PROLOGUE

A journey of a thousand miles must begin with a single step.

-Lao Tsu
The term “cancer” is derived from the Latin cancer and from the Greek Karkinos both meaning, “crab”. Cancer is a multistep, multifactorial, multifaceted and multimechanistic disease involving three distinct stages i.e. initiation, promotion and progression (Klaunig and Kamendulis, 2004). Initiation involves a nonlethal mutation in DNA that produces an altered cell followed by at least one round of DNA synthesis to “fix” the genetic damage produced by initiation. The promotion stage is characterized by the clonal expansion of initiated cells by the induction of cell proliferation resulting in the formation of an identifiable focal lesion. The promotion stage requires continuous presence of the stimuli, and thus it is a reversible process. Progression is the final stage in the carcinogenesis process. This stage is characterized by accumulation of additional genetic damage, leading to the transition of cells from a benign to malignant phenotype, and is an irreversible process. Spread of the potentially malignant cells from primary site to the distant site through blood or lymphatic system is called metastasis.

Cancer, a dreaded disease, is a global health problem taking toll of millions of lives. The international agency for research on cancer estimated 10.9 million new cases, 6.7 million deaths, and 24.6 million persons living with cancer in the year 2002. Among all the cancers, breast cancer is the second most frequent cancer in the world (1.15 million new cases) and is by far the most common malignant disease among women (Parkin et al, 2005). In India, it is the second leading malignancy in females (Agarwal et al, 2002). At The Gujarat Cancer & Research Institute, breast cancer is a major female malignancy with an annual incidence rate of 20% of all cancer cases.

Variety of risk factors are associated with breast cancer including age, geographical variations, effects of migration, age at menarche, childbearing, breastfeeding, menopause, endogenous hormones, oral contraceptives, hormonal therapy, diet, anthropometry, exercise, ionizing radiation, family history and genetic factors etc. (Key et al, 2001; McPherson et al, 2000). The classical risk factors for breast cancer, such as age at menarche, age at
menopause and parity, are not amenable to change for the purpose of reducing the risk. However, other lifestyle risk factors can be modified and these changes reduce the risk of breast cancer and also have other health benefits. Diet related factors are thought to account for about 30% of cancers in developed countries (Key et al, 2001) and the large international variation in the risk of breast cancer may be explained by dietary causes. Certain dietary patterns, such as high fat and carbohydrate consumption, low vegetables, fruits and fiber may increase risk of breast cancer. The nutrition and lifestyle factors may exert their carcinogenic effect indirectly either by cell stimulation or by inhibition of DNA repair mechanisms, effecting estrogen metabolism as enhancers for tumour growth. (Gerber et al, 2003). The International agency for research on cancer estimates that 25% of breast cancer cases worldwide are due to lifestyle factors (IARC, 2002). Several epidemiological studies have investigated association between dietary intakes of various vitamins and minerals and the risk of breast cancer. Thus lifestyle factors are major risk factor for the burden of breast cancer.

Increasing evidences suggest that oxidative stress, defined as an imbalance between oxidants and antioxidants in favor of the former, results in overall increase in the cellular levels of reactive oxygen species (ROS). ROS lead to many biochemical changes and is an important contributing factor in several human chronic diseases including breast cancer. ROS are produced by both endogenous and exogenous sources, which directly attack to the important constituents of, cell i.e. proteins, lipids and DNA (figure-1). Under normal physiological conditions, cells are capable of counterbalancing the production of ROS with antioxidant defense mechanisms (figure-1). The defense mechanism includes two types of antioxidants, enzymatic and non-enzymatic.
Enzymatic Antioxidants:
The first line of defense against ROS mediated injury are enzymatic antioxidants mainly Superoxide Dismutase (SOD), Catalase, Glutathione peroxidase (GPx) and Phase II enzymes glutathione-s-transferase (GST) and glutathione reductase (GR) which are synthesized in response to higher production of ROS. SOD is a primary enzymatic antioxidant, mainly dismutates $O_2^{-}$ to $H_2O_2$, which is further converted to $H_2O$ with the help of Glutathione peroxidase (GPx) and catalase. SOD also inhibits the production of hydroxyl (OH) radical. It also acts as antiproliferative agent, anticarcinogen.
and of initiation and promotion/transformation stages of carcinogenesis. Catalase functions as promotion/transformation inhibitor in carcinogenesis (Ray and Husain, 2002). GST and GR are second line of defense after SOD, catalase and GPx and are involved in the detoxification of many ROS. GSTs are large family of multifunctional detoxification enzymes that catalyse the conjugation of wide variety of electrophilic toxins and carcinogens with reduced Glutathione (GSH). GR catalyses the reduction of oxidized glutathione (GSSG) to form reduced GSH in the presence of NADPH. This reaction maintains a high GSH/GSSG ratio in the cell (Wlodek, 2002).

