

## Oxidative, Inflammatory, Angiogenic Markers and Vitamin D Status in Pre- and Postmenopausal Breast Cancer Egyptian Patients

Safinaz Ebrahim El-Toukhy<sup>1</sup>, Omar M.E. Abdel-Salam<sup>2</sup>,  
Amr M M Ibrahim<sup>1</sup> and Eman R. Youness<sup>1\*</sup>

<sup>1</sup>Department of Medical Biochemistry, National Research Centre, Cairo, Egypt.

<sup>2</sup>Department of Toxicology and Narcotics, National Research Centre, Cairo, Egypt.

\*Corresponding author E-mail: hcoctober2000@yahoo.com

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We aimed to investigate the oxidative, inflammatory, angiogenic biomarkers and vitamin D status in serum of Egyptian patients with cancer breast. Seventy patients with known cancer breast (non-metastatic and metastatic) were evaluated and compared to and healthy women. We observed significant decreases in serum malondialdehyde, nitric oxide, total antioxidant capacity (TAC) and uric acid in patients with non-metastatic and metastatic cancer. Subjects with metastatic cancer exhibited significantly lower nitric oxide and TAC compared with non- metastatic cancer. Meanwhile, significant increases in serum VEGF, HGF, and MMP-9 occurred in both pre- and post-menopausal patients with either non-metastatic or metastatic cancer with significantly higher values in metastatic disease. Significant increase in serum TNF- $\alpha$  was observed with significantly higher values in metastatic disease. Serum 25 hydroxy vitamin D (VITD) decreased in both types of cancer with significantly lower values in pre-menopausal compared to post-menopausal patients. Pre-menopausal subjects showed significantly lower serum VITD level compared to their post-menopausal counterparts, but there were no differences between those who were -ve for PR receptor and +ve patients. These results suggest that vascular and inflammatory markers VEGF, HGF, MMP-9 and TNF- $\alpha$  increased in serum in advanced stages of breast cancer and could monitor disease progression and/or severity.

**Keywords:** Angiogenesis; Breast Cancer; Inflammation; Oxidative Stress; Vitamin D.

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Breast cancer is the most common cause of cancer-related deaths in women particularly in industrialized nations, and it is a significant public health problem. It is the leading cause of cancer related death for women aged between 35 and 55 years worldwide <sup>1</sup>. Distant metastases are the principal cause of death. An essential process in forming distant metastases is the degradation of the extracellular matrix allowing tumor cells

to invade local tissue, intravasate and extravasate blood vessels and build new metastatic formations <sup>2</sup>. This process is primarily influenced by the activity of proteinases secreted by the tumor. Currently, at least four classes of proteinases are known: serine proteinases, aspartic proteinases, cysteine proteinases and matrix metalloproteinases<sup>3</sup>. Collectively, these proteinases are capable of breaking down all components of the extracellular

matrix. Under physiological conditions (e.g. tissue remodeling, angiogenesis, ovulation, wound healing) there is a precise regulation between proteolytic degradation and regulatory inhibition of proteolysis<sup>4</sup>. This physiological balance seems to be disrupted in cancer. Matrix metalloproteinases (MMPs) are up regulated in almost every type of cancer and their expression is often associated with a poor prognosis for patients<sup>5</sup>.

Methods have been developed to assess the expression or concentrations of certain angiogenic factors. Among these angiogenic factors, vascular endothelial growth factor (VEGF) has been studied most extensively and is probably the most essential factor for differentiation and development of the vascular system. VEGF is a highly specific and selective mitogen for vascular endothelial cells<sup>6</sup>. It induces proliferation and migration of endothelial cells *in vitro*<sup>7</sup> while inhibiting apoptosis<sup>8</sup>. *In vivo*, VEGF is necessary for vasculogenesis<sup>9</sup>, promotes angiogenesis, and enhances vascular permeability<sup>10</sup>. In several different experimental conditions, overexpression of VEGF was accompanied by marked tumor growth and neovascularization<sup>11</sup>. On the other hand, therapeutic blockade of VEGF has been shown to inhibit primary and metastatic tumor growth in animal models<sup>12,13</sup>.

Although significant improvements in therapy have occurred recently, most deaths from breast cancer are still caused by metastases that are resistant to conventional treatment. Therefore, novel approaches to the management of breast cancer need to be developed. VEGF have been implicated as the major angiogenic factor in human cancers. VEGF promotes angiogenesis and invasion and increases vascular permeability<sup>14</sup>. MMP-2 and MMP-9 are related to tumor invasion and metastasis by their capacity for tissue remodeling via extracellular matrix as well as basement membrane degradation and induction of angiogenesis<sup>15</sup>. Vitamin D has emerged as the most prolific topic in the last decade with work connecting it with risk reduction in various epithelial cancers. Vitamin D exerts a wide range of immunogenic and antiproliferative activities in the body<sup>16</sup>.

This study aims to explore the inflammatory and angiogenic markers as well as to evaluate the oxidative and vitamin D status in

Egyptian females with breast cancer in order to assess the prognostic significance of these markers.

## PATIENTS AND METHODS

Seventy consecutive patients with known cancer breast (non-metastatic and metastatic), with a mean age of  $50.6 \pm 1.04$  years (range, 33.2 - 67 years) treated in the National Oncology Institute were studied after they had given informed consent. Thirty five patients had non-metastatic cancer. Seventeen patients were pre-menopausal (mean age  $42.9 \pm 1.01$  years; range 35.6-49 years) and 18 were post-menopausal (mean age  $58.4 \pm 0.92$  years; range 53.0-67 years). Thirty five patients had metastatic cancer. Seventeen patients were pre-menopausal (mean age  $42.6 \pm 1.18$  years; range 33.2-49 years) and 18 were post-menopausal (mean age  $56.6 \pm 0.83$  years; range 51.3-64 years). The control group comprised 17 pre-menopausal (mean age  $42.3 \pm 1.1$  years; range 33.7-50 years) and 17 post-menopausal (mean age  $56.4 \pm 1.03$  years; range 50.3-66.1 years) healthy women.

### Biochemical analyses

#### Determination of lipid peroxides

Malondialdehyde was determined by measuring thiobarbituric reactive species using the method of Ruiz-Larrea *et al.* 1994 in which the thiobarbituric acid reactive sub-stances react with thiobarbituric acid to produce a red colored complex having peak absorbance at 532 nm<sup>17</sup>.

#### Determination of nitric oxide metabolites

Nitric oxide was determined in serum according to the method of Miranda<sup>18</sup>. The level of total nitrite/nitrate in serum samples was calculated using the standard curve constructed with the prepared serial dilutions of sodium nitrite.

#### Determination of total antioxidant capacity

Total serum antioxidant activity was determined by the reaction of antioxidants in the sample with a defined amount of exogenously provide hydrogen peroxide ( $H_2O_2$ ). The antioxidants eliminate a certain amount of the provided  $H_2O_2$ . The residual  $H_2O_2$  is determined colorimetric ally by an enzymatic reaction which involves the conversion of 3, 5, dichloro-2-hydroxy benzensulphonate to a colored product<sup>19</sup>.

