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Research Article

UTILITY OF ASSESSMENT SERUM LEVELS OF VEGF, bFGF AND PDGF-BB AS SUGGESTED ANGIOGENESIS BIOMARKERS PANEL TOOL IN PATIENTS WITH BREAST CANCER

[1]Nahla Anber, [2]Ahmed EL-Sebaie, [3]Shaker Mousa, [4]Osama Elbaz

[1] Fellow of Biochemistry, Emergency Hospital, Mansoura University, Mansoura, Egypt.

[2] Clinical Pathology Department, Faculty of Medicine, Mansoura University, Mansoura, Egypt.

[3] Pharmaceutical Research Institute at Albany College of Pharmacy and Health Sciences, Rensselaer, NY, USA.

[4] Clinical Pathology Department, Faculty of Medicine, Mansoura University, Mansoura, Egyp.

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ABSTRACT

Introduction: Breast cancer is the commonest cause of cancer death in women worldwide. A shift in the angiogenic balance allows the up-regulation of several pro-angiogenic factors, which by ways of mutual interactions stimulate tumor angiogenesis. Angiogenic factors are produced directly by tumor through the cancer cells or indirect by inflammatory cells that infiltrate tumor. Tumoural angiogenesis is essential for the growth and metastases of breast cancer cells.

Aim: The aim of the present study was to asses vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF-BB) serum levels in breast cancer patients as promising suggested panel of tool of some angiogenesis markers assisted by Bio- Plex Pro assays for diagnosis and prognosis performance in breast cancer, also as predictors to identify subgroups of patients fitting into different treatment to improve clinical outcome

Method: Serum samples were collected from 68 breast cancer female patients; 32 pre-treatment and 36 under conventional chemo-radiotherapy and 10 healthy female donors as controls. Under treatment patients classified according to therapy related number of cycles regimen received to 1-4 (n=10), 5-8 (n=18), and > 8 cycles (n=8). The levels of the angiogenic markers, VEGF, bFGF and PDGF-BB, were assessed by Bio-Plex Pro assays that quantify multiple protein biomarkers.

Results: There was a significant increase in VEGF and PDGF-BB (P1< 0.05) and non significant elevated bFGF (p1>0.05) in breast cancer pretreatment patients compared to controls. In patients under treatment there was a non significant elevated level in median values for each marker than the base line measurement (P2 > 0.05). A highly significant reduction serum levels for each of bFGF and PDGF-BB (P3< 0.001) and significant reduction (P3< 0.05) for VEGF after conventional adjuvant therapy treatment compared with pretreated patient serum levels. Those patients received 1-4 cycles regimen elevated non-significant median values (P4 > 0.05) for each of FGF and PDGF-BB, while VEGF showed significant elevated serum level (P4 < 0.05) in compare to baseline measurement. Those patients received 5-8 cycles regimen treatment identified non significant reduction (P5 > 0.05) in median values with baseline level of control subjects. Those patient received more than 8 cycle regimen reviled significant reduction for bFGF (P6 < 0.05) and significant elevated value (P6 > 0.05) for VEGF and non-significant (P6 > 0.05) reduction serum level for PDGF-BB compared with base line measuring value.

Conclusions: our results confirmed that suggested panel tool of VEGF, bFGF and PDGF-BB serum levels assessed by Bio-Plex ® Pro Assays for Angiogenesis Factors Quantification is useful for the early detection of breast cancer within screening programs, suitable for prospective studies for breast cancer diagnosis and /or recurrence of tumor, in prognosis and monitoring members of high- risk breast cancer families. Also, our suggested panel angiogenesis marker tool is useful for identifying subgroups of patients fitting into different treatment to improve clinical outcome.

Key words: Breast cancer; Bioplex; VEGF; bFGF; PDGF-BB

INTRODUCTION

Angiogenesis is defined as a physiological process where a new blood vessels form from pre-existing vessels. Angiogenesis in normal and pathological conditions is multi- step process governed by positive and negative endogenous regulators. This process depends on endothelial cell migration, proliferation and differentiation [1]. In case of malignancy, this interaction is out of balance, and

*Corresponding author: Nahla Hamed Anber | E-mail: nahla.anber@yahoo.com

balance shift taken place. A shift in the angiogenic balance allows the up-regulation of several pro-angiogenic factors [2-6], which by ways of mutual interactions stimulate tumor angiogenesis [7, 8]. Angiogenic factors are produced directly by tumor through the cancer cells or indirect by inflammatory cells that infiltrate tumor [9]. Tumoural angiogenesis is essential for the growth and metastases of breast cancer cells. Many studies confirmed that by experimental studies [10-15] and others by clinical setting [16-18]. Also, many studies identified a quantitative relation between hematogenous spread of tumor cells and intratumoral microvessel density (MVD) and correlation between increased MVD and higher incidence of metastasis and a poor prognosis in various malignancies, including breast cancer [9, 19] .

So far, VEGF circulating levels considered a surrogate marker for angiogenesis, at the same time as target for anticancer treatment [20]. VEGF is a potent angiogenic cytokine in normal tissue and tumors. Many studies have demonstrated elevated serum levels of VEGF in different type of solid tumors included breast cancer [21-24]. VEGF is over expressed in breast cancer when compared to normal breast tissue and serum [25-27] and levels of VEGF in those patients correlate with disease-free and overall survivals [28, 29].

During the last decade, it is clearly appear that other-angiogenic factors may be involved in tumor evasion of anti-VEGF treatment [30]. Special focus has been developed toward many others of angiogenic markers and their role involved in angiogenic pathways, such as bFGF and PDGF-bb [20]. bFGF is a potent pro-angiogenic factor belongs to a large family of growth factors [31,32], affecting endothelial cell migration and proliferation [33,34]. Serum bFGF is elevated in malignant tumors as compared to healthy controls, and have been identified in many studies of breast cancer [35, 36]. PDGF-BB belongs to the family which is playing an important role in cell growth [37], chemotaxis [38], and in the regulation of the tumor stroma [39-42]. It is an important component in angiogenesis, where it promotes pericyte recruitment and the stabilization of microvasculature [43, 44]. The correlation between levels of angiogenesis regulated factors, clinical pathology and prognosis is very significant, especially in breast cancer [45-47]. The clinical relevance of prognostic parameters (lymph node status, tumor size, grade of malignancy, estrogen reseptors, progestron reseptors, and HER2 status) are relatively inadequate to precisely define the prognosis of individual with breast cancer [48]. Also, molecular profile identification of different tumors is useful for detecting subgroups of patients fitting into different schemes of treatment [48, 49].

Treatment of breast cancer patients depends on the stage of the tumor. Staging describes the extent of the cancer (i.e. whether it is invasive or non-invasive, the size of tumor, involved lymph nod), and whether it has metastasized. At stage II and III of breast cancer, primary treatment begins with surgically removing of the tumor and a small margin of healthy tissue around it. Adjuvant therapies used after surgery to get rid of any cancer cells that may be left behind and to reduce the risk of the cancer come back. Adjuvant therapies are, chemo, hormone, targeted therapy, and radiation therapy can all be used as adjuvant treatments. In some cases, neo-adjuvant therapy may be used before surgery to shrink the tumor so less tissue needs to be removed. Recently, there has been an increased development of novel agents targeting multiple angiogenic pathways (e.g. VEGFR, PDGFR, and bFGFR) [20].

