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A combined analysis of serum Growth Differentiation Factor-15 and Cancer Antigen 15-3 enhances the diagnostic efficiency in breast cancer

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ABSTRACT

Introduction

Existing diagnostic biomarkers of breast cancer (BC) are limited by poor sensitivity. In this study, we evaluated the role of serum GDF-15 in early BC diagnosis, independently and in combination with CA15-3, a known blood biomarker of BC.

Material and methods

A total of 113 diagnosed, pre-therapy BC patients and 54 healthy controls were recruited. Clinical characteristics, TNM staging, and hormone receptor status of the patients were recorded. Serum GDF-15 and serum CA15-3 were measured by sandwich ELISA and chemiluminescence assay, respectively.

Results

The serum GDF-15 levels were significantly ($p<0.001$) elevated in BC patients compared to healthy controls and in patients with larger tumor size, advanced disease stage, and distant metastasis. ROC analysis revealed that at the cut-off of 525.77 pg/mL, GDF-15 had greater sensitivity than CA15-3. GDF-15 and CA15-3 performed better in combination than individually, with the combined test having an AUC of 0.85 and sensitivity and specificity of 0.63 and 0.98, respectively.

Further, serum GDF-15 had a better predictive ability for early-stage BC compared to CA15-3. GDF-15 could independently diagnose BC patients after adjusting for age.

Conclusion

We conclude that serum GDF-15 is a promising, robust marker for detecting early-stage BC. However, larger prospective studies are necessary to validate this claim.

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Abbreviations:

APE: Apurinic/apyrimidinic endonuclease
BC: Breast cancer
CA15-3: Cancer antigen 15-3
CEA: Carcinoembryonic antigen
EGFR: Epidermal growth factor receptor
GDF-15: Growth Differentiation Factor-15
MIC-1: Macrophage inhibitory cytokine-1
NSE: Neuron-specific enolase
TGF-β: Transforming growth factor-β

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INTRODUCTION

Breast cancer (BC), a heterogeneous disease, has differentially expressed cell surface receptors.

It is the commonest cancer in females (11.6% of all cancers), causing 626,679 deaths worldwide, making it the second leading cause of cancer mortality (1). Early diagnosis is crucial to prevent poor outcomes. Currently, BC diagnosis relies upon mammography, but it has limited sensitivity (2,3). Some blood-based biomarkers can detect cancer early on before the appearance of clinical symptoms, e.g. CA125 for ovarian cancer (4). At present, blood-based biomarkers of BC such as CA15-3 and BR27.29 are not reliable due to their low sensitivity (5). Thus, there is an unmet need for early diagnostic biomarkers that could predict disease outcomes and prevent the poor prognosis of BC.

CA15-3 concentrations are elevated in BC as well as in pancreatic, ovarian, lung, colon and liver cancers (6,7). Nieder et al. 2017 (8) have observed that it can be used as a prognostic marker for monitoring therapeutic response as elevated levels increased with tumour size and disease progression. But higher values have been reported in benign conditions, implying low sensitivity (6). A meta-analysis conducted by Fu et al. (9) showed that elevated CA15-3 was significantly associated with malignant breast tumours.

GDF-15, also called macrophage inhibitory cytokine-1 (MIC-1), is a member of the transforming growth factor-β (TGF-β) superfamily. It has a molecular weight of 25-kDa. GDF-15 is vital in cancer proliferation, apoptosis, migration, angiogenesis, and immune modulation (10). An increase in serum GDF-15 is associated with pathological grade, staging, lymph node involvement, and other clinical outcomes and prognosis of multiple cancers such as ovarian, hepatocellular, prostate and lung cancer

(10-15). Furthermore, some studies reported that an increase in GDF-15 expression is associated with proliferation, migration, invasion and stemness of BC (16-18). However, the role of GDF-15 in BC diagnosis is unexplored.

The current study proposes to evaluate serum GDF-15 and CA15-3 as a diagnostic markers with clinicopathological features of BC. Serum GDF-15 could significantly delineate early-stage BC patients from healthy controls; diagnostic accuracy analysis further showed that it could be used as an early diagnostic marker, with high sensitivity and specificity, which improved in combination with CA15-3. Serum GDF-15 was also significantly higher in metastatic compared to non-metastatic BC.

MATERIALS & METHODS

2.1 Ethics statement

The study was carried out in compliance with the ethical principles for medical research involving human subjects, in accordance with the declaration of Helsinki. Ethical approval was granted from the Institutional Ethics Committee (IEC) of AIIMS, Jodhpur. Written informed consent was taken from each participant before enrolling in the study.

2.2 Study population

Sera from 113 BC patients were collected between August 2018 and March 2020 from the outpatient clinics of the Department of Radiation Oncology and the Department of Surgical Oncology at AIIMS, Jodhpur. 54 healthy subjects were recruited by a physical examination. The inclusion criteria for BC patients were 1) aged >18 years, 2) primary BC patients who had not received any chemotherapy, i.e. newly diagnosed BC without prior treatment, 3) diagnosed through mammography and histopathological examination. Participants were ineligible if they were histologically diagnosed with other conditions such as benign tumour upon pathological review, prior history of any other malignancy, anti-cancer medication or were under palliative care. All the clinicopathological characteristics of BC, including TNM staging and IHC receptor status, were acquired from the hospital medical records only after taking consent from the patients.

