

Oxidant / antioxidant parameters in breast cancer patients and its relation to VEGF, TGF- β or Foxp3 factors

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Abstract: Background: Oxidative stress has been suggested as an important factor for initiation and progression of human breast cancer. The development and dissemination of solid tumors depends upon the formation of a vascular network to supply oxygen and nutrients (angiogenesis) that may be regulated by oxidative stress. The data regarding the status of oxidant / antioxidant parameters in high risk and breast cancer patients and its relation to VEGF, TGF- β or Foxp3 limited and conflicting. The aim of this study was to investigate the alterations in some oxidant and antioxidant enzyme defenses in the blood of patients with malignant breast tumor and benign breast disease. The relationship between oxidant/ antioxidant parameters and serum VEGF, TGF- β and Foxp3 levels among patients with benign and malignant breast disease were also evaluated. **Method:** Ninety female serum samples of invasive breast carcinoma (all are grade II), high risk patients with benign breast lesions (fibroadenoma) patients and healthy control groups were analyzed for measurement of glutathione reduced (GSH), catalase (CAT), superoxide dismutase (SOD), Malondialdehyde (MDA) as well as nitric oxide (NO) levels were analyzed spectrophotometrically. The serum vascular endothelial growth factor (VEGF), transforming growth factor beta (TGF β) concentrations and human Fork head Box Protein p3 (Foxp3) levels were measured using ELISA technique. **Result:** Statistically, a highly significant decrease ($p < 0.001$) in the serum levels of reduced glutathione (GSH) and catalase (CAT) were recorded in the high risk and breast cancer groups. Elevation in the serum superoxide dismutase (SOD), Malondialdehyde (MDA) and nitric oxide (NO) were also reported in both groups. These changes were more pronounced in the breast cancer group than the high risk group as compared to the control group values. Meanwhile, VEGF, TGF- β and FOXP3 revealed significantly elevated levels in the invasive breast carcinoma and high-risk groups as compared to healthy control group ($p < 0.001$) that was more detectable in patients with breast carcinoma. Furthermore, a significant correlation between the serum levels of reduced glutathione (GSH), catalase (CAT), superoxide dismutase (SOD), Malondialdehyde (MDA) and nitric oxide (NO) with VEGF, TGF- β and FOXP3 levels were observed. **Conclusion:** Reduction of most antioxidant levels except for SOD levels that may be a compensatory up regulation in response to elevation of oxidative stress especially in breast cancer patients were paralleled by high levels of VEGF, TGF-B and Foxp3 with different degrees in high risk and breast cancer patients. Oxidant/antioxidant changes may be promising biomarkers which play an important role for the early detection of breast cancer.

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1. Introduction

Breast cancer is the most frequent malignancy in females with approximately 9% of worldwide cancer deaths. Breast cancer risk assessment research has been ongoing for decades. Large population studies have enabled scientists and clinicians to identify risk factors that influence a woman's risk of being diagnosed with breast cancer (Spaeth, 2018). With all the accumulated evidence of breast cancer risk, several

obstacles were still documented in implementing appropriate screening measures for high-risk women.

Reactive oxygen species (ROS) are produced by living organisms as a result of normal cellular metabolism and environmental factors, such as air pollutants or cigarette smoke (Birben *et al.*, 2012). ROS are highly reactive molecules that can damage cell structures such as carbohydrates, nucleic acids, lipids, and alter their functions (Rahal, 2014). Regulation of reducing and oxidizing (redox) state is

critical for cell viability, activation, proliferation, and organ function. The shift in the balance between oxidants and antioxidants is termed "oxidative stress", can lead to DNA damage, lipid peroxidation and may be involved in the initiation and greatly linked to the progression of breast cancer (**Federico et al., 2007** and **Acharya et al., 2010**).

Although the incidence of breast cancer is increasing, knowledge about its early detection, progression and spread is inadequate. Despite the recent advances in surgery, chemotherapy and radiotherapy the disease continues to be the second largest killer in females (**Yeh et al., 2005**). The basic difficulty in treatment is its late detection and rapid spread to other organs. The present clinical approaches for cancer diagnosis and management are useful only in advanced stages of disease (**Feng et al., 2012**). Thus, the searching for a relation between some biochemical parameters such as oxidant and antioxidant enzymes and other used techniques for diagnosis may be helpful as a biomarker that could help for the early detection of breast cancer patients.

Reactive oxygen metabolites (ROM), including superoxide anion (O_2^-) hydrogen peroxide (H_2O_2) and hydroxyl radical ($\cdot OH$), play an important role in carcinogenesis. On the other hand, some antioxidants can protect the cellular defense mechanisms and molecular damage caused by the ROM such as catalase (CAT), glutathione peroxidase and superoxide dismutase (SOD) (**Ray et al., 2000** and **Prabhasheela, 2011**).

Human erythrocytes possess a NO synthase (NOS) that can be activated by oxidative stress to produce nitric oxide (NO) (**De Caterina et al., 1995**). Nitric oxide (NO) is a multifunctional gaseous molecule synthesized from L-arginine that can produce a free radical which acts as a signaling molecule in the body. It is a highly toxic molecule which related to angiogenesis and tumor progression plays a central role in cell migration in metastasis and dissemination of cancer. NO used as prognostic and diagnostic factors and it may be used in the strategies for treatment and control the cancers like breast cancer. (**Ahmad and Sah, 2017**).