#### Determination of serum uric acid

Uric acid concentration was measured

by the direct enzymatic method, in which uric acid was oxidized by uricase coupled with peroxidase. Uricase converts uric acid to allantoin and hydrogen peroxide. The hydrogen peroxide formed further reacts with a phenolic compound and 4 aminoantipyrine by the catalytic action of peroxidase to form a red colored quinoneimine dye complex. Intensity of the colour formed is directly proportional to the amount of uric acid present in the sample<sup>20</sup>.

#### **Quantification of serum VEGF, HGF, MMP-9, TNF- $\alpha$ , 25 hydroxy Vitamin D**

Commercially available immunoassay kits were used according to manufacturer's guidelines. Vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) levels were determined with human VEGF immunoassay kit from R&D system (R&D System, Minneapolis, MN, USA); tumour necrosis factor- $\alpha$  levels were determined by TNF- $\alpha$  ELISA kits (Pierce Co., Rockford, IL, USA); matrix metalloproteinase-9 (MMP-9) levels were determined using human matrix metalloproteinase-9 ELISA kit (Ray Biotech Human ELISA Kit (Ray Biotech, Norcross, Georgia, USA); 25 hydroxy Vitamin D was performed using the enzyme-linked immunosorbent assay (ELISA) (Immundiagnostik EIA, Bensheim and Biomedica, Wien, Austria).

#### **Statistical analysis**

Results are presented as means  $\pm$  SE. For statistical analysis, group comparisons were performed by one way ANOVA followed by Duncan's multiple range test. Differences between groups and correlation coefficients were considered significant if  $P < 0.05$ . GraphPad Prism 6 for Windows (GraphPad Prism Software Inc., San Diego, CA, USA) was used.

## **RESULTS**

#### **Lipid peroxidation**

In premenopausal patients serum malondialdehyde (MDA) levels were significantly lower in patients with non-metastatic (mean 46.81, range 40.9 to 55.3  $\mu\text{mol/l}$ ;  $P < 0.001$ ) and metastatic cancer (mean 43.6, range 39.9 to 46.8  $\mu\text{mol/l}$ ;  $P < 0.001$ ) than in normal individuals (mean 70.0, range 65.2 to 76.3  $\mu\text{mol/l}$ ). Similarly, serum MDA levels were significantly higher in normal subjects (mean 72.67, range 68.5 to 79.2  $\mu\text{mol/l}$ ) compared with

patients with either non-metastatic (mean 47.0, range 40.9 to 55.3  $\mu\text{mol/l}$ ;  $P < 0.001$ ) or metastatic cancer (mean 44.0, range 40.3 to 46.8  $\mu\text{mol/l}$ ;  $P < 0.001$ ). There were no significant differences between serum MDA levels in premenopausal and postmenopausal patients with either non-metastatic or metastatic disease (figure 1 & table 1).

#### **Nitric oxide**

There was a significant decrease in serum nitric oxide in premenopausal patients with non-metastatic (mean 13.94, range 11.2 to 16.5  $\mu\text{mol/l}$ ;  $P < 0.001$ ) and metastatic cancer (mean 8.74, range 6.8 to 10.8  $\mu\text{mol/l}$ ;  $P < 0.001$ ) by 29.0% and 56.3%, respectively, compared to the control group (mean 19.5, range 16.8 to 21.9  $\mu\text{mol/l}$ ). Similarly, serum nitric oxide levels were significantly lower in postmenopausal patients with non-metastatic (mean 14.04, range 11.2 to 16.5  $\mu\text{mol/l}$ ;  $P < 0.001$ ) and metastatic cancer (mean 8.56, range 6.6 to 10.5  $\mu\text{mol/l}$ ;  $P < 0.001$ ) compared to normal subjects (mean 19.78, range 17.8 to 21.5  $\mu\text{mol/l}$ ) (figure 1 & table 1).

A significant decrease in serum nitric oxide by 37.3% and 39.0% was observed in pre- or postmenopausal patients with metastatic disease compared to non-metastatic cancer patients (figure 1 & table 1).

#### **Uric acid**

Serum uric acid showed a significant decrease in premenopausal patients with non-metastatic (mean 3.21, range 2.9 to 3.9 mg/dl;  $P < 0.001$ ) and metastatic cancer (mean 2.90, range 2.1 to 3.6 mg/dl;  $P < 0.001$ ) by 32.3% and 35.5%, respectively, compared to the control group (mean 4.74, range 3.7 to 6.2 mg/dl). Serum uric acid levels were also significantly lower in postmenopausal patients with non-metastatic (mean 3.13, range 2.6 to 3.9 mg/dl;  $P < 0.001$ ) and metastatic cancer (mean 2.88, range 2.1 to 3.8 mg/dl;  $P < 0.001$ ) compared to normal subjects (mean 4.85, range 3.9 to 6.2 mg/dl). There were no significant differences between serum uric acid levels in premenopausal and postmenopausal patients with either non-metastatic or metastatic disease (figure 1 & table 1).

#### **Total antioxidant capacity**

Serum TAC was significantly lower in premenopausal patients with non-metastatic (mean 1.63, range 1.42 to 1.85  $\mu\text{mol/l}$ ) and metastatic cancer (mean 1.19, range 0.96 to 1.39  $\mu\text{mol/l}$ ;  $P <$