2.3 Sample preparation

Blood samples for serum CA15-3 and GDF-15 were collected by venipuncture in clot activator vacutainer for serum separation. The serum was separated immediately by centrifugation and stored at -80°C. Sera were only thawed once, just before the analysis. Serum GDF-15 was measured in batches using GDF15 Human ELISA Kit purchased from Invitrogen, (Thermo Fisher Scientific, #EHGDF15) having a 2 pg/mL detection limit and coefficient of variation (CV) of <10% (intra-assay) and <12% (inter-assay) following the manufacturer's protocol. Serum CA15-3 was detected by chemiluminescent enzyme immunoassay on Diasorin Liaison.

2.4 Statistical analysis

Data were analyzed in SPSS version 22.0 and R (version 3.5.3) using RStudio (19). Continuous variables were expressed as median and interquartile range (IQR). Since serum GDF-15 and CA15-3 were not normally distributed, non-parametric tests were employed viz. Mann Whitney U for two groups and Kruskal Wallis Rank Sum test for three or more groups. Multiple comparisons (Dunn Test) were carried out if significance was found in more than two groups. To evaluate the diagnostic ability of GDF-15 and to merge diagnostic information of the predictors, Receiver Operating Characteristic (ROC) curve and binary logistic regression were used. For all analytical purposes, a two-tailed $p<0.05$ was considered to be statistically significant.

RESULTS

3.1 Clinico-pathological characteristics

A total of 113 BC patients were enrolled in this study which included 111 females and two males. Table 1 summarizes the somatometric characteristics of the patient population. The median age of the BC patients was 51 (IQR 19.5) years. 24 (21.24%) had comorbidities such as thyroid dysfunction, diabetes mellitus, hypertension and asthma among these patients. 88 (77.88%) patients did not have any comorbidities. There were 12 (10.62%) diabetics. Based on their menstruation history, 38 (34.23%) were pre-menopausal, and 73 (65.77%) were post-menopausal. The patients were staged as follows: 35 (30.97%) were early-stage (stages I-II), 77 (68.14%) were of advanced stage (stages III-IV). In these patients, 85 (75.22%) were non-metastatic and 27 (23.89%) were metastatic (Table 2).

Table 1 Somatometric variables of the patient population according to serum GDF-15 and CA15-3

Variables	n=113	GDF-15 (pg/mL)			CA15-3 (U/mL)		
		Median	IQR	*p*-value	Median	IQR	*p*-value
Age							
<50 years	47	537.70	513.32	0.012*	24.01	35.01	0.313
≥50 years	66	706.40	686.06		20.17	28.13	
Menstruation status							
Pre-menopausal	38	603.70	589.95	0.354	26.82	40.51	0.027*
Post-menopausal	75	658.67	640.11		18.89	24.60	
Comorbidity							
Present	24	851.21	907.18	0.110	22.96	54.13	0.420
Absent	88	603.70	479.22		21.03	21.99	
Diabetes Mellitus							
Present	12	1363.80	1338.10	0.001*	39.33	151.71	0.333
Absent	100	593.16	489.61		21.60	5.31	

** Statistically significant at p<0.05 after comparison by Mann Whitney U test.*
IQR: Interquartile range.

Table 2 Tumor characteristics according to serum GDF-15 and CA15-3

Variables	n=113	GDF-15 (pg/mL)			CA15-3 (U/mL)		
		Median	IQR	*p*-value	Median	IQR	*p*-value
Tumor size							
T1-T2	37	558.65	348.77	0.018*	16.42	14.51	<0.001*
T3-T4	75	707.12	701.59		28.27	41.27	
Nodal status							
N0	48	618.47	495.28	0.384	18.60	13.09	0.031*
N1-3	64	627.10	710.20		28.27	41.14	
Metastasis							
M0	85	603.70	457.51	0.014*	19.07	19.16	0.001*
M1	27	927.49	1461.40		36.26	177.85	
Staging							
Early	35	551.03	322.01	0.006*	15.20	14.24	<0.001*
Advanced	77	707.12	807.73		28.27	39.68	
IHC receptor status							
ER+	53	659.81	840.30	0.266	20.43	23.52	0.888
ER-	35	634.40	363.78		21.21	25.00	
PR+	33	884.54	1146.90	0.059	21.29	23.58	0.772
PR-	55	629.95	445.30		20.22	22.40	
Her2+	33	707.80	663.15	0.739	27.84	23.96	0.103
Her2-	55	613.02	469.43		19.56	22.12	

TNBC status							
TNBC	23	604.89	315.84	0.221	22.16	25.61	0.792
Non-TNBC	65	706.40	646.70		20.17	22.62	

** Statistically significant at p<0.05 after comparison by Mann Whitney U test.*
IHC: Immunohistochemistry, IQR: Interquartile range.