In tumor cells NOS activity has been associated with tumor grade, proliferation rate and expression of important signaling components associated with the initiation of cancer. The level of NOS can be affected on the tumor cells, it has genotoxic and angiogenic properties, the high levels of NOS expression may be cytotoxic for tumor cells, whereas low-level activity can promote tumor growth by up regulating VEGF and p53 (**Ahmad and Sah, 2017**). Nitric oxide (NO) has been at the center of multiple contradictory findings regarding its role in cancer biology. With greater understanding, it is well established that the

biphasic effects of NO are concentration dependent (**Basudhar et al., 2016**). The cellular source, the duration of exposure and the amount of NO are critical factors for its suppressive as opposed to its stimulatory capacity, particularly in the complex microenvironment of solid tumors (**Mills, 2001**).

The oxidation of lipids induces the formation of aldehydes and lipid peroxides (Malondialdehyde or MDA). These molecules in high concentrations are considered the more damaging species because they easily react with proteins, DNA and phospholipids, that lead to the propagation and amplification of oxidative stress (**Federico et al., 2007**). MDA is low molecular weight aldehyde that can be produced from free radical attack on polyunsaturated fatty acids. Increased plasma MDA levels have been reported in breast cancer by several studies (**Gönenç et al., 2006**; **Pande et al., 2011**; **Kilic et al., 2014**).

Antioxidants are chemicals that known as "free radical scavengers" and preventing them from causing damage (**Valko et al., 2007**). Antioxidants prevent free radical induced tissue damage by preventing the formation of radicals, scavenging them, or by promoting their decomposition (**Bouayed and Bohn, 2010**). Glutathione reduced (GSH) is produced naturally by the liver and considered the most abundant intracellular thiol that preventing damage to important cellular components caused by reactive oxygen species such as free radicals and peroxides. In healthy cells and tissue, more than 90% of the total glutathione pool is in the reduced form (GSH) and less than 10% exists in the disulfide form (GSSG). An increased GSSG-to-GSH ratio is considered indicative of oxidative stress. Low glutathione is commonly observed in cancer HIV/AIDS and high oxidative stress (**Ithayaraja, 2011**).

The catalase enzyme is an endogenous antioxidant enzyme that neutralizes reactive oxygen species by converting H_2O_2 into water and oxygen (**Young and Woodside, 2001**). Together with other antioxidant enzymes, including superoxide dismutase and glutathione peroxidase, catalase is a primary defense against oxidative stress.

Meanwhile, SOD was the first characterized antioxidant enzymes (**McCord and Fridovich, 1969**). The superoxide dismutase family contains three members (Sod1, Sod2 and Sod3) that protect cells and/or tissues against intracellular or extracellular ROS damage. However, the role of Sod family members has not been well studied in cancer, and where it has been studied, the roles are controversial. Some studies showed a protective role against chemical or hormone-induced tumor formation (**Kim et al., 2005**). In contrast, other studies have demonstrated that expression of Sod maintains the metabolic activity of cancer cells when they are detached from the

extracellular matrix and that it increases tumor promotional nuclear factor (NF κ B) signaling (**Davison et al., 2013**). SOD are able to catalyze the detoxification of superoxide anion (O₂) and protect the cell against ROS-induced damage and catalyzes the dismutation of superoxide to hydrogen peroxide. The hydrogen peroxide must then be removed by catalase or glutathione peroxidase. SOD might play a role in the regulation of vascular tone, because endothelial derived relaxing factor (nitric oxide) is neutralized by superoxide (**Young and Woodside 2001**). SOD also acts as anti-carcinogens and inhibitors of the initiation and promotion /transformation stage in carcinogenesis (**Prabasheela et al., 2011**). Vascular endothelial growth factor (VEGF) is one of the most potent promoters of angiogenesis and vascular permeability in breast cancer. Likewise, the immune-modulating cytokine transforming growth factor beta1 (TGF- β 1) is dominantly viewed as a critical factor in the regulation of cell differentiation and induction T regulatory cells (Tregs) that help tumor progression. As the transcription factor fork head box protein P3 (FOXP3) is the prominent marker for Treg activity, it might be an indicator of breast tumorigenesis (**Chen et al., 2013**). The present study was designed to investigate the alterations in some oxidant and antioxidant enzyme defenses in the blood of patients with malignant breast tumor and benign breast disease. The relationship between oxidant/ antioxidant parameters and serum VEGF, TGF- β and Foxp3 levels among patients with benign and malignant breast disease were also evaluated.

2. Materials and Methods

This study was carried out on ninety female serum samples as follows: 30 samples of invasive breast carcinoma (grade II) along with equal number of samples from high risk patients with benign breast lesions (fibroadenoma) and 30 samples of healthy females were also recruited as control. Patient samples were collected at the Egyptian National Cancer Institute (NCI), Cairo University during the routine medical care of the patients after informed verbal consent. Diagnosis was carried out by experienced oncologists that made according to the World Health Organization (WHO) Classification of Tumors, combining history and clinical examination. Blood samples were obtained by venipuncture from peripheral blood of females. Samples were collected in a sterile, heparinized Falcon tubes that allowed clotting for half an hour and centrifuged at 12000 rpm for 15 min at room temperature. The sera were separated and stored at -80°C until the time of analysis.

Biochemical measurements

Standard procedures were adopted for the estimation of GSH, CAT, SOD, MDA and NO levels in the current work by using kits provided by Bio-diagnostic company, Egypt.

