Even if you are on the right track, you will get run over, if you just sit there.

-Will Rogers
Significance of the study:
The incidence of breast cancer is increasing worldwide. Therefore, it is the present day concern of women all over the world. According to the recent statistics, an increasing trend for breast cancer incidence is also observed in India (Agarwal et al, 2002). Therefore, more attention is now focused on prevention as an ultimate strategy for the management of breast cancer. Breast cancer is a multifactorial disease with a large number of etiological factors. There are certain risk factors like age, genetic factors, previous history of breast cancer etc. which can not be controlled. However physical activity, hormone levels and life style factors including dietary factors (rich in antioxidants) are amendable risk factors. Apart from these classical risk factors, oxidative stress has emerged as an important etiological factor in the pathogenesis of breast cancer. The oxidative stress in breast cancer poses a risk mainly due to excess ROS production (Ambrosone, 2000). ROS directly or indirectly damage important cellular components. In addition to that ROS have multifactorial effects on the regulation of cell growth and capacity of malignant cells to invade. Thus, it is important to rule out status of the protectors against ROS in a study of preventive approach which starts from one's own, indispensable antioxidant defense system. The term “Antioxidant” is used to define cells’ own protective mechanisms against the deleterious effects of ROS. Antioxidant defense system comprise of enzymatic and non-enzymatic antioxidants. Several components of these system are micronutrients or are dependent upon dietary micronutrients (Evans and Halliwell, 2001). Thus, the dietary micronutrients are of prime importance, as the antioxidant defenses act as a co-ordinated system and insufficiency of one of the components may affect the efficiency of others.

Epidemiological and laboratory studies have documented that a high consumption of antioxidant rich fruits and vegetables can reduce risk of cancer. In the past several years, vitamins and phenolic substances derived from the daily diet have received considerable attention because of their potential chemotherapeutic activities. Diet rich in vegetables and fruits that
contain a variety of antioxidants clearly have cancer preventive effects (Kohlmeyer et al, 1995; Rijken et al, 1999; Thompson et al, 1999). A reasonable amount of data supports a beneficial effect of supplementation with high doses of antioxidants used in combination with conventional cancer therapy (Conklin, 2000; Lamson and Brignall, 1999; Prasad et al, 1999; Prasad et al, 2001) But, there are some contradictory results also regarding the use of antioxidants for cancer prevention. A large randomized double-blind trial of daily supplementation with α-tocopherol or β-carotene alone showed no reduction in the incidence of lung cancer among male smokers. Jacqueline et al (1999) showed that supplementation of certain antioxidants resulted in changes in the erythrocyte enzyme activities of GR and catalase and serum α-tocopherol concentration in humans. Thus, the role of enzymatic and non-enzymatic antioxidants is complex and change in one antioxidant may result in the changes in concentration of other antioxidants or enzymes. Depending upon the oxidative status of the cells antioxidants can be protective against cancer or cancer promoting mainly by damaging biological membranes, lipoproteins, DNA and proteins.

Therefore, considering the importance of oxidative stress and antioxidants as an important etiological factors in addition to classical risk factors in breast carcinogenesis, the current study was aimed to analyse both enzymatic and non-enzymatic antioxidants, lipid profile and oxidative stress related markers i.e. lipid peroxidation and thiol in healthy controls, patients with BBD and breast cancer patients. In addition to that the levels of these parameters were also analysed after initiation of anticancer therapy to monitor the treatment response. Oxidative stress also affects the proteins, but to protect them there is cytoplasmic antioxidant hsp70, a major molecular chaperone. In the current study, hsp70 expression was also analysed in breast cancer patients. ROS by reacting with cellular components like lipids and proteins modify the proteolytic – antiproteolytic balance. This imbalance damages cellular membrane, which lead to enhancement of proteolysis and destruction of ECM proteins and in consequence to invasion and metastasis. MMPs and TIMPs
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play an important role in the ECM degradation and ultimately invasion and metastasis. In the current study MMP-2, MMP-9 and their inhibitors TIMP-2 and TIMP-1 were also analysed from the subjects.

**Sociodemographic and clinical factors:** In the current study, the subjects were interviewed using a health habit questionnaire for life style and reproductive history. To rule out the association of different risk factors in the current study, OR were computed. OR is an important statistical tool and can be considered to approximate the risk ratio. Logistic regression is a widely used technique to calculate OR, in case-control studies as well as in cohort studies. OR is simply a ratio of odds; they refer to the ratio of the odds of an event occurring in the exposed group versus the unexposed group. The more frequent the outcome becomes, the more the odds ratio. OR will overestimate the risk ratio when it is more than 1 or underestimate the risk ratio when it is less than 1. Thus, OR can be used to get an idea of how strongly a given variable may be associated with the outcome of interest compared to other variables. In the current study, OR were calculated for age, menarche age, menopausal status, monthly income and education (Table-6). The data revealed that increasing age and postmenopausal status were significantly associated with increased risk of breast cancer. Higher BMI were also associated with increased risk of breast cancer. In contrast higher education and higher monthly income were associated with decreased risk of breast cancer. Increasing menarche age has less risk of breast cancer but it was not significant. Age at menopause, age at first pregnancy, use of birth control pills etc. is also important risk factors for breast cancer. However in the current study we could not collect reliable confirm history for these risk factors so they were not included in the data.

In accordance with the current results, Baia et al (2001) showed that women having higher education showed lower risk of breast cancer. Tavani et al (1997) showed increased risk of breast cancer with higher education level. In contrast to our results, Robert et al (2004) found that women living in higher
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Socioeconomic status have higher risk of breast cancer as compared to women living in lower socioeconomic status. In accordance with the results of the current study, Baquet and Commiskeyl (2000) found that women of lower socioeconomic class present with higher stage disease as compared to the women of higher socioeconomic class and suggested that socioeconomic status was related with breast cancer incidence and mortality rates. Heck and Pamuk (1997) studied relation between education and post menopausal breast cancer. The authors showed direct relationship between educational level and breast cancer risk. Gao YT et al (2000) showed that early menarcheal age, nulliparity and later age at first live birth were associated with increased risk of breast cancer among both pre and post menopausal women while, never having breast fed and later age at menopause were associated with elevated risk only among postmenopausal women.

Circulating enzymatic and non-enzymatic antioxidants, lipid profile parameters, lipid peroxidation and thiol levels in breast cancer patients:

The enzymatic antioxidants SOD, CAT, GR are considered as the first line of endogenous defense against oxidative stress. The resultant secondary oxidation products may still damage DNA, protein and lipids and require further detoxification. The second line of defense against ROS is provided by GST, which belongs to phase II enzyme family. GSTs detoxify not only lipid peroxidation products and oxidized bases, but also other endogenous products, including \(\text{o-quinones}\) (Yager and Liehr, 1996). The current study analysed enzymatic antioxidants; GST and GR from plasma as well as from RBCs. SOD and CAT were studied from plasma as well as RBCs, as they are exposed continuously to the oxidative stress.