3.2 Serum GDF-15 and CA15-3 were elevated in breast cancer

We detected increased levels of serum GDF15 (median [IQR] 625.46 [530.94] pg/mL) in BC patients compared with healthy subjects (median [IQR] 385.31 [202.57] pg/mL; p<0.001) (Figure 1). Moreover, when all patients with BC were grouped according to TNM classification, the level of serum GDF-15 gradually increased with the staging of BC (p<0.001) (Figure 1); also, serum GDF-15 levels were significantly higher in the early-stage group (stage I-II) (median [IQR] 551.03 [322.01] pg/mL) in comparison with healthy controls (median [IQR] 385.31 [202.57] pg/mL; p=0.009), suggesting that an elevated serum GDF15 might present in the early stage of BC. There was a gradual increment in serum GDF-15 levels, with higher levels in advanced patients (Stage III-IV) (median [IQR] 707.12 [807.73] pg/mL; p=0.006) compared with early-stage (I-II) patients, implying the positive correlation of GDF-15 with BC progression.

Further analysis showed that the level of serum GDF-15 was higher in BC patients with larger tumour size (T3-T4) (p=0.018), progesterone receptor (PR) positive status (p=0.059), and distant metastasis (M1) (p=0.014) compared to small tumour size (T1-T2), PR negative status or in the absence of distant metastasis (M0), respectively (Table 2). Serum GDF-15 level was also significantly higher in patients with diabetes (p=0.001). Additionally, data also indicated a significant association between the level of serum GDF-15 and age (p<0.001); with advancing age, an increasing trend was observed. However, no statistical association of serum GDF-15 with nodal involvement, menstruation or receptor status was reported.

Likewise, we observed that serum CA15-3 concentration was significantly higher in BC patients (median [IQR] 21.640 [26.51] U/mL) than in healthy controls (median [IQR] 13.614 [9.37] U/mL; p<0.001). Both early-stage (median [IQR] 15.200 [14.24] U/mL; p=0.316) and advanced-stage patients (median [IQR] 28.275 [39.68] U/mL; p<0.001) showed elevated CA15-3 levels (Figure 2), but the increase in early-stage was statistically not significant, unlike serum GDF-15. CA15-3 was also significantly higher in BC patients with larger tumor size (T3-T4) (p<0.001), nodal involvement (p=0.031), metastasis (p=0.001), advanced-stage (p<0.001), and post-menopausal status (p=0.027) (Table 1 and Table 2).

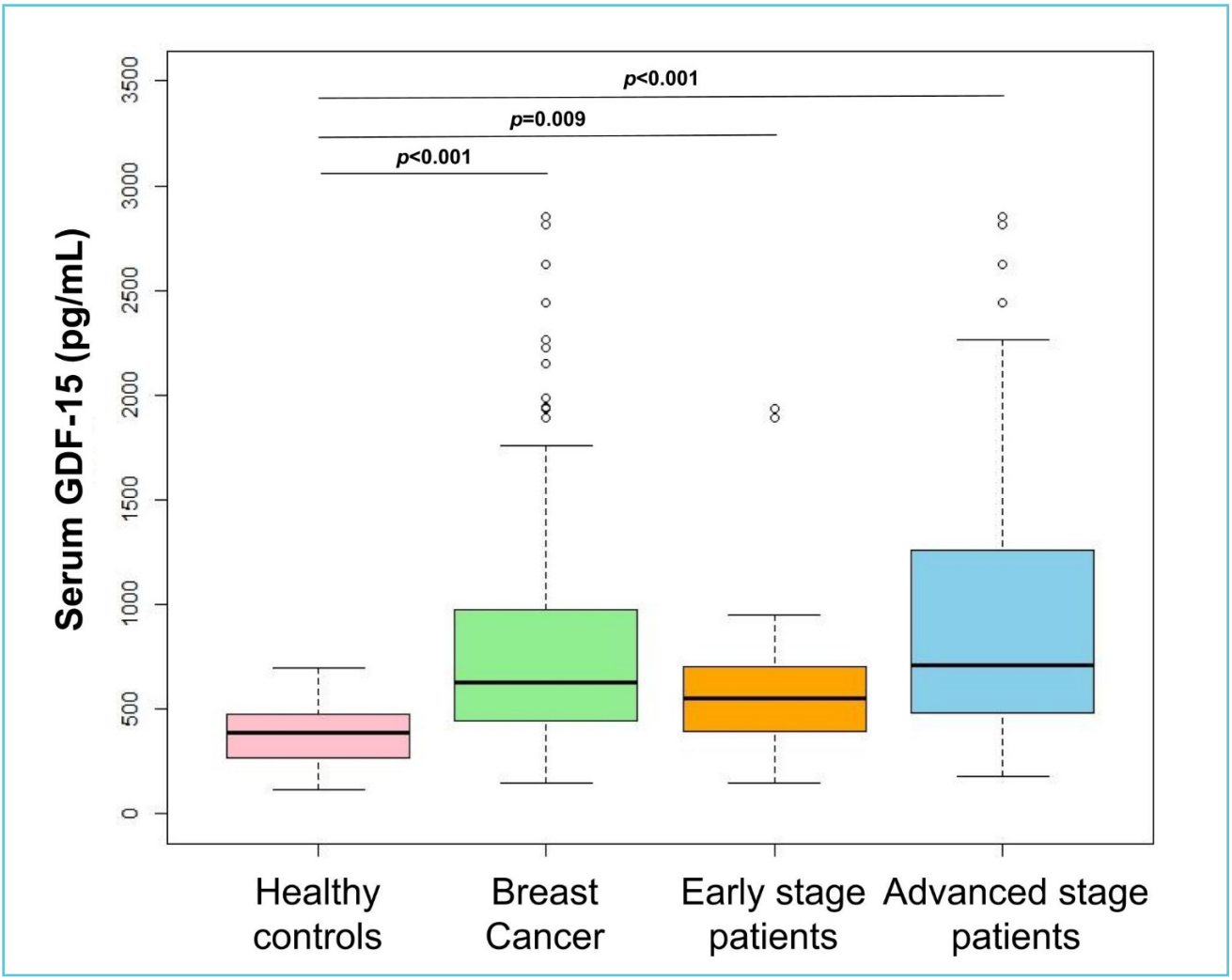
3.3 Diagnostic performance of serum GDF-15 compared to CA 15-3

