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ORIGINAL ARTICLE

Evaluation of Serum Integrin αvβ3 & Vitronectin in the Early Diagnosis of Breast Cancer

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SUMMARY

Background: **This study aimed to evaluate the relationship of serum vitronectin and integrin alpha V beta 3 (αvβ3) levels with various clinicopathological parameters of breast cancer and to assess the diagnostic value of these markers alone or in combination with the conventional breast cancer biomarker CA15.3.**
Methods: **This study included 50 early diagnosed stage I - II primary breast cancer patients, 20 patients with fibroadenoma benign lesions, and 20 apparently normal healthy controls. Integrin αVβ3, vitronectin, and CA15.3 levels were measured using ELISA technique.**
Results: **Serum levels of integrin αVβ3 and vitronectin were significantly higher in the malignant group than those in the benign group and the control group with ($p < 0.001$). Significant positive correlation between integrin αvβ3 and vitronectin concentrations was found. Both markers showed significant statistically difference with lymph node, histological grade, tumor stage, and tumor size ($p < 0.05$). Integrin αvβ3 exhibited the highest sensitivity (70%) and specificity (68%), then vitronectin with 67% and 68%, respectively, followed by CA15.3 showing the least sensitivity and specificity (65% and 62%, respectively). All assessed parameters revealed comparable area under the receiver-operating characteristic curve (AUC) 95% confidence interval (CI) range of 0.581 - 0.822.**
Conclusions: **Integrin αvβ3 is a promising biomarker alone or in combination with vitronectin and CA15.3 for diagnosis and prognosis of early stage breast cancer.**
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KEY WORDS

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INTRODUCTION

Breast cancer is the most common invasive cancer that affects women worldwide [1]. The current available approach for the early detection of breast cancer is cancer antigen 15.3 (CA15.3) and mammography, both have several limitations and poor diagnostic accuracy [2,3]. So, there is an urgent need to investigate other biomarkers or procedures that could help in picking up early breast cancer patients and enhance CA15.3 sensitivity and specificity.
The tumor microenvironment, including the extracellular matrix (ECM), is an important factor contributing to both the maintenance of tissue homeostasis and the pro-

motion of tumorigenesis [4]. Major components of the ECM include collagens, laminin, fibronectin, and vitronectin. Signaling by the ECM largely occurs through the integrin family of proteins [5].

Integrins are part of a major family of cell adhesion receptors that are implicated in angiogenesis, neovascularization, initiation, progression and metastasis of solid tumors [6]. As a receptor with transmembrane domains, integrins are expressed on tumor cells and tumor associated host cells which could facilitate receptor mediated signaling between tumor cells and tumor associated cells to promote tumor growth and invasion [6]. A wide variety of integrins have been shown to contribute to tumor progression. Integrin αvβ3 has been shown to be highly expressed on some tumors such as breast cancer tumors and is implicated with increased bone metastasis [7], acting as a marker for luminal progenitor cells in the mammary ductal epithelium. Its signaling can help in maintaining cancer stem cell populations in tumors and regulate other cancer stem cell markers [8]. Expression of αvβ3 integrin allows endothelial cells to bind to matrix proteins enhancing angiogenesis and promoting adhesion to tumor associated endothelium [9]. To our knowledge, this is the first study evaluating serum integrin αvβ3 in breast cancer patients using ELISA technique.

Vitronectin is a multi-domain, 75-kDa adhesive glycoprotein found in both the ECM and plasma [10] that executes its regulatory functions by binding to a wide array of ligands. It is divided into three main domains plus a connecting region: The N-terminal somatomedin B domain (SMB), the connecting region, a central hemopexin homology domain, and a C-terminal heparin-binding domain [10]. Immediately following the SMB domain is the integrin binding sequence that mediates cell adhesion by vitronectin [11]. Vitronectin plays a key role in the development and progression of solid tumors including breast cancer through enhancing migration, invasion, and participation in differentiation of breast cancer stem cells [12].

In this study, we evaluated the relationship of vitronectin and integrin αvβ3 with various clinicopathological parameters of breast cancer. This study aimed to explore whether vitronectin/integrin αvβ3 alone or in combination with the conventional breast cancer biomarker CA15.3 could be used as new biomarker panels to recognize cancer cells with high sensitivity and specificity for the early detection of breast cancer.