Enzymatic antioxidants:

The comparison of RBC enzymatic antioxidants between controls and breast cancer patients showed that GST activity was significantly lower in breast cancer patients as compared to the controls (Figure-14). RBC GR and SOD
activity were significantly higher in breast cancer patients as compared to the controls. RBC catalase activity was comparable in controls and breast cancer patients. Plasma GST activity was lower and plasma GR activity was significantly higher in breast cancer patients as compared to the controls (Figure-15). The OR for lowest versus the highest quartiles also showed that increasing quartiles of RBC GST with increasing activity were significantly associated with reduced risk of breast cancer whereas, increased quartiles of RBC GR and SOD with increased activities were significantly associated with increased risk of breast cancer (Table-9). Thus, lower plasma as well as RBC GST activity and increased plasma as well as RBC GR and RBC SOD activities showed the increased risk of breast cancer. The altered levels of phase II enzyme may suggest imbalance in GSH/GSSG ratio which has been reflected by low levels of plasma thiol in the current study. Increased rate of free radical production frequently elicits, as a response, an increase in the activity of antioxidant enzymes. In the present study, higher levels of catalase and SOD indicated higher production of $\text{H}_2\text{O}_2$ due to higher production of $\text{O}_2^-$. In addition, the higher production of ROS in breast cancer patients is manifested by higher plasma GR and RBC GR, RBC SOD and RBC catalase. ROC curve also showed that plasma and RBC GR as well as RBC GST activities can significantly discriminate between controls and breast cancer patients (Figure-18, 19). The Receiver's Operating Characteristic (ROC) curve is defined as a plot of test sensitivity versus its 1-specificity. It is an effective method to evaluate the quality or performance of diagnostic tests. ROC plots provide a pure index of accuracy by demonstrating the limits of a test's ability to discriminate between two groups. AUC is a measure of the overall performance of a diagnostic test and is interpreted as the average value of sensitivity for all possible values of specificity. It can take on any value between 0 and 1. If, AUC is closer to 1, the overall diagnostic performance of the test is better, and a test with an AUC value of 1 is one that is perfectly accurate. The practical lower limit for the AUC of a diagnostic test is 0.5. AUC greater than 0.5 have at least some ability to discriminate between patients.
with disease from those without disease. The 95% CI gives the range of values in which the true value lies.

The results of the present study are in accordance with data reported by Ray et al (2000) showing significantly higher SOD and lower catalase activity in breast cancer patients as compared to controls. It was further hypothesized that higher activity of SOD may be in response to increased rate of free radical production and significantly reduced activity of catalase may be due to high rate of free radical input, which might result in reduced enzyme activity leading to autocatalysis of oxidative damage process. Kumaraguruparan et al (2002, 2005) showed increased SOD, catalase and GST activities in breast tumour tissue as compared to the adjacent normal tissues. The extent of the increase was 1.17 and 1.18 fold for SOD and catalase, respectively. The increase in tissue SOD levels were in accordandance with our results. Breast tumour tissue levels of GST showed 2-3 fold increase which is in contrast to the results of present study for plasma as well as RBC GST levels. Abou et al (2000) showed higher GST activity in breast cancer patients. Sharma et al (2003) showed marked decrease in RBC SOD and catalase in ovarian, cervical and uterine cancer patients as compared to controls. Subapriya et al (2002) showed reduced SOD, catalase and GST in oral cancer patients as compared to controls. Saygili et al (2003) showed significantly higher RBC GST and GR activity in colorectal cancer patients as compared to healthy subjects. The increased RBC GR activity in colorectal cancer patients are in accordance with results of the present investigation for breast cancer patients. In contrast to the results of current study, Bakan et al (2003) reported that serum Cu-Zn SOD activity was lower in CLL patients while serum GR activity was not different in CLL patients as compared to controls. Sabitha and Shyamaladevi (1999) reported significantly decreased RBC SOD, catalase, GST and GR in oral cancer patients as compared to the controls. Tas et al (2005) described elevated SOD activity and decreased catalase activity in breast tumour tissue as compared to normal tissue. Thus, the data of the current study is in accordance with various studies. SOD is the primary enzymatic antioxidant
because they are involved in elimination of oxy- radicals. Metabolism of estradiol produces higher amount of hydroxy radicals through redox cycling of catechol estrogens. Hence the SOD levels may be increased in response to higher ROS generated by this redox cyclic of estrogen. The higher ROS state indicates oxidized state and the GR levels might be elevated to maintain the balance between oxidized and reduced state. Preliminary results of the present study suggested in-depth analysis to evaluate role of GST-µ null genotype in etiology of breast cancer.

**Non-enzymatic antioxidants:**
Mayne (2003) suggested that measurement of plasma non-enzymatic antioxidants β-carotene, vitamin-E and vitamin-C are considered as valid ways to measure exposure of dietary antioxidants and also called "Nutritional Biomarkers". The nutritional biomarkers can be designated into different categories. Biomarkers can be used as (i) a means of validation of dietary instruments; (ii) surrogate indicators of dietary intake; and (iii) integrated measures of nutritional status for a nutrient. The biomarker serves as an integrated measure of metabolism of the nutrient of interest. The nutritional biomarker can be used as a measure of internal dose, which is an indication of the amount of nutrients available to the tissues after absorption and metabolism (Potischman and Freudenheim, 2003). Handelmann et al (1996) showed that assessment of serum/plasma micronutrient status is more reliable and biologically meaningful than dietary estimation of nutrition, as plasma levels reflect the dietary intake, absorption, utilization and other metabolic aspects including depletion of serum/plasma and tissue nutrients due to oxidative stress. An advantage of the present study of non-enzymatic antioxidant is that it did not rely solely on dietary recall or records but on direct measurement of plasma levels of antioxidants as it may give a more accurate approximation of the amount available to the target tissue than intake estimates.
In the current study, the non-enzymatic antioxidants (β-carotene, vitamin-A, vitamin-E and vitamin-C) between controls and breast cancer patients showed that β-carotene, vitamin-E and vitamin-C levels were significantly lower in breast cancer patients as compared to controls. Significantly lower levels of β-carotene, vitamin-E and vitamin-C were observed in breast cancer patients as compared to controls. It may be postulated that the decrease may suggest response to higher ROS production in the patients. Vitamin A levels were significantly elevated in breast cancer patients as compared to the controls (Figure-16). Higher levels of vitamin-A may suggest abrupt retinoic acid signaling. The OR also indicated that higher quartiles with increasing levels of β-carotene, vitamin-E and vitamin-C were significantly associated with reduction in breast cancer risk whereas, increasing quartiles with increasing levels of vitamin-A were significantly associated with increased risk of breast cancer (Table-11). ROC curve also revealed that plasma β-carotene, vitamin-A and vitamin-E levels could significantly discriminate between controls and breast cancer patients (Figure:18, 19).