Our study assessed serum GDF-15 as a non-invasive, diagnostic biomarker for BC compared to CA15-3 by ROC curve analysis. Using 54 normal samples as controls, the calculated AUC of GDF-15 was 0.790 (Figure 3A), compared to CA15-3, which had an AUC of 0.747 (Table 3, Figure 3B). To establish serum GDF-15 as a marker for BC, we used the *cutpointr* package to calculate optimal cut-off values. We found the serum GDF-15 cut-point for our study population to be 525.77 pg/ml by maximizing the sum of sensitivity and

specificity. The Youden's Index of GDF-15 in the diagnosis of BC was 0.528 at the 525.77 pg/ml, with a sensitivity and specificity of 65.77% and 87.04%, respectively and an accuracy of 72.73%. Also, CA15-3 had a sensitivity, specificity, and accuracy of 47.75%, 92.59%, and 62.42%, respectively. The combination of both GDF-15 and CA15-3 had an improved AUC of 0.846, with a sensitivity and specificity of 63.06% and 98.15%, respectively. Further, the combined AUC was significantly better than CA15-3 alone (p=0.003 by DeLong's test for comparison of two correlated empirical ROC curves) (Figure 3C).

In early-stage BC (n=34), GDF-15 had a sensitivity and specificity of 73.53% and 68.52%, respectively, with an AUC of 0.726 (p<0.001) (Table 3, Figure 4A). The serum GDF-15 cut-off point for early-stage BC was 426.35 pg/mL.

Figure 1 **Comparison of serum GDF-15 concentrations in breast cancer patients compared to healthy controls**