Experimental analysis for quantitative level of angiogenic marker is playing important role for making angiogenic marker in rotten work as a diagnostic and prognostic marker in breast cancer. The measurement of biomarkers in blood is preferable to the measurement in the tumor tissue [46, 50], and the serum levels of those growth factors may be an indicator of both their cellular and soluble concentrations [51, 52]. Enzyme-linked immunosorbant assays (ELISA) considered the primary tequnice for qualitative assessment kits for single marked analysis [53, 54]. Recently more advanced experimental analysis of multi-circulating serum level of markers are valid. This device has a great advantage through quantitative assessment level of many circulating markers in the same minute sample and in a short time in compare with ELISA system.

The aim of the present study was to asses VEGF, bFGF and PDGF-BB serum levels in breast cancer

patients as promising suggested panel of tools of some angiogenesis markers assisted by Bio- Plex Pro assays as predictors for diagnosis and prognosis performance in breast cancer, also as predictors to identify subgroups of patients fitting into different treatment to improve clinical outcome.

MATERIAL and METHODS

Materials:

This study consisted of blood samples obtained from 68 female patients were 20 to 50 years old. All patients had histological confirmed with invasive grade II and III stage ductal adinocarcinoma breast cancer.

All patients where passed the primary treatment included breast- sparing surgical removal of the tumor with adequate margin of normal tissue, treatment of draining lymphatics and restoration of function.

Control group comprised (10) apparently healthy female ranged in age 18 to 50. Control subject were free from any disease associated with an increased angiogenic activity such as diabetic retinopathy, heart disease or lung disease, which could affect anti-angionic markers. Informed consent had been obtained from participating subjects according to the Ethics Committee of Faculty of Medicine, Mansoura University.

Blood samples collected from (32) patients after 2-3 months of breast- sparing surgical and from (36) patients under conventional adjuvant therapy treatment begins after 2-3 months of breast-sparing surgical (conventional adjuvant therapy either being chemo-treatment or hormonal therapy), and from (10) healthy control subjects.

Blood samples had been collected without anticoagulant into serum separator vacutainers and allowed to coagulate for 20 to 30 min at room temperature. Sera were separated by centrifugation (2,000 rpm, 10min), and all specimens were aliquot immediately, frozen and stored in a –70ºC freezer.

Bio-Plex ® Pro Assays for Angiogenesis Factors Quantification:

Bio-Plex Pro human angiogenesis array for VEGF, bFGF, and PDGF-BB was run according to the manufacture instructions (Cat # M50007W214, Bio-Rad Laboratories, USA), that quantify multiple protein bio-factors. Principle Technology of The Bio-Plex® suspension array system is built around the three core elements of xMAP technology: A) Fluorescently dyed microspheres (beads), each with a distinct color code or spectral address. This allows simultaneous detection of different types of molecules in a single well of a 96-well microplate. B) A dedicated flow-cytometer with two lasers and associated optics to measure the different molecules bound to the surface of the beads. C) A high-speed digital signal processor that efficiently manages the fluorescence data.

Assay Format of Bio-Plex Pro™ growth factor assays are essentially immunoassays formatted on magnetic beads. The assay principle is similar to that of a sandwich ELISA. Capture antibodies directed against the desired biomarker are covalently coupled to the beads. Coupled beads react with the sample containing the biomarker of interest. After a series of washes to remove unbound protein, a biotinylated detection antibody is added to create a sandwich complex. The final detection complex is formed with the addition of streptavidin- phycoerythrin (SA-PE) conjugate. Phycoerythrin serves as a fluorescent indicator, or reporter.

This multiplex tequnice enables us to quantify multiple protein biomarkers in a single well of a 96-well plate in just 3 to 4 hr, also these robust immunoassays require as little as 12.5 µl serum and the use of magnetic (MagPlex®) beads allowed us to automate wash steps on a Bio-Plex Pro wash station. Magnetic separation offers greater convenience, productivity, and reproducibility compared to vacuum filtration.

STATISTICAL METHODS

Statistical analysis was performed using SPSS 16. Quantitative data were summarized in the format of median and range. Nonparametric ranking statistics (median test) were used to analyze the relationship between each of studied markers in different groups. Spearmans correlation coefficient (*r*) and *P*- values were used to investigate the relationship between each two of studied markers in the serum. For all statistical analysis, $P < 0.05$ was considered statistically significant and $P< 0.001$ was considered statistically highly significant. Receiver Operating Characteristic (ROC) curve analysis used to

identified accurate cut- off value capable of discriminating between healthy women and cancer patients in the absence of international accepted cut-off values for serum VEGF, bFGF, and PDGF-bb concentration. Accuracy of studied factors was performed also by ROC curve represented by area under the curve. Ninety- five percent confidence intervals were calculated for sensitivity and specificity. Mann- Whitney test was used to compare the serum level of studied angiogenesis markers in breast cancer patients and control healthy women. The median levels of expression of analyzed markers were compared using Krushal- Wallis test.

Results

We obtained and analyzed serum samples from 68 breast cancer female patients, after corresponding breast-sparing surgery, and 10 control healthy female volunteers. Serum samples obtained from 32 patients before receiving corresponding conventional adjuvant therapy regiment and from 36 patients were under the same treatment. Those patient under therapy treatment were classified according to number of therapy cycles regimen into: subgroup received 1-4 cycles (n=10), 5-8 cycles (n= 18) and those received more than 8 cycle (n=8).

Pretreatment patients - control case analysis for each marker serum level overlapping and serum ranged levels (median values) in both group were carried out and were reported in table (1) and figure (1; A, B, C). VEGF serum level showed a partial overlapping, but median values showed significantly elevated level ($P1< 0.05$); on the other hand, bFGF serum level showed a considerable overlapping and median values were not significantly elevated ($P1 > 0.05$); and PDGF-BB serum level reviled no clear observed overlapping with significant increase serum level were obtained than the base line measurement ($P1 < 0.05$).

ROC curve calculation and analysis result represented in table (2), figure (2; A, B, C). Cut-off point concentration for bFGF, PDGF-bb and VEGF reported 50.47, 1878.5 and 18.58 pg/ml, respectively, with good accuracy for FGF (0.669) and high accuracy values for PDGF and VEGF (0.859, 0.852, respectively). In term of sensitivity, each of PDGF-BB and VEGF showed acceptable values (78.1%, 65% respectively), while bFGF showed low sensitivity (31.3%). In the term of specificity all of factors identified highly value (100%).