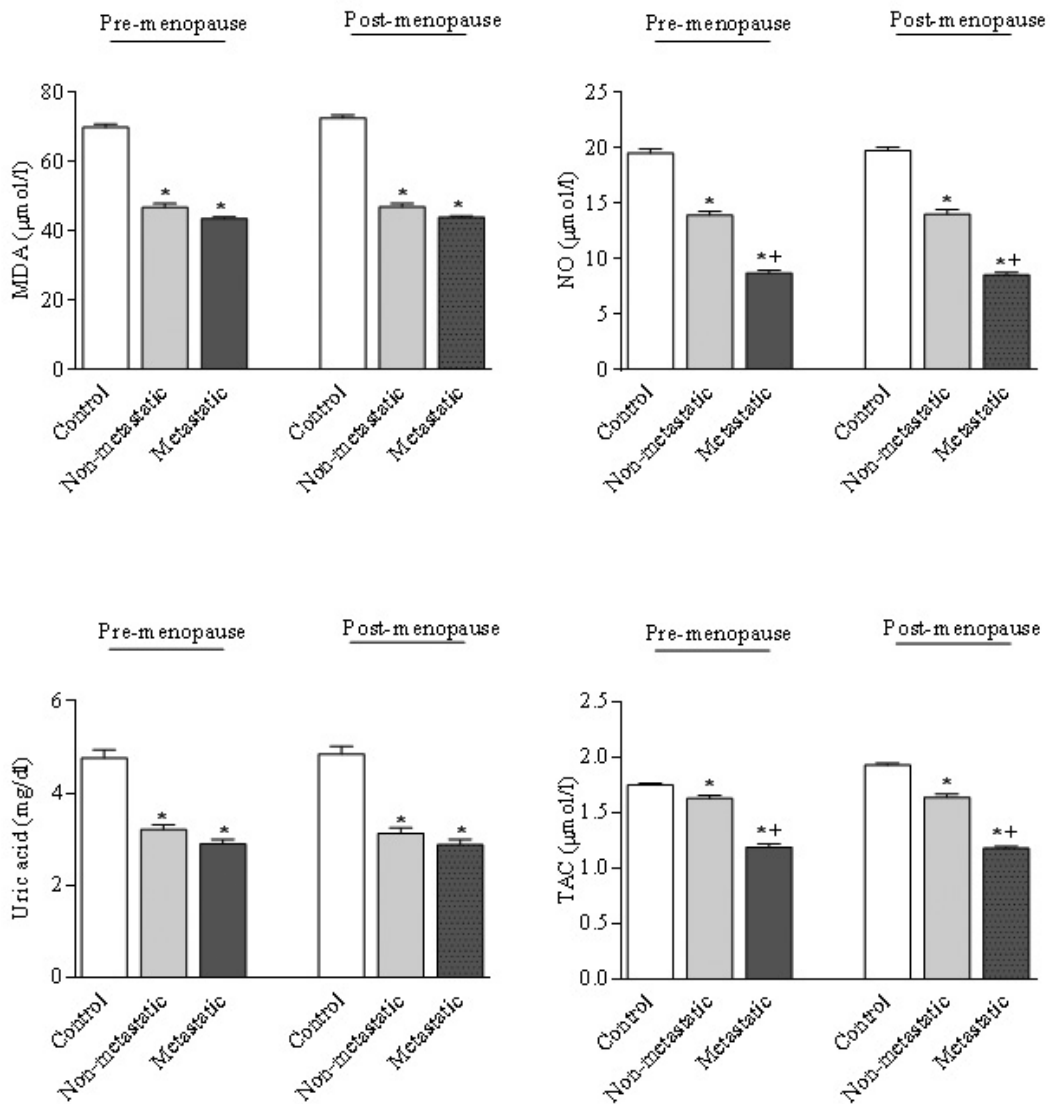
0.001) as compared to the control group (mean 1.75, range 1.62 to 1.83  $\mu\text{mol/l}$ ). Serum TAC values were also significantly lower in post-menopausal patients with non-metastatic (mean 1.64, range 1.42 to 1.85  $\mu\text{mol/l}$ ;  $P < 0.001$ ) and metastatic disease (mean 1.18, range 0.95 to 1.37  $\mu\text{mol/l}$ ;  $P < 0.001$ ) compared to their corresponding control subjects (mean 1.93, range 1.82 to 2.09  $\mu\text{mol/l}$ ).

Patients with metastatic disease (either premenopausal or post-menopausal) exhibited

significantly lower TAC values compared to those with non-metastatic cancer (figure 1 & table 1).

**VEGF**

Pre-menopausal patients with non-metastatic or metastatic disease had significantly higher serum VEGF values (mean 227.5, range 202.8 to 239.2  $\text{pg/ml}$  and mean 372.6, range 349.5 to 400.6  $\text{pg/ml}$ , respectively) than did the control subjects (mean 116.39, range 102.4 to 122.8  $\text{pg/ml}$ ). In addition, post-menopausal patients with non-

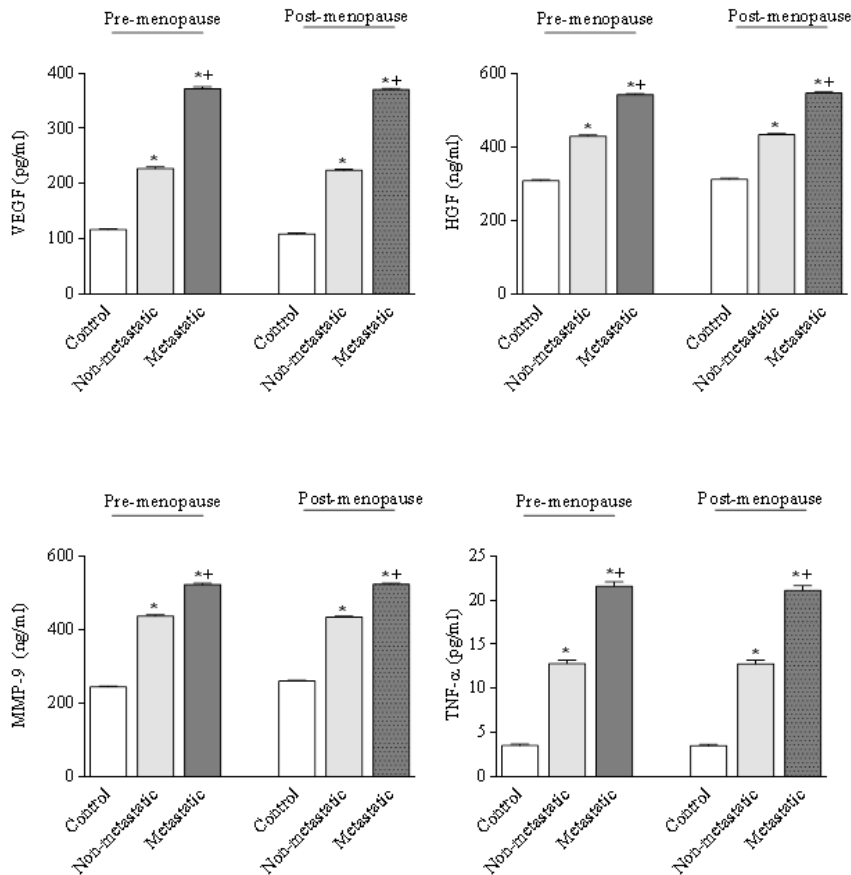


**Fig. 1.** Serum malondialdehyde (MDA), nitric oxide, uric acid and total antioxidant capacity (TAC) in patients with breast cancer. \*:  $p < 0.05$  vs. the corresponding control group. +:  $p < 0.05$  vs. the corresponding non-metastatic group

**Table 1.** Serum malondialdehyde (MDA), nitric oxide, uric acid and total antioxidant capacity (TAC) in patients with breast cancer

	Control	Non-metastatic breast cancer	Metastatic breast cancer
MDA (mmo/l)			
Pre-menopause	69.94 ± 0.96	46.81 ± 1.14* (-33.1%)	43.64 ± 0.49* (-37.6%)
Post-menopause	72.67 ± 0.9	47.0 ± 1.00* (-35.3%)	44.0 ± 0.45* (-39.4%)
Nitric oxide(μmol/l)			
Pre-menopause	19.55± 0.38	13.94 ± 0.39* (-28.7%)	8.74 ± 0.26*+ (-55.3%)
Post-menopause	19.78 ± 0.30	14.04 ± 0.41* -29.0%	8.56 ± 0.24*+ (-56.7%)
Uric acid(mg/dl)			
Pre-menopause	4.74 ± 0.18	3.21 ± 0.12* (-32.3%)	2.9 ± 0.10* (-38.8%)
Post-menopause	4.85 ± 0.17	3.13 ± 0.12* (-35.5%)	2.88 ± 0.12* (-40.6%)
TAC (μmol/l)			
Pre-menopause	1.75 ± 0.02	1.63 ± 0.03* (-5.7%)	1.19 ± 0.03*+ (-32%)
Post-menopause	1.93 ± 0.02	1.64 ± 0.03* (-15.5%)	1.18 ± 0.02*+ (-38.9%)

Data are expressed as mean ± SEM. \*:p<0.05 vs. the corresponding control group. +:p<0.05 vs. the corresponding non-metastatic group. The percentage change from the corresponding control group is shown in parenthesis.