Antioxidants

Reduced glutathione (GSH) content was determined using Ellman's reagent according to the method described by **Tietze (1969)** and expressed as nmol/100mgprotein. Measurement of catalase (CAT) activity in serum was performed according to **Aebi (1984) and Fossati et al., (1983)** where catalase reacts with a known quantity of H₂O₂. However, SOD activity was determined as described by **Nishikimi et al., (1972)**. Oxidation of Hydroxylamine Hydrochloride was used to generate the superoxide anion. This anion reduces nitro-blue-tetrazolium (NBT) to formazan, which is monitored at 560 nm. SOD of the sample removes the superoxide anion and inhibits the reduction. The level of this reduction was used as a measure of SOD activity.

Oxidative damage assay

Oxidative damage in the serum of patients and healthy controls was assessed by measuring LPO products in serum using the thiobarbituric acid (TBA) method (**Ohkawa et al., 1979**). MDA, which is a stable end- product of fatty acid peroxidation, reacts with TBA under acidic conditions to form a complex that has a maximum absorbance at 532 nm. However, Serum nitric oxide (NO) was determined spectrophotometrically at 540 nm using Griess reagent after reduction of nitrate to nitrite by vanadium trichloride (**Montgomery and dymock, 1961**) and expressed in serum as μ mol/l.

ELISA methods

Human VEGF and TGF- β levels were determined by enzyme linked immunoassay technique using quantitative kit manufactured by e-Bioscience Bender Med-systems GmbH, Austria and DRG Instruments GmbH, Germany Division of DRG International, Inc Frauenbergstr respectively. However, the human Fork head Box Protein p3 (Foxp3) levels were measured according to the manufacturers protocol of ELISA kit provided by Glory Science co.

Statistical analysis

All data were presented as a mean \pm standard error. Data analysis was performed with one-way ANOVA using SPSS (Version 23). Post hoc test was used to assess differences between means. A significant difference for all statistical analysis in this study was considered at the level of $P \leq 0.05$ and high significantly different at the level of $P \leq 0.001$ as compared to the control group.

3. Results

This study was conducted on three groups; invasive breast carcinoma (grade II) group (n=30), high risk patients with benign breast lesions (neoplastic fibrocystic atypical hyperplasia disease) group (n=30) and control group (n=30). The females included in this study were aged from 36-48 years old. The patients were diagnosed, and followed-up in the National Cancer Institute, Cairo University, Egypt.

Evaluation of serum oxidant and antioxidant enzyme activities

Statistically, a highly significant decrease ($p < 0.001$) in the mean value of serum GSH and catalase levels were recorded in the high risk and breast cancer groups as compared to the control group values (Table 1 and Fig. 1). The decrease in these parameters was more pronounced in the breast cancer group than the high risk group where the GSH level

was 3.22 ± 0.07 and 5.98 ± 0.19 respectively. However, the catalase levels in the breast cancer group and the high risk group were 3.37 ± 0.08 and 4.27 ± 0.09 respectively.

Table (1): Serum GSH and CAT levels in the control and the different studied groups.

Groups	GSH	CAT
control	9.2 ± 0.19	5.1 ± 0.11
High risk	$5.98 \pm 0.19^{**}$	$4.27 \pm 0.09^{**}$
Breast Cancer	$3.22 \pm 0.07^{**}$	$3.37 \pm 0.08^{**}$

Values are expressed as mean \pm SE of 30 female per group.

* means significantly different as compared to the control group at $P \leq 0.05$

** Means high significantly different as compared to the control group value of $P \leq 0.001$.

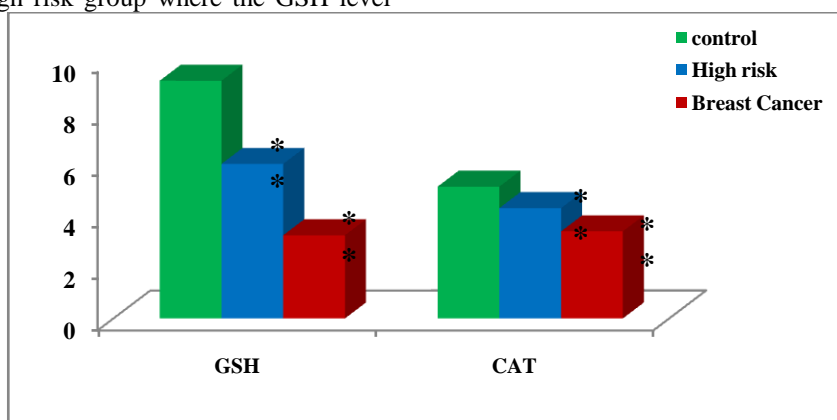


Fig (1): The mean values of serum reduced glutathione (GSH) and catalase (CAT) in the control and different studied groups.

* means significantly different as compared to the control group at $P \leq 0.05$

** Means high significantly different as compared to the control group value of $P \leq 0.001$.

Spectrophotometrically, the mean value of serum SOD levels were significantly elevated in both of the high risk and breast cancer groups as represented in fig. (2). This elevation was remarkably shown in the

breast cancer group ($194.5 \pm 4.3^{**}$). Meanwhile, the high risk patient also recorded a significant increase in the mean value of serum SOD level (83.5 ± 22.58) when compared to control group ($p < 0.001$).