MATERIALS AND METHODS

Patients

This study has been performed on 90 adult females classified into three groups, group 1: (n = 50) early diagnosed primary breast cancer patients most of them with invasive ductal carcinoma type, aged from 38 - 55 with a mean of 46.1 ± 5.2 years, they did not receive chemotherapy, radiotherapy or had surgical resection of the tumor; group 2: (n = 20) patients diagnosed with benign fibroadenoma lesions aged from 35 - 52 years with a mean of 43.3 ± 1.2 years, and, group 3: (n = 20) apparently healthy volunteers were included as normal controls aged from 35 - 51 years with a mean of 42.1 ± 2 years. The study was approved by the ethical committee of the Faculty of Pharmacy, Ain Shams University. Informed written consent was obtained from all study subjects.

The exclusion criteria were any cancer other than breast cancer, late stages of breast cancer, presence of kidney or liver disease, any ocular diseases, bone diseases including osteoporosis and rheumatoid arthritis [13]. All patients were recruited from outpatient clinics at the National Cancer Institute (NCI) from November 2015 to February 2017.

All cases and controls were subjected to full history taking and clinical examination. Laboratory investigations were carried out in the form of routine laboratory investigations (CBC, liver and kidney function tests, CA 15.3, and CEA). Imaging techniques were performed for patients with cancer in the form of: i) breast mammography for benign and malignant breast lesions. ii) chest X-ray for cancer patients. iii) liver and bone scan for cancer patients to exclude metastasis. iv) histopathological study and grading for breast lesions. v) immunohistological study (estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (Her2) receptor).

Serum collection and storage

Eight milliliter blood samples were withdrawn into yellow gel vacutainers then, after 30 minutes, subjected to centrifugation at 4,000 rpm for 10 minutes. Sera were divided into two aliquots, the first aliquoted sera were used for assay of liver function tests and kidney function tests and the other ones were kept frozen at -80°C until used for assay of integrin αvβ3, vitronectin CA-15.3, and CEA.

Methods

a) Enzyme-linked immunosorbent assay (ELISA)

Sera were analyzed using assay kits for vitronectin and integrin αvβ3 supplied by R&D system (Sunnyvale, CA, USA). For CA15.3 it was supplied by R&D system (South San Francisco, CA, USA), whereas CEA was supplied by Thermo Fischer scientific (Waltham, MA, USA) and all were done according to manufacturer's instructions.

b) Histopathological evaluation

Tumor grading was evaluated according to Nottingham combined histologic grade (Elston-Ellis modification of the Scarff Bloom Richardson grading system) [14]. Tumor staging was evaluated according to American Joint Committee on Cancer (AJCC) [15]. Receptors study defined by immunohistochemistry (IHC) expression of ER or PR and Her2 [16].

Statistical methods

Data was analyzed using IBM SPSS advanced statistics (Statistical Package for Social Sciences), version 22 (IBM Corp., Armonk, NY, USA). Numerical data was described as mean, standard deviation and median, IQR or range as appropriate, while qualitative data was described as number and percentage. Testing for normality was done using Kolmogrov-Smirnov test and Shapiro-Wilk test. For not normally distributed variables comparisons between the median of the two independent groups was tested using Mann Whitney *U* test. Comparisons between the median of more than two independent groups was tested using Kruskal-Wallis test that was followed by a post hoc test if significant. To evaluate the linear relationship between the numerical measurements, Spearman's rho correlation coefficient was calculated. Receiver operating characteristics (ROC) curve was done to estimate the best cutoff point followed by the calculation of sensitivity, specificity, with their 95% CI. Logistic regression was used to estimate the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the optimal combination of biomarkers. A p-value less than or equal to 0.05 was considered statistically significant. All tests were two tailed.

