Diagnostic and prognostic value of serum nitric oxide, tumor necrosis factor-α, basic fibroblast growth factor and copper as angiogenic markers in premenopausal breast cancer patients: a case-control study

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ABSTRACT

Many studies demonstrate that increased microvessel density (MVD) surrounding primary tumour is associated with decreased overall survival in patients with breast cancer. This study compares the diagnostic and prognostic values of the angiogenic serum factors nitric oxide (NO), tumour necrosis factor-α (TNFα), basic fibroblast growth factor (bFGF) and copper with those of serum CA15-3 as the standard tumour marker in breast cancer patients. Microvessel density was estimated in CD31-immunostained sections from breast cancer patients. Before surgery, NO, TNFα, bFGF, copper and CA 15-3 were measured in serum samples from 30 premenopausal breast cancer patients in comparison with 15 healthy controls. The diagnostic values of the assayed parameters were compared using receiver operating characteristic (ROC) curve analysis. Univariate survival analysis of patients was assessed using the Kaplan-Meier method. Breast cancer tissues showed higher MVD than did normal breast tissues adjacent to the tumour (P=0.008). Before surgery, tumour MVD correlated significantly with serum NO, TNFα, bFGF and copper (r=0.458, P=.011; r=0.379, P=.039; r=0.513, P=.004 and r=0.613, P=0.000, respectively). Serum NO, TNFα, bFGF, copper and CA 15-3 levels in patients were significantly elevated compared with controls (P=0.011, P=0.004, P=0.039, P=0.000 and P=0.001, respectively). Kaplan-Meier analysis revealed that patients with elevated serum TNFα, CA 15-3 and copper (P=0.035, P=0.040, P=0.0339, respectively) had an overall survival significantly shorter than those who had lower levels of these parameters. These data suggest that serum TNFα, CA 15-3 and copper are useful predictive markers for overall survival in premenopausal breast cancer patients.

KEY WORDS: Angiogenesis.
Breast cancer.
Diagnosis.
Survival.

Introduction

Breast cancer in women is commonly characterised by poor prognosis, with increasing rates of incidence and death, with mortality frequently the result of tumour metastasis.[1] Growth, progression and metastasis of breast cancer are angiogenesis-dependent processes,[2] which emphasises the need to develop new measures and strategies for its prevention.[1]

Angiogenesis is the formation of new capillaries from pre-existing vascular structures, and involves at least two distinct phases. The initial phase is characterised by activation of quiescent endothelial cells to a proliferative and migratory phenotype. Among the mediators of this transition are inflammatory cytokines such as tumour necrosis factor-α (TNFα), interleukin-8 (IL-8) and possibly soluble adhesion molecules such as E-selectin. Growth factors such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) are also potent stimulators of endothelial cell proliferation and migration.

The later phase involves the redifferentiation of the migrating and proliferating endothelial cells into vascular tubes. This necessitates a reversal of endothelial cell phenotype to a more quiescent state typical of mature vascular structures. It has been suggested that nitric oxide (NO), produced in response to bFGF, may act as a molecular 'switch' counteracting the growth-promoting actions of this angiogenic mediator, terminating the proliferative actions of angiogenic growth factors and promoting endothelial cell differentiation into vascular tubes.[3]

Nitric oxide, a reactive nitrogen species, is a mediator of angiogenesis. It is an endothelial survival factor, inhibiting apoptosis and enhancing endothelial cell proliferation, perhaps in part by increasing the expression of VEGF. It may also suppress the production of angiostatin, an endogenous antagonist of angiogenesis.[4]

Tumour necrosis factor-α is a 17 kDa polypeptide.[5] Endogenous TNFα produced in the tumour microenvironment enhances tumour development and spread. It may induce other pro-angiogenic factors such as

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Table 1. Relationship between tumour MVD and clinicopathological parameters in breast cancer patients.

		Axillary node status			Tumour size (cm)			Clinical stage	
		0	1–5	>5	<2	2–5	>5	II	III
		n=6	*n*=16	*n*=8	*n*=6	*n*=17	*n*=7	*n*=19	*n*=11
MVD	mean	81.69	98.83	126.25	87.50	98.41	124.53	90.89	122.09
	SE	4.64	12.21	11.84	6.40	6.40	13.55	4.89	9.04
	P value	0.04*			0.011*			0.004*	

SE: standard error; ER: oestrogen receptor; PR: progestrone receptor.

*differences considered statistically significant at $P<0.05$.

VEGF, bFGF, IL-8 and matrix metalloproteinase (MMPs) that promote cancer progression.[6]

Basic fibroblast growth factor, an 18 kDa protein, is an angiogenic protein both *in vivo* and *in vitro*.[7] It is a regulator of cell proliferation, differentiation and function and has an important role in normal development and maintenance of tissues and in wound healing.[8] It has been identified in a wide variety of malignant tissues and cells.[9]

Copper, an essential trace element present in the diet, is an important co-factor of angiogenesis. It stimulates proliferation and migration of human endothelial cells.[10] The concentration of copper controls angiogenesis, turning it 'on' when sufficient or 'off' when deficient. Copper acts as an obligatory co-factor for some angiogenic factors including TNFα and bFGF.[11] Addition of copper to the rabbit cornea is sufficient to induce new vessel formation.[12,13] Furthermore, copper-deficient rabbits are unable to mount an angiogenic response.[12,14]