In accordance to our results, Toniolo et al (2001) showed that serum β-carotene levels were lower in breast cancer patients as compared to controls and retinol levels were higher in breast cancer patients as compared to controls. Authors also documented that there was a progressive increase in the risk of breast cancer for decreasing serum concentration of β-carotene. Ray and Husaini (2001) showed lower levels of plasma vitamin-E and vitamin-C in breast cancer patients as compared to controls. They also documented marked decrease in vitamin-E and vitamin-C levels in stage IV breast cancer patients. Thus, the current results showing lower levels of β-carotene, vitamin-E and vitamin-C in breast cancer patients are in accordance with published data. Vitamin-A levels were higher in breast cancer patients as compared to controls. Tamini et al (2005) showed that though the levels of plasma β-carotene, retinol and α-tocopherol were higher in breast cancer patients, no significant difference was observed between controls and breast cancer patients. OR showed an inverse relationship for β-carotene with breast...
cancer risk. In contrast to the result of the current study for vitamin-A, Ching et al. (2002) showed that increased quartiles of plasma levels of vitamin-A were significantly associated with reduction in breast cancer risk. The result from our laboratory (Raval et al., 2001) documented lower levels of plasma β-carotene and vitamin-E and higher vitamin-A in oral and pharyngeal cancer patients as compared to controls. ROC curve in the study also showed that plasma β-carotene, vitamin-A and vitamin-E can significantly distinguish between controls and cancer patients. In contrast Copper et al. (1999) documented comparable plasma vitamin-A levels in untreated oral and pharyngeal cancer patients.

The lower levels of plasma non-enzymatycic antioxidants also suggested higher oxidative stress. The lower levels of β-carotene may suggest that it does not work as prooxidant and quenches the oxyradicals produced in breast cancer patients. The lower levels of vitamin-C and vitamin-E may be in response to prevent oxidation of PUFA against ROS attack by quenching them. Vitamin-C plays an important role in recycling of α-tocopheroxyl radical to α-tocopherol and thus indirectly involved in neutralization of ROS. Thus lower levels of non-enzymatic antioxidants also suggest higher oxidative stress in breast cancer patients. Plasma vitamin-A levels were significantly higher in breast cancer patients which may be due to disruption in cellular vitamin-A homeostasis.

**Lipid profile parameters:**
The inefficient antioxidant defense, evident by altered antioxidant levels permits the ROS to attack vital cellular components including DNA, proteins and lipids. Lipids being the major cellular component are more susceptible to damage from ROS resulting in lipid peroxidation. In the present study, comparison of plasma lipid profile in controls and breast cancer patients represented significantly lower levels of plasma cholesterol as well as HDL and significantly higher levels of plasma TG and VLDL in breast cancer patients as compared to the controls. Plasma LDL levels were lower in breast cancer patients as compared to the controls (Figure-17). The odds ratio for lipid
profile indicated that higher quartiles of cholesterol and HDL were associated with significantly reduced risk of breast cancer whereas, higher quartiles of TG and VLDL revealed significantly increased risk of breast cancer (Table-12). ROC curve showed that plasma HDL, VLDL and TG could significantly discriminate between controls and breast cancer patients (Figure-18, 19). The altered lipid profile status in the current study may be due to abnormal lipid metabolism associated with tumour pathogenesis and tumour host interactions. Malignant cells appear to metabolize lipids differently from the normal cells. Cholesterol is an essential constituent of lipoprotein fractions like LDL, HDL and VLDL. 75% of the plasma cholesterol is transported in the form of LDL. Cells sequester cholesterol from LDL fraction of lipoproteins. LDL receptors are necessary for metabolizing circulating LDL levels and nearly 80% of the plasma LDL is cleared by LDL receptors (Kesaniemi et al, 1983). Thus, high activity of LDL receptor attributes for lowering the circulating cholesterol levels. The individuals having deficient LDL receptors remove plasma LDL at much lower rate and have considerably high LDL levels. It may be possible that lower levels of LDL in the present study may also be responsible for lower circulating cholesterol. Lower levels of LDL and HDL in the present study may be due to higher amount of ROS, as LDL is more susceptible to oxidation by ROS resulting in to lipid peroxidation (Padayatty et al, 2003). HDL is important as it counteract the oxidative damage of LDL thereby preventing lipid peroxidation. The mechanism for high levels of triglycerides and low levels of HDL in breast cancer is not known. It has been suggested that lipoprotein lipase may regulate the clearance of TG from blood to tissue (Carbo et al, 1994). The activity of lipoprotein lipase in white adipose tissue decreases in cancer hosts contributing to hypertriglyceridemia. Since precursor particles of HDL are thought to be derived from lipolysis of TG, and the LPL activity is decreased in cancer, increased plasma TG levels may be one of the factors for lower concentration of HDL in the present study. It is also possible that higher levels of TG may lead to decreased levels of sex hormone binding globulin resulting in higher amount of free estradiol which is likely to increase breast cancer risk (Takatani et al, 1991).
Ray and Husain (2001) showed significantly increased plasma total cholesterol and TG levels and significantly lower HDL levels in breast cancer patients as compared to the controls suggesting that higher levels of cholesterol and TG may play an important role in carcinogenesis. In accordance with the low levels of plasma cholesterol in the current study Elchholzer et al. (2000) showed low plasma cholesterol in lung, prostate and colon cancer. Gaard et al (1997) also showed lower levels of plasma cholesterol in colon cancer patients. They showed that decrease in total cholesterol may be due to pre-existing cancer. Moschovi et al (2004) observed low levels of HDL and elevated LDL and TG levels. They suggested that higher LDL and TG levels may suggest an increase in their synthesis and decrease in their clearance from the plasma or both. Lipoprotein lipase and lecithin-cholesterol acyl transferase are the enzymes that are implicated in chylomicron metabolism, so their probable inactivity lead to decreased clearance of TG and LDL. They suggested that the increased production of these substances may be the effect of an acute phase reaction mediated by cytokines released from the dysregulated immune system in the ALL patients. The authors mentioned lower serum levels of HDL that may be the result of impaired hepatic synthesis perhaps due to blastic infiltration. Cytokines may also play a role, but the most probable explanation is provided by the fact that the activity of LDL receptors is clearly decreased. The deregulation of these receptors is responsible for the reduced rate of LDL catabolism, which leads to higher levels of serum LDL, and inhibition of HDL production. Earlier data from our laboratory (Patel et al, 2004) also documented lower levels of cholesterol, HDL, VLDL and TG in oral cancer patients.

**Oxidative stress related markers (LPx and Thiol):**

Imbalance of oxidants and antioxidants in favour of the former leads to the production of ROS. Accumulation of ROS results in oxidation of lipids giving rise to lipid peroxidation of biological membrane PUFAs. MDA, which is the end product and an index of lipid peroxidation, is used as an indicator of oxidative stress as it is mutagenic and genotoxic that may contribute to the
development of cancer. On the other hand glutathione (GSH) is the major low-molecular-weight thiol in cells that controls cellular thiol-disulfide redox state, which is essential for its normal redox signaling oxidized form, GSSG (Yee, 2003). Oxidation-reduction reactions during oxidative perturbations will cause a redistribution of GSH and GSSG; the resultant quantitative shift in the ratio of GSH to GSSG in favor of GSSG directly reflects an oxidized redox status and is a convenient expression of oxidative stress within a cell. Therefore, lipid peroxidation and glutathione were considered as oxidative stress related markers in the present study. The lipid peroxidation levels were higher in breast cancer patients as compared to controls while thiol levels were significantly lower in breast cancer patients as compared to controls (Figure-15). ROC curve for thiol indicated that it can significantly discriminate between controls and breast cancer patients (Figure-19). OR also revealed that higher quartiles with increasing levels of thiol were significantly associated with reduction in breast cancer risk (Table-10). The higher levels of lipid peroxidation in the current study may be due to lower levels of non-enzymatic antioxidants and higher levels of enzymatic antioxidants in response to higher production of ROS. The decreased levels of thiol may be due to imbalance in the GSH/GSSG ratio which is reflected in the current study by altered GST and GR activity. GR is glutathione replenishing enzyme which converts GSSG to GSH. The activity of GR may be increased to maintain the redox state in the cell for conversion of GSSG to GSH.