RESULTS

Integrin αvβ3

Integrin αvβ3 was significantly higher in the malignant group than in both the benign and control groups ($p < 0.001$). However, integrin failed to reveal any significant difference between benign group and control group as illustrated in Figure 1a. The relationship between serum integrin αvβ3 and clinicopathological parameters in malignant patients was analyzed. No association with age, menstrual status, PR and ER status ($p < 0.5$) were found. On the other hand, integrin αvβ3 revealed a significant difference between tumor grade, lymph node status, tumor stage I - II ($p < 0.001$) and tumor size ($p = 0.010$) (Table 1). Integrin αvβ3 showed a sensitivity of 70% and a specificity of 68% at a cutoff level of 5.02 ng/mL. PPV was 69.8% and NPV was 64.8% which provided 67.8% diagnostic accuracy. Combining integrin αvβ3 with CA 15.3 markedly increased the sensitivity to 72% and the PPV to 70.5% for breast cancer diagnosis (Table 2). Regarding the area under ROC for the whole range of sensitivities and specificities, our results showed that it was 0.72 (95% CI 0.621 - 0.822) for integrin αvβ3 (Figure 2a) and 0.70 (95% CI 0.581 - 0.805) for CA15.3 (Figure 2c).

Vitronectin

Vitronectin was significantly higher in the malignant group than in the control group ($p < 0.001$) and the benign group ($p < 0.05$). Moreover, the benign group was significantly higher than the control group ($p < 0.01$) as illustrated in Figure 1b. No association was found between age, menstrual status, tumor stage I - II, PR and ER status and serum levels ($p > 0.5$). On the other hand, there is a significant difference between and tumor grade ($p < 0.001$), lymph node status ($p < 0.001$), and tumor size ($p = 0.002$) (Table 1). Vitronectin showed a sensitivity of 67% and a specificity of 68% at a cutoff level of 50.37 ng/mL. The PPV was 66.7% and NPV was 57.5% which provided a diagnostic accuracy 63.2%. In addition, combining vitronectin with CA 15.3 markedly maximized the sensitivity to 74% and the PPV to 77.0% for breast cancer diagnosis (Table 2). As for the area under ROC for the whole range of sensitivities and specificities, vitronectin was 0.71 (95% CI 0.597 - 0.818) (Figure 2b).

CA 15.3

Serum CA 15.3 showed a significant increase in the malignant group compared to the control group ($p < 0.01$). However, there was no association at all between CA 15.3 and all clinicopathological parameters of malignant patients (Table 2). Also, CA 15.3 failed to show a significant difference between the malignant group and benign group and failed to show a significant difference between the benign group and control group as illustrated Figure 1c. CA 15.3 showed a sensitivity of 65% and a specificity of 62% at a cutoff level of 21.07 U/mL. The PPV was 64.4%, NPV was 61.3% with total accuracy 63.3%. Furthermore, the combination of the studied markers enhanced the sensitivity to 77% and the specificity to 73% with a PPV of 78% (Table 3).

CEA

Regarding CEA levels, the study showed normal levels and revealed no significant difference between the studied groups as illustrated in Figure 1d. CEA was only used as a confirmatory test for exclusion of metastasis.

Correlation between the studied markers

Spearman's rho correlation was used in the analyses between integrin αvβ3 and vitronectin. The results observed significant positive correlation between integrin αvβ3 ($r = 0.758$; $p < 0.001$) with vitronectin within the malignant group (Table 3).

DISCUSSION

The objective of the present study was to assay promising non-invasive diagnostic markers that would help in screening early breast cancer. CA 15.3 which is the current conventional tumor marker is mostly elevated in stage III - IV breast cancer patients with low sensitivity and specificity when used to distinguish early stages; thus, it is recommended to be used as a biomarker for already confirmed diagnosed breast cancer [17]. In this study, we attempted to evaluate new diagnostic markers, vitronectin and integrin αvβ3, to show their usefulness in the clinical assessment of breast cancer, especially for the most frequent type: invasive ductal carci-

Table 1. Association between the studied markers and the patients' characteristics.