Angiogenesis, which is induced by tumour epithelial angiogenic factors, can be quantified by measuring microvessels.[15] Several endothelial markers have been identified for assessing the degree of angiogenesis in endothelial cells including platelet-endothelial cell adhesion molecule (PECAM-1 [CD31]),[16] CD34[17] and factor VIII (FVIII).[18] CD31 is a cell adhesion molecule on platelet and endothelial cells. It has the advantage over FVIII of being present also on immature blood vessels. Consequently, counts of microvessels as a marker of angiogenesis using CD31 are 30% higher than those using FVIII.[16]

The angiogenic activity of a tumour is widely reported to be related to the proliferative activity of tumour cells. Moreover, angiogenic growth factor serum levels have been found to be higher in breast cancer patients than in healthy women.[19]

The clinical relevance of angiogenesis in cancer is illustrated by research data that correlate tumour expression of angiogenic growth factors with prognosis.[20] Most, but not all, studies have demonstrated that increased microvessel density (MVD) surrounding primary tumours is associated with decreased disease-free survival and overall survival in patients with lymph node-negative and lymph node-positive breast cancer.[21] In patients with renal cell carcinoma, for instance, increased bFGF expression correlates with reduced survival.[22] Additionally, expression of VEGF in breast cancer correlates with a decrease in relapse-free survival.[23]

CA 15-3 is a circulating breast cancer-associated antigen and is the most widely used serum marker for the disease.[24] However, CA 15-3 concentrations are increased in approximately 10% of patients with stage I disease, 20% with stage II disease, 40% with stage III disease and 75% with stage IV disease.[25] As well as lacking sensitivity for early disease, CA 15-3 also lacks specificity for breast cancer. Increased concentrations of the marker can be found in a small proportion of apparently healthy individuals (approximately 5%), in patients with certain benign diseases, especially liver disease, and in patients with other types of advanced adenocarcinoma.[26]

As TNFα, bFGF, copper and NO were reported to be activators of tumour angiogenesis, the aim of this study is to compare the diagnostic and prognostic values of serum TNFα, bFGF, copper and NO with those of serum CA 15-3 as the most commonly used breast cancer tumour marker in premenopausal females.

Materials and methods

Forty-five subjects were included in this study. They were divided into two groups: Group I included 30 premenopausal patients with invasive ductal carcinoma of clinical stages II and III[27] (recently detected, not receiving surgery or chemotherapy; mean age: 33.15±1.33 years). Patients were recruited from the Department of Surgery and the Department of Cancer Management & Research of the Medical Research Institute, Alexandria University. Group II included 15 healthy premenopausal female volunteers of comparable age (32.80±2.05), menstrual cycle and socioeconomic status.

A full history was recorded and each patient underwent a thorough clinical examination. This included laboratory investigations, mammography of breast and

Table 2. Correlations between studied serum parameters in breast cancer patients before surgery.

	NO (μmol/L)	TNFα (pg/mL)	bFGF(pg/mL)
NO (μmol/L)			
TNFα (pg/mL)	*r*=0.373*		
bFGF (pg/mL)	*r*=0.225	*r*=0.384*	
Copper (μg/dL)	*r*=0.291	*r*=0.447*	*r*=0.504*

r: Spearman correlation.

*correlation considered significant at $P<0.05$

	Tumour grade			ER				PR			
	I	II	III	–ve	+	++	+++	–ve	+	++	+++
	n=5	*n*=18	*n*=7	*n*=10	*n*=8	*n*=5	*n*=7	*n*=9	*n*=9	*n*=5	*n*=7
	83.60	99	117.14	97.40	108.38	118.8	90.71	99.67	110.67	97.00	98.86
	4.93	7.31	10.92	39.39	12.04	13.17	3.97	10.40	12.11	12.21	6.41
	0.036*			0.282				0.873			

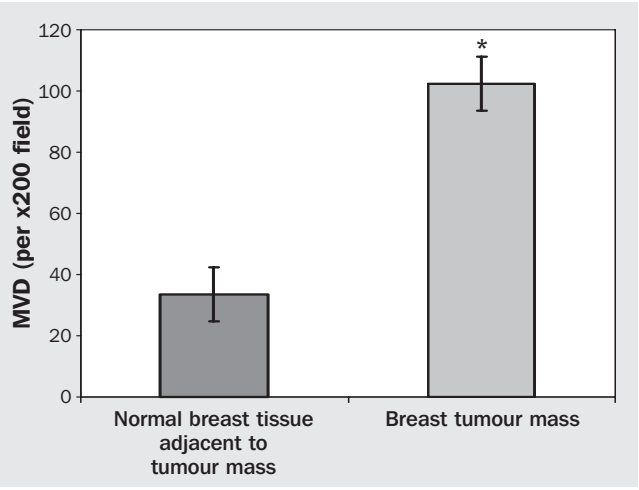

Fig. 1. MVD difference between normal breast tissue adjacent to tumour mass and the breast tumour mass.

ultrasonography of abdomen and liver, radiological investigations including X-ray of chest, computed tomography (CT) scan and bone scan when needed, and fine-needle aspiration cytology (FNAC) of breast mass to establish the pathological diagnosis in the patients.