**Fig. 2.** VEGF, HGF, MMP-9, VITD and TNF- $\alpha$  in patients with breast cancer. Data are expressed as mean ± SEM. \*:p<0.05 vs. the corresponding control group. +:p<0.05 vs. the corresponding non-metastatic group

metastatic or metastatic breast cancer exhibited significantly higher serum VEGF (mean 224.1, range 202.8 to 235.7 pg/ml and mean 371.2, range 349.5 to 382.0 pg/ml, respectively) compared to their corresponding normal control values (mean 108.5, range 101.6 to 119.4 pg/ml).

There was no difference between pre- or post-menopausal patients as regards their serum VEGF levels. However, patients with metastatic disease whether pre- or post-menopausal had significantly higher serum VEGF levels compared to their non-metastatic counterparts (figure 2 & table 2).

#### TNF- $\alpha$

Serum TNF- $\alpha$  showed marked and significant increases by 264.5% and 513.6% in pre-menopausal women with both non-metastatic (mean 12.83, range 9.3 to 15.8 pg/ml) or metastatic disease (mean 21.6, range 18.4 to 24.9 pg/ml) compared with the healthy controls (mean 3.51, range 2.6 to 4.4 pg/ml). Similarly, in post-menopausal patients, significant elevations in serum TNF- $\alpha$  by 266.2% and 505.4% were observed in patients with both non-metastatic (mean 12.78, range 9.3 to 15.8 pg/ml) or metastatic disease (mean 21.13, range 18.4 to 24.9 pg/ml) compared with the control group (mean 3.49, range 2.7 to 4.6 pg/ml). Moreover, patients with metastatic cancer whether pre- or post-menopausal exhibited

significantly higher levels of TNF- $\alpha$  in serum by 68.3% and 65.3% compared to their corresponding control values (figure 2 & table 2). Figure 3 shows the correlation analysis between serum VEGF and TNF- $\alpha$  in patients with breast cancer. There is significant +ve correlation between serum VEGF and TNF- $\alpha$  in pre-menopausal patients with either metastatic or non-metastatic disease and in post-menopausal patients with metastatic cancer.

#### MMP-9

Premenopausal women with non-metastatic or metastatic disease had significantly higher MMP-9 serum values (mean 437.3, range 410.2 to 458.3 ng/ml and mean 523.3, range 495.3 to 539.5 ng/ml, respectively) than in healthy subjects (mean 244.2, range 110.7 to 336.9 ng/ml). Serum MMP-9 were also significantly elevated in post-menopausal patients with either non-metastatic (mean 434.3, range 410.3 to 453.6 ng/ml) or metastatic cancer (mean 524.2, range 537.6 to 498.4 ng/ml) than in normal individuals (mean 259.9, range 129.5 to 331.2 ng/ml).

There was significantly increased serum MMP-9 in pre- and post-menopausal patients with metastatic disease compared to corresponding non-metastatic patients (figure 2 & table 2). Figure 4 shows the correlation analysis between serum VEGF and MMP-9 in patients with breast cancer. There is significant +ve correlation between serum

**Table 2.** Serum VEGF, HGF, MMP-9, VITD and TNF- $\alpha$  in patients with breast cancer.

	Control	Non-metastatic breast cancer	Metastatic breast cancer
VEGF (pg/ml)			
Pre-menopause	116.39 $\pm$ 1.33	227.5 $\pm$ 2.56* (95.5%)	372.64 $\pm$ 3.56*+ (220.1%)
Post-menopause	108.55 $\pm$ 1.54	224.14 $\pm$ 2.00* (106.5%)	371.19 $\pm$ 2.11*+ (241.9%)
HGF (ng/ml)			
Pre-menopause	307.97 $\pm$ 2.6	429.12 $\pm$ 3.15* (39.3%)	542.99 $\pm$ 2.39** (76.3%)
Post-menopause	311.25 $\pm$ 3.29	433.32 $\pm$ 2.82* (39.2%)	546.97 $\pm$ 2.66** (75.7%)
MMP-9 (ng/ml)			
Pre-menopause	244.16 $\pm$ 2.3	437.28 $\pm$ 3.37* (79.1%)	523.3 $\pm$ 3.00*+ (114.3%)
Post-menopause	259.86 $\pm$ 2.36	434.28 $\pm$ 3.10* (67.1%)	524.18 $\pm$ 2.78*+ (101.7%)
VITD (ng/ml)			
Pre-menopause	21.73 $\pm$ 0.72	8.69 $\pm$ 0.15* (-60.0%)	8.62 $\pm$ 0.14* (60.3%)
Post-menopause	22.02 $\pm$ 0.84	14.47 $\pm$ 0.19* (-34.3%)	13.86 $\pm$ 0.29* (-37.1%)
TNF- $\alpha$ (pg/ml)			
Pre-menopause	3.52 $\pm$ 0.14	12.83 $\pm$ 0.39* (264.5%)	21.6 $\pm$ 0.49*+ (513.6%)
Post-menopause	3.49 $\pm$ 0.13	12.78 $\pm$ 0.41* (266.2%)	21.13 $\pm$ 0.52*+ (505.4%)

Data are expressed as mean  $\pm$  SEM. \*:p<0.05 vs. the corresponding control group. +:p<0.05 vs. the corresponding non-metastatic group. The percentage change from the corresponding control group is shown in parenthesis.

VEGF and MMP-9 in pre-menopausal patients with non-metastatic disease and in post-menopausal patients with either metastatic or non-metastatic disease.