Characteristics	No.	Integrin αvβ3	p-value	Vitronectin ng/mL	p-value	CA 15.3 U/mL	p-value
Age							
≤ 45	24 (48%)	4.99 (4.63 - 6.64)	0.214	49.83 (45.31 - 73.97)	0.095	21.92 (18.79 - 24.94)	0.086
> 45	26 (52%)			59.19 (50.40 - 78.69)		23.40 (21.02 - 27.91)	
Menstruation							
Postmenopausal	23 (46%)	5.73 (4.56 - 7.51)	0.755	60.73 (48.42 - 77.32)	0.428	23.9 (21.09 - 27.26)	0.096
Premenopausal	27 (54%)	5.34 (4.75 - 6.49)		54.03 (64.28 - 74.91)		21.84 (19.12 - 25.27)	
Size							
≤ 2.5	20 (40%)	4.92 (4.55 - 5.46)	0.01 *	50.03 (43.41 - 55.61)	0.002 *	21.17 (18.28 - 27.15)	0.243
> 2.5	30 (60%)	6.23 (4.86 - 7.62)		69.4 (49.53 - 85.28)		22.68 (21.09 - 25.70)	
Grade							
I	16 (32%)	4.64 (4.17 - 5.02)	< 0.001 *	45.75 (41.13 - 50.40)	< 0.001 *	20.97 (18.28 - 25.90)	0.134
II	34 (68%)	6.23 (5.07 - 7.62)		65.99 (50.76 - 83.41)		22.72 (21.59 - 26.12)	
Stage							
I	22 (44%)	4.75 (4.45 - 5.12)	< 0.001 *	46.68 (41.73 - 50.43)	< 0.001 *	21.09 (19.12 - 25.28)	0.189
II	28 (56%)	6.98 (5.65 - 8.29)		74.25 (55.78 - 85.37)		22.74 (21.98 - 27.26)	
Type							
* IDC	47 (94%)	5.28 (4.65 - 6.98)	0.097	55.11 (46.68 - 74.91)	0.202	22.65 (19.62 - 26.50)	0.533
* ILC	3 (6%)	7.93 (5.51 - 8.38)		86.25 (47.75 - 92.11)		22.31 (20.44 - 22.32)	
Lymph node							
Negative	32 (64%)	4.81 (4.31 - 5.51)	< 0.001 *	46.68 (41.73 - 52.44)	< 0.001 *	21.52 (19.12 - 27.36)	0.778
Positive	18 (36%)	6.98 (5.12 - 8.29)		72.35 (55.11 - 85.37)		22.70 (20.82 - 25.69)	
ER							
Negative	12 (24%)	5.37 (4.5 - 7.3)	0.725	59.39 (46.7 - 74.45)	0.910	22.17 (19.55 - 24.99	0.768
Positive	38 (76%)	5.43 (4.74 - 7.25)		54.9 (47.29 - 75.63)		22.54 (20.24 - 26.69)	
PR							
Negative	12 (24%)	5.37 (4.5 - 7.3)	0.725	59.39 (46.7 - 74.45)	0.910	22.17 (19.55 - 24.99)	0.768
Positive	38 (76%)	5.43 (4.74 - 7.25)		54.9 (47.29 - 75.63)		22.54 (20.24 - 26.69)	
Her2							
Negative	42 (84%)	5.17 (4.65 - 6.83)	0.074	51.64 (46.6 - 75.6)	0.458	22.37 (19.55 - 25.40)	0.507
Positive	8 (16%)	6.87 (5.65 - 8.1)		65.1 (55.8 - 74.8)		24.21 (20.83 - 27.53)	

The data represented by median and IQR - Interquartile range.
*** - p < 0.05, statistically significant.**
*** IDC - invasive ductal carcinoma.**
*** ILC - invasive lobular carcinoma.**

Table 2. Diagnostic accuracy of the studied markers, either single or combined.

Marker	Cutoff value	AUC	Sensitivity	Specificity	PPV	NPV	Total accuracy	95% CI
Integrin αvβ3 ng/mL	5.02	0.72	70%	68%	69.8%	64.8%	67.8	(0.621 - 0.822)
Vitronectin ng/mL	50.37	0.71	67%	68%	66.7%	57.5%	63.2	(0.597 - 0.811)
CA15.3 U/mL	21.07	0.70	65%	62%	64.4%	61.3%	63.3	(0.581 - 0.805)
Integrin αvβ3 & CA15.3	-	-	72%	62.5%	70.5%	64.1%	67.8	-
Vitronectin & CA15.3	-	-	74%	72. %	77%	69%	73.3	-
Integrin αvβ3, Vitronectin &	-	-	75%	73%	78%	70%	73.3	-

(PPV) - positive predictive value, (NPV) - negative predictive value, (CI) - confidence interval.

Table 3. Correlation studies between the studied markers in malignant group.