All 30 breast cancer patients underwent modified radical mastectomy,[28] then received adjuvant combination chemotherapy (5-fluorouracil, adriamycin and cyclophosphamide [FAC])[29] for six cycles. The patients were re-evaluated after three and six cycles of chemotherapy to estimate clinical response. They were followed up for 36 months for assessment of overall survival.

Blood samples were collected from the controls and patients before surgery (BS). Immediately after withdrawing, blood samples were allowed to coagulate and then centrifuged for 20 mins at 3500 x*g* at 4°C. The separated serum samples were aliquoted and stored frozen at –80°C. After thawing, each serum sample was assayed once.

Serum NO levels were determined as the concentration of nitrite plus nitrate. Nitrate was reduced to nitrite by nitrate reductase and the concentration of nitrite was measured spectrophotometrically at 430 nm using the Griess reaction.[30]

Serum TNFα and CA 15-3 levels were assayed using commercially available IRMA kits,[31,32] while bFGF levels were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit.[33] The kits were obtained in ready-to-use form from Biosource International, USA.

Frozen serum aliquots were delivered to the Department of Nutrition at the High Institute of Public Health, University of Alexandria, where the serum copper levels were measured using atomic absorption photometry.[34]

In the patient group, formalin-fixed, paraffin wax-embedded tissue sections from each case were examined to evaluate tumour grade, stage, size and axillary lymph node status, then stained immunohistochemically to evaluate microvessel count, oestrogen (ER) and progesterone (PR) receptor status. The results were correlated with serum NO, bFGF, TNFα and copper.

Microvessel count: assessment of angiogenesis

Paraffin blocks of tumour tissue were cut (5 μm) and attached to charged glass slides. Immunostaining was performed using the Envision Dual Link System-HRP (Dako) and monoclonal mouse anti-human CD31 (clone jc70A; Dako). Briefly, sections were dewaxed in two changes of xylene, hydrated to buffer and equilibrated to room temperature. Excess buffer was removed before a dual endogenous

Table 3. Area under the ROC curves for serum nitric oxide, bFGF, TNFα, copper and CA15-3 in breast cancer patients before surgery.

	Area under the curve (%)	*P* value*	Cut-off	Sensitivity (%)	Specificity (%)
NO (μmol/L)	100	0.000	10.9	100	100
bFGF (pg/ml)	94.7	0.000	13.5	83	69
TNFα (pg/mL)	80	0.001	4.90	77	73
CA15-3 (u/mL)	72.2	0.016	25	43	68
Copper (μg/dL)	70.2	0.028	75.75	80	60

*Area under the ROC curve considered significant at $P<0.05$.

Table 4. The relationships between serum NO, TNFα, bFGF, copper and CA 15-3 levels and clinicopathological parameters in breast cancer patients before surgery.

		n	Nitric oxide (Mean±SE)		TNFα (Mean±SE)		bFGF (Mean±SE)	
Axillary node status	0	6	26.74±4.76	*P*=0.973	6.77±1.73	*P*=0.02*	23.40±4.15	*P*=0.706
	1–5	16	24.56±1.61		13.81±8.45		18.86±1.30	
	>5	8	25.66±3.04		21.90±6.60		18.70±2.31	
Tumour size (cm)	< 2	6	22.61±1.67	*P*=0.757	5.13±0.80	*P*=0.005*	18.48±1.83	*P*=0.467
	2–5	17	25.67±2.01		10.52±3.07		19.56±1.97	
	>5	7	26.66±3.76		24.10±6.72		21.19±1.81	
Clinical stage	II	19	25.15±1.76	*P*=0.735	9.26±2.77	*P*=0.030*	19.48±1.70	*P*=0.307
	III	11	25.54±2.65		18.42±4.86		20.15±1.73	
Tumour grade	I	5	20.10±2.61	*P*=0.639	6.78±1.02	*P*=0.894	19.60±2.63	*P*=0.057
	II	18	24.52±1.82		12.54±3.13		19.02±1.27	
	III	7	30.99±2.77		16.97±7.70		21.61±3.96	
ER	–ve	10	23.73±2.33	*P*=0.622	15.78±4.23	*P*=0.127	18.85±1.77	*P*=0.238
	+	8	24.48±3.34		11.31±5.29		22.48±1.88	
	++	5	29.72±4.34		17.71±10.12		17.85±5.74	
	+++	7	25.29±2.32		5.95±0.85		19.17±1.47	
PR	–ve	9	21.91±1.76	*P*=0.205	13.08±4.4	*P*=0.776	17.29±1.92	*P*=0.369
	+	9	26.70±3.75		16.26±6.99		23.29±2.97	
	++	5	22.40±1.91		6.94±1.18		17.98±3.07	
	+++	7	29.89±2.29		11.38±3.73		19.52±1.07	

SE: standard error.

*considered significant at $P<0.05$.

enzyme block was applied for 5–10 min. Slides were placed in fresh buffer, removed and the excess was wiped off. The monoclonal antibody (CD31) was applied and incubated for 30 min. Sections were placed in fresh buffer and then goat-polyvalent antibody was applied to cover the specimen and incubated for 30 min. Slides were washed (x4) and placed in a buffer bath for 5 min. Sufficient substrate-chromogen solution (DAB) was added and incubated for 5–10 min. Slides were rinsed gently with distilled water and then counterstained in haematoxylin. Slides were rinsed in a bath of distilled water for 2–5 min, then dehydrated, cleared and mounted.