#### HGF

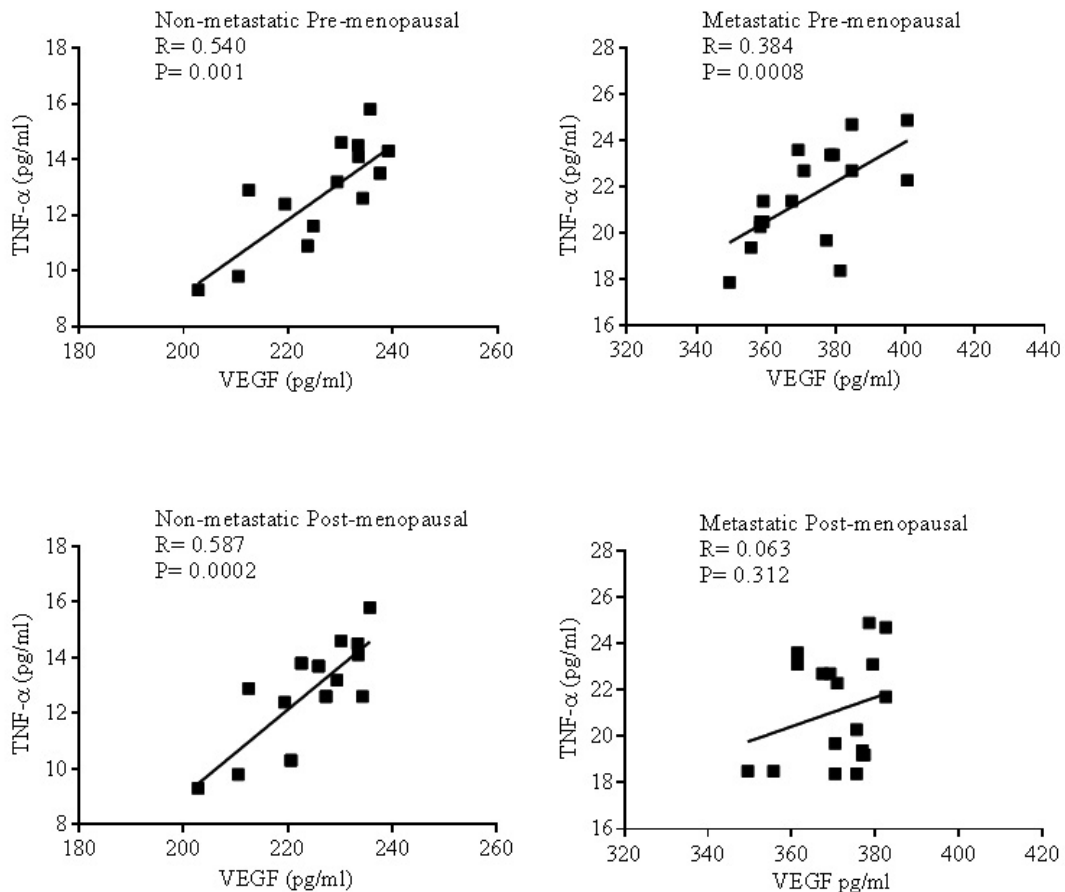
Serum HGF levels were significantly higher in pre-menopausal patients with non-metastatic or metastatic disease (mean 429.1, range 410.3 to 455.7 ng/ml and mean 543.0, range 528.4 to 559.3 ng/ml, respectively) than in healthy subjects (mean 307.9, range 290.6 to 323.4 ng/ml).

Significant elevations in serum HGF levels were also observed in post-menopausal patients with non-metastatic or metastatic breast cancer (mean 433.3, range 413.2 to 455.7 ng/ml and mean 546.9, range 428.4 to 562.9 ng/ml, respectively) compared to normal individuals (mean 311.2, range 219.3 to 335.5 ng/ml).

No significant differences were observed between pre- or post-menopausal subjects as regards their serum HGF levels. In contrast, there were significantly increased HGF levels in pre- and post-menopausal patients with metastatic disease than their non-metastatic counterparts (figure 2 & table 2). Figure 5 shows the correlation analysis between serum VEGF and HGF in patients with breast cancer. There was significant inverse correlation in post-menopausal patients with metastatic cancer.

#### VITD

Serum VITD was significantly decreased by 60.0% and 60.3% in pre-menopausal women with both non-metastatic (mean 8.68, range 7.5 to 9.7 ng/ml) and metastatic breast cancer (mean 8.62, range 7.8 to 9.6 ng/ml) compared with the control group (mean 31.7, range 15.4 to 25.2 ng/



**Fig. 3.** Correlation analysis between serum VEGF and TNF- $\alpha$  in patients with breast cancer

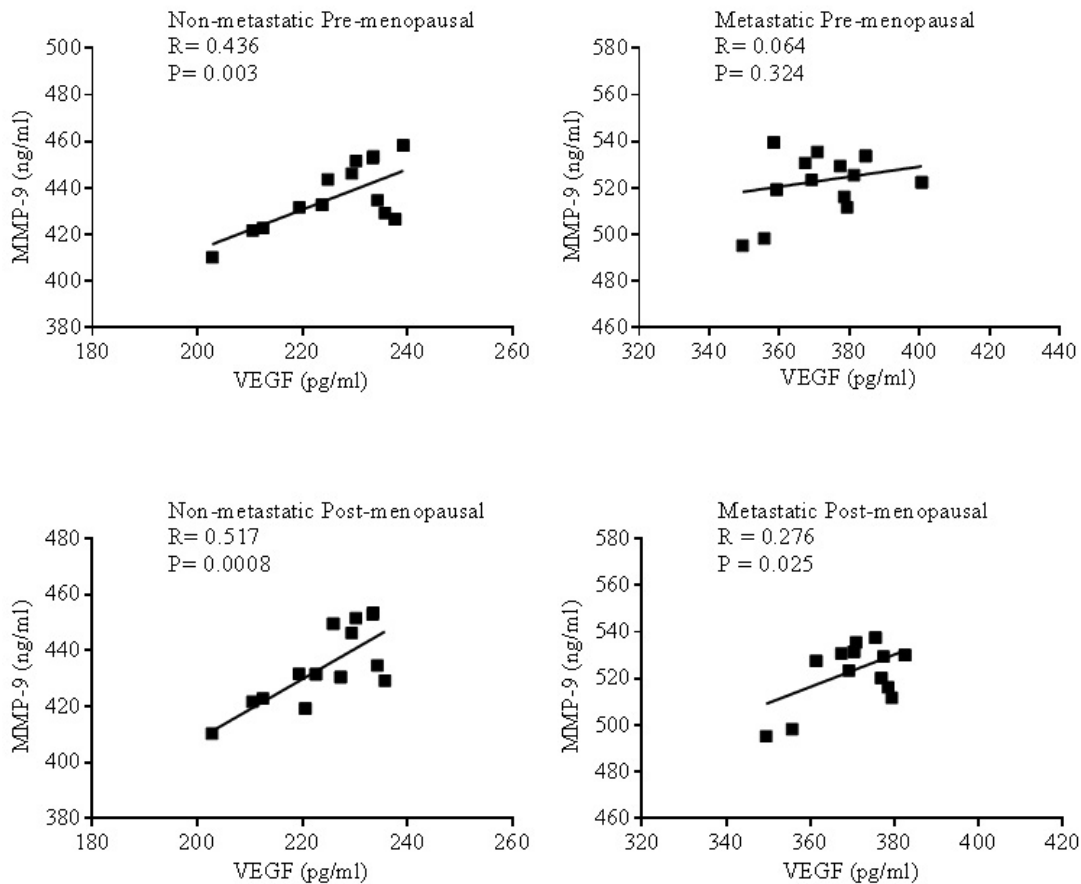
ml). Serum VITD was also significantly lower by 34.3% and 37.1% in post-menopausal women with both non- metastatic (mean 14.47, range 9.3 to 15.8 ng/ml) and metastatic breast cancer (mean 13.86, range 11.5 to 15.3 ng/ml) compared with the control group (mean 22.02, range 15.4 to 26.3 ng/ml). There were no significant differences between serum VITD in metastatic or non-metastatic patients whether pre- or post-menopausal (figure 6).