In accordance with the present study, Ray et al (2000) showed higher serum levels of lipid peroxidation in breast cancer patients as compared to the controls. Kumaraguruparan et al (2005) in breast tumour tissue reported, significantly higher levels of lipid peroxidation in the form of TBARS and lower levels of GSH as compared to adjacent normal tissue. Tas et al (2005), found higher MDA levels in breast tumour tissue as compared to normal tissue. Bakan et al (2003) found lower serum GSH and higher MDA in CLL patients as compared to controls. Mukundan et al (1999) showed significantly lower levels of plasma and RBC GSH in cervical cancer patients as compared to
levels of plasma and RBC GSH in cervical cancer patients as compared to controls. Sabitha and Shyamaladevi (1999) reported significantly higher serum MDA levels in oral cancer patients as compared to controls with significant decrease in RBC enzymatic antioxidants suggesting enormous production of free radicals in the system. Coban et al (1998) showed higher GSH in the malignant breast tissue as compared to normal tissue while, there was no significant difference in lipid peroxidation levels in tumour and normal tissue. Thus, the alterations in the LPx levels and thiol are supported by previous reports.

Circulating enzymatic and non-enzymatic antioxidants, lipid profile parameters, lipid peroxidation and thiol levels in patients with BBD:

The current investigation compared all the parameters between controls, patients with BBD and breast cancer patients to compare their status between malignant and non-malignant diseases. Comparison of enzymatic antioxidants between controls and patients with BBD showed significantly decreased activity of RBC GST and increased SOD activity in patients with BBD as compared to the controls. RBC GR activity was comparable and RBC catalase activity was lower in patients with BBD as compared to controls. Plasma GST activity was decreased and GR activity was increased in patients with BBD as compared to controls (Figure-20, Table-13). Plasma non-enzymatic antioxidants β-carotene and vitamin-E levels were lower in patients with BBD as compared to controls while vitamin-C levels were significantly lower in patients with BBD as compared to controls. Plasma vitamin-A levels were significantly higher in patients with BBD as compared to controls. Plasma lipid profile levels showed significantly lower cholesterol, LDL, VLDL and TG levels in patients with BBD as compared to the controls. Plasma HDL levels were also lower in patients with BBD as compared to the controls (Table-14, Figure-21). Plasma thiol levels were comparable while plasma lipid peroxidation was higher in patients with BBD as compared to controls (Table-13). Comparison of enzymatic antioxidants between breast cancer patients and patients with BBD showed that plasma and RBC GR activity was
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significantly higher in breast cancer patients as compared to patients with BBD. Plasma levels of β-carotene and vitamin-C levels were significantly lower while plasma vitamin-A levels were significantly higher in breast cancer patients as compared to patients with BBD. Plasma cholesterol and LDL levels were higher and VLDL and TG levels were significantly higher in breast cancer patients as compared to patients with BBD. Plasma HDL levels were lower in breast cancer patients as compared to patients with BBD. Plasma thiol levels were significantly lower in breast cancer patients as compared to patients with BBD (Table-15).

There are very few studies showing the comparison of the above markers in patients with BBD as compared to controls and breast cancer patients as compared to patients with BBD. Kumaraguruparan et al (2002) showed depleted plasma GSH levels in breast cancer patients as compared to the patients with BBD and in patients BBD as compared to controls and higher LPx in breast cancer patients as compared to patients with BBD. However, in our study plasma thiol levels were lower in breast cancer patients as compared to patients with BBD. Thiol levels were comparable between controls and patients with BBD. LPx levels were higher in patients with BBD as compared to controls and comparable levels were obtained between patients with BBD and breast cancer patients. Plasma Vitamin-C and vitamin-E levels were also lower in their study, which is in accordance with the current data. RBC SOD, catalase and GST levels were decreased in patients with BBD as well as breast cancer patients as compared to the controls. Seven et al (1998) showed elevated RBC SOD activity and lower TBARS levels in breast cancer patients as compared to BBD. Polat et al (2002) showed higher SOD and MDA levels and lower catalase in patients with BBD as compared to the controls. Beno et al (2000) documented decreased β-carotene, vitamin-A, vitamin-E and vitamin-C levels in gastric and colorectal precancerous lesions. β-carotene, vitamin-A, vitamin-E and vitamin-C levels were lower in patients with oral leukoplakia (Ramaswamy et al, 1996). The data should be confirmed in the large sample size. However, the alterations in enzymatic and non-
enzymatic antioxidant, lipid profile parameters as well as in the levels of lipid peroxidation showed imbalance in the antioxidant capacity of the patients with BBD.

**Correlation with clinicopathological parameters:**
Multivariate analysis was carried out to correlate enzymatic and non-enzymatic antioxidants, lipid profile, thiol, LPx and clinicopathological features like age, menopausal status, lymphnode involvement, early and advanced stage of the disease, BR score and nuclear grade in the breast cancer patients. Multivariate analysis for enzymatic antioxidants showed that plasma GST and GR, RBC GST and RBC catalase were significantly associated with nuclear grade of the tumour **(Table-20,21)**. Multivariate analysis between plasma non-enzymatic antioxidants and clinicopathological parameter indicated that Vitamin-E was significantly associated with lymphnode involvement and BR score and Vitamin-C was significantly associated with nuclear grade of the tumour **(Table-22)**. Multivariate analysis between cholesterol, HDL, LDL, VLDL, Triglycerides and clinicopathological features showed that plasma VLDL and Trglycerides were significantly associated with nuclear grade of the tumour **(Table-23)**. Multivariate analysis for thiol and lipid peroxidation and clinicopathological features did not showed any association between plasma thiol and LPx and clinicopathological features **(Table-21)**. Yigitbasi et al (2000) found significant association of SOD activity with grade of the tumour, which is in accordance with the present study. Multivariate analysis of the parameters with clinicopathologic parameters is not studied in recent earlier reports.

**Correlation of the markers in breast cancer patients:**
In the present study, the correlation of enzymatic and non-enzymatic antioxidants in breast cancer patients was carried out by Pearson’s correlation. RBC SOD and catalase variations showed positive association with each other **(Table-17)**. Plasma and RBC GST were positively and significantly associated with the changes in plasma and RBC GR respectively **(Table-16)**.
Vitamin-E values were negatively and significantly associated with the in vitamin-C (Table-18). The alterations in vitamin-E levels were positively and significantly associated with the changes in lipid peroxidation while, vitamin-C levels were significantly and negatively associated with lipid peroxidation (Table-19). The results of the current study showed positive association of SOD and catalase. SOD converts oxyradicals in $H_2O_2$. This is further eliminated by catalase by forming $H_2O$. The positive association of SOD and catalase may mean that in breast cancer patients they work in concert with each other to eliminate oxyradicals. Plasma and RBC GST were significantly and positively associated with plasma and RBC GR. GST is a glutathione depleting enzyme and GR is glutathione replenishing enzyme and their activities are interrelated for detoxification of toxic substances as well as for the maintenance of reduced state in the breast cancer patients. The significant negative association of vitamin-E with vitamin-C in the current study may be due to the interrelated quenching capacity of both vitamin-E and vitamin-C in breast cancer patients. By quenching free radicals, Vitamin-E is reduced to $\alpha$-tocopheroxyl radical. Vitamin-C regenerates the $\alpha$-tocopheroxyl radical to $\alpha$-tocopherol by reducing itself to ascorbyl radical.