Assessment of neovascularisation was performed according to the method described by Weidner *et al*.[36,37] Briefly, the entire tumour area was scanned using a x10 objective to select areas of most intensive vascularisation. Three separate, non-overlapping fields were selected from these areas and all CD31-positive microvessels were counted in each field. Counts were performed using a x20 objective (field area: 0.74 mm^2). The microvessel count was scored by averaging the counts from three fields, and calculated per cm^2.

Statistical analysis

Statistical analysis was performed using the SPSS 11.5 software package. Non-parametric Spearman's test was used to investigate correlations between different parameters. The non-parametric Kruskal-Wallis test was used for relating studied parameters to tumour size, lymph node status, grade, PR and ER, whereas the Mann-Whitney U-test was used to relate studied parameters to clinical stage and for studying differences between the patient and control groups. Univariate survival analysis of the studied parameters was assessed using the Kaplan-Meier method. Statistical differences between survival curves were evaluated using the log-rank test. $P<0.05$ was regarded as significant.

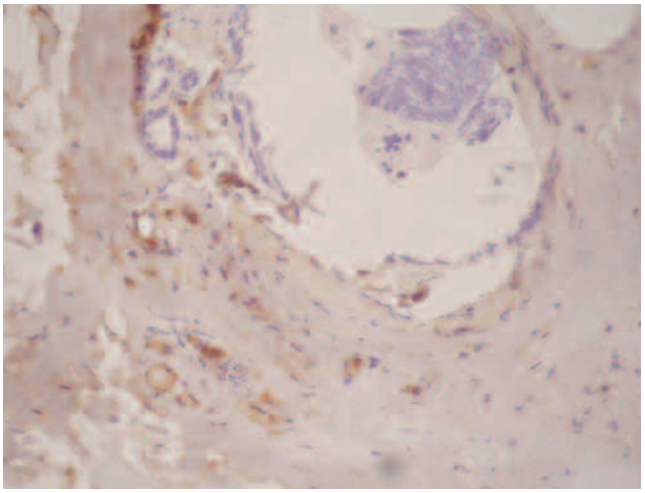

Fig. 2. Normal breast tissue showing brown-stained microvessels (IHC-CD31, original magnification x400).

	Copper (Mean±SE)		CA15.3 (Mean±SE)	
	97.33±19.91	P=0.078	20.63±3.0	P=0.013*
	102.59±8.95		28.56±2.28	
	134.50±9.36		37.80 ±5.58	
	85.33±7.01	P=0.016*	19.74±3.58	P=0.007*
	111.97±9.53		29.03±2.28	
	126.57±16.06		39.21±7.44	
	96.74±7.95	P=0.011*	23.16±1.77	P=0.03*
	133.05±10.39		37.65±5.21	
	97.20±15.15	P=0.30	30.48±9.02	P=0.857
	100.39±7.76		31.32±3.19	
	144.07±14.68		28.28±3.74	
	115.80±13.73	P=0.390	33.55±4.75	P=0.108
	118.81±12.68		26.44±4.39	
	113.8±23.97		38.5±6.58	
	89.14±5.14		23.38±3.32	
	103.06±15.22	P=0.882	41.05±5.39	P=0.130
	116.11±14.43		26.28±3.25	
	106.80±12.85		20.94±4.14	
	113.57±12.78		31.79±3.51	

Results

Microvessel count: tumour versus normal tissue

As shown in Figure 4, the microvessel count was 102.33±5.25/cm^2 (mean±SE) in all tumours and 36.25±0.228/cm^2 (mean±SE) in the normal adjacent tissue of the same patient. This difference in MVD was significant (P=0.008).

Relationships between tumour MVD and studied parameters

As shown in Table1, MVD was significantly higher in node-positive tumours than in node-negative tumours (P=0.04). Larger tumours showed higher MVD (P=0.011). The MVD was associated with the tumour clinical stage, with stage III tumours showing higher MVD than stage II tumours (P=0.004). The MVD was associated with the extent of tumour differentiation, with MVD from women with well-differentiated tumours (grade I) lower than that in women with higher tumour grades (grade II or III; P=0.036). On the other hand, MVD lacked association with ER or PR status.

Correlation between MVD and serum NO, TNFα bFGF and copper

Serum levels of NO, bFGF, TNFα and copper correlated significantly with MVD (r=0.458, P=0.011; r=0.513, P=0.004; r=0.379, P=0.039 and r=0.613, P=0.000, respectively) (Figs. 5–8).

Correlation between studied serum parameters

As shown in Table 2, in breast cancer patients before surgery there were significant correlations between the levels of serum TNFα and the levels of NO, bFGF and copper. Also, there was significant correlation between the level of serum bFGF and serum copper.

Mean±standard error for all studied parameters

In women with breast cancer, serum concentrations of NO (25.29±1.454 µmol/L), TNFα (12.61±2.59 pg/mL), bFGF (19.72±1.232 pg/mL), copper (110.05±7.00 µg/dL) and CA 15-3 (30.11±2.48 u/mL) were significantly higher than those in the control group (NO: 5.54±0.483 µmol/L; TNFα: 4.12±0.72 pg/mL; bFGF: 10.83±0.50 pg/mL; copper: 84.60±5.65 µg/dL and CA 15-3: 16.41±1.11 u/mL) (P=0.011, P=0.004, P=0.039, P=0.000 and P=0.001, respectively).