**Non-Enzymatic Antioxidants:**
The non-enzymatic antioxidants, β-carotene, vitamin-A, vitamin-E and vitamin-C belong to the second line of defense. They are involved in a number of biological functions such as immune stimulation, inhibition of nitrosamine formation and alterations of metabolic activation of carcinogens. They can prevent genetic changes by inhibiting DNA damage induced by the ROS. β-carotene is an established and excellent scavenger of singlet oxygen. Vitamin-A is a fat soluble vitamin which is essential for growth, vision, reproduction and differentiation of epithelial tissue. Vitamin-E, a major chain breaking antioxidant scavenges peroxyl radical intermediates in lipid peroxidation and is responsible for protecting polyunsaturated fatty acids (PUFA) present in cell membrane and low-density lipoprotein (LDL), against lipid peroxidation. Because of its antioxidant property, vitamin-E neutralizes ROS and reduces oxidative DNA damage and genetic mutations. Vitamin-C is an important water soluble antioxidant in biological fluids. It readily scavenges ROS and hypochlorous acid. It can act as co-antioxidant by regenerating α-tocopherol from the α-tocopheroxyl radical produced during scavenging of ROS. It can also protect lipid and lipoprotein against oxidative damage (Irshad and Chaudhuri, 2002). Thus, these antioxidants are thought to play an important role in various stages of carcinogenesis through their contribution to immuno-competence, membrane and DNA repair and decreasing oxidative DNA damage.
Lipids and lipoproteins:
Lipids are major cell membrane constituents for various biological functions including cell growth and division of normal and malignant tissues. During neoplastic process there is a large requirement of lipids including total cholesterol, lipoproteins and triglycerides for new membrane biogenesis. Cells fulfill these requirements either from the circulation, by synthesis through the metabolism or from degradation of major lipoprotein fractions like VLDL, LDL or HDL. ROS directly attack lipids, which lead to the peroxidation of polyunsaturated fatty acid (PUFA). Lipid hydroperoxides derived from the PUFA are prominent intermediates of peroxidative reactions. They may undergo reductive degradations, which can either diminish or enhance cytotoxic potentials, depending on the severity of stress. In addition, the lipid hydroperoxides or related intermediates trigger signal transduction pathways calling for either greater cytoprotection (upregulation of detoxifying enzymes) or deliberate termination (apoptotic death) (Girotti, 1998).

Heat shock proteins (hsp50):
Exposure of cells to environmental stress conditions—including heat shock, oxidative stress, heavy metals, or pathologic conditions, such as ischemia and reperfusion, inflammation, tissue damage, infection, and mutant proteins associated with genetic diseases—induces the expression of heat shock proteins (hsp50). These hsp50 are known to function as molecular chaperones. Molecular chaperones, a class of proteins interact with diverse protein substrates to assist in their folding, play a critical role during cell stress to prevent the appearance of folding intermediates that may lead to misfolded or otherwise damaged molecules. Consequently, hsp50 assist in the recovery from stress either by repairing damaged proteins (protein refolding) or by degrading them, thus restoring protein homeostasis and promoting cell survival. hsp50 are classified into six major families according to their molecular size: hsp 100, hsp 90, hsp 70, hsp 60, hsp 40 and small heat shock proteins (Jolly and Morimoto, 2000). The hsp 70 family of proteins functions mainly as chaperons, interacting transiently with many proteins in an ATP dependent
manner. hsp70 is strictly a stress inducible protein. It is present at basal levels in unstressed cells, but following stress, it is strongly induced and is over expressed in the cytosol and nucleus. Expression of inducible heat shock proteins is known to correlate with increased resistance to apoptosis and has been implicated in chemotherapeutic resistance of tumours and carcinogenesis (Creagh et al, 2000).