Circulating enzymatic and non-enzymatic antioxidants, lipid profile parameters, lipid peroxidation and thiol levels in PT and follow-up:

The most commonly used treatment modality for breast cancer is surgery followed by chemotherapy or radiotherapy. The effects of radiotherapy or chemotherapy are mediated by production of free radicals in the cells. Radiotherapy and chemotherapy are, long term treatment which largely relies on their oxidative damage to cancerous cells (Coia and Moyland, 1998). Hence, it is important to measure pretreatment and post treatment levels of antioxidants and other markers. To compare the association of the biomarkers with the response to anticancer therapy, paired 't' test was calculated. Paired 't' test compared levels of the parameters of each post-treatment follow-up paired with its PT value.
In the current study, the patients were grouped as CR and NR according to their clinical status at the time of follow-up. The comparison of paired 't' test between PT and CR levels indicated that RBC GST activity was higher and RBC GR activity was lower in CR as compared to PT. RBC SOD activity was comparable between PT and CR. RBC catalase activity was significantly lower in CR as compared to PT (Figure-22). Plasma GST activity was higher and plasma GR activity was significantly higher in CR as compared to PT (Figure-23). Plasma β-carotene levels were higher, Vitamin-E levels were lower and vitamin-C levels were comparable in CR as compared to PT. Plasma vitamin-A levels were lower in CR as compared to PT (Figure-24). Plasma VLDL and TG levels were significantly declined, Plasma cholesterol levels were lower, plasma HDL levels were higher and plasma LDL levels were comparable in CR as compared to PT (Figure-25). Plasma Thiol values were significantly higher and LPx were also higher in CR as compared to PT (Figure-23). The comparison of paired 't' test between pretreatment levels and NR levels indicated that RBC GST and GR activity was increased in NR as compared to the PT. However the increase in the activity was not statistically significant. RBC SOD, RBC catalase and plasma GST activity were comparable in PT and NR. Plasma GR activity was significantly higher in NR as compared to PT. Plasma β-carotene, Vitamin-E and Vitamin-C levels were comparable between PT and NR. Plasma vitamin-A levels were lower in NR as compared to PT. Plasma cholesterol and LDL levels were significantly lower in NR as compared to PT. Plasma HDL and VLDL levels were comparable and plasma TG levels were decreased in NR as compared to PT. Plasma Thiol levels were lower in NR as compared to PT and plasma LPx levels were higher in NR as compared to PT. Previous study from our laboratory (Patel et al, 2002) showed significantly elevated GST and declined GR activity in CR as compared to oral cancer patients. Yilmaz et al (2004) suggested that MDA levels can be useful in diagnosis and follow-up of prostate cancer patients. In cervical cancer patients, plasma and erythrocyte GSH levels were measured before and after anticancer treatment, which showed lower GSH after radiotherapy as compared to the control levels, which is contradictory to the current study.
However, there was no remarkable change in pre and post treatment levels in plasma and erythrocyte GSH levels (Mukundan et al, 1999). In contrast to the current study, Liu and Yang (2003) showed higher levels of TG in breast cancer patients who were on adjuvant chemotherapy however, the clinical status (CR or NR) of the patients was not mentioned in their study. Higher plasma GST and GR activities as well as higher RBC GST and lower GR activity may be in response to increased cellular toxicity due to chemotherapy in the CR. However, the altered levels as well as the higher GSH levels show presence of reduced state in CR. In contrast to the CR, NR showed increased plasma GR activity with decreased thiol levels, which suggested persistent oxidative stress in NR. RBC catalase activity in CR was significantly lower suggesting lower levels of ROS.

**hsp70 in breast cancer patients:**
Oxidative stress leads to a massive induction of heat shock proteins. This is partly mediated by the damaged proteins, which occupy chaperone-binding sites, and liberate heat shock factor-1 (HSF-1), the transcription factor responsible for hsp induction. Thus, a decrease in GSH levels showing higher oxidized state, may lead to a direct activation of HSF-1. A mild change of redox homeostasis also leads to the activation of HSF-1 (Creagh et al, 2000). Apart from the enzymatic and non-enzymatic antioxidants, traditional chaperone the small hsps and hsp70 can also serve as cytoplasmic “antioxidants”. They protect their target proteins by covering their sensitive sites. Sometimes enzymatic and non-enzymatic antioxidant mechanisms are not effective enough, and the oxidative damage is prevailed. In this case, hsps capture denatured proteins and hold them until their refolding or degradation. An increasing number of evidence indicate that this system may serve as an additional antioxidant mechanism scavenging oxidative agents.