Receiver operating characteristic curve analysis

The ROC curves were constructed to compare the diagnostic value of biochemical parameters with CA 15-3 in such a way that the higher curve corresponds to a better diagnostic test (Fig. 9).

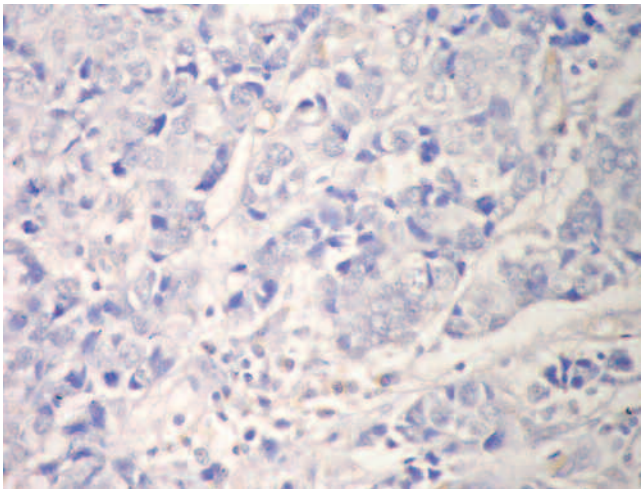

Fig. 3. Low microvessel count in a case of invasive ductal carcinoma grade II (IHC-CD31, original magnification x400).

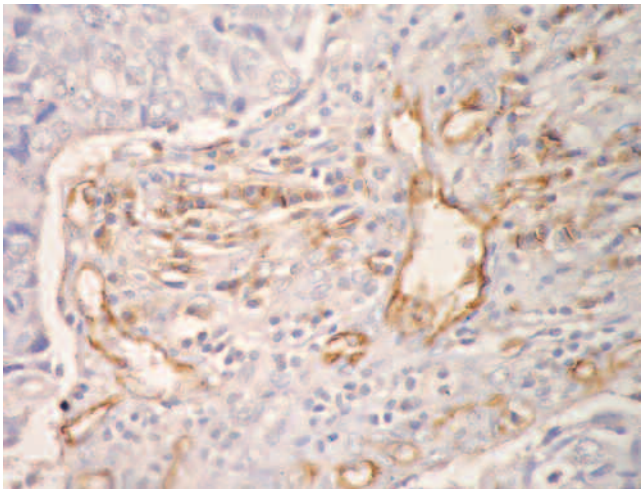

Fig. 4. Proliferating microvessels surrounding malignant ductal cells (IHC-CD31, original magnification x400).

Relationships between serum and clinicopathological parameters

As shown in Table 4, TNFα and CA 15-3 levels were significantly higher in node-positive tumours than in node-negative tumours (P=0.02 and P=0.013 respectively). Larger tumours showed significantly higher TNFα, copper and CA 15-3 concentrations compared with smaller tumours (P=0.005, P=0.016 and P=0.007, respectively). Levels of TNFα, copper and CA 15-3 correlated with clinical stage where patients with clinical stage III disease had significantly higher serum concentrations (P=0.03, P= 0.011 and P=0.03, respectively) than those with clinical stage II disease. In contrast, serum NO and bFGF showed no difference with respect to clinicopathological parameters.

Relationship between biochemical parameters and overall survival

Kaplan-Meier analysis revealed that patients with elevated serum TNFα, CA 15-3 and copper (P=0.035, P=0.040 and P=0.0339, respectively) had a survival significantly shorter than those who had lower levels of these parameters (Table 5, Figs 10–12). However, serum NO and bFGF did not correlation with mortality.

Table 5. Correlation between serum levels of TNFα, copper and CA15.3 and overall survival (months) among patients with breast cancer.

	Cut-off value	Survival time (mean±SE)	*P* value
TNFα	<4.9 pg/mL (Negative)	35.33±0.41	
	≥4.9 pg/mL (Positive)	31.24±1.03	0.035*
Copper	<75.75 µg/dL (Negative)	34.83 ±0.82	
	≥75.75 µg/dL (Positive)	31.66±0.97	0.0339*
CA15-3	<25 u/mL (Negative)	34.43±0.97	
	≥25 u/mL (Positive)	30.31±1.43	0.040*

SE: standard error.

*considered significant at P<0.05.

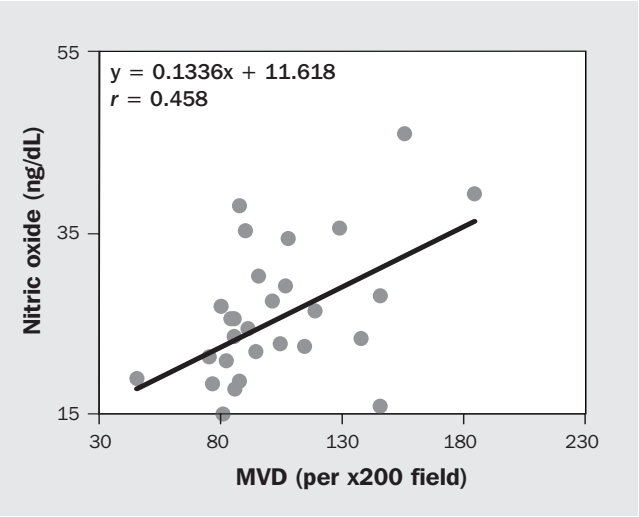

Fig. 5. Correlation between MVD and serum nitric oxide.