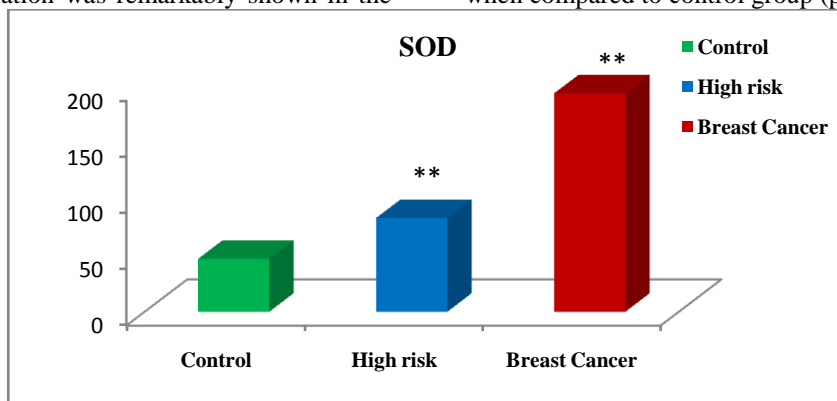


Fig (2): The mean values of serum superoxide dismutase (SOD) in the control and different studied groups.

* means significantly different as compared to the control group at $P \leq 0.05$

** Means high significantly different as compared to the control group value of $P \leq 0.001$

The serum Malondialdehyde (MDA) level in the three studied groups by using spectrophotometer measurement were demonstrated in **table (2)** and **fig. (3)**. Breast cancer patients recorded a highly significant increase ($p < 0.001$) in the mean value of serum MDA level (16.66 ± 0.46) as compared to the control group. Moreover, the high risk patients recorded also a highly significant increase ($p < 0.001$) in the MDA levels (12.35 ± 1.79) when compared to the control group but lesser than the breast cancer group values.

Table (2): Serum MDA and NO levels in the control and the different studied groups.

Groups	MDA	NO
control	9.3 ± 0.34	47.34 ± 1.2
High risk	$12.35 \pm 0.32^{**}$	$84.36 \pm 2.6^{**}$
Breast Cancer	$16.66 \pm 0.46^{**}$	$194.63 \pm 2.3^{**}$

Values are expressed as mean \pm SE of 30 female per group.

* means significantly different as compared to the control group at $P \leq 0.05$

** Means high significantly different as compared to the control group value of $P \leq 0.001$.

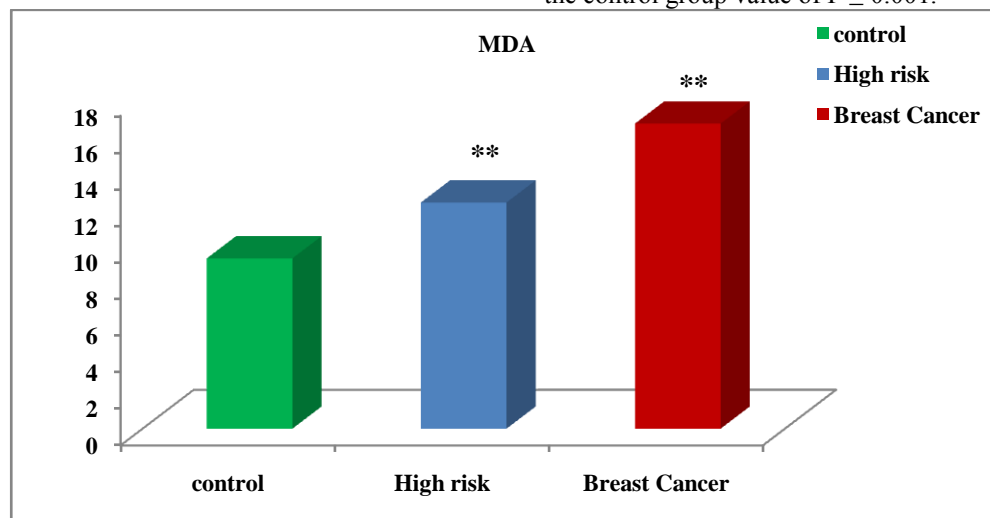


Fig (3): The mean values of serum Malondialdehyde (MDA) in the control and different studied groups.

* means significantly different as compared to the control group at $P \leq 0.05$

** Means high significantly different as compared to the control group value of $P \leq 0.001$.

On the other hand, breast cancer patients revealed a high significant increase ($p < 0.001$) in the mean value of serum NO level (194.63 ± 2.3) when compared to high risk and control groups. Moreover,

the high risk group also recorded a high significant elevation ($p < 0.001$) in the mean value of serum NO level (84.36 ± 2.6) when compared to control group as shown in **table (2)** and **Fig. (4)**.

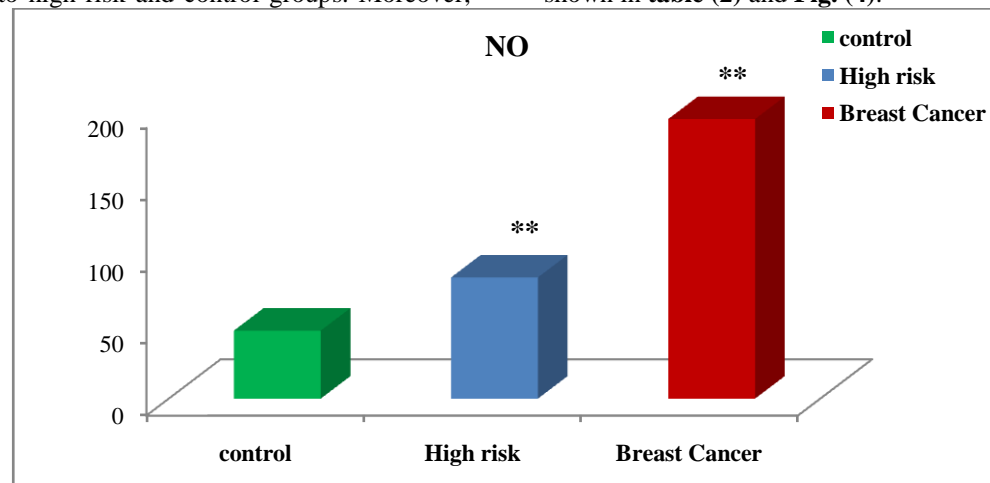


Fig (4): The mean values of serum nitric oxide (NO) in the control and different studied groups.