Correlation analysis study for relationship between studied angiogenic markers in pretreated patient group were identified in table (3). Highly significant positive correlation between each of them were identified ($p1=p2=p3< 0.001$)

Under treatment patient - control case analytical Comparative study results were reported in table (1) and figure (1; A, B, C). Each of VEGF and PDGF-BB markers serum level revealed a considerable overlapping, while bFGF identified partial overlapping of serum level with non significant elevated levels in median values for each marker than the base line measurement ($P2 > 0.05$).

ROC curve statistical results and analysis represented in table (4), figure (3; A, B, C). bFGF factor revealed acceptable accuracy represented by AUC (0.643) at cut off point serum level equal 16.37 pg/ml with high sensitivity (100%) and acceptable specificity (69.4%). Also, PDGF showed accuracy value (0.506) and cut- off level at 535.14 pg /ml with high sensitivity (100%) and acceptable specificity (61.1%). On the other hand, serum VEGF showed higher accuracy value than other factors (0.701), cut off point at 20.01pg/ml with high specificity (100%) but with low sensitivity value (39.9%).

Correlation study between our studied markers in under treatment patient reported in table (5). Each of those angiogenic markers revealed a highly significant positive correlation with each other factor ($p < 0.001$).

Under treatment- pretreatment patients case study were reported in table (1) and figure (1; A, B, C). Comparative study results reviled complete no overlapping for each of studied markers with highly significant reduction serum levels for each of bFGF and PDGF-BB ($P3< 0.001$) and significant reduction ($P3< 0.05$) for VEGF after conventional adjuvant therapy treatment.

Under treatment therapy related subgroups - control case study were represented by table (6), figure (4). Those patients received 1-4 cycles regimen identified considerable overlapping of our studied markers serum levels with elevated non-significant median values ($P4 > 0.05$) for each of bFGF and PDGF-BB, while VEGF showed significant

elevated serum level (P4 < 0.05) in compare to baseline measurement. Those patients received 5-8 cycles regimen treatment identified a considerable overlapping for each of studied markers serum level and no-significant reduction (P5 > 0.05) in median values with baseline level of control subjects. Patient received more than 8 cycle regimen reviled partial serum levels overlapping for bFGF and VEGF, but bFGF median values showed significant reduction (P6 < 0.05) and VEGF median values showed no significant elevated value (P6 > 0.05). PDGF-BB serum level showed acceptable overlapping with non-significant (P6 > 0.05) reduction compared with base line measuring value.

ROC curve calculated results using serum levels of studied angiogenic markers of healthy women and under treatment therapy related subgroups identified in table (7), figure (5; A, B, C). Patient subgroup received 1-4 cycles regimen results revealed cut off point for bFGF, PDGF-BB and VEGF at serum level 21.197, 1879.75 and 20.70 pg /ml, respectively, with corresponding good accuracy for each of bFGF and PDGF-BB (0.51, 0.540, respectively) and higher accuracy for VEGF (0.77). In term of sensitivity; bFGF identified higher percentage of sensitivity (90%) while PDGF-bb and VEGF showed lower sensitivity (40% for each). In term of specificity, bFGF identified 70 % specificity value, while PDGF-BB and VEGF showed considerable higher specificity (100% for each). Patient with 5-8 cycles regimen subgroup showed the most highlight appear marker values were VEGF that identified cut off point at serum level 20.01 pg/ml with corresponding good accuracy (0.636) and high specificity (100%), while sensitivity have been reported in low value (38.9%). PDGF-BB serum level also identified cut off point 1836.29 pg/ml, with higher specificity (100%), while low accuracy (0.467) and low sensitivity value (38.9%). Also, at patient subgroup received more than 8 cycles, cut off point serum concentration of PDGF-BB reported 1955.79 pg/ml, and acceptable accuracy value (0.5) with highly specificity value (100%), while reported low value of sensitivity (37.5%). VEGF identified cut off point 10.81 pg/ml with high accuracy (0.76) and sensitivity (100%), while specificity revealed low value (37.5%).

Under treatment therapy related subgroups – pretreated patient case study identified in table (6), figure (4). Comparative study patient subgroup received 1-4 cycles' regimen revealed considerable overlapping for PDGF-bb and VEGF and partial overlapping for bFGF serum levels with non-significant reduction in each of angiogenic markers serum levels (P7 > 0.05 for each). While those patients received 5-8 cycles treatment showed: partial overlapping but with significant reduction in serum values of bFGF; PDGF-BB identified no overlapping with highly significant reduction in median values (p8<0.001); and VEGF although showed considerable overlapping, but reviled significant reduction value (p8< 0.05). Those patients received more than 8 cycles regimen showed: no overlapping between medians values with a highly significant reduction in bFGF serum level (p9<0.001); partial overlapping but with significant reduction in PDGF-bb (p9< 0.05); while VEGF serum level showed considerable overlapping with no significant difference (p9> 0.05) when compare to pretreated patients.

Statistical and analytical Comparative study was carried on for assessed serum levels of our studied markers in therapy related subgroups represented in figure (4; A, B, C) and table (8). Comparative study between subgroups received 1-4 and those received 5-8 cycles regimen identified considerable overlapping medians values for bFGF and complete overlapping for PDGF-BB and VEGF with non significant reduction serum levels for each of markers (p10>0.05). In Patients subgroup received 5-8 compared with those received more than 8 cycles identified partial overlapping serum bFGF median values with non significant reduced serum level (p11>0.05), while each of PDGF-BB and VEGF showed complete no overlapping with elevated non significant serum level(p11>0.05). Also, comparative study between patient subgroups received 1-4 cycles and those received more than 8 cycles identified partial non significant reduced bFGF serum level, while each of PDGF-BB and VEGF showed complete overlapping medians values serum levels with non significance difference values (p12>0.05).

Table 1: **Angiogenic Markers serum levels in control subjects and patients with breast cancer; before and under- treatment versus controls**

Angiogenic Markers	Cases median: pg/ml (rang)			Significance P- value		
	Controls (N: 10)	Pre- treatment (N: 32)	Under treatment (N: 36)	P_1	P_2	P_3
FGF	32.85 (17.76-49.27)	37.79 (14.99-88.32)	26.73 (6.18-65.35)	0.111	0.170	0.000**
PDGF	1263.19* (537.01-1762.15)	2742.30 (334.59-11450.44)	1145.13 (36.31-3995.28)	0.001*	0.958	0.000**
VEGF	11.55* (3.28-19.33)	25.11 (5.98-189.82)	16.76 (4.68-84.42)	0.001*	0.053	0.007*

P_1: Pre-treatment versus Controls. (t- test)

P_2: Under- treatment versus Controls. (t- test)

P_3: Pre-treatment versus under treatment

*Significant (p< o.o5)