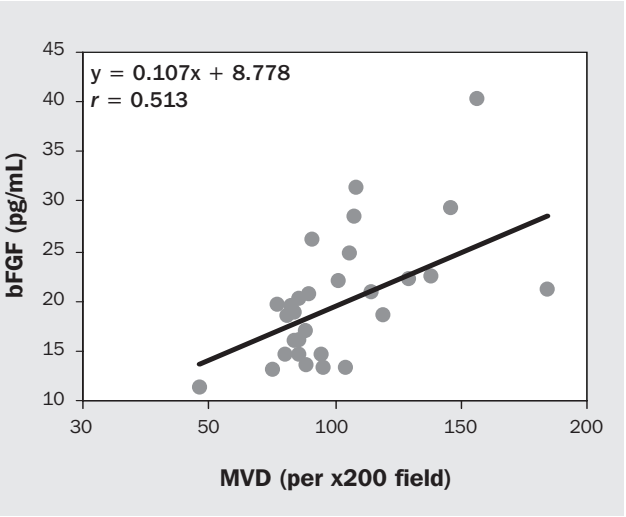

Fig. 6. Correlation between MVD and serum bFGF.

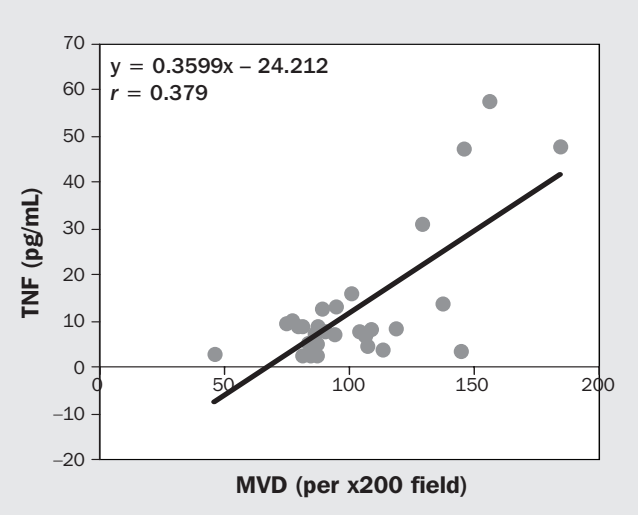

Fig. 7. Correlation between MVD and serum TNFα.

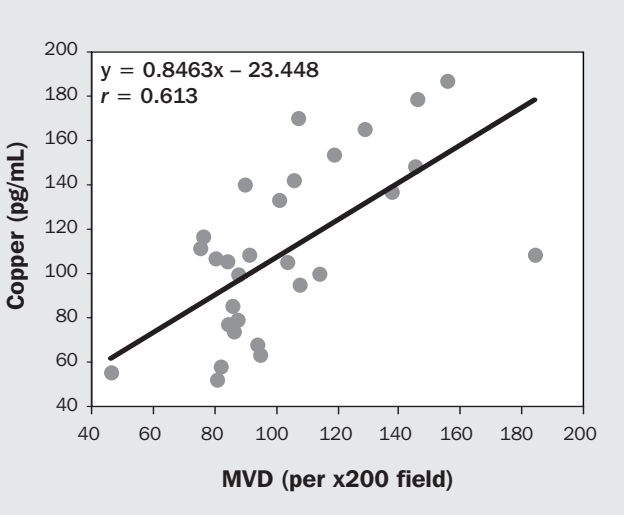

Fig. 8. Correlation between MVD and serum copper.

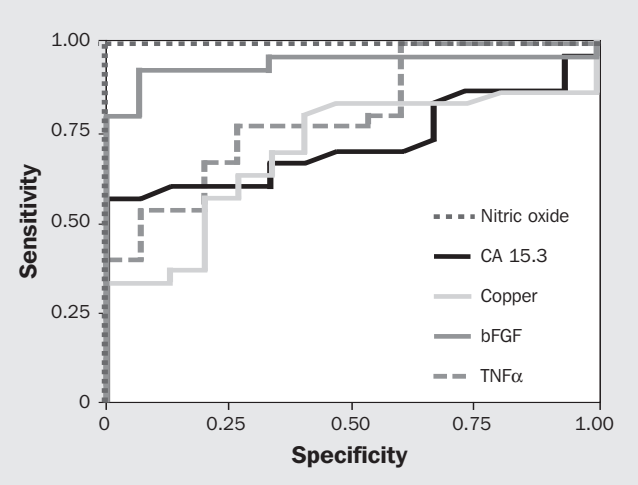

Fig. 9. Graphical representation of the ROC curves for serum nitric oxide, bFGF TNFα, copper and CA 53-3 in breast cancer patients before surgery.