**Changes in oxidative and inflammatory biomarkers according to hormonal receptor status**

Out of 17 pre-menopausal patients with non-metastatic cancer 16 were +ve for the estrogen receptor and 16 were +ve for the progesterone receptor. In Post-menopausal patients with non-metastatic cancer, 18/18 were +ve for the estrogen receptor and 16/18 were positive for the progesterone receptor.

Out of 17 pre-menopausal patients with metastatic cancer, 10 were –ve and 7 were positive for the progesterone receptor. Out of 18 post-menopausal patients with metastatic cancer, 10 were –ve and 8 were positive for the progesterone receptor. Only one pre-menopausal patient and one post-menopausal patient with metastatic cancer were +ve for the estrogen receptor. Statistical comparisons were therefore made between patients with metastatic disease (pre- and post-menopausal) according to their progesterone receptor (PR) status.

No significant differences were observed between PR –ve and PR +ve patients (whether pre- or post-menopausal) as regards MDA, nitric oxide, or uric acid in serum. PR +ve patients showed lower TAC compared with PR –ve subjects. This difference reached statistical significance only in pre-menopausal patients (Table 3).



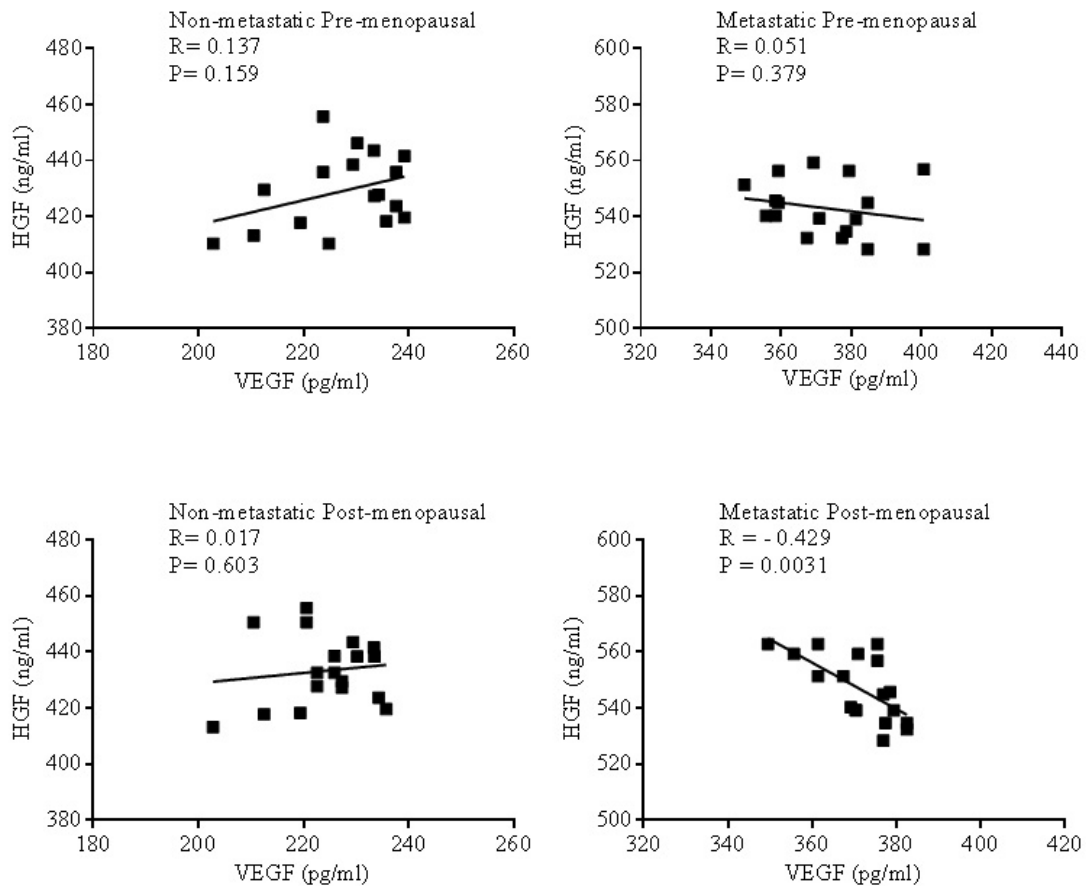
**Fig. 4.** Correlation analysis between serum VEGF and MMP-9 in patients with breast cancer



**Table 3.** Serum malondialdehyde (MDA), nitric oxide, uric acid and total antioxidant capacity in patients with metastatic breast cancer according to the progesterone receptor status

	Progesterone receptor -ve	Progesterone receptor +ve
MDA (mmo/l)		
Pre-menopause	44.14 ± 0.73	42.91 ± 0.60
Post-menopause	43.25 ± 0.60	44.9 ± 0.52
Nitric oxide(μmol/l)		
Pre-menopause	8.55 ± 0.30	9.0 ± 0.34
Post-menopause	8.33 ± 0.36	9.8 ± 0.28
Uric acid(mg/dl)		
Pre-menopause	9.46 ± 0.33	10.73 ± 0.42
Post-menopause	9.27 ± 0.31	10.00 ± 0.34
TAC (μmol/l)		
Pre-menopause	1.11 ± 0.04	1.32 ± 0.02* (p = 0.012)
Post-menopause	1.14 ± 0.05	1.22 ± 0.03 (p = 0.063)

Data are expressed as mean ± SEM.



**Fig. 5.** Correlation analysis between serum VEGF and HGF in patients with breast cancer

There were also non-significant differences in serum VEGF, HGF, MMP-9, or TNF- $\alpha$  between PR -ve and PR +ve patients (Table 4). Pre-menopausal patients, however, showed significantly lower serum VITD level compared to their post-menopausal counterparts, but no differences were observed between those

who were -ve for PR receptor and +ve patients (Table 4).