**Highly significant (p<0.001)

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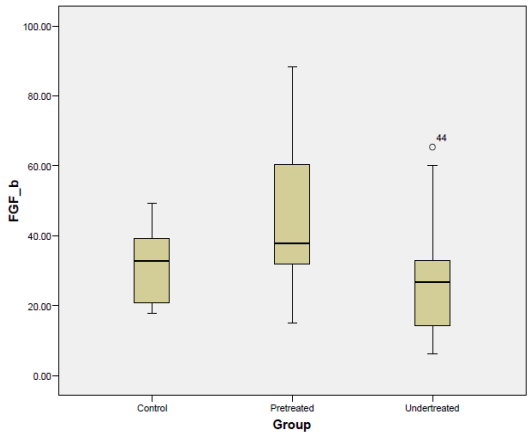

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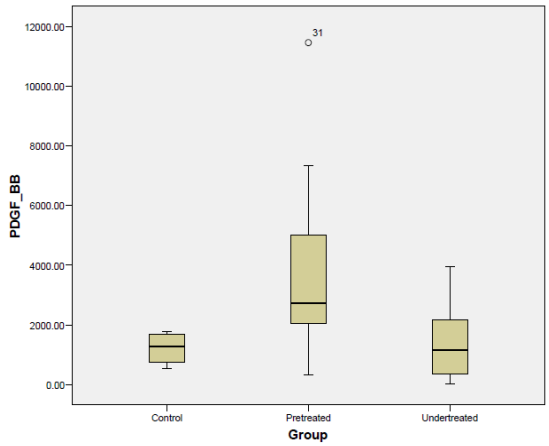

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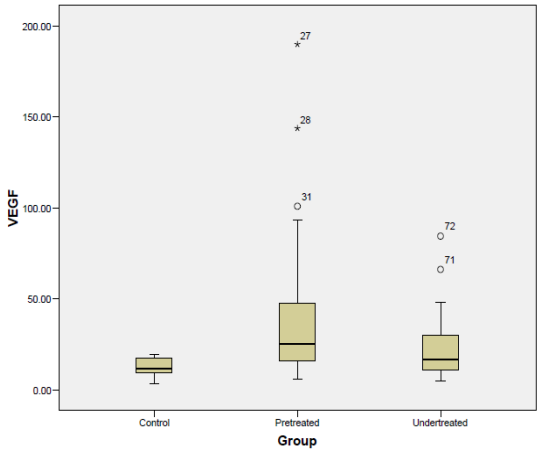

Figure 1: Angiogenic markers serum levels in studded groups. Box plots of (a) basic fibroblast growth factor (bFGF). (B) platelet derived growth factor(PDGF-BB) (C) vascular endothelial growth factor (VEGF). The lower boundary of the box is the 25th percentile and the upper boundary is the 75th percentile. The bold line inside the box represents the median. * Cases with values more than 1.5 box lengths from the upper or lower edge of the box (extreme values). The largest and smallest observed values that are not extreme values are also shown

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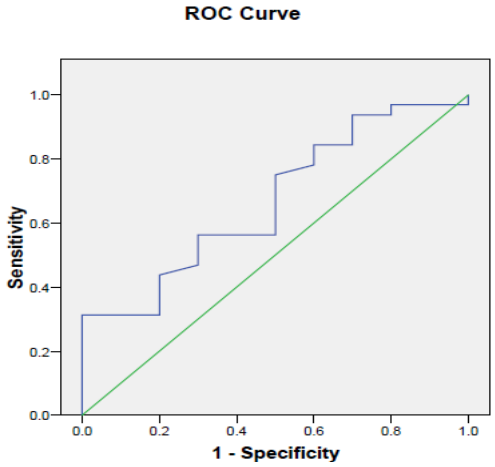

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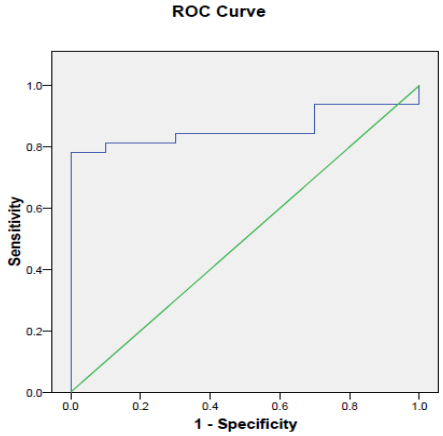

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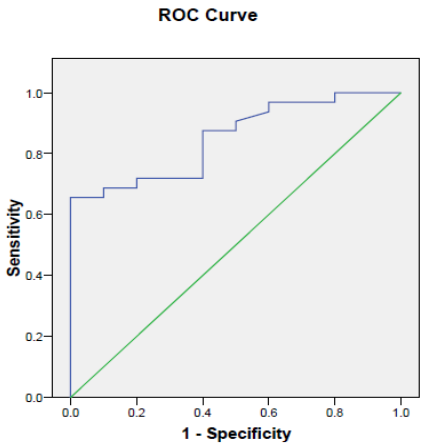

Figure 2: Receiver operating characteristic curve (ROC) of angiogenic markers in pretreated patients with breast cancer. (A) Basic fibroblast growth factor serum levels. (B) Platelet- derived growth factor. (C) Vascular endothelial growth factor.

Table 2: Angiogenic markers accuracy, cutoff point serum level, sensitivity and Specificity in breast cancer patients pretreated.

	Accuracy	Cut off point (pg /ml)	Specificity %	Specificity %	Asymptotic 95% Confidence Interval
bFGF	0.669	50.47	31.3 %	100 %	0.483- 0.854
PDGF-BB	0.859	1878.5	78.1 %	100 %	0.748- 0.971
VEGF	0.852	19.58	65 %	100 %	0.734- 0.969

Table 3: Correlation between Angiogenic marker serum levels in pretreated patients with breast cancer.

Angiogenesis Markers	Person Correlation	bFGF	PDGF-BB	VEGF
bFGF	Correlation Coefficient (r)	1	0.687**	0.696**
	Sig. (2-tailed) *(P)*		0.000	0.000
PDGF-BB	correlation Correlation (r)	0.696**	0.643**	1
	Sig. (2-tailed) *(P)*	0.000	0.000	
VEGF	correlation Correlation (r)	0.687**	1	0.643**
	Sig. (2-tailed) *(P)*	0.000		0.000

**. Correlation is significant at the 0.01 level (2-tailed).
r = 0.5 – 0.6 (weak positive correlation)
r = 0.7 (good positive correlation
r = 0.8 – 0.9 (strong positive correlation)