In the present study, the expression of hsp70 was analysed by western blot method from malignant breast tissues and their corresponding adjacent normal tissues. The expression of hsp70 was significantly higher in malignant
breast tissues as compared to adjacent normal tissues. Paired ‘t’ test analysis also indicated higher levels of hsp70 in breast tumour tissues as compared to adjacent normal tissues (Table-24). Further Multivariate analysis for hsp70 with clinicopathologic features showed no significant association. Ehrenfried et al (1995) showed differential hsp70 protein expression in human gastric, pancreatic and colon cancer. The authors suggested that increased hsp70 expression occurs constitutively and were not result of physiological and environmental stress. They also showed that increased expression of hsp70 may enhance resistance to certain chemotherapeutic drugs. Vargas-Roig et al (1998) also suggested that hsp70 is involved in drug resistance in breast cancer patients treated with combination therapy. Lazaris et al (1997) showed that hsp70 positive immunoreactivity was significantly correlated with axillary lymphnode metastasis and it is a probable marker of the biological stress. Chuma et al (2003) showed that hsp70 was the most abundantly upregulated gene in early hepatocellular carcinoma and it could be a sensitive marker for the differential diagnosis of early hepatocellular carcinoma. Wang et al (2005) showed that the expression rate of hsp70 is significantly higher in colonic cancer as compared to adjacent mucosal membrane and over expression of hsp70 can be used as diagnostic or prognostic marker for colonic cancer. Nakajima et al (2002) showed that reduction of hsp 70 expression was significantly associated with poor prognosis of esophageal SCC. Noguchi et al (2002) showed that hsp70 overexpression in esophageal SCC exhibited a significantly better prognosis and was associated with sex, tumour differentiation, lymphnode metastasis and lymphatic invasion. The authors suggested that hsp70 expression might be used to assess the progression, lymphnode metastasis and lymphatic vessel invasion of esophageal SCC. Higher expression of hsp70 in the current study is suggestive of higher oxidative stress in breast cancer patients. The tumours showing presence of inducible form suggest that these patients may develop resistance to further treatment.
Matrix Metalloproteinases in breast cancer patients: Although, a considerable amount of efforts has been placed on discovering the etiology of cancer, the majority of basic research has been focused on understanding the molecular mechanisms of tumour formation and metastasis. Metastatic spread of tumours continues to be a major obstacle to successful treatment of malignant tumours. Approximately 30% of patients diagnosed with solid tumours have a clinically detectable metastasis and for the remaining 70% metastasis are continually being formed throughout the life of tumour. Oxidative stress has also been repeatedly implicated in tumour invasion and metastasis. Proteolytic –antiproteolytic balance directly depends on ROS (Skrzydlewska et al, 2005). Several cytokines (TNF, IL-1) and growth factors (bFGF, VEGF) are capable of producing ROS in target cells and the ROS thus produced, further participate in mediating the effects of cytokines and growth factors (Beinert et al, 2000; Farrow and Evers, 2002; Mantovani et al, 2003). ROS may also serve as common signaling molecules regulating the activity of transcription factors, NF-kB and AP-1, in response to cytokines and other stimuli (Chung et al, 2002; Hsu et al, 2000). Oxidants can also stimulate transcription of other transcription factor genes such as c-jun, c-fos and c-myc in various cell types (Factor et al, 1998; Hironaka et al, 2003; Hocker et al, 1998). The regulation of gene expression by these factors will ultimately lead to a series of cellular changes such as proliferation, growth suppression, differentiation, senescence and apoptosis. These transcriptional factors are involved either directly in the induction of the expression of MMP genes (by binding to enhancer regions in these genes) or indirectly in modulating the expression of proteins which ultimately affect proteinase release (Birkedal et al, 1993). ROS also influences protease activation and distribution of enzymes and their inhibitors, which is the most important factor for proteolytic-antiproteolytic balance. It has been shown that ROS can activate procollagenase (Okamoto et al, 1997; Saari et al, 1990). In addition to oxidative stress, tumour microenvironment is also important for successful growth of tumour at secondary site. There is an increasing evidence that MMPs have an expanded role as they are important for the creation and
maintenance of a microenvironment that facilitate growth and angiogenesis of tumours at primary and metastatic sites. MMPs are important for disruption of stromal barriers and are key candidate mediators of invasion. MMPs and TIMPs have been repeatedly associated with a metastatic phenotype. The role of MMP-2, MMP-9, TIMP-1 and TIMP-2 is studied extensively in various malignancies including colorectal cancer (Mook et al, 2004), lung cancer (Aljada et al, 2004; Simi et al, 2004), prostate cancer (Ross et al, 2003), oral cancer (Tsai et al, 2003), cervical cancer (Nair et al, 2003) and breast cancer (Giannelli et al, 2004; Schrohl et al, 2004).

The expression of MMPs can be determined by various techniques i.e. IHC, ELISA, Western blot, zymography. Among which, substrate zymography is widely used technique, which identifies MMPs by the degradation of their preferential substrate and by their molecular weight. This technique also determines different forms of MMPs i.e. latent and active forms of MMPs. This technique is widely used as it is simple, sensitive, quantifiable, cost effective and functional assay to analyze MMPs in biological samples. In this method, SDS used for electrophoresis dissociates most of the enzyme-substrate complex and its eventual substitution with Triton X-100 in the gel followed by incubation in appropriate buffer, restores the enzyme activity and the activity of zymogen form.

The present study explored MMP-2 and MMP-9 activity from malignant breast tissue and adjacent normal tissue by gelatin zymography and plasma total MMP-2, total MMP-9, TIMP-1 and TIMP-2 levels by Enzyme Linked Immunosorbant Assay (ELISA) method. Using gelatin zymography for malignant and adjacent normal breast tissue, all the four forms of MMP-2 and MMP-9 i.e. proMMP-2, Active MMP-2, ProMMP-9 and active MMP-9 were clearly separated. ProMMP-2 levels were significantly lower while active MMP-2, total MMP-2 (ProMMP-2 and active MMP-2), and activation ratio of MMP-2 levels were significantly higher in malignant breast tissues as compared to adjacent normal tissues (Figure-35). ProMMP-9, active MMP-9, total MMP-9
and activation ratio of MMP-9 were significantly higher in malignant breast tissues as compared to adjacent normal tissues (Figure-36). Paired sample analysis showed that active MMP-2 and MMP-9 levels were significantly higher in malignant breast tissues as compared to adjacent normal tissues (Figure-38). Latent MMP-2 levels were lower and latent MMP-9 levels were comparable in malignant tissues as compared to adjacent normal tissues. Activation ratio of MMP-2 and MMP-9 were significantly higher in the malignant breast tissues which were considered to have lymphnode metastasis (Figure-39). ROC curves for all four forms of MMP-2 and MMP-9 suggested that, proMMP-2, active MMP-2 and active MMP-9 could significantly discriminate between malignant and adjacent normal tissues (Figure-37).

Waas et al (2002) found elevated levels of all the four forms of MMPs in colorectal cancer as compared to normal mucosa by zymography. In accordance with the current results, Hrabec et al (2002) analysed MMP-2 and MMP-9 in lung cancer patients by gelatin zymography and showed that expression of both type IV collagenase was remarkably higher in carcinoma samples than in the lung parenchyma. MMP-9 levels were over two fold higher in lung cancer tissues as compared to normal lung tissue. For MMP-2, in their study they found 3.8 to 17 fold higher levels of latent and active forms of MMP-2 in lung cancer samples as compared to lung parenchyma. Baker and Leaper (2003) studied latent and active forms of gelatinases in paired colorectal tumour and normal tissues and showed that both active and latent MMP-2 and MMP-9 lysis bands were greater in tumours than in normal colorectal tissue. Their results are also in accordance with current results except for the levels of latent MMP-2. Yoshizaki et al (2001) detected latent MMP-2 band in tumours lymphnodes and adjacent normal tissues of tongue using gelatin zymography. They have observed active MMP-2 band in all tumours and metastatic lymphnode. Lengyel et al (2001) noted gelatinolytic activity of active MMP-2 only in malignant ovarian tissue but absent in benign tumours. Zymographic analysis performed by Sier et al (2000) on urine sample of bladder carcinoma patients and healthy subjects showed that
gelatinolytic activity of MMP-2 and MMP-9 were enhanced in bladder cancer patients than urine samples of normal subjects. Gelatinolytic activity of active MMP-2 and active MMP-9 were also observed to be higher in malignant tissue than their corresponding adjacent normal lung tissue (Schutz et al, 2002). Data from our laboratory (Patel et al, 2005) also showed that active and latent forms of MMP-2 and MMP-9 have increased gelatinolytic activity in malignant oral tissues as compared to their corresponding adjacent normal tissue which is in agreement with the current results. Hong et al (2000) also showed similar findings on zymogram. ROC curve for MMP-9 could significantly discriminate between control and head and neck SCC patients (Ranuncolo et al, 2002).

