He is wise who knows the sources of knowledge - who knows who has written and where it is to be found.

-Hodge
Cancer is a complex, polygenic and multifactorial disease, resulting from successive accumulation of molecular alterations in genome of somatic cells. The subsequent changes lead to genetic instability, initiation of tumourigenesis, and progression to an increasingly malignant, invasive, and resistant phenotypes. The vast catalog of cancer cell genotypes is a manifestation of six essential alterations in the cell physiology that collectively dictate malignant growth (Figure-3). These include: self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. These novel capabilities are acquired by cells during tumourigenesis and are shared by most of the human tumours (Hanahan and Weinberg, 2000).

**Figure-3: Acquired capabilities of cancer**
Worldwide there were 10.9 million new cases, 6.7 million deaths, and 24.6 million persons living with cancer in the year 2002. Among all cancers, breast cancer ranked second when both sexes were considered together. However, breast cancer is the most frequently occurring malignancy of females worldwide with an estimated 1.15 million new cases in 2002 comprising 23% of all cancer cases. Incidence rate of breast cancer is increasing in most countries and the changes are usually increased where rate was low previously. The breast cancer incidence is increasing at the rate of about 0.5% annually and it is estimated that there would be around 1.4 million new cases of this malignancy in the year 2010 (Parkin et al, 2005).

Recently, Yang et al (2004) reported incidence rate of various cancers in the Asian Pacific Rim, which includes Asia, and the Pacific islands of the pacific rim. The authors reported that there were over 3 million new cancer cases, 2 million cancer deaths and 5.4 million people living with cancer in the year 2000. Breast cancer is currently ranked sixth in this area according to the number of new cases. It is the leading cause of cancer among women (200,000 new cases) along with cause of 64,000 deaths annually. Within Asian Pacific Rim, relatively high incidence rates were observed in Japan and parts of South-Eastern Asia, which includes India. The incidence and mortality rate for breast cancer in India, are 19.1, and 9.9, per 100,000 cases, respectively.

In India, carcinoma of breast is the second most common cancer among women, and an increasing trend in its incidence has been observed in most of the metropolis (Agarwal et al, 2001). According to the Hospital Based Cancer Registry of The Gujarat Cancer and Research Institute (GCRI), there is an increasing trend in the number of breast cancer cases (Figure-4). According to the hospital based cancer registry (HBCR), it is the second leading female malignancy at the institute comprising 947 breast cancer cases annually for the year 2001 (Annual Report, 2001).
THE MAMMARY GLAND
The breast contains two compartments: (1) the glandular portion, which is involved in production and transportation of milk, and (2) the stromal and connective tissues (Hubert, 2003). The glandular part of the mammary gland has 15–20 lobes, and within each lobe there are many smaller lobules ending in dozens of tiny bulbs that produce milk (Figure-5). All the lobules are linked by thin tubes called ducts, and all ducts lead to the nipple. The cells forming the ducts and lobules are epithelial cells. The main function of these cells is to produce and to secrete various constituents of milk. In addition, epithelial cells are surrounded by a layer of myoepithelial cells, attached on a basal membrane, whose role is to maintain the tubular structure of ducts and lobules. Surrounding the lobules and ducts, connective and fat tissues are composed of fibroblasts, with their abundant extracellular matrix and adipocytes. In addition, both blood vessels and lymph vessels irrigate the mammary gland, and nerve fibers, mostly sensory and sympathetic are also present.
THE DEVELOPMENT OF MAMMARY GLAND

The development of the mammary gland occurs predominantly after birth, and the mature breast undergoes cyclic changes regulated by both the menstrual cycle and the gestation / parturition processes. To acquire their functionality, the epithelial cells receive proper signals from hormones (estrogens, progesterone) as well as from nearby cells and components of its microenvironment (growth factors). Before epithelial cells can produce milk, they develop into lobuloalveoli (functional units) through morphogenesis involving cell proliferation, invasion, and differentiation. The cyclic development of the mammary gland reflects the fact that it is only needed during well-defined periods of life. Thus, mammary epithelium undergoes repeated cycles of growth, differentiation, and regression. Continuous cycling leads to the formation of a ductal tree. Multiplication of breast epithelial cells occurs constantly, stimulated by estrogens during menstrual cycle and pregnancy. After parturition, functional differentiation of the epithelial cells takes place. During lactation, these cells produce large amounts of milk, and after weaning, the gland regresses by a process of extensive cell death and
tissue remodeling. Thus, the mammary gland is a cellular ecosystem in which each represented cell type is subjected to a constant turnover. This is particularly the case for the epithelial cells, which are subjected to various hormonal and growth factor stimulation throughout their lifetime, with correlative changes in morphology and metabolism.

**BREAST TUMOURS**

Majority of breast tumours are of epithelial origin. The term “breast cancer” encompasses numerous types of tumours that are classified with respect to their origin and histological features. There are two main classes, *in situ* carcinoma and invasive cancer: *in situ* carcinomas are characterized by tumour cells localized either in the ducts (ductal carcinomas *in situ*) or in the lobules (lobular carcinomas *in situ*), without invasion through the basement membrane into the surrounding stroma. In contrast, in invasive carcinomas, the basement membrane is partially or totally destroyed and cancer cells progressively invade surrounding tissues, eventually leading to metastatic cells.