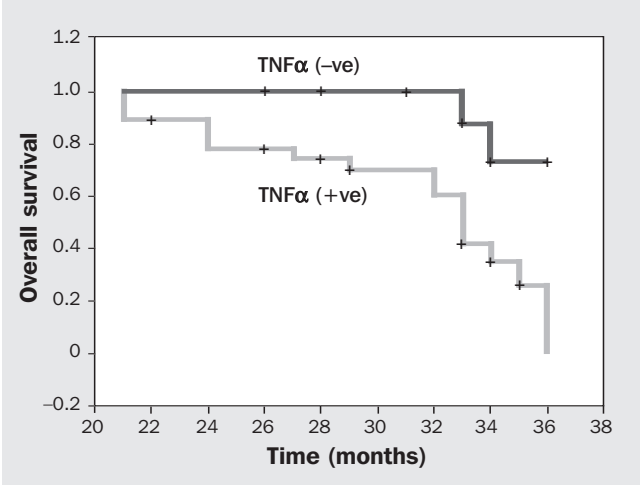

Fig. 10. The correlation between serum TNFα level and overall survival among patients with breast cancer.

Discussion

Primary malignant breast tumours are among those neoplasms that exhibit the greatest angiogenic activity. The significance of tumour angiogenesis as a prognostic indicator has been documented in various human tumours.[38] The development of immunohistochemical techniques using monoclonal antibodies against endothelial mitogens such as FVIII-related antigen have allowed the semiquantitative analysis of microvessel proliferation in tumour tissues.[35] Using this evaluation method, Weidner *et al*.[37] first reported that tumour angiogenesis is an independent prognostic indicator in primary breast cancer. Furthermore, Hork *et al*.[39] confirmed its value by an immunocytochemical method using CD31, another monoclonal antibody for platelet/endothelial cell adhesion molecules.

Regarding the correlation between tumour MVD and clinicopathological parameters, the results presented here support the study of Choi *et al*.[40] but contradicted the study of Charpin *et al*.[41] In this study, a significant correlation between serum NO and tumour MVD was found. This means that NO may play a role in angiogenesis in breast cancer. The role of NO in tumour angiogenesis was studied *in vitro* and *in vivo*. Ziche *et al*.[42] reported that tumour cells transfected with inducible NOS grew more slowly *in vitro* but exhibited increased metastatic spread *in vivo*, associated with evidence of increased tumour vascularity. At the same time, the results of the present study were in agreement with the study of Konukoglu *et al*.,[43] who found an association between preoperative serum NO and angiogenesis.

The results of the present study show that serum NO was significantly higher in the patients with breast cancer than in the control group. These results were in accordance with the results obtained by Coskun *et al*.[44] With respect to the prognostic value of serum NO, the results showed that NO lacked any correlation with clinicopathological parameters and did not correlate with overall survival. These results are consistent with those from the study by Gunel *et al*.[45] but not the study by Martin *et al*.[46]

Tumour necrosis factor-α is a highly pleiotropic polypeptide affecting many cell systems.[47] Preliminary results have shown enhanced serum TNFα production in patients with breast cancer and other solid tumours. The results obtained here show a significant correlation between serum TNFα tumour MVD. The involvement of TNFα in tumour angiogenesis has been indicated *in vitro* and *in vivo* studies. Malik *et al*.[48] showed that over-expression of TNFα increased metastatic activity of tumour cell lines. Orosz *et al*.[49] found that treatment of mice with TNFα promoted development of liver metastases. Moore *et al*.[50] found that mice deficient in TNFα were resistant to skin carcinogenesis. TNFα was able to increase matrix metalloproteinase-9 concentration by approximately 30% in the supernatant of MCF-9 breast cancer cell lines, thereby stimulating tumour growth and metastasis.[51] TNFα stimulates production of plasminogen activator by endothelial cells which, via plasmin production, leads to proteolysis of the extracellular matrix and the release of matrix-bound angiogenic factors.[52]

The present results showed that serum TNFα level were significantly higher in breast cancer patients than in the control group. Regarding the diagnostic and prognostic value of serum TNFα, these results are in accordance with the study carried out by Sheen-Chen *et al*.[53]

The results for bFGF showed significantly correlation with tumour MVD. These observations support the other studies which found that bFGF is an angiogenic protein and one of the most potent inducers of angiogenesis *in vivo* as well as *in vitro*. Evidence suggests that the bFGF-related genes *hst/K-fgl, int-2, FGF-5, FGF-6* and *KGF*, all of which cause transformation *in vitro*, may also play a role in malignancy *in vivo*,[54] and that bFGF exported into the extracellular milieu stimulates migration of the same cell that secretes it via a true autocrine mechanism.[55] Rowe *et al*.[56] purified a bFGF-like growth factor from pooled breast tumours that was mitogenic for T47D cells.