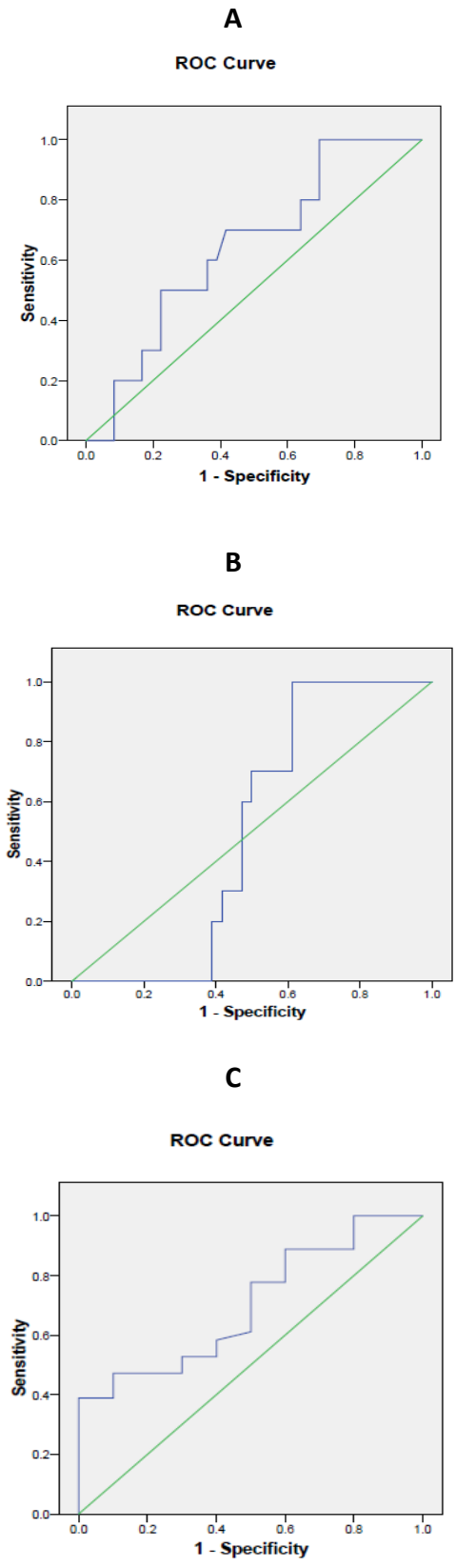

Figure 3: Receiver operating characteristic (ROC) curve of angiogenic markers in under-treatment breast cancer patients. (A) Basic fibroblast growth factor serum levels. (B) Platelet- derived growth factor. (C) Vascular endothelial growth factor.

Table 4: Angiogenic markers accuracy, cutoff point serum level, sensitivity and Specificity in breast cancer patients under-treatment.

Angiogenic Markers	Accuracy	Cut off point (pg/ml)	Sensitivity %	Specificity %	Asymptotic 95% Confidence Interval
bFGF	0.643	16.37	100 %	69.4 %	0.464 – 0.822
PDGF-BB	0.506	536.14	100 %	61.1%	0.348 – 0.663
VEGF	0.701	20.01	38.9 %	100 %	0.531 – 0.871

Table 5: Correlation between Angiogenic Markers in under treatment patients with breast cancer

Angiogenic Markers	Pearson correlation	bFGF	PDGF-BB	VEGF
bFGF	Correlation Coefficient *(r)*	1.000	0.687**	0.696**
	Sig. (2-tailed) *(P)*		0.000	0.000
PDGF-BB	Correlation Coefficient *(r)*	0.567**	1.000	0.643**
	Sig. (2-tailed) *(P)*	0.000		0.000
VEGF	Correlation Coefficient *(r)*	0.696**	0.643**	1.000
	Sig. (2-tailed) *(P)*	0.000	0.000	

**. Correlation is significant at the 0.01 level (2-tailed).

r = 0.5 – 0.6 (weak positive correlation)
r = 0.7 (good positive correlation)
r = 0.8 – 0.9 (strong positive correlation

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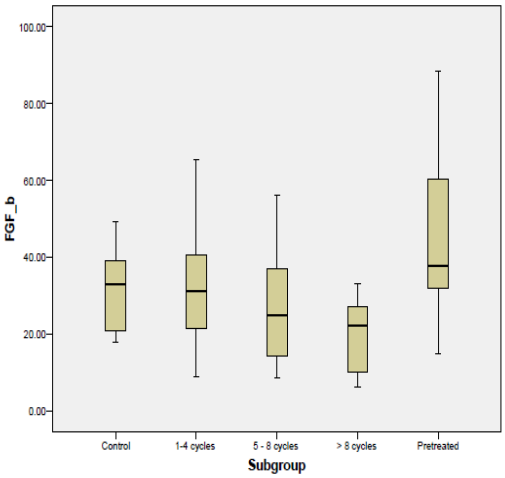

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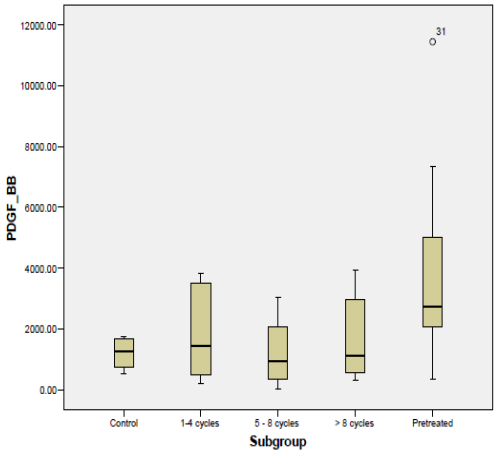

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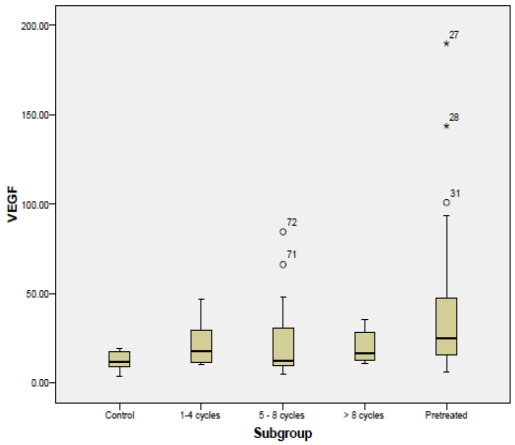


Figure 4: Angiogenic serum levels in studded groups and subgroups. Box plots of (a) basic fibroblast growth factor (bFGF). (B) platelet derived growth factor(PDGF-BB) (C) vascular endothelial growth factor (VEGF). The lower boundary of the box is the 25th percentile and the upper boundary is the 75th percentile. The bold line inside the box represents the median. * Cases with values more than 1.5 box lengths from the upper or lower edge of the box (extreme values). The largest and smallest observed values that are not extreme values are also shown

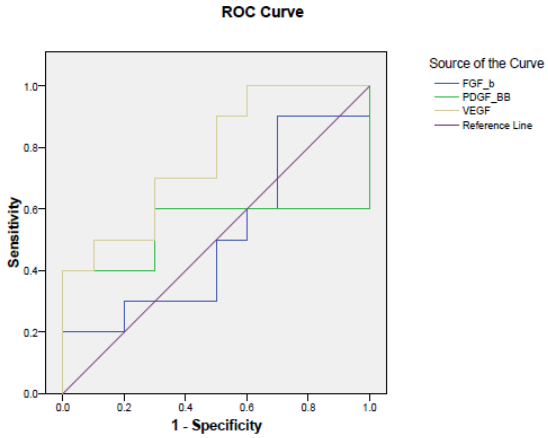
Table 6: Angiogenic Markers serum levels in studied groups and subgroups.