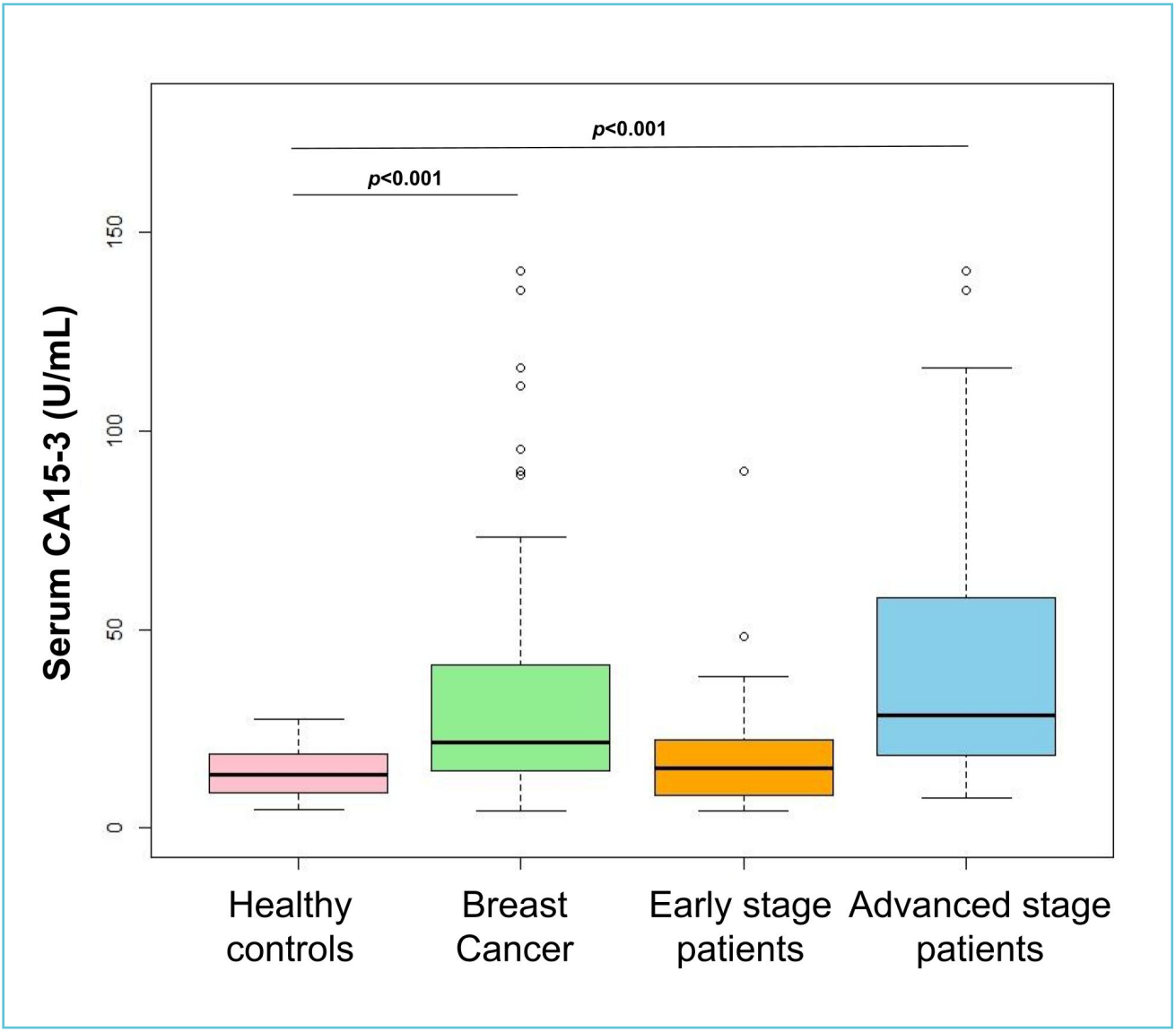
The breast cancer population is further grouped into early-stage and advanced-stage, and compared with the healthy control group. Between group comparison by Kruskal Wallis rank sum test revealed a highly significant difference (p<0.001). Further, a pairwise comparison by Dunn test with Holm correction showed that serum GDF-15 was significantly higher in breast cancer (adjusted p<0.001), early-stage breast cancer (adjusted p=0.009) and advanced-stage breast cancer (adjusted p<0.001) compared to healthy controls.

Serum CA15-3 had a specificity of 90.74% for early-stage BC with an AUC of 0.562 (p=0.326) (Figure 4B). Furthermore, the sensitivity and specificity of a combined GDF-15 and CA15-3 were 67.65% and 72.22%, respectively (AUC 0.734, p<0.001, Figure 4C), implying that serum GDF-15 overall is a better predictive marker for early-stage BC than CA15-3.

In metastatic BC patients, serum GDF-15 was found to have a sensitivity, specificity, and AUC of 50.00%, 82.14%, and 0.660, respectively (p=0.014) (Table 3).

In comparison, CA15-3 had better sensitivity (80.77%, AUC 0.720, p=0.001) than GDF-15 and the combination of GDF-15 and CA15-3 (50.00%, AUC 0.685, p=0.004).

Figure 2 **Comparison of serum CA15-3 levels in breast cancer patients in comparison to healthy controls**