* means significantly different as compared to the control group at $P \leq 0.05$

** Means high significantly different as compared to the control group value of $P \leq 0.001$.

By using ELISA technique, breast cancer patients recorded a highly significant increase in the mean value of serum Foxp3, TGF- β and VEGF protein levels as compared to both of the high risk and the control group ($p < 0.001$). Moreover, A highly

significant elevation ($p < 0.001$) in the mean value of these parameters were also recorded in the high risk group as compared to the control group ($p < 0.001$) as revealed in table (3) and figs (5 and 6).

Table (3): Foxp3, TGF- β and VEGF levels using ELISA technique in the different studied groups

Groups	Foxp3	TGF- β	VEGF
Control	0.73 \pm 0.06	64.81 \pm 4.4	75.3 \pm 0.97
High risk	1.73 \pm 0.05 **	191.6 \pm 3.9**	292.6 \pm 3.7**
Breast cancer	3.65 \pm 0.11 **	407.4 \pm 4.3 **	1013.2 \pm 134.7**

Values are expressed as mean \pm SE of 30 female per group.

* means significantly different as compared to the control group at $P \leq 0.05$

** Means high significantly different as compared to the control group value of $P \leq 0.001$.

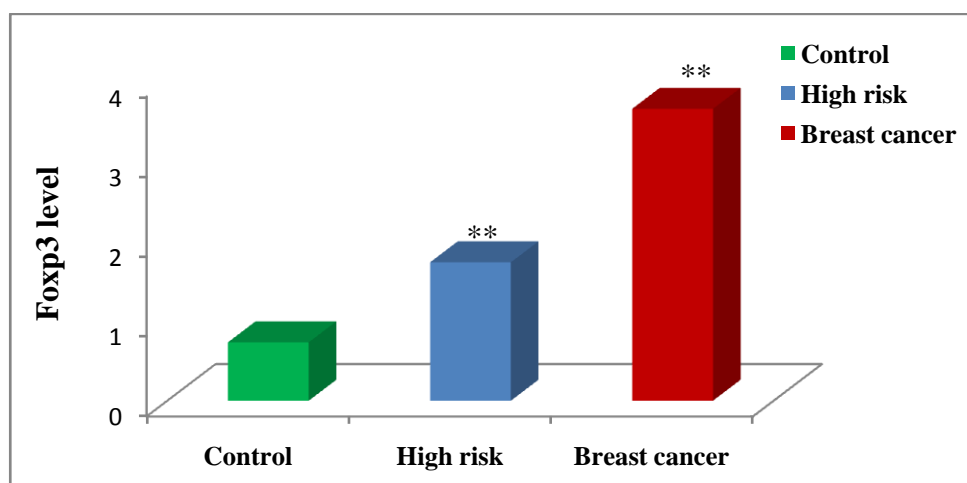


Fig (5): Foxp3 levels using ELISA technique in the different studied groups.

* means significantly different as compared to the control group at $P \leq 0.05$. ** Means high significantly different as compared to the control group value of $P \leq 0.001$

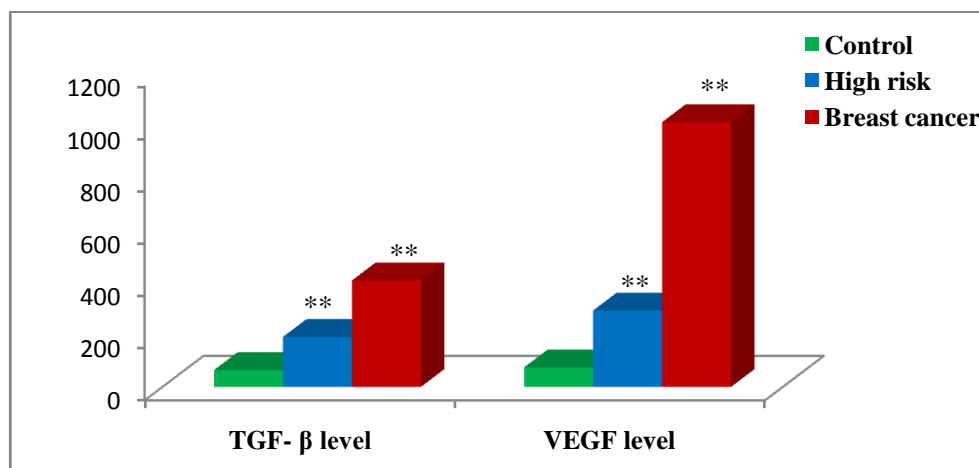


Fig (6): TGF- β and VEGF levels using ELISA technique in the different studied groups.