Group				Integrin αvβ3 (ng/mL)	Vitronectin (ng/mL)	CA 15.3 (U/mL)
Malignant	Spearman's rho	Vitronectin (ng/mL)	R	0.758	1.000	
			p-value	< 0.001 *		
			N	50	50	
	Spearman's rho	Integrin (ng/mL)	R	1.000		
			p-value			
			N	50		
	Spearman's rho	CA 15.3 (U/mL)	R	0.103	0.187	1.000
			p-value	0.479	0.194	
			N	50	50	50

*** - p < 0.05, statistically significant correlation.**

noma.

In clinical practice, measurement of CEA proved to be a well-established biomarker, most useful in the determination of distant metastases and in monitoring the response to treatment of relapsed patients [18]. This was matched with the results of our study that showed normal levels of CEA with no significant difference between the studied groups, as the study was conducted on confirmed early diagnosed primary breast cancer patients.

Serial determination of CEA and MUC-1 antigen may be beneficial in monitoring response to therapy and for early detection of recurrence or metastasis. The main disadvantages of these markers are lack of sensitivity for low-volume disease and lack of specificity. They are therefore of no value in either screening or diagnosing early breast cancer [19].

In this study, we observed and firstly reported assayed serum integrin αvβ3 in breast cancer and benign tumors in serum. Our results demonstrated that integrin αvβ3 was of higher significance in early stage breast cancer (I - II) than benign and control cases. This may be attributed to up-regulation and overexpression of integrin αvβ3 in breast cancer [7], or because of the migratory response of breast cancer cells during the activation of integrin αvβ3 while interacting with specific extracellular matrix proteins [20]. Ligated αvβ3 can interact with fibroblast growth factor receptor (FGFR) to prevent apoptosis through the intrinsic apoptosis pathway. Also, integrin αvβ5 interacts with vascular endothelial growth factor receptor 2 (VEGFR2) to promote resistance against extrinsic apoptosis nevertheless thus enhancing angiogenesis [21]. Furthermore, integrin αvβ3 is implicated in enhancing tumorigenesis via interaction with