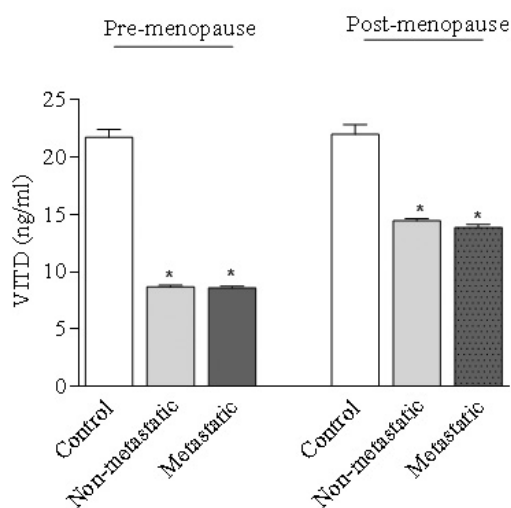
## DISCUSSION

Free radicals have been implicated as a possible cause for cancer. In this study, we

**Table 4.** Serum VEGF, HGF, MMP-9, VITD and TNF- $\alpha$  in patients with metastatic breast cancer according to the progesterone receptor status

	Progesterone receptor -ve	Progesterone receptor +ve
VEGF (pg/ml)		
Pre-menopause	374.18 $\pm$ 4.72	370.44 $\pm$ 5.27
Post-menopause	369.28 $\pm$ 3.21	373.57 $\pm$ 2.24
HGF (ng/ml)		
Pre-menopause	542.84 $\pm$ 3.22	543.21 $\pm$ 3.55
Post-menopause	545.73 $\pm$ 3.58	548.51 $\pm$ 3.90
MMP-9 (ng/ml)		
Pre-menopause	520.01 $\pm$ 4.42	528.0 $\pm$ 2.80
Post-menopause	519.44 $\pm$ 4.11	530.11 $\pm$ 2.20
VITD (ng/ml)		
Pre-menopause	8.76 $\pm$ 0.21	8.41 $\pm$ 0.12
Post-menopause	13.75 $\pm$ 0.33	14.0 $\pm$ 0.49
TNF- $\alpha$ (pg/ml)		
Pre-menopause	21.89 $\pm$ 0.78	21.18 $\pm$ 0.38
Post-menopause	20.69 $\pm$ 0.62	21.69 $\pm$ 0.86

Data are expressed as mean  $\pm$  SEM.



**Fig. 6.** Serum VITD in patients with breast cancer. Data are expressed as mean  $\pm$  SEM. \*:p<0.05 vs. the corresponding control group

assessed the oxidant/antioxidant status of patients with cancer breast by measuring MDA, nitric oxide, total antioxidant capacity and uric acid in the serum. We observed significant and marked decrease in serum malondialdehyde, an end product of lipid peroxidation which indicates a decrease in free radicals in these patients. This finding confirms our previous observation suggesting reduced oxidative stress in breast cancer patients undergoing chemotherapy<sup>21</sup>. In their study, Saintot et al. 1996 found that malondialdehyde plasma concentration were lower in breast cancer patients before therapy than in controls<sup>22</sup>. This decrease in lipid peroxidation was related to tumour size and progression i.e., tumour aggressiveness. Another study showed that the presence of nodes and/or metastases was directly associated with low plasma concentrations of cholesterol and malondialdehyde<sup>22</sup>. Moreover, Gonenc et al. 2006 found decreased serum and tissue malondialdehyde

levels in breast cancer patients compared to benign breast disease<sup>23</sup>. The observed decrease in serum malondialdehyde in breast cancer patients could be also the result of chemotherapeutic agents. Our findings are in contrast with studies that reported increased plasma malondialdehyde in cancer breast<sup>24,25</sup>. Other studies found increased lipid peroxidation in breast cancer compared with non-malignant tissue and also increased superoxide dismutase and glutathione peroxidase enzyme activities<sup>26</sup>.

The measurement of antioxidant capacity (TAC) defined as the moles of radicals neutralized per 1 L of tested sample, is widely used test in biological studies that is thought to reflect the sum of endogenous antioxidants<sup>27</sup>. Other researchers suggested that the determination of individual antioxidants might be more informative of the oxidant/antioxidant status of the tissue than a single test<sup>28</sup>. In this study, TAC significantly decreased in subjects with metastatic cancer compared to the control group, which might indicate consumption of endogenous antioxidants and/or reduced intake of exogenous antioxidants. Other studies also indicated decreased TAC in breast cancer patients compared to the healthy control<sup>29</sup>. Feng *et al.* 2012 found decreased TAC in serum and breast tissue of patients with benign breast lesions and breast cancer<sup>30</sup>. Significantly decreased TAC has also been reported during chemotherapy for malignancies in children which has been attributed to reduced dietary intake of antioxidants and an increase in free radicals by the effect of anticancer agents<sup>31</sup>. In the present study, TAC decreased in patients with either non-metastatic or metastatic cancer with the lowest values being observed in subjects with metastatic disease. Whether this is due to consumption of endogenous antioxidants in the disease process, reduced dietary intake in advanced disease or the result of chemotherapy is not clear. Since the test is affected by decreased dietary intake of antioxidants it might therefore not be suitable for monitoring disease or indicate disease severity.

In this study, we found that nitric oxide also decreased in serum of breast cancer patients, with those having metastatic disease exhibiting the lowest levels, which confirms our previous studies. Similar results were provided by Güler *et al.* 2006 who demonstrated decreased plasma

nitric oxide after chemotherapy<sup>32</sup>. Other studies reported increased serum nitric oxide in breast cancer<sup>29</sup>. The gaseous molecule nitric oxide is derived from L-arginine via the enzyme nitric oxide synthase (NOS) that exists in constitutive (endothelial and neuronal) and an inducible (NOS2) isoform. The latter is responsible for the sustained generation of large amounts of nitric oxide during inflammatory conditions by neutrophils and phagocytes<sup>33</sup>. Increased expression of both endothelial (eNOS) and NOS2 was detected in invasive and in situ breast cancer<sup>34</sup>. Nitric oxide has an important role in different stages of cancer *eg.* angiogenesis, cell invasion, intravasation and metastasis<sup>35,36</sup>. And the increased expression of NOS2 has been shown to be associated with its increased expression is associated with disease aggressiveness, and predict poor outcome in ER (-) breast cancer<sup>37,38</sup>. High levels of nitric oxide are associated with the production of a range of reactive nitrogen species such as peroxy nitrite and nitrogen dioxides and trioxides, which subsequently interact with more diverse targets, and result in chemical stress<sup>33</sup>. Rabender *et al.* 2015 found that NOS of tumor cells, in contrast to normal tissues, generates peroxy nitrite and superoxide anion than nitric oxide, with important consequent on tumor growth<sup>39</sup>. Nitric oxide might also inhibit tumorigenesis by inhibiting MMP-9 activity<sup>40</sup>. Indeed, both tumorigenic and anti-oncogenic activities have been detected for nitric oxide with low-intermediate concentrations stimulating whilst high concentrations inhibiting oncogenic signaling<sup>41</sup>.