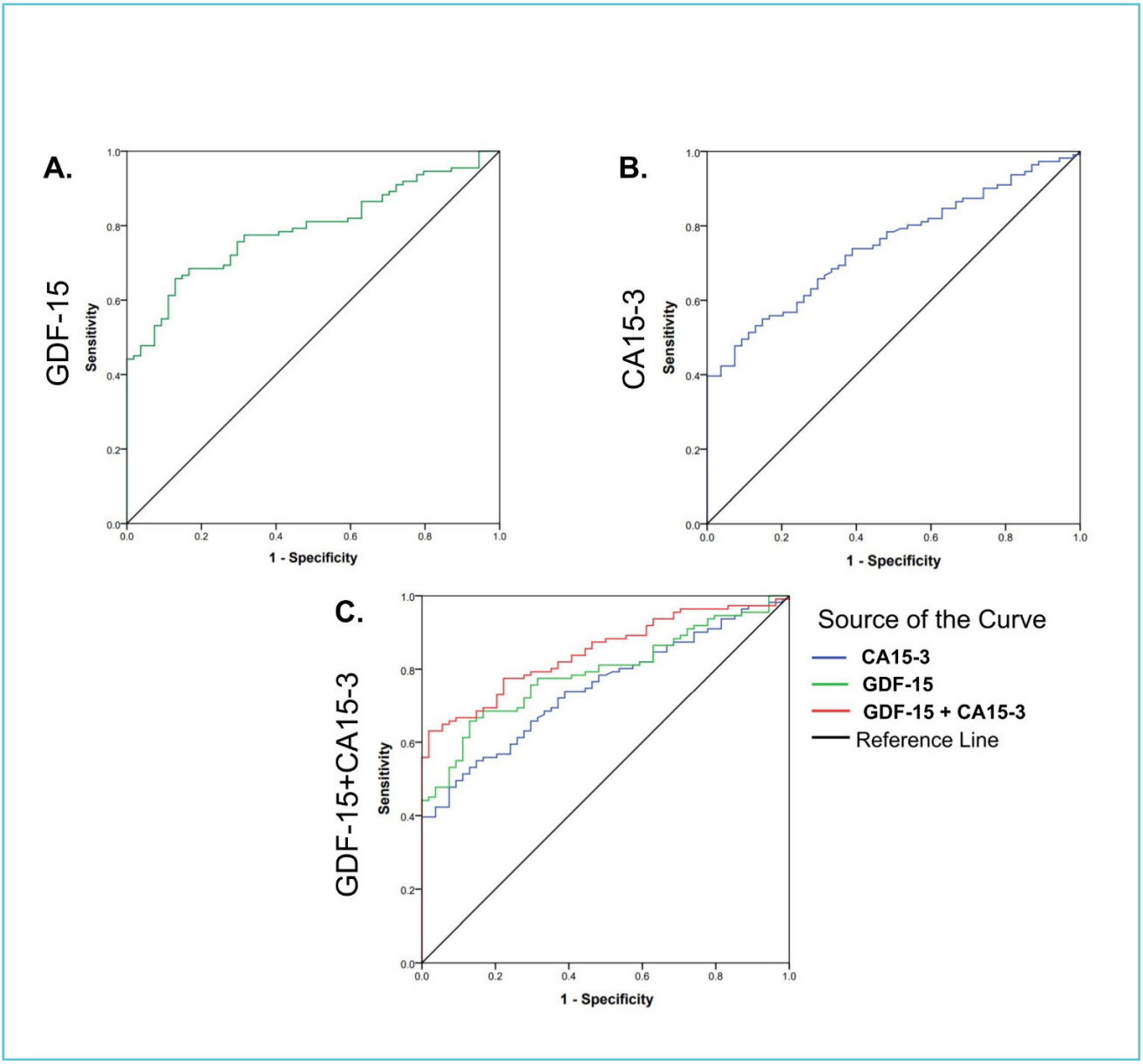


Between group comparison by Kruskal Wallis rank sum test revealed a highly significant difference (p<0.001). Multiple comparison by Dunn test with Holm correction showed that serum GDF-15 was significantly higher in breast cancer (adjusted p<0.001) and advanced-stage breast cancer (adjusted p<0.001) compared to healthy controls.

3.4 Serum GDF-15 is an independent predictor of BC patients

We further carried out a binary logistic regression, which showed serum GDF-15 as an independent predictor that could differentiate BC patients from controls after adjusting for age (Table 4). The adjusted serum GDF-15 had an OR of 5.76 (95% CI 1.98-16.79, p=0.001).

Figure 3 **Receiver Operating Characteristic (ROC) curve analysis for A. Serum GDF-15, B. Serum CA15-3, and C. the combination of both markers in breast cancer.**



The combined ROC curve has been plotted from the combined probabilities derived from logistic regression of serum GDF-15 and serum CA15-3 in the study population. The reference line signifies an AUC of 0.50. The combined markers had a sensitivity of 63.06% and a specificity of 98.15%, and the AUC (0.846) was significantly higher compared to serum CA15-3 alone (AUC 0.747, p=0.003).

Table 3 Area Under the Curve for serum GDF-15 and CA15-3 in breast cancer and early-stage breast cancer patients

Patient population	Marker	AUC	Std. Error	95% CI	*p*-value
Breast Cancer	GDF-15	0.790	0.034	0.723-0.857	<0.001*
	CA15-3	0.747	0.037	0.674-0.820	<0.001*
	GDF-15+CA15-3	0.846	0.029	0.789-0.903	<0.001*
Early-stage breast cancer	GDF-15	0.726	0.058	0.612-0.840	<0.001*
	CA15-3	0.562	0.066	0.434-0.691	0.326
	GDF-15+CA15-3	0.734	0.058	0.621-0.847	<0.001*
Metastatic breast cancer	GDF-15	0.660	0.067	0.529-0.792	0.014*
	CA15-3	0.720	0.059	0.604-0.836	0.001*
	GDF-15+CA15-3	0.685	0.068	0.551-0.819	0.004*

** Statistically significant at p<0.05*
Null hypothesis: true area = 0.50
Std. error has been calculated under the nonparametric assumption.