* means significantly different as compared to the control group at $P \leq 0.05$

** Means high significantly different as compared to the control group value of $P \leq 0.001$

As shown in fig (7), similar sensitivity for the determination of NO and SOD spectrophotometrically was demonstrated for the patient diagnosis in all studied groups. However, the rate of homology was 93.9 between the parameters and detection of Foxp3 levels using ELISA technique. MDA measurement formed a very close clade with the SOD, NO and Foxp3 levels detected by ELISA representing a great homology that was approximately 89.4 % similarity levels.

VEGF and TGF-B showed a degree of similarity measured by 86.4 and 79.3 respectively. However, the others parameters formed clades with lesser degree of similarity (fig.7). A significant conclusion can be drawn from the figure demonstrated that the measurement of oxidant parameters (NO, SOD and MDA) spectrophotometrically were in great similarity to detection of Foxp3 using ELISA technique that could be of great significance to early diagnosis of breast cancer.

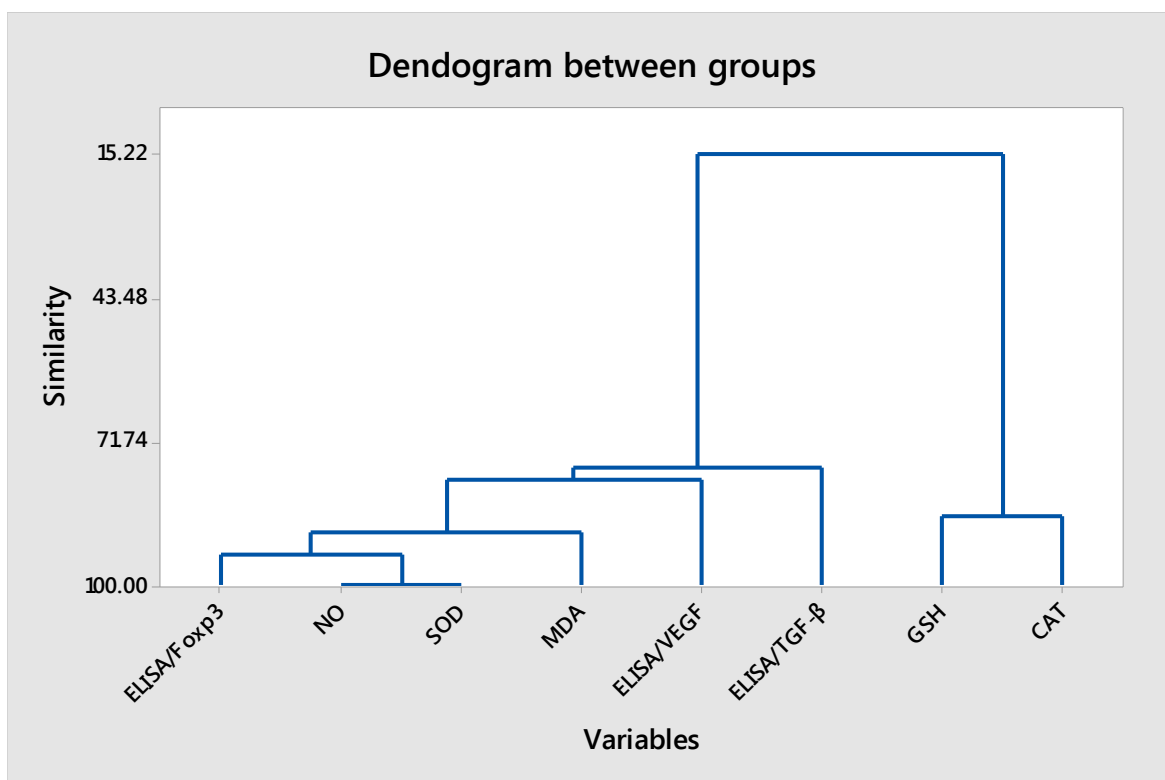


Fig (7): Dendrogram with homologous using Minitab constructed with oxidant/ antioxidant parameters and VEGF, TGF-B Foxp3 using ELISA technique in different studied groups.

4. Discussion

The risk of breast cancer is multifactorial and is an interaction between environmental, lifestyle, hormonal, and genetic factors (Spaeth, 2018). Benign breast disease is more prevalent than malignancy of the breast and Prevalence rate is 68% among all breast disease and 6.9% among all diseases of women. Majority of them require treatment in their life time (Vijayalakshmi *et al.*, 2016). Our results suggest that some biomarkers can be used as indicator of prognostic in patients with breast cancer.

Formation of reactive oxygen species is a normal consequence of variety of essential biochemical reactions and an adequate range of anti-oxidative defenses within and outside the cell has also been considered very important to offer protection against oxidative damages (Sun, 1990). Therefore, balance

between free radical activity and anti-oxidant defense system becomes an important requirement to prevent the damage to the cellular membrane (Yeolekar and Nargund, 1994). Damage to DNA, proteins, cell membrane and mitochondria play an important role in breast carcinogenesis. Although the incidence of breast cancer is on increase; knowledge about its early detection, progression and spread remains inadequate. No specific biochemical marker has been identified yet.

In view of this, the availability of suitable blood-based biochemical markers would be of immense help for diagnosis of breast cancer. Previous investigators in this area have identified disturbances in the levels of many oxidant and antioxidant parameters however remain inconclusive and contradictory (Huang *et al.*, 1991 and Punnonen *et al.*, 1994). The present study

were undertaken with the purpose of finding and describing the changes, if any, in serum levels of some oxidant and antioxidant enzymes in the blood of high-risk in comparison to breast cancer patients and healthy control subjects.

