E., Baiocco, E., Chiofalo, B., Patrizi, L., Zampa, A., Piaggio, G., & Cicchillitti, L. (2018). Serum DNA integrity index as a potential molecular biomarker in endometrial cancer. *Journal of Experimental and Clinical Cancer Research*, *37*(1), 1–9.
Waks, A. G., & Winer, E. P. (2019). Breast Cancer Treatment. In *JAMA - Journal of the American Medical Association* (Vol. 321, Issue 3, p. 316). American Medical Association.
Yuejiao Zhong, Qingyu Fan, Zhaofei Zhou, Yajing Wang, K. H. & J. L. (2020). Plasma cfDNA

as a Potential Biomarker to Evaluate the Efficacy of Chemotherapy in Gastric Cancer. *Cancer Management and Research*, *12*, 3099–3106.

Zhu, G., Guo, Y. A., Ho, D., Poon, P., Poh, Z. W., Wong, P. M., Gan, A., Chang, M. M., Kleftogiannis, D., Lau, Y. T., Tay, B., Lim, W. J., Chua, C., Tan, T. J., Koo, S. L., Chong, D. Q., Yap, Y. S., Tan, I., Ng, S., & Skanderup, A. J. (2021). Tissue-specific cell-free DNA degradation quantifies circulating tumor DNA burden. *Nature Communications*, *12*(1), 1–11.

Zulato, E., Del Bianco, P., Nardo, G., Attili, I., Pavan, A., Boscolo Bragadin, A., Marra, L., Pasello, G., Fassan, M., Calabrese, F., Guarneri, V., Conte, P. F., Indraccolo, S., & Bonanno, L. (2022). Longitudinal liquid biopsy anticipates hyperprogression and early death in advanced non-small cell lung cancer patients treated with immune checkpoint inhibitors. *British Journal of Cancer*, *127*(11), 2034–2042.