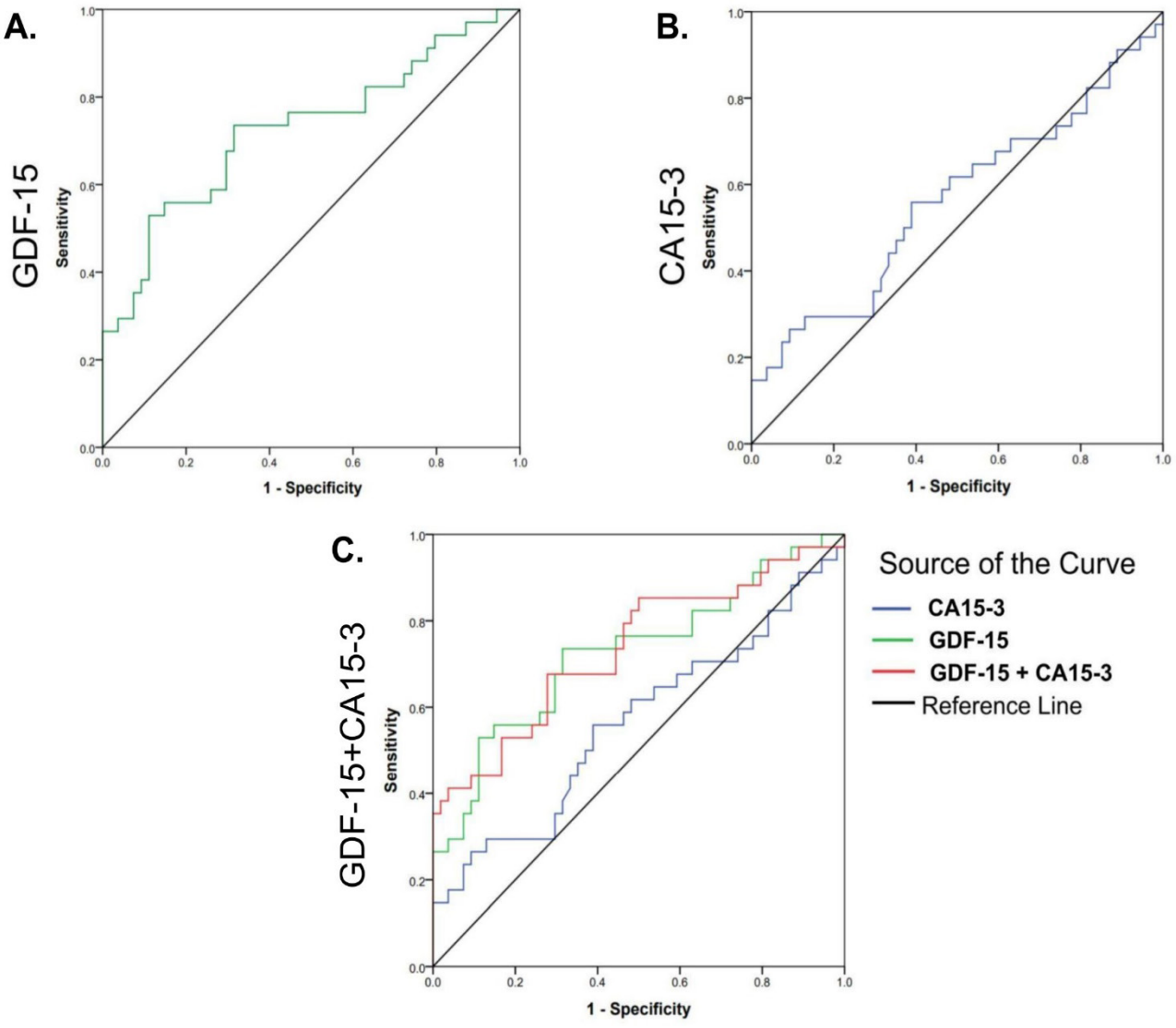
 # DeLong's test for comparison of two correlated Empirical ROC curves. While GDF-15 alone was not significantly better (p=0.389) than CA15-3 for breast cancer, the combined AUC of the two markers showed significant improvement over CA15-3 alone (p=0.003) but not GDF-15 (p=0.057). For early-stage breast cancer, the AUC of GDF-15 alone (p<0.001) and a combination of GDF-15 and CA15-3 (p<0.001) were comparable.

Table 4 Predictive ability of serum GDF-15 in breast cancer, adjusted for age

Predictor	Unadjusted			Adjusted		
	Coefficient	OR (95% CI)	*p*-value	Coefficient	OR (95% CI)	*p*-value
GDF-15	2.021	7.548 (3.474-16.400)	<0.001*	1.751	5.763 (1.978-16.793)	0.001*
Age	-	-	-	0.195	1.216 (1.136-1.301)	<0.001*

** Statistically significant at p<0.05*

Figure 4 **Receiver Operating Characteristic (ROC) curve analysis for A. Serum GDF-15, B. Serum CA15-3, and C. the combination of both markers in early-stage breast cancer.**



The combined ROC curve has been plotted from the combined probabilities derived from logistic regression of serum GDF-15 and serum CA15-3 in the study population. The reference line signifies an AUC of 0.50. Serum GDF-15 had a higher sensitivity (73.53%, p<0.001) compared to CA15-3 alone and the combination of GDF-15 and CA15-3.