The results of the present study showed that serum bFGF was significantly higher in the breast cancer patients than in the control group, and support the study carried out by Granato *et al*.[7] However, bFGF did not correlate significantly with clinicopathological parameters or patient survival. These results support the study by Sliutz *et al*.[57]

Regarding the angiogenic activity of serum copper, the present results support other work showing that copper plays a role in angiogenesis. Goodman *et al*.[20] suggested

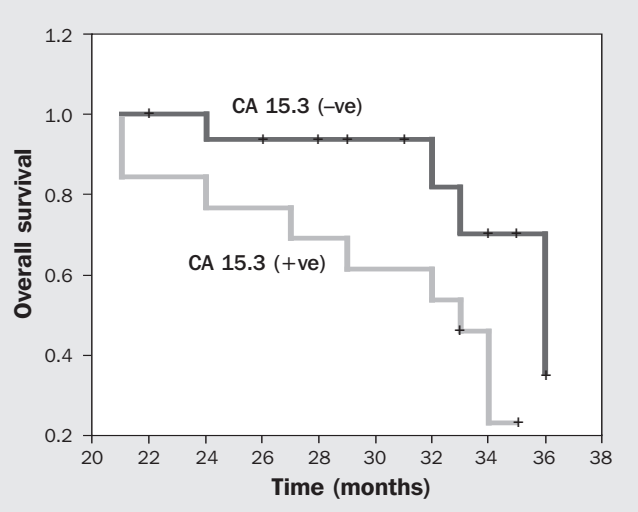

Fig. 11. The correlation between serum CA 15-3 level and overall survival among patients with breast cancer.

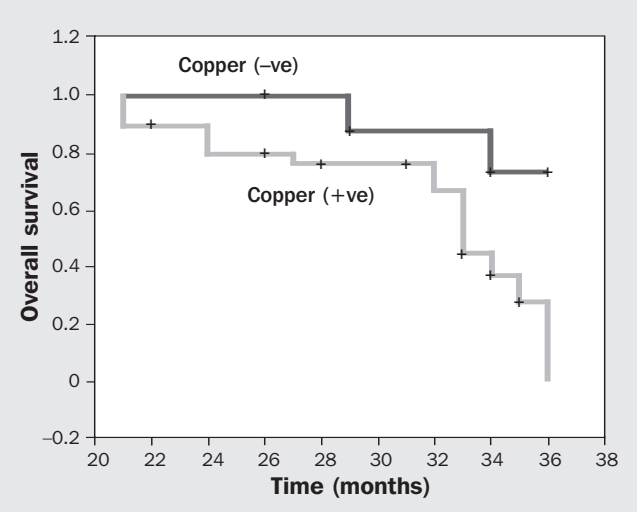

Fig. 12. The correlation between serum copper level and overall survival among patients with breast cancer.

several mechanisms through which copper may exert its angiogenic effect: i) copper may act through binding of angiogenic growth factors and increasing their affinity for endothelial cells, as seen with angiogenin; ii) copper may control the secretion of angiogenic cytokines, as demonstrated with FGF1 and IL-1a; and iii) copper may induce expression of angiogenic growth factors such as VEGF. Thus, therapy aimed at depleting copper may be a successful antineoplastic strategy to target multiple angiogenic growth factors.[20]

Results from the present study support those from Dabek *et al*.[58] In their study, total serum copper was measured in pre- and post-menopausal breast cancer patients and controls. Premenopausal cancer patients had higher serum copper levels than their controls. The reason for this increase among cancer patients remains unclear. It may result from increased liver production of copper-containing ceruloplasmin as an inflammatory response to the cancer or from a tumour-induced decrease in catabolism of the serum ceruloplasmin.[59] Several published studies suggest that Cu^{2+} can react with flavones and catechol oestrogens, producing reactive oxygen species that damage DNA.[60]

The present results suggest the possibility of using serum copper as a prognostic marker to predict clinical outcome in breast cancer and support the study by Lowndes and Harris.[61]

The present results showed that serum CA 15-3 was elevated in the breast cancer patient group compared to the control group, and this was statistically significant. In addition, CA 15-3 concentration increased significantly with tumour size, number of lymph nodes involved and clinical stage. Also, patients with high CA 15-3 concentrations (≥25 u/mL) showed worse overall survival. These results supported the study of Gion *et al*.[62]

The correlation shown between serum TNFα and serum bFGF agrees with Hagemann *et al*.[63] who stated that tumour-associated macrophages (TAMs) induce cancer cell invasion through TNFα-dependent activation of the NF-κB pathway. The activated TAMs were found to produce VEGF, bFGF and TNFα.[64]

The correlation shown between serum TNFα and serum NO agrees with Murohara *et al*.[65] They reported that the angiogenic and inflammatory effects of VEGF can be mediated by NO, which is produced by VEGF-activated eNOS in vascular endothelial cells (VEC). Also, Luo *et al*.[66] found that TNFα also contributes to tumour initiation by stimulating the production of genotoxic molecules (e.g., NO and ROS), which can lead to DNA damage and mutations,.

Regarding the correlation between serum copper and serum TNFα and bFGF, the present results agree with those of Pan *et al*.[10] They reported that copper deficiency induced by tetrathiomolybdate (TM) significantly impaired tumour growth and angiogenesis in two animal models of breast cancer. *In vitro*, TM decreases the production of VEGF and basic fibroblast growth factor. The mechanism of the antiangiogenic effect of copper deficiency is suppression of NF-κB, contributing to a global inhibition of NF-κB-mediated transcription of proangiogenic factors such as TNFα.[67]

In conclusion, in premenopausal breast cancer patients, the angiogenic serum markers NO, bFGF, TNFα and copper are useful diagnostic markers. In addition, serum TNFα, CA 15-3 and copper are useful predictive markers for overall survival. □

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