Markers	Groups				
	Controls (N: 10)	Pre- treatment (N: 32)	Under treatment (N: 36)		
			1-4 cycles (N=10)	5-8 Cycles (N=18)	>8cycles (N= 8)
bFGF	32.85 (17.76-49.27)	37.79 (14.99-88.32)	31.08 (9.02-65.350)	24.90 (8.52-56.02)	22.06 (6.18-33.05)
PDGF-BB	1263.19 (537.01-1762.15)	1145.13 (36.31-3995.28)	1428.38 (204.94-3828.37)	948.56 (36.31-3028.0)	1108.90 (313.25-3945.28)
VEGF	11.55 (3.28-19.33)	16.76 (4.68-84.42)	17.68 (9.97-46.61)	12.29 (4068-84.42)	16.72 (11.04-35.13)

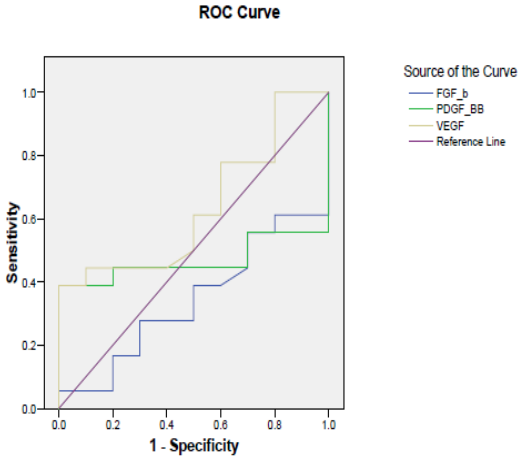
	bFGF	PDGF-BB	VEGF
P4	0.945	0.762	0.041*
P5	0.172	0.774	O.240
P6	0.033*	1.000	0.062
P7	0.079	0.025	0.128
P8	0.001*	0.000**	0.015**
P9	0.000**	0.028*	0.112

P4: control versus 1-4 cycles subgroup
P5: control versus 5-8 cycles subgroup
P6: control versus >8 cycles subgroup
P7: pretreatment versus 1-4cycles subgroup
P8; pretreatment versus 5-8 cycles subgroup
P9; pretreatment versus > 8 cycles subgroup
*Significant (p< o.o5)
**Highly significant (p<0.001)

A



B



C

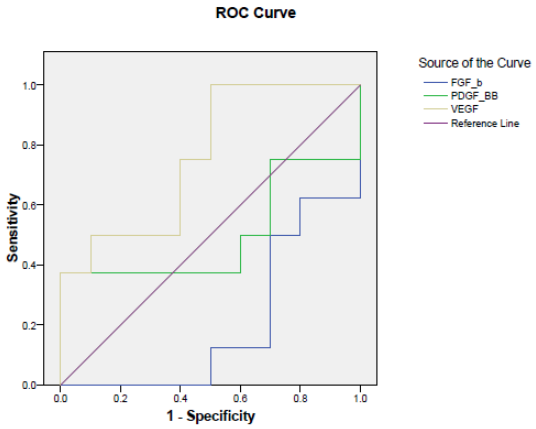


Figure 5: Receiver operating characteristic (ROC) curve of angiogenic markers in breast cancer patients under- treatment subgroups. (A) Subgroup received (1-4) cycle. (B) Subgroup received (1-4) cycles. (C) Subgroup received > 8 cycle.

Table 7: Angiogenic markers accuracy, cutoff point serum level, sensitivity and Specificity in breast cancer patients under-treatment subgroups.

Subgroups	Factors	Accuracy	Cut off point (pg/ml)	Sensitivity %	Specificity %	Asymptotic 95% Confidence Interval
1-4 cycles	**b FGF**	0.510	21.197	90 %	70 %	0.245-0.775
	PDGF-BB	0.540	1879.75	40 %	100 %	0.251-0.829
	VEGF	0.770	20.70	40 %	100 %	0.563-0.977
5-8 Cycles	bFGF	0.342	52.64	%5.6 %	100 %	0.140-0.543
	PDGF-BB	0.467	1836.29	38.9 %	100 %	0.247-0.686
	VEGF	0.636	20.01	38.9 %	100 %	0.426-0.846
> 8 cycles	bFGF	0.200	50.27	0 %	100 %	0.007-0.407
	PDGF-BB	0.500	1955.79	37.5 %	100 %	0.194-0.806
	VEGF	0.763	10.81	100 %	50 %	0.0537-0.988

Table 8: Angiogenesis markers serum levels in under treatment therapy related subgroups.

Factors	Cases median: pg/ml (rang)			Significance P- value		
	1-4 cycles (N:10)	5-8 cycles (N: 18)	➢ 8 cycles (N:8)	P_{10}	P_{11}	P_{12}
b FGF	31.08 (9.02-65035)	24. 90 (8052-56.02)	22.06 (6.18-33.05)	0.280	0.056	0.266
PDGF-BB	1428.38 (204.94-3828.37)	948.56 (36.31-3028.00)	1108 (313.25-3945.28)	0.415	0.965	0.470
VEGF	**17.68 (9.96-46.61)**	**12.29 (4.68-84.42)**	**16.76 (4.68-84.42)**	**0.502**	**0.965**	**0.374**

P_{10}: 1-4 cycles versus 5-8 cycles subgroups

P11: 1-4 cycles versus > 8 cycles subgroups

P12: 5-8 cycles versus > 8 cycles subgroups

DISCUSSION

Angiogenesis in normal and pathological conditions is multi-step process governed by positive and negative endogenous regulators. Many factors are involved in different step of angiogenesis, such as endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) or platelet-derived growth factor (PDGF-BB) [20]. When angiogenic balance shift taken place, the up-regulation of several pro-angiogenic factors take place. Angiogenesis is an important and critical process for tumor growth and progression [48].

High levels of angiogenic markers suggested having a biological aggressive disease with high risk of recurrent and low benefit from conventional adjuvant therapy [55]. There is need for identifying a new predictive or prognostic marker that could prove useful in the stratification of patients in the direction of correct diagnosis and of more individualized treatment strategies.

To the best of our knowledge, there are no published studies about serum level of some angiogenic markers assisted by Bio-Plex Pro assays as promising suggested rapid and sensitive panel tool as predictors for diagnosis and/or predictors for prognosis and recurrent of breast cancer after breast-sparing surgery. Also, to define subgroups of patients fitting into different treatment to improve clinical outcome,

We performed a pretreated adjuvant therapy-control case study to verify the first objective, the potential of VEGF, bFGF and PDGF-BB serum levels panel tools as diagnostic markers in breast cancer patients or recurrent of tumor growth and tumor residual after surgery treatment. In the present study comparative statistic analysis revealed significant elevated serum level of VEGF and PDGF-BB and non-significant elevated serum level of bFGF. Also, ROC analysis identified the cutoff point serum level for each marker that predict the preclinical or clinical tumor life span that recurrent cancer cell produce angiogenic factors, and identify when those factors become determinant in stimulating and supporting tumor growth. All of those subjects with detected serum level equal to or more than cut of point, for each of our studied markers, were detected only in pretreated therapy patients. They represent (10/32) 32% for bFGF, 24/32 (75%) for PDGF-BB and (21/32) 65% for

VEGF. PDGF-BB and VEGF identified high accuracy, good sensitivity and high specificity, while bFGF showed good accuracy, high specificity, but in term of sensitivity highlight low value in those patients.