**Matrix Metalloproteinases and Tissue Inhibitor of Metalloproteinases (MMPs and TIMPs):**

Oxidative stress has also been implicated in tumour invasion and metastasis (Wang et al, 1998). Due to extensive local invasion and distant metastasis, management of breast cancer is still a major problem. The important requisite for neoplastic cell invasion during tumourigenic processes is the remodeling events that occur within the stroma or extracellular matrix (ECM). Excess matrix degradation is one of the hallmarks of cancer and is an important component of the tumour progression process. Many proteinases are capable of degrading ECM components, the most important being serine proteases, cathepsins and matrix metalloproteinases (MMPs). Among these, MMPs appear to be particularly important for matrix degradation.

Currently, the MMPs comprise a large family of over 20 secretory transmembrane proteins that together can degrade or proteolyse major components of the ECM and basement membrane. The gelatinases, which are also known as type IV collagenases, degrade gelatin (denatured collagen), and types IV, V, VII, IX and X collagen. Type IV collagen is particularly abundant in basement membranes. Degradation of type IV collagen by gelatinases occurs within the triple helical regions (Duffy et at, 2000). This subgroup has two distinct members, known as gelatinase A (MMP-2) and gelatinase B (MMP-9). The activity of MMPs is regulated in three ways: gene transcription, proenzyme activation and by their natural inhibitors, Tissue Inhibitor of Metalloproteinases (TIMPs). There are four members of the mammalian TIMP family, TIMP-1, TIMP-2, TIMP-3, and TIMP-4. TIMP-1 and
TIMP-2 preferentially block the pro forms of MMP-9 and MMP-2, respectively. Increased TIMP-1 levels have traditionally been associated with reduced invasion and metastasis. Some controversy has arisen on the role of TIMP-1, however; recent studies have shown that TIMP-1 may increase the invasive capacity of tumour cells due to its growth factor like activity. TIMP-2, similar to TIMP-1, is associated with decreased metastatic potential. (Anitha et al, 2001). The inhibitory activity of TIMPs might be important in inhibiting tumourigenesis and subsequent malignant progression. However, the effects of TIMPs on tumourigenesis are multifunctional and paradoxical. Although, the inhibitory effect of TIMPs on tumour growth and metastasis is achieved by over-expression of the TIMP gene into tumour cells, TIMPs also have growth stimulatory and anti-apoptotic effect.

**Figure-2: Role of Matrix Metalloproteinases in cancer**

MMPs are important in the late stage of tumour progression leading to metastasis. MMP-2, MMP-9, TIMP-1 and TIMP-2 are known to be closely associated with the metastatic potentials of tumour cells. Therefore, studies on biomarkers addressing the mechanisms underlying invasiveness of breast
cancer, like MMPs and TIMPs can define metastatic potentials and prognosis of breast cancer, which may be of significant clinical utility.

Considering the importance of oxidative stress in carcinogenesis, invasion and metastasis of breast cancer, the major aim of the study was to analyse levels of enzymatic and non-enzymatic antioxidants, lipid peroxidation, thiol and lipid profile parameters from healthy individuals, patients with Benign breast diseases (BBD) and breast cancer patients by highly sensitive and specific spectrophotometric methods. Evaluation of hsp70 from breast cancer patients by western blot method. Analysis of plasma MMP-2, MMP-9, TIMP-1 and TIMP-2 levels from healthy individuals, patients with BBD and breast cancer patients as well as tissue levels of MMP-2 and MMP-9 by zymography. The objectives of the study were as follows:

1. Evaluation of enzymatic antioxidants like plasma GST and GR and RBC GST, GR, SOD and catalase in healthy controls, patients with BBD and breast cancer patients.

2. Evaluation of non-enzymatic antioxidants like β-carotene, vitamin-A, vitamin-E and vitamin-C from plasma of healthy controls, patients with BBD and breast cancer patients.

3. Analysis of plasma lipid profile levels including cholesterol, very low density lipoprotein (VLDL), low density lipoprotein (LDL), high density lipoprotein (HDL) and triglycerides (TG) in healthy controls, patients with BBD and breast cancer patients.

4. Oxidative stress related markers like plasma thiol and lipid peroxidation levels in healthy controls, patients with benign breast diseases BBD and breast cancer patients.

5. Expression of heat shock protein hsp70 from malignant and adjacent normal breast cancer tissues.
6. Analysis of plasma MMP-2, MMP-9, TIMP-1 and TIMP-2 levels in the controls, patients with BBD and breast cancer patients.

7. Analysis of MMP-2 and MMP-9 from tumour tissues and adjacent normal tissues of breast cancer patients as well as from lymphnode tissues of breast cancer patients.

The above objectives were explored to assess their role in identification of high risk group, early diagnosis, staging, prognostication and treatment monitoring of breast cancer patients.