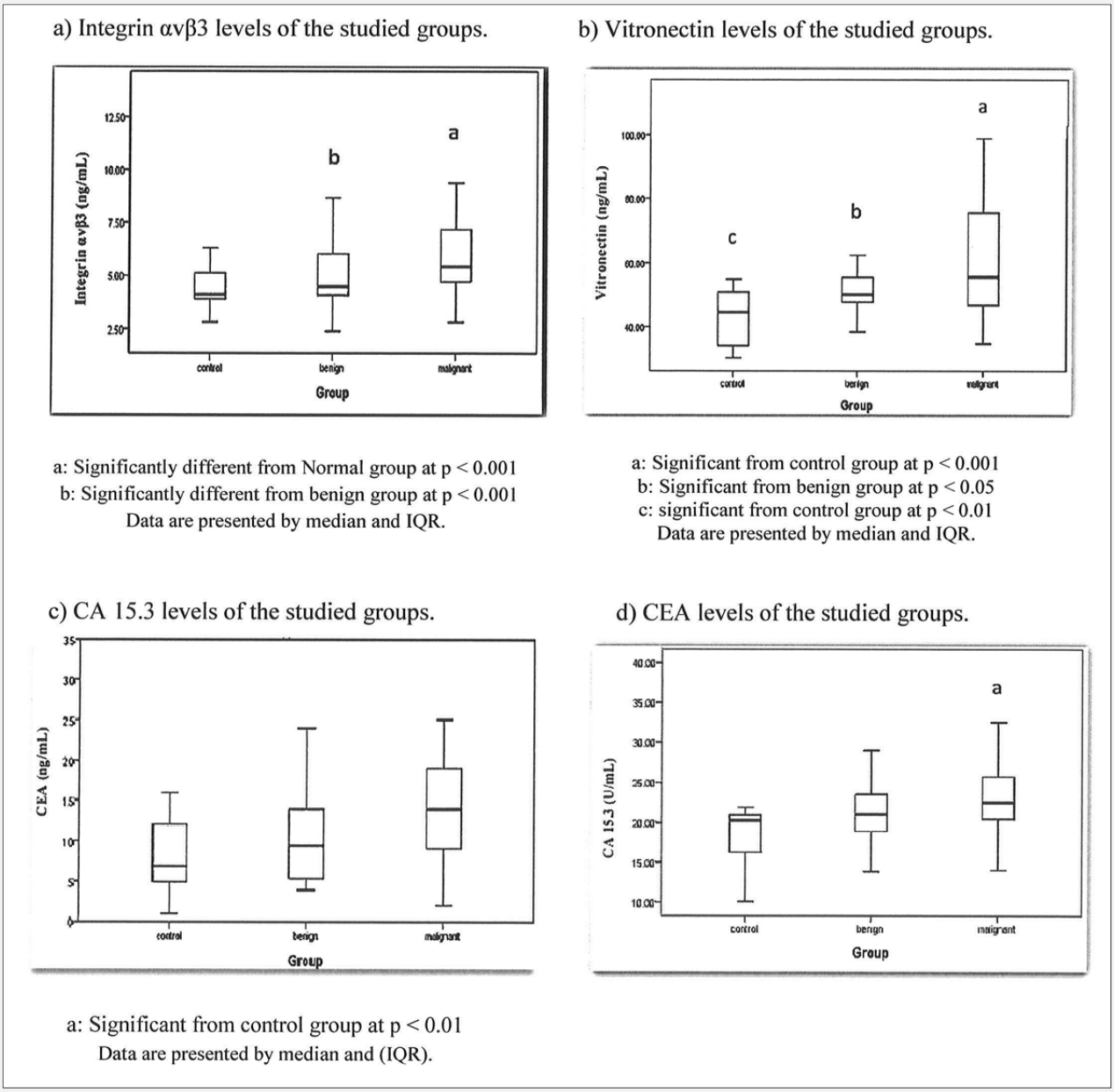

Figure 1. Serum levels of the studied markers.

oncogenes through activation of focal adhesion kinase (FAK), sarcoma (Src), and adaptor protein p130 Crk-associated substrate (p130CAS) which leads to invasion, proliferation, and survival of tumor cells [6].

Regarding vitronectin, the results of this study showed that it was significantly higher in the sera of breast cancer patients compared to the benign and control group. Vitronectin has been shown to be altered in breast cancer tissue and to serve in clearly distinguishing early stage breast cancer stage I - II breast cancer patients from nonmalignant subjects including normal and benign subjects and that matched with other studies [22, 23]. Kadowaki et al. showed that a rise in serum of malignant breast lesions could be through the catabolic reactions of vitronectin that are associated with breast cancer [24], or as a secondary product of tumor-stroma interactions [25]. Vitronectin is derived from abnormal and fragile growing new vessels that surround the ma-

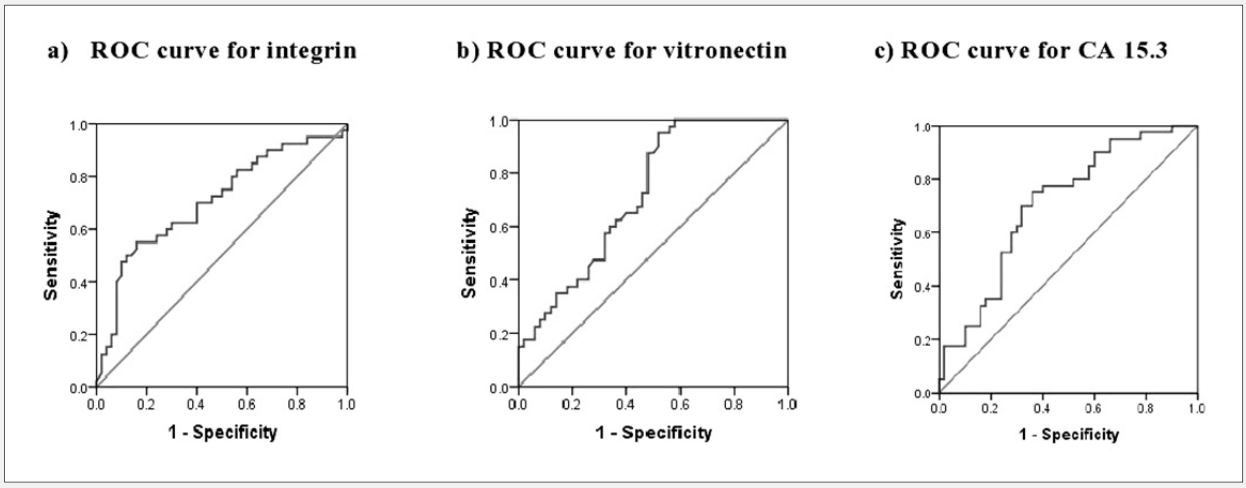

Figure 2. ROC curve for the studied markers.