In addition, relationship statistic analysis identified highly significant positive correlation between those angiogenic markers in pretreated therapy patient group. We suggested marked residual tumor tissue after surgery or recurrent of tumor growth. Our suggestion is confirmed by other studies reported that up-regulation of angiogenic markers is correlated with malignant tumors and contribute to tumor growth [56, 57].

Also, many studies agree with our suggestion revealed that the aim of the primary surgery is cytoreduction to microscopic disease [58], which has an impact on survival in cancer [59, 60] and a positive association between serum angiogenic markers and the present of residual tumor after surgery [59,61]. The significant elevated pro-angiogenic markers were identified in those patients whom complete resection was not successfully possible [20].

In our work, under treatment - pretreated therapy patient case study were carried on to evaluate the promise role of angiogenic markers suggested panel tools as predictors for the efficacy of choosing adjuvant therapy and suggested prognoses and management of breast cancer, as well as, to assess the patient groups in terms of the different nature and ferocity of the disease and reaction to the drug user. Our results detected significant serum level reduction with in each of our studied markers in under treatment compared with pretreated patients groups.

We suggested that, these results did not clearly highlight the efficacy of conventional adjuvant therapy regiment used, where those patient received, for suggested many reasons. The prognostic factors for breast cancer, that considered the principle for diagnosis and for choosing the adjuvant therapy regimen, has been reported to be not fully predicting individual clinical outcome mostly among stage II and III patients [48]. Also, our results showed elevated non significant serum level of those markers in under-treatment patients compared to base line measurement level. Furthermore, highly significant positive correlations between VEGF, bFGF and PDGF-BB have been identified in our work. For all of those reasons, we suggested that, breast cancer patients with elevated angiogenic markers serum levels assisted after primary surgery treatment and before receiving adjuvant therapy, have not the same clinical prognosis. Therefore, assessment serum levels of studied angiogenic markers in under-treatment therapy related subgroups- pretreated patients case were statistically analyzed and reported. These results identified and confirmed our suggestion. Despite the fact that these patients have the same diagnosis and went a primary surgery treatment, but do not have the same reaction toward the medication received. It clearly appear that, adjuvant therapy 1-4 cycles were not effective, while 5-8 cycles considered to be effective, on the other hand, those patients needed more than 8 cycles identified a considerable resistant against this regimen.

In addition, under-treatment therapy related subgroups-control case have been statistically studied and analyzed in order to provide additional information about therapy regimen efficacy for prognoses and optimal management of breast cancer prevalence using our suggested angiogenic markers panel tool. A considerable valuable result has been reported. Those patients treated with 1-4 cycle therapy revealed elevated non-significant value for each of bFGF and PDGF-BB, while VEGF serum level identified significant higher level. Those patients received 5-8 cycles showed no significant reduction value between two groups. Treated subgroup patients received more than eight cycles identified elevated non-significant VEGF and PDGF-BB values, while bFGF serum level identified significant reduction level. Angiogenic markers serum values discriminate between patients and healthy subjects, where serum levels equal to or more than cut off point is identified only in breast cancer patients.

Our results confirmed our hypothesis, where those breast cancer patients with elevated serum level of angiogenic markers have a biological aggressive case. Those patients identified low benefit from conventional adjuvant therapy which give a great chance for recurrent of tumor growth and bad prognosis. *Tange et al. (2007)* reported that 27 patients, with elevated VEGF serum level determined before adjuvant therapy, showed a

significant decrease with therapy-responsive and stable disease, while those patients starting with significant higher level VEGF before conventional adjuvant therapy were correlated with response to adjuvant therapy at the end of 5-6 cycles [62]. At the time of progression, VEGF, bFGF and PDGF-BB are significantly increased. After two months of therapy, FGF values found to be highly significantly decreased and VEGF and PDGF-BB were significantly trend toward lower values [63]. *Tang et al.(2007)* study explaining our result and confirmed our suggestion, those patients treated with 1-4 cycles not response to conventional adjuvant therapy treatment, while intensive treatment could be indicator of the controlled disease status, otherwise, those patients needed more than 8 cycles represent some resistance and bad prognosis. On the other hand, some studies identified the benefit of anti-angiogenic therapy within those patients with elevated VEGF serum level. *Calleri et al. (2009)* identified by experimental studies that low dose of cytoxan (targeting anti-VEGF) causes a decreased in microvessel density, which leading to induction of hypoxia and induction of devising endothelial cell apoptosis [64]. Also, those patients with highly elevated angiogenic markers have not the same response toward the same conventional chosen therapy. Our result confirmed that our suggested panel tool of angiogenic markers could identify different subgroups of breast cancer patients with different benefits.

In order to elucidate the potential relevance of our suggested angiogenic markers panel tool, the impact of tumor biology regarding their biological variation in circulation were discussed and explained. Angiogenesis markers regulate pathways in embryonal development, inducing mesenchymal epithelial signaling and multiple organ system [65, 66]. Those markers signaling regulate cell proliferation, differentiation, and survival, as well as angiogenesis and wound healing [67]. Pro-angiogenic factors up-regulation takes place when angiogenic balance shift taken place [2- 6]. In different situations, such as tumor angiogenesis, those markers act as negative regulators of proliferation and positive regulators of differentiation leading to tumor growth [68-71]. Also, angiogenesis markers play a pivotal role in tumor progression and metastasis [72, 73].

In tumor situation, there is a complex interplay between cancer cells, endothelial cells (ECs) and other cells, where angiogenic markers have a crucial role in this interaction [55]. In our study, serum level of VEGF, EGF and PDGF correlated with suggested residual tumor tissue left behind the primary surgery treatment. Also, under treatment therapy related subgroups identified breast cancer patient with bad prognosis. Even those patients received conventional adjuvant therapy 5-8 cycles suggested to be in high risk with silent cases, where angiogenic markers revealed elevated serum level without reaching back the baseline measurement of control subjects.

VEGF is the most investigated and efficient angiogenic marker characterized twenty years ago [74]. Many studies identified the great role of VEGF in the angiogenic response essential for ductal tumors, mostly breast cancer [25-27, 75]. Other studies confirmed VEGF serum level correlation with cancer recurrence [28, 29], and its relevant biological role in the progression of breast cancer [76, 77]. Some studies identified VEGF serum level prognostic value in heterogenous patient's population regardless type of adjuvant therapy administered [78].

In addition to VEGF, a lot of evidence has been accumulated in last 10 years that supports the contribution of PDGF-bb in developing angiogenesis in both normal and tumoral conditions [55, 79, 80,].