Activation ratio of MMP-2 and MMP-9 is worthwhile to study activation of MMP-2 and MMP-9. It is more meaningful way to express gelatinolytic activity as latent and active forms of MMP-2 and MMP-9 shows elevation with different patterns in which only total MMP-9 was observed sometimes and may not able to discrete latent and active forms of MMP-9. Very few studies have calculated activation ratio of MMP-2 and MMP-9 to compare relative activity of active MMP-2 and MMP-9. In accordance with the results of the current study, Schmidt et al (1999) showed higher activation ratio of MMP-2 and MMP-9 in head and neck squamous cell carcinoma. Koshiba et al (1998) reported higher activation ratio of MMP-2 in pancreatic cancer. Takashi et al (2002) also found higher activation ratio of MMP-2 in renal carcinoma. Recent data from our laboratory (Patel et al, 2005) documented higher activation ratio of MMP-2 and MMP-9 in oral tumour tissues as compared to the adjacent normal tissues. In the current study, activation ratio of MMP-2 and MMP-9 was significantly higher in node positive breast tissues as compared to node negative breast tissues suggesting higher activity of active forms of MMP-2 and MMP-9 in lymphnode positive tissues which may suggest increased invasion and metastasis.
In the current study, MMP-2 and MMP-9 activities were correlated with clinicopathologic features like age, menopause status, lymphnode involvement, early and advanced stage of the disease, BR score and nuclear grade. ProMMP-2 was associated with lymphnode involvement in adjacent normal tissues, active MMP-2 levels were associated with lymphnode involvement and BR score in malignant tissues. Total MMP-2 activity was associated with lymphnode involvement in adjacent normal tissue and early and advanced stage of the disease in malignant tissues. Activation ratio of MMP-2 was associated with nuclear grade in the adjacent normal tissues (Table-26). Multivariate analysis for all forms of MMP-9 with clinicopathologic features showed that ProMMP-9 was associated with lymphnode involvement in adjacent normal tissues (Table-27). Waas et al (2002) also correlated active and latent forms of MMP-2 and MMP-9 with different clinicopathologic parameters. Their study showed that both active and latent forms of MMP-2 levels were inversely correlated with stage of the disease while, latent and active forms of MMP-9 in tumour tissue did not correlate with any of the clinicopathological parameters which contradict the results of the current study. In a immunohistochemical study by Aglund et al (2004) for gelatinase A and B in endometrial cancer, 52% of the cases were positive for MMP-9 and 72% for MMP-2. Both, MMP-2 and MMP-9 were correlated with histologic grade. MMP-9 correlated with clinical stage of the disease whereas MMP-2 did not. The authors did not found any association with either depth of invasion, menopausal status or the age of the patient.

There are very few studies showing circulating levels of MMP-2 and MMP-9 using zymography. Rocca et al (2004) studied zymographic detection of MMP-2 and MMP-9 in serum of patients and its clinical correlation. The densitometric analysis for this study showed that serum activities of both gelatinases were significantly higher in breast cancer as compared to controls. Both the gelatinases were inversely associated with estrogen receptors and MMP-2 was inversely associated with nuclear grade. Plasma MMP-9 activities were also studied by gelatin zymography by Ranuncolo et al (2003) in follow-
up and in the assessment of prognosis in breast cancer patients. Similar study by the authors was done from euglobulin fraction of plasma of head & neck cancer patients. In this method, they used euglobulin plasma fraction which was prepared by mixing plasma with deionised water and the plasma was acidified to pH 5.5 with acetic acid. This euglobulin fraction was highly effective to enhance MMP detection. However, they found only 92 kDa (MMP-9) and 62 kDa (MMP-2) bands only. They did not find 130 kDa and 225 kDa gelatinolytic bands, which were frequently found in all plasma samples. In addition, this is a cumbersome process, requires precipitation of plasma at pH 5.5. It was also demonstrated that most gelatinases circulates as latent enzymes. The acid treatment as well as the addition of SDS to samples converts latent MMPs to catalytically active forms, without proteolytic cleavage of the N-terminal inhibitory sequence. The activity may be increased due to acetic acid precipitation. This method doesn’t show any precise role of acetic acid precipitation for euglobulin fraction, as well as how this is important to reveal the activities of MMPs.

In the current study, total activity of MMP-2, MMP-9, TIMP-1 and TIMP-2 were analyzed by ELISA from plasma samples obtained from controls, patients with BBD and breast cancer patients. Total MMP-2 levels were lower in patients with BBD and breast cancer patients as compared to controls. Plasma total MMP-9 levels were higher in patients with BBD and breast cancer patients as compared to controls. TIMP-1 levels were lower in patients with BBD and breast cancer patients as compared to the controls. TIMP-2 levels were higher in patients with BBD and breast cancer patients as compared to controls (Figure-40). Okamoto et al (2003) assessed difference in the levels of MMP-2, MMP-9, TIMP-1 and TIMP-2 in the normal ovary and ovarian tumours of different histology using ELISA. They found that proMMP-9 levels were prominently increased and proMMP-2 and TIMP-1 levels were moderately increased in benign tumours as compared to normal. In contrast, TIMP-2 levels were markedly decreased in malignant tumours as compared with normal ovary except ovarian clear cell carcinoma. Komorowski et al
(2002) showed lower plasma levels of MMP-2 and TIMP-2 and higher levels of MMP-9 and TIMP-1 in thyroid cancer patients as compared to normal subjects, which is in contrast with the current study.

Kawata et al (2002) measured MMP-2 and MMP-9 from serum and tissue homogenate by ELISA and showed that both MMPs in serum were unrelated to lymphnode metastasis. In the current study Lower plasma MMP-2 and higher plasma MMP-9 were obtained. Their inhibitors TIMP-2 and TIMP-1 were higher and lower respectively. It is known that MMP-2 activity is regulated by TIMP-2 and MMP-9 activity is regulated by TIMP-1. The negative correlation of MMP-2 and TIMP-2 and MMP-9 and TIMP-1 was obtained in the current study. Chen et al (2004) studied MMP-9 and TIMP-1 in serum of esophageal squamous dysplasia and showed that MMP-9 activity was decreased in subjects with dysplasia as compared to subject without dysplasia. In case of TIMP-1 the significant difference was not observed. Simi et al (2004) focused on the simultaneous measurement of MMP-9 and TIMP-1 mRNA expression by multiplex RTPCR showing higher MMP-9 and TIMP-1 mRNA in NSCLC. Holten-Anderesen et al (2004) studied plasma TIMP-1 levels in patients with colorectal adenomas showing no difference in TIMP-1 levels as compared to healthy subjects. In their previous study (Holten-Anderesen et al, 2002), authors found higher plasma levels of TIMP-1 in early stage cancer regardless of primary tumour location and showed that plasma TIMP-1 has high sensitivity and specificity in diagnosis of colorectal cancer. Schrohl et al (2004) studied tumour tissue levels of TIMP-1 in cytosolic extracts of large number of primary breast tumours by ELISA and showed that higher levels of cytosolic TIMP-1 are significantly associated with poor prognosis. Similar observations were also observed in breast cancer at both m-RNA and protein levels (McCarthy et al, 1999; Nakopoulou et al, 2002; Ree et al, 1997; Schrohl et al, 2003). Aljada et al (2004) showed overexpression of TIMP-1 by IHC associated with adverse outcome in patients with NSCLC. Jumper et al (2004) also found higher levels of serum TIMP-1 in patients with lung cancer as compared to controls. Rhee et al (2004) suggested that TIMP-1 is an important contributor to epithelial neoplastic progression and supports the
concepts that TIMP-1 exerts differential regulation on tissue in a stage dependent manner. Majority of the studies showed higher levels of TIMP-1 in cancer patients, which is not in accordance with the present study. Higher levels of TIMPs in others studies may be a sign of MMP independent growth activity of TIMP-1. TIMP-1 may promote or suppress tumour growth depending on the characteristics of the individual tumour cells and the potential for crosstalk with other signaling pathways (Jiang et al, 2002). In the present study higher plasma MMP-9 and lower TIMP-1 levels indicate that TIMP-1 regulate cell survival by mmp dependent manner.