The sensitivity of the cell to ROS is attenuated by an array of enzymatic and nonenzymatic antioxidants. This antioxidant system includes both endogenous and exogenous and enzymatic and non-enzymatic antioxidants. Glutathione (GSH), is a tripeptide and the major endogenous antioxidant produced by the cells, which helps to protect cells from ROS such as free radicals and peroxides (**Pompella et al., 2003**). It is now well established that ROS and electrophilic chemicals can damage DNA, and that GSH can protect against this type of damage (**Valko et al., 2007**).

In the present study, the level of GSH in the high risk and breast cancer patient's serum were significantly decreased compared to the control group with a considerable difference between both of high risk and breast cancer GSH levels. The decrease in GSH concentration can be explained by the decreased GSH synthesis and/or increased GSH consumption in the removal of peroxides and xenobiotics as reported by **Yeh et al., (2006)**. The lower GSH levels in patients with breast cancer support the hypothesis that glutathione status is inversely related in malignant transformation **Kumaraguruparan et al., (2002)**. Several studies have reported decreased levels of GSH in the blood of patients with breast cancer compared to control subjects (**Lamari et al., 2008**).

Our results come in accordance with **Shams et al., (2012)** who also revealed a significant decrease of reduced glutathione (GSH) in both benign and malignant tumor cases in relation to the control group. In contrast, levels of reduced glutathione were found to be elevated in malignant lesions by **Mishra et al., (2004)**. On the other hand, **Kumaraguruparan et al., (2002)** reported similar significant reduction of the concentrations of plasma glutathione, as well as levels of antioxidant enzymes such as SOD, catalase, glutathione peroxidase, glutathione-transferase in both fibroadenoma and adenocarcinoma patients compared to control subjects with tendency of blood GST to decrease with cancer progression.

Catalase (CAT) enzyme is considered as a primary antioxidant enzymes, since it act as anti-carcinogens and inhibitors at initiation and promotion/transformation stage in carcinogenesis (**Halliwell and Gutteridge, 1985**). In the present study, there is a significant decrease in the serum mean value of CAT in the high-risk and breast cancer patients when compared to the control group. This decrease was more pronounced in the breast cancer group than the high risk group. These results are in

line with **Yuksel et al., (2007)** that confirmed the presence of a significant decrease in CAT activities in patients with fibroadenoma and fibrocystic disease than the control group. On the other hand, they also showed that no significant association between CAT activities and different stages of the malignant tumors.

Furthermore, **Sinha et al., (2009)** and **Kasapović et al., (2010)** reported that the decrease in catalase activity enhance the free radical activity in breast cancer patients and weak defense mechanism. However, **Tas et al., (2005)** reported that CAT activity was lower in breast cancer tissue than in the control tissue and related this to the role of CAT in detoxifying H_2O_2 into H_2O completely that might be accumulated in breast cancer tissue and resulting in higher production of the OH^- radical, which was supported by the higher production of MDA contents.

Superoxide dismutase (SOD) was the first characterized antioxidant enzymes (**McCord and Fridovich, 1969**). It is considered as a primary antioxidant enzyme; since it involved in direct elimination of reactive oxygen metabolites so act as anti-carcinogens and inhibitors of carcinogenesis (**Prabhasheela et al., 2011**). In the present study, the mean value of serum SOD levels recorded a highly significant increase in the breast cancer and high risk groups as compared to the control group.

Our results are in consistent with **Koksoy et al., (1997)** and **Ray et al., (2000)** who reported significantly increased SOD activities in benign and malignant breast diseases. It was believed that higher H_2O_2 production in BC patients might be due to higher production O_2^- and elevated SOD activity. The results of this study suggested that reactive oxygen metabolites may play a pathogenetic role in both benign and malignant tumor development, which is reflected by the change in SOD activity.

On the contrary, **Sinha et al., (2009)** and **Prabhasheela et al., (2011)** found that SOD activity were lower in breast cancer patient's when compared to control. They have demonstrated in their study that the reduction in SOD activities increases the toxic effect of O_2 and might lead to cellular damage. However, the low activity of SOD was in accordance with the previous record by **Kumaraguruparan et al., (2002)** who reported an enhanced lipid peroxidation with concomitant depletion of antioxidants in breast cancer patients as compared to normal subjects and patients with fibroadenoma similar pattern of changes was seen in fibroadenoma (high risk) patients as compared to the corresponding normal subjects. This study has revealed an imbalance in the redox status in patients with fibroadenoma and breast cancer patients.

In recent study, **Qebesey et al., (2015)** also reported that SOD activity was decreased in all grades of breast cancer tissues in human when compared to

the control, and attributed this to the increase in lipid peroxidation that accompanied by decreased antioxidant status. However, **Yuksel et al., (2007)** reported that the SOD activities between among the controls and the patients with malignant and benign tumors were not statistically significant. The specific SOD activity did not differ between controls and the patients with fibroadenoma or fibrocystic disease. Furthermore; there was no significant correlation among the SOD activities and the tumor stages. Meanwhile, **SadatiZarrini et al., (2016)** demonstrated that there was no evidence of remarkable discrepancy in the status of glutathione peroxidase (GPX), catalase (CAT) and superoxide dismutase (SOD) levels in various stages of breast cancer. It seems that the severity of oxidative stress in different stages of breast cancer is similar to some extent.