lignant tumor, and accumulates in new vessel walls. This study revealed that there is a positive correlation between integrin αvβ3 and vitronectin concentrations. Vitronectin and its receptor integrin αvβ3 are implicated in the pathogenesis of several tumor types, including the brain, breast, and prostate, where cancer stem cells (CSCs) have been identified and characterized [26]. Vitronectin mediates its effects predominantly through integrin αvβ3 and results in altered β-catenin localization [27]. Interactions of both lead to induce osteogenic differentiation of mesenchymal stem cells [28] and support endoderm maturation from the inner cell mass in mouse development [29].

Comparing both marker levels and the different prognostic factors in the breast cancer group, showed significant differences between both integrin αvβ3 and vitronectin levels and tumor size, lymph node, histological grade, and tumor stage. In addition to urokinase plasminogen activator (uPA), vitronectin is another ligand that can bind urokinase plasminogen activator receptor (uPAR) [30]. Vitronectin was found to play important roles in tumor cell adhesion, migration, invasion, and growth of solid tumors via interaction with (uPAR) [31, 22]. Other studies found that high expression of integrin αvβ3 enhance cell migration, angiogenesis [32], tumor size progression, and lymph node metastasis [33] through endothelial cell activation, basement membrane degradation, and prevention of apoptosis through the intrinsic apoptosis pathway [34]. However, there was no correlation between integrin αvβ3 and vitronectin levels and patient menstrual status, age, ER expression, PR expression, and HER2 overexpression.

Crosstalk between integrin subgroups and EGF family receptors can promote tumor initiation, proliferation, and invasion [6]. Integrin α6β4 complexes with HER2 to enhance activation of downstream signaling that lead to loss of cell polarity and induce hyperproliferation [5]. Other studies found that in glioblastoma, integrin αvβ3 is overexpressed at the invasive margins of the tumor increased which is associated with enhanced cell motility and apoptosis resistance [35]. In pancreatic tumor, the increased expression of αvβ3 integrin is associated with increased activation of MMP-2 and lymph node metastasis [18]. In breast cancer αvβ3 expression is associated with disease progression and metastasis [19]. In addition, levels of αvβ3 were significantly increased in the MDA-MB-231 cell line. Furthermore, αvβ3 expression is involved in the regulation of breast cancer cell response to chemotherapy and serves as a marker to chemosensitivity [36]. Other studies found that 99mTc-NC100692 scintigraphy which is used for a range of human integrin receptors including $\alpha_v\beta_3$ and $\alpha_v\beta_5$, and it was also established in an *in vitro* study that it may be effective in detecting malignant breast lesions [37]. Our results were found to be more or less comparable in an AUC in ROC analysis with different sensitivity and specificity for each marker alone. Integrin αvβ3 exhibited the highest sensitivity of 70% and specificity of 68%, then vitronectin with a sensitivity of 67% and specificity of 68% followed by CA15.3 that showed the least sensitivity of 65% and specificity of 62%. So, integrin αvβ3 is a promising marker in the diagnosis of early breast cancer. A more potent diagnostic role was illustrated when the three markers were combined with each other. They displayed an improvement in diagnostic sensitivity and specificity with 75% and 73%, respectively, and 78% PPV, when compared to individual biomarkers or combinations of two of the three bio-

markers, suggesting that integrin αvβ3, vitronectin, and CA15.3 panel have good diagnostic performance.

CONCLUSION

We demonstrated that integrin αvβ3 is a promising biomarker alone or in combination with vitronectin and CA15.3 for diagnosis of early stage breast cancer. As is known, tumor stage, size and lymph node numbers are considered well-established prognostic factors in breast cancer that affect prognosis and treatment strategy [38, 39]. They were significantly correlated with the studied markers. To our knowledge, this is the first study to use combined measurement of serum levels of these markers in the screening and diagnosis of early stage breast cancer.

Declaration of Interest:
The authors have stated that they have no conflicts of interest.

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