Ross et al (2003) studied MMP-2 and TIMP-2 in prostate cancer patients by IHC and showed higher expression of MMP-2 and TIMP-2, which was further significantly correlated with advanced tumour stage. Katayama et al (2004) by IHC in early stage oral SCC patients found higher scores in the expression of TIMP-2 in patients with lymphnode metastasis and pointed out that higher expression of TIMP-2 is the most independent factor for worse prognosis in early stage of oral SCC. Thus for TIMP-2, conflicting data reflected the fact that, TIMP-2 participate in both, activation and inhibition of MMP-2 in a dose dependent manner. Thus increasing levels of TIMP-2 showed increased MMP-2 activation to a certain limit, after which the activation and proteolytic activity of MMP-2 would be blocked by inhibitory activity of TIMP-2. As explained earlier for MMP-9 and TIMP-1, TIMP-2 in the current study may also regulate cell survival in MMP-dependent manner. Although it is clearly difficult to distinguish wheather MMP dependent and MMP independent activities are involved in a given process. Therefore, simultaneous study of MMPs and TIMPs is important in order to gain a better understanding of their role in cancer.

To determine role of MMP-2, MMP-9, TIMP-1 and TIMP-2 in treatment monitoring , their levels were investigated in follow-up samples of breast cancer patients which were grouped in CR and NR according to their treatment outcome. In the present study, plasma MMP-2, TIMP-1 and TIMP-2
levels were higher and plasma MMP-9 levels were lower in CR as compared to PT. In NR, the levels of MMP-2, MMP-9 and TIMP-1 were higher and TIMP-2 levels were lower as compared to PT. However, the sample size from NR was small (n=9), therefore the significance of the difference in the MMP and TIMP levels must be confirmed only after analyzing the levels of MMPs and TIMPs in large sample size. The alterations in levels of MMP-2, MMP-9, TIMP-1 and TIMP-2 may be explained by the activity of TIMPs, which regulate cell survival by MMP dependent manner. In CR, TIMP-1 and TIMP-2 may be increased due to the decreased MMP-9 and increased MMP-2 levels. The alterations in levels of TIMPs in CR as well as NR may be due to their complex role, as TIMPs have both anti MMP activity, which favors tumour suppressing effects and growth stimulatory effects or apoptosis regulatory activity, which exerts protumour effects.

There are very few reports showing analysis of MMPs and TIMPs in post-treatment follow-up samples. Ranuncolo et al (2003) analysed plasma MMP-9 activity by gelatin zymography from plasma euglobulin fraction. In the 38 months follow-up study MMP-9 levels were significantly decreased in breast cancer patients as compared to PT level. Giannelli et al (2004) analysed proMMP-9 and TIMP-1, proMMP-2 and TIMP-2 before and after surgery in breast cancer patients. After surgery proMMP-9 levels were decreased and TIMP-1 levels were strongly increased. There was no significant difference for proMMP-2 and TIMP-2 levels as compared to PT.

Apart from anti MMP activity of TIMPs, they functions also in favor of tumour growth either in an MMP independent or MMP dependent manner including growth promotion and antiapoptotic effects, inhibition of angiostatin and endostatin converting MMPs or up regulation of VEGF and therefore stimulation of angiogenesis and involvement of activation of promMMP-2. The net effect of TIMPs in tumourigenesis may depend upon bioavailability of local amount of TIMPs in tumour microenvironment i.e. the time when TIMP is presented to tumour cells and the presence of putative TIMP receptors on
tumour cells. The balance between anti-MMP and anti-apoptotic effect on tumour growth may depend on the bio-availability of TIMP protein in tumour microenvironment. In this regard, higher levels of TIMPs may have tumour suppressing effects due to its dominant anti MMP activity (Jiang et al, 2002). In addition, lower levels of TIMP-2 may also favor proMMP-2 activation by MT1-MMP thereby favoring tumour progression (Butler et al, 1998; Shofuda et al, 1998). The timing of TIMP function is also very important. TIMP-1 has a significant tumour stimulating effect during onset, but suppress the tumour growth during late stage of tumour progression (Gudez et al, 2001).

**In the nutshell, the present investigation indicated that**, among established risk factors for breast cancer, increasing age and postmenopausal status were significantly associated with increased risk of breast cancer. In addition to that the current study enlightened the role of oxidative stress in breast carcinogenesis, invasion and metastasis. The present investigation found, significant alterations in enzymatic and non enzymatic antioxidants, lipid profile, lipid peroxidation and thiol levels in breast cancer patients as compared to the controls. Significantly elevated SOD activity in breast cancer patients as compared to the controls indicate presence of higher ROS in breast cancer patients. Decreased plasma as well as RBC GST activity and increased plasma as well as RBC GR activity shows the imbalance in phase II enzyme system in response to detoxification metabolism. The altered levels of GST and GR have been reflected by low levels of plasma thiol suggesting higher oxidative stress and redox imbalance in breast cancer patients in the current study. Plasma non-enzymatic antioxidants including β-carotene, vitamin-E and vitamin-C levels were decreased and plasma vitamin-A levels were increased in breast cancer patients. Lower levels of β-carotene, vitamin-E and vitamin-C suggest that these non-enzymatic antioxidant are not likely to be sufficient enough to counteract the ROS production in breast cancer patients. Plasma lipid profile levels were altered and levels of lipid peroxidation were higher in breast cancer patients. Significantly lower levels of cholesterol and HDL as well as higher levels of TG and VLDL suggested
that these constituents are altered due to their increased utilization by neoplastic cells for new membrane biogenesis. Thus altered enzymatic and non enzymatic antioxidants, lipid profile, lipid peroxidation and thiol levels indicate the association of higher oxidative stress in breast cancer patients. The cytoplasmic antioxidant hsp70 was significantly increased in malignant breast tissues as compared to adjacent normal tissues showing the role of ROS in breast carcinogenesis. MMPs have an expanded role as they are important for the creation and maintenance of a microenvironment that facilitate growth and angiogenesis of tumours at primary and metastatic sites. In the current study, higher levels of active MMP-2 and MMP-9 as well as activation ratio of MMP-2 and MMP-9 were obtained in malignant tissues as compared to adjacent normal tissues. Higher activation ratio of MMP-2 and MMP-9 in patients with nodal involvement suggested higher activity of MMP-2 and MMP-9 is associated with lymphnode metastasis. Circulating levels of MMP-2, MMP-9, TIMP-1 and TIMP-2 showed lower levels of MMP-2 and TIMP-1 and higher levels of MMP-9 and TIMP-1 levels in breast cancer patients as compared to the controls. This fact suggested that TIMPs functions in MMP dependent manner in breast cancer and are important in tumour invasion and metastasis. The follow-up analysis of MMP-2, MMP-9, TIMP-1 and TIMP-2 suggested that MMP-2, MMP-9 and TIMP-1 can be used as treatment monitors for breast cancer patients.