Uric acid, the end product of purine metabolism that results from the oxidation of xanthine and hypoxanthine by xanthine oxidase is considered an important factor in the development and mortality from cancer. Uric acid because of its pro-inflammatory properties is thought to represent a link between inflammatory states like obesity and metabolic syndrome and the occurrence of cancer. Uric acid, however, is a double edged sword because the molecule is a potent antioxidant which scavenges peroxy, hydroxyl and superoxide radicals and inhibits oxidative damage to cell biomolecules lipids, proteins, and nucleic acids<sup>42,43</sup>. Uric acid might thus serve a protective role to reduce cancer<sup>44</sup>. In the present study, serum uric acid levels were significantly lower in breast cancer

patients compared to healthy controls. Uric acid levels were unaffected by the menopausal status. This reduction in serum uric acid might be due to drugs that inhibit uric acid synthesis eg. allopurinol, often given in the course of chemotherapy to prevent sudden increments in serum and tissue uric acid from tissue damage. In healthy subjects, administering uric acid was found to decrease oxidative stress and increase serum TAC during acute physical exercise<sup>45</sup>. The decrease in serum uric acid might also contribute to the decrease in TAC observed in our study.

Our study also shows depressed serum VITD levels in metastatic and non-metastatic breast cancer patients. Significantly lower values were however detected in pre-menopausal compared to post-menopausal patients. In cells, VITD binds to its nuclear vitamin D receptor and mediates signaling pathways that involve cellular proliferation, apoptosis, angiogenesis, and metastasis and hence its significance in cancer development and progression<sup>46,47</sup>. In recent years, serum VITD has been a focus of much interest in relation to breast cancer. Low levels of VITD in serum of patients with breast cancer were observed at diagnosis compared to controls and showed significant decrease after neoadjuvant chemotherapy<sup>48,49</sup>. In a meta-analysis study by Li *et al.* 2014 higher VITD levels were significantly associated with improved disease free survival for patients with cancer breast<sup>50</sup>. In their study, Almeida-Filho *et al.* 2017 found an association between the extent of VITD deficiency in post-menopausal women with breast cancer and tumours with worse prognosis i.e., high grade, locally advanced, metastatic, ER (-) and PR (+ve) tumours<sup>51</sup>. The authors suggested that low serum VITD is a risk factor for ER (-) tumours with axillary nodes and high cellular proliferation. In their study, Cheney *et al.* 2018 in a follow-up study of 7 years, however, observed no significant relationship between serum VITD levels and cancer risk<sup>52</sup>. Moreover, Charehbili *et al.* 2016 found significantly decreased serum VITD levels during post-neoadjuvant chemotherapy<sup>53</sup>. However, basal and end of treatment VITD levels were not related to the pathological response following neoadjuvant chemotherapy. Similar data were provided by<sup>48</sup>.

The development and progression of breast cancer is dependent not only on the intrinsic properties of cancer cells but also on

microenvironmental factors<sup>54</sup>. Studies indicated that the inflammatory cytokine TNF- $\alpha$  have an important role in tumour growth and metastasis, via inducing proangiogenic factors and matrix metalloproteinases<sup>54,55,56</sup>. Our results are in agreement with other studies indicating increased serum TNF- $\alpha$  in breast cancer patients<sup>57,58</sup>. Saglam *et al.* 2009 found that plasma TNF- $\alpha$  levels were higher in ERBB2+ breast cancer patients than in controls<sup>57</sup>. Ma *et al.* 2017 reported significantly higher serum TNF- $\alpha$  levels in stage III breast cancer patients than in controls. Serum TNF- $\alpha$  was associated with lymph node metastasis<sup>58</sup>.

The matrix-degradative enzymes, matrix metalloproteinases (MMPs) have been implicated in cancer progression and metastasis<sup>59,60</sup>. MMP-9 (also -1, -12 and -15) is significantly elevated in high-grade as compared with low-grade tumors and high expressions of MMP-9 (also -1, -12, -14 and -15) associates with poor overall survival<sup>61</sup>. In the present study, we found significant increase in serum MMP-9 in both pre- and post-menopausal patients with either non-metastatic or metastatic cancer with significantly higher values in those with metastatic disease. Similar data were provided by Rashad *et al.* 2013<sup>29</sup>.

Angiogenesis is the formation of new blood vessels and is a prerequisite for tumour growth and dissemination. Vascular endothelial growth factor (VEGF) is an important regulator of the growth and proliferation of blood vessels<sup>62,63</sup>. Adams *et al.* 2000 found that patients with metastatic disease had higher plasma and serum VEGF levels compared to normal controls<sup>64</sup>. Samy *et al.* found that serum VEGF were increased in patients with breast cancer compared to controls<sup>65</sup>. Serum VEGF increased in advanced stages and in ER (+ve) patients. Byrne *et al.* found that plasma VEGF levels were increased in pre-menopausal patients with early breast cancer compared to controls<sup>66</sup>. The authors, found no correlation between plasma and intra-tumoural VEGF. Studies, however, suggested that clinicopathological prognostic parameters and tumour microvessel density were not affected by circulating VEGF levels<sup>64,65</sup>. Moreover, Bachelot *et al.* 2003 reported that serum and plasma levels of VEGF are not useful indicators for prognosis in patients with metastatic breast cancer<sup>67</sup>. Our study found significant increase in serum angiogenic factor VEGF in both

non-metastatic and metastatic cancer patients with significantly higher values in those with metastatic disease. This suggests that the level of VEGF in serum increases in advanced disease. We found no difference, however, in serum levels of VEGF in relation to menopausal status of patients. We also observed significant positive correlation between serum VEGF and the inflammatory cytokine TNF- $\alpha$  in pre-menopausal patients with non-metastatic or metastatic disease and in post-menopausal patients with non-metastatic cancer, suggesting a link between the inflammatory response and the level of VEGF in serum. On the other hand, there was also significant association between serum VEGF and MMP-9 in non-metastatic cancer <sup>67</sup>.

Hepatocyte growth factor (HGF) is a potent stimulator of hepatocyte growth and also a tumour-disseminating factor <sup>68</sup>. Yang *et al.* 2015 reported high expression of HGF in breast cancer cells which was related to lymph node metastasis, prognosis and also to the sensitivity to chemotherapy <sup>69</sup>. Significant increase in plasma HGF levels was found both in patients with benign breast lumps and in those with breast cancer patients when compared to controls <sup>70</sup>. We also found significant increase in HGF in serum of patients with non-metastatic or metastatic cancer. Higher levels of HGF were observed in patients with metastatic disease. There was, however, no difference in serum HGF levels in relation to menopausal status. Moreover, a negative correlation was present between serum VEGF and HGF in patients with metastatic disease post-menopausal.

In the present study, patients with metastatic breast cancer disease (pre- and post-menopausal) were further evaluated according to their progesterone receptor (PR) status. It is estimated that about 50% of breast cancer patients express PR and which parallels ER expression <sup>71,72</sup>. We found no significant differences between PR -ve and PR +ve patients for the alterations in the oxidative, inflammatory, angiogenic biomarkers in serum of breast cancer patients.

In summary, the present study in patients with breast cancer suggests that vascular and inflammatory markers VEGF, HGF, MMP-9 and TNF- $\alpha$  were increased in serum in advanced stages and could monitor disease progression and/or disease severity.

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None

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