A lot of evidences support the implication of PDGF-BB in tumor growth and development of specific lesions as a result of inflammatory diseases and atherosclerosis [81, 82]. Previous studies have demonstrated that the expression of PDGF-BB appeared to have an influence on clinical outcome and overall survival in cancer patients [83, 84].

As well as a relation to stage and residual tumor, serum PDGF-BB reported to have a prognostic value as well as clinicopathological parameters [84, 85, 86]. bFGF also reported in many studies as a potent pro-angiogenic factor with important role in breast cancer [87- 89]. bFGF serum level have been reported in other studies as a useful marker for early detection of sporadic cancer, within screening programs and in monitoring members of high risk breast cancer families [90]. On the other hand, some studies in contrast with our results

reported no direct interaction between FGF expression and angiogenesis in breast cancer [91, 92].

Tumor angiogenesis as a result of up regulation of angiogenesis marker is performed through ways of mutual interactions [93]. Those angiogenic markers are considered the most potent endothelial cell mitogen, also a regulator of vascular permeability and a powerful prognostic tool [48, 78, 94-97]. In addition, they promote tumor associated angiogenesis by autocrine and/or paracrine mechanisms, as well as, migration during tumor invasion [98].

Cross talk results between VEGF, bFGF and PDGF-BB in our work revealed the following: at pretreatment therapy patients, elevated non-significant value for bFGF and significant elevated valued for each of VEGF and PDGF-BB, under treatment therapy patient group received 1-4 cycles showed elevated non significant level for each of PDGF-BB and bFGF and significant elevated value for VEGF; on the other hand, those patients received more than 8 cycles identified with elevated non-significant VEGF serum level in contrast with significant reduction of bFGF serum value. In addition to those comparative results, each of angiogenic marker serum level clearly discriminates between healthy women and breast cancer patients. An important result must be taken in consideration that neither pretreated patients, nor under treatment patients' angiogenic marker serum level reached back the baseline serum level of control subjects.

Mutual interaction between VEGF, bFGF and PDGF-BB reported in many studies explained and clarified our results and supported our suggestion. There is evidence of cross-take between those markers [99-102]. VEGF induces angiogenesis by binding to its specific receptors, VEGFR and VEGFR2 [103]. PDGF induces angiogenesis by binding to its specific receptors PDGFRα and up-regulation VEGF production and modulating the proliferation and recruitment of perivascular cells [81,104]. On the other hand, VEGF enhances endothelial PDGF-β expression, whereas FGF-2 enhances perivascular PDGFRβ expression [105]. Increased PDGFRβ activity is associated with expression of VEGF-A and VEGFR-2 and resulted in increased vessel formation [106].

A significant relationship exists between VEGF and FGF during angiogenesis, where VEGF appears earlier during angiogenesis process than doe's bFGF [107]. Induction of bFGF induced angiogenesis is partially dependent on the activation of VEGF and the presence of endogenous VEGF and VEGF-C [108]. Also, bFGF has been reported to have indirect effect on VEGF pathways in breast cancer [90].

bFGF was found to activate hypoxia-induced VEGF release through augmentation of the phosphoinositide 3-kinase pathways and upregulation of neuroplipin-1 (NPRR-1) as well as hypoxia-inducible factor-1α expression [109].

It was suggested that bFGF acts as a sensitizer of endothelial cells to respond to PDGF-β signaling, and this signaling feeds back to peri-vascular cells to enhance their response to bFGF stimulation. These events lead to the development of new vessels, accelerated tumor growth and metastasis [110]. The link between PDGF-BB and tumor-associated angiogenesis is supported by expression of tumor cells, and over expression was found to be correlated with MVD and poor survival in a large variety of human cancer [111-113]. Although, both PDGF-bb and bFGF expression is induced by HIF-1 under hypoxia conditions [91], PDGF-BB reported higher in MVD tumor, whereas bFGF inversely reported to MVD [91].

Overall, our obtained results confirmed that elevated angiogenesis markers suggested as biological aggressive disease with high risk of recurrence and low benefit from conventional adjuvant therapy. Those angiogenic markers could be identified as new predictive or prognostic markers that could be useful in stratification of patients in the direction of correct diagnosis and of more individualized treatment strategies. We recently published that VEGF considered as a risk marker that can indicate that colorectal cancer is more likely to occur in silent cases, whereas tumor markers carcinoembrionic antigen (CEA) and carsinogenic antigen(CA19.9) can indicate the presence of cancer [114]. Zhanget et al. (2006) explained the role of adjuvant chemotherapy to kill micro metastasis that have high fraction, so sensitivity to cytotoxic therapy [115]. Advantage of targeted therapy was discussed by Watanabe 2008, who reported that targeted therapies "target cancer cell" minimize damage to

noncancerous cells and increase neovascular damage as another possible mechanism that improves angiogenic markers-targeted agents combining with conventional cytotoxic therapy [116]. Carrato 2008 also reported that anti-angiogenesis therapy inhibits angiogenesis marker receptor signaling in tumor cells may potentiate the effects of cytotoxic drugs by inhibiting anti-apoptotic regulators or some other survival mechanisms in tumor cells [117]

In many pathological conditions, mostly in proliferative lesions, therapeutic inhibition of only one angiogenic factor not effective for inhibiting angiogenesis, which reported to result in a slight decrease in MVD. In contrast, combined inhibition of VEGF, PDGF-BB and bFGF results in a marked inhibition of angiogenesis and uncompleted blood vessel maturation [81]. Timke et. al. 2008 found that anti-angiogenesis therapy are inhibitors for tyrosin kinase effect on ECs, enhanced apoptosis, reduced cell proliferation, reduced migration of ECs and stopped tube formation [118].

CONCLUSION

Overall our results confirmed that suggested panel tool of VEGF, PDGF-BB and bFGF serum levels assessed by Bio-Plex ® Pro Assays for Angiogenesis Factors Quantification is useful for the early detection of breast cancer within screening programs, suitable for prospective studies for breast cancer diagnosis and /or recurrence of tumor, in prognosis and monitoring members of high- risk breast cancer families. Also, our suggested panel angiogenesis marker tool is useful for identifying subgroups of patients fitting into different treatment to improve clinical outcome.

Treatment strategy should be taken in consideration to avoid unnecessary conventional adjuvant therapy in high-risk patients with high serum level of angiogenesis markers, in addition to use anti- angiogenesis markers both as a target for therapy and potentially predictive markers for novel therapeutic strategies. That suggested panel tool gives an evaluating angiogenesis marker set in serum that therefore may play important role in selecting breast cancer patients for combination therapy consisting individual chosen anti-angiogenic drugs.

Understanding of the codependence of chronic inflammation and angiogenesis have been considered as a potential benefit of targeting angiogenesis and chronic inflammation through identification of the role of molecular changes as predictor causes of breast cancer advancement and metastatic development. Accordingly approaches for newly diagnostic markers

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