Interestingly, the current study showed that the expression of TGF-B, VEGF and FOXP3 were positively correlate with SOD levels in benign and breast cancer patients that agreed with **Yu et al., (2008)** who revealed a positive relationship between VEGF-C and Sod3 in breast tumors. Furthermore, **Wang et al., (2014)** determined the mechanism by which VEGF regulates the response to oxidative stress, via regulation of Sod3 expression. Importantly, Sod3 downstream of VEGF is required for tumor growth and metastasis in the mammary orthotopic xenograft model, providing evidence that VEGF mediates breast cancer metastasis in part through regulating ROS. It is possible that VEGF stimulates antioxidant effects via regulating several redox-related enzymes. Thus, these studies further support our findings and suggest that the increased levels of SOD may be relevant to increased SOD3 levels that needs further studies.

Damage due to oxidative stress and free radicals is one of the important factors in carcinogenesis. So we considered it is important to analyze the tissue levels of lipid peroxides in the term of MDA and level of free radicals in the term of NO in breast cancer patients to assess their role in cancer development and progression.

Malonaldehyde (MDA) is one of the most important oxidative stress markers that have been identified in breast cancer. MDA has been used extensively as a marker of lipid peroxidation, which considered a significant endogenous source of DNA damage and mutation that contribute to human genetic disease (**Lykkesfeldt, 2007**). MDA is claimed to be an inhibitor to protective enzymes. Hence, it could have both mutagenic and carcinogenic effects. It is also implicated as a key molecule in DNA adducts formation (**Shams et al., 2012**).

In the current study, breast cancer patients recorded a highly significant increase in the MDA levels compared to the control group. These results were consistent with **Ray et al. (2000) and Tas et al., (2005)**. They reported increased plasma MDA concentration in the breast cancer patients and as a result cellular membrane degeneration and DNA damage occurred. Meanwhile, **Qebesey et al., (2015)** and **Hussain and Ashafaq, (2018)** found that the MDA level was increased gradually from Stage I to Stage IV in breast cancer patients. On the contrary, **Gerber et al., (1989)** and his colleagues reported decreased plasma MDA concentration in BC patients, these findings suggest a decreased lipid per-oxidation in BC patients. Moreover, the high risk patients recorded also a highly significant increase in the MDA levels when compared to the control group but lesser than the breast cancer group values. This come in accordance with **Shams et al., (2012)**. They detected higher plasma and erythrocyte MDA levels in patients with benign and malignant breast tumors in comparison to the healthy group.

Presentation of nitric oxide in human serum is a well-known phenomenon that points to a crucial role of nitric oxide in physiological and pathological processes. It exhibits a dual role, with regard to the complex mechanism of tumor invasion and metastasis. It could either mediate tumorocidal activity or promote tumor growth (**Gönenç et al., 2006**). Its presence has been assessed in various human malignant tumors (**Jansson et al., 1998**).

In the present study, breast cancer patients revealed a high significant increase in the serum NO level when compared to high risk and control groups. These results were in concordance with that reported by **Switzer et al., (2011) and Amin et al., (2012)** as they demonstrated the presence of high levels of NO production in the breast cancer patients as nitric oxide promotes cancer progression by activating several oncogenic signaling pathways such as extracellular signal-regulated kinases and phosphoinositide3-kinases. Moreover, **Qebesey et al., (2015)** also found a significant increase in the tissue levels of NO in all grades of breast carcinoma when compared to the healthy control that positively correlated to the tumor grade.

On the other hand, the high risk group in our study also recorded a high significant elevation in the serum NO level when compared to the control group. However, these values still significantly lower than the No values in the breast cancer group. Interestingly, the current study revealed a great relationship between the changes in the No levels and the levels of VEGF, TGF-B and Foxp3 in the different studied groups. This could be explained by that nitric oxide produced in tumors has been implicated in

enhanced vascular permeability, and increased tumor blood flow (**TamirTannenbaum, 1996**) that could reflect the increase in the VEGF and TGF-B. Moreover, **Ambs and Glynn (2011)** confirmed the release of variable amounts of NO into the tumor microenvironment that could stimulate the tumor micro-vascularization.

Meanwhile, several lines of evidence suggest an important role for NO in the generation and function of Tregs as CD⁴⁺CD²⁵⁺/Foxp3⁺ adaptive Tregs (**Tang et al., 2006**) that may reflect possible effect of NO on the Foxp3⁺. On the other hand, **Olson and Garbán (2008)** examined the effect of NO in the regulation of FOXP3 in human breast cancer and hypothesized that NO might suppress the expression of FOXP3 mRNA by interfering with the GR/ER-dependent transcriptional activation of FOXP3 promoter.

Higher production of NO and enhanced lipid peroxidation in breast tumors observed in the present study may be attributed to overproduction of ROS. High levels of ROS have been reported to damage many biomolecules and exert diverse cellular and molecular effects including mutagenicity, cytotoxicity, and changes in gene expression that lead to initiation and promotion of carcinogenesis (**Rayand Husain, 2002**). Moreover, ROS have been found to modulate signaling events in the cell and play a functional role in the pathogenesis of malignancy, including breast cancer.

5. Conclusion

In conclusion, the present study suggest a functional interplay among oxidative stress, antioxidants, and VEGF, TGF-B and Foxp3 with different degrees in patients with benign and breast cancer. Our study can be useful to establish blood based biochemical index for diagnosing and monitoring the course of breast cancer. It may be more reliable to study the changes in the serum than the tissue in both cancerous and normal cells. Evaluation of such markers would certainly be useful and supportive for investigations in precancerous and cancerous condition. Further oxidant/antioxidant changes must be traced relatively to cancer grade in addition to conventional methods of breast cancer diagnosis.

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