DISCUSSION

BC is among the most prevalent cancers among women. Currently, mammography is the standard method of early-stage diagnosis of BC (20). However, existing diagnostic markers have low sensitivity; and the development of a highly sensitive and non-invasive approach for early BC diagnosis is needed to complement existing detection methods, which will consequently improve the outcome of the disease (20,21). In this study, we identified a novel early-stage diagnostic marker of BC with a higher sensitivity than CA 15-3, an already known marker of BC. Further, a combination of these two markers showed better diagnostic performance. To our knowledge, this is the first study reporting the clinical value of serum GDF-15 in the diagnosis of early-stage BC.

Earlier studies have reported significantly higher expression of GDF-15 in BC (17). But the role of serum GDF-15 in BC diagnosis has not been optimally explored. Windrichova et al. (14) assessed 130 patients with different cancers, including BC, and observed circulating GDF-15 to be increased in metastatic cancer patients, with 65% sensitivity and 90% specificity, with the cut-off value of 1480 pg/mL. Another study has reported that increased serum GDF-15 in BC patients is associated with metastasis (17). We found that serum GDF-15 could differentiate metastatic BC from non-metastatic cases with a sensitivity, specificity and AUC of 50.00%, 82.14% and 0.66, respectively. In-vitro and in-vivo studies have reported that an increase in GDF-15 levels is associated with proliferation, invasion, migration, drug resistance and stemness of BC (16-18). We also observed a significant difference in the serum levels of GDF-15 and CA15-3 in BC patients compared to the control group.

There are various diagnostic markers for screening of BC, such as CA27-29, CA15-3, carcinoembryonic antigen (CEA), which usually are not elevated in the control subjects but are found to be elevated in cancer patients. These markers are progressively increased in disease progression and recurrence and can monitor response to therapy (22). Among the known blood markers of BC, only CA15-3 is specific since it showed no elevation in patients in the control group (23). In other studies, elevated CA15-3 levels have been associated with tumour size, axillary node involvement, and advanced stages, making it a highly predictive prognostic marker (6,24,25).

Additionally, some studies report the role of CA15-3 in combination with other markers like CEA, CA125, Apurinic/apyrimidinic endonuclease (APE), epidermal growth factor receptor (EGFR), Neuron-specific enolase (NSE) for BC, but all these have less sensitivity and specificity (23,24,26). In our study, we also found the specificity of CA15-3 to be very high. But the main disadvantage of these markers is the lack of sensitivity for low volume disease. So, it is of no value in either screening or diagnosing early BC. Bayo et al. (23) have previously reported an AUC of 0.918 from a combined model, including CA15-3. The cut-off point was 0.697; this model had high sensitivity (85.7%) and specificity (82.3%).

In our study, serum GDF-15 in combination with CA15-3 had a better diagnostic value with sensitivity and specificity of 63.06% and 98.15%, respectively. We also observed that serum GDF-15 could also be used as an independent early diagnostic marker of BC and had a better predictive ability than CA15-3. Various other studies have reported that serum GDF-15 could be used as a diagnostic and prognostic marker of multiple types of malignancies such as liver, lung, prostate cancer (11-13,15). Previous studies showed that IHC positive GDF-15 tissue is correlated with high-grade tumours. GDF-15 expression was also positively associated with lymph node

metastasis (17). Reports have also demonstrated the association of GDF-15 with the advancement of BC. We observed that serum GDF-15 was significantly higher in distant metastatic patients than non-metastatic BC patients.

Welsh et al. (27) studied ten different tumour types and found GDF-15 to be increased in metastatic colorectal, prostatic, and BC compared to controls. Further, Wollmann et al. (28) found higher GDF-15 expression in breast tumour tissue samples compared to matched adjacent control tissues. Thus, even though having a small number of samples, these studies showed elevated GDF-15 expression in more than half serum or tissue specimens. Sasahara et al. (18) also reported higher GDF-15 expression in BC tissue than controls and higher GDF-15 expression in HER2-positive tumours. Finally, Peake et al. (17) found significant positive associations between GDF-15 expression and high tumour grade and ER-negative and HER2-positive status.

Our study takes these findings further and evaluates serum GDF-15 levels in circulation to differentiate BC from healthy controls. It can indeed be utilized in clinical settings as an adjunctive marker for these patients with better sensitivity and sensitivity.

There are some limitations of this study. Firstly, we could not follow up with the patients. Therefore, survival data analysis was not possible, which would have allowed us to present the prognostic role of GDF-15 in the BC patients. Secondly, IHC data of receptor status was missing for some patients. Thirdly, along with BC, some patients had comorbidities such as hypertension and diabetes mellitus, which could have affected the serum GDF-15 levels as GDF-15 is known to be increased in diabetes mellitus (29,30).

CONCLUSION

Biomarkers have long been prevalent in the clinical practice related to BC. However, early diagnosis can aid in better prognostic outcomes in these individuals. Serum GDF-15 has shown much promise as an early diagnostic marker in other cancers. Based on the existing evidence and our findings, we suggest that it can also be utilized as an adjunct marker in BC diagnosis, especially to detect early-stage (stage I-II) BC from the non-cancer group. The current study also suggested a cut-off (525.77 pg/ml) value differentiating BC and control groups. Future studies on a larger scale are needed to establish the robustness of serum GDF-15 use in a clinical setting.

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