**Figure-6: Types of breast tumours**

![Diagram of breast tumours](image-url)
The group of invasive carcinomas includes more than 10 different types (Figure-6). The invasive ductal carcinomas (IDC) (65–80% of all breast cancers) and the invasive lobular carcinomas (ILC) (5–15% of the cases) are of the two main types, while other forms such as mucinous, medullar, tubular, and apocrine carcinomas are less frequent (0.1–4%). Besides in situ and invasive carcinomas, Paget’s disease of the nipple constitutes a particular form of malignant epithelial tumours, where malignant cells infiltrate the epidermis. In addition, a number of benign tumours of numerous types have also been described, and among them epithelial atypical hyperplasia is considered as a precancerous form. The developmental steps of invasive ductal carcinoma are shown in figure-7. The steps include histologic progression of the disease from normal epithelium to malignant stage. In breast cancer this progression consists of atypical ductal hyperplasia, preinvasive ductal carcinoma in situ (DCIS), and IDC (Chinoy, 2003; Lakhani, 1999). Thus, breast tumours can be seen as cellular ecosystems in which cell populations are constantly renewed to give rise to a progressively more aggressive tumour. This selective process takes place under the influence of hormones and growth factors, and breast cancer cells are therefore affected by a network of intercellular interactions.

Figure-7: Developmental steps of invasive ductal carcinoma

![Developmental steps of invasive ductal carcinoma](image-url)
RISK FACTORS FOR BREAST CANCER:
The etiology of breast cancer is still poorly understood. Risk factors that modulate the development of breast cancer include age, geographical location, socioeconomic status, reproductive events, exogenous hormones, life style factors (Diet, obesity, physical activity), family history of breast cancer, history of benign breast diseases (BBD) exposure to ionizing radiation etc (Key et al, 2001). Among these factors certain factors can be controlled for preventive approach to breast cancer. Table-1 shows the risk factors for breast cancer.

Table-1 Risk factors for breast cancer

<table>
<thead>
<tr>
<th>Factors can not be controlled</th>
<th>Factors can be controlled</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>Diet</td>
</tr>
<tr>
<td>Genetic factors</td>
<td>Physical activity</td>
</tr>
<tr>
<td>Benign breast disease</td>
<td>Obesity</td>
</tr>
<tr>
<td>Radiation exposure</td>
<td>Exogenous hormones</td>
</tr>
<tr>
<td>Height</td>
<td>Alcohol and smoking</td>
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</table>

Age: Age plays an important role in breast cancer risk. The incidence of breast cancer increases rapidly with age during the reproductive years and then increases at a slower rate after the age of about 50 years (Key et al, 2001; McPherson et al, 2000).

Geographical location: Migration studies showed that breast cancer incidence increases in people who migrate from a region of lower incidence to that of higher. Asian women who commonly have low breast cancer incidence in their natives had increased risk of breast cancer when they migrated to USA (Deapen et al, 2002; Lacey et al, 2002).

Socioeconomic status: Women with higher socioeconomic status or from urban area have slightly increased risk of breast cancer (Robert et al, 2004).
Reproductive events: Female reproductive hormones play an important role in breast cancer development. Epidemiological studies have consistently identified a number of risk factors, which are associated with increased exposure of endogenous estrogens. Early age at menarche, nulliparity or late age at full term pregnancy and late age at menopause increase the risk of developing breast cancer (Feigelson and Henderson, 1996). Early age at menarche (less than 12 years) has been associated with increase in breast cancer risk (Berkey et al, 1999; Titus-Ernstoff et al, 1998). Delayed menopause maximizes the number of ovulatory cycles and therefore may lead to an increased breast cancer risk. It is also reported that the breast cancer risk was increased by 3% for each year of delayed menopause (Collaborative group report, 1996; Collaborative group report, 1997). In contrast surgically induced menopause before age 35 decreases breast cancer risk. These women have only 40% of the risk as compared to women having natural menopause (McPherson et al, 2000). Early age at first pregnancy has a protective effect against breast cancer (Pathak et al, 2000) Both early age at first full term pregnancy as well as higher parity decreases breast cancer risk to half of the risk of nulliparous women. Early age at second pregnancy further reduces the risk of breast cancer (McPherson et al, 2000).

Exogenous hormones: Hormonal usage after menopause increases breast cancer risk depending on the duration of exposure and whether the estrogen is used alone or in combination with progestins (Ross et al, 2000). A large meta analysis has demonstrated that long term hormone replacement therapy (HRT) is responsible for the cumulative increase in breast cancer risk as compared to never users of HRT (Collaborative group report, 1997). Use of oral contraceptive is also associated with increased risk of breast cancer. The risk of breast cancer is increased around 25% in the current users of oral contraceptives and the excess risk falls after cessation of use (Collaborative group report, 1996).
Family history: Family history however, is a heterogeneous risk factor with different implications depending on the number of relatives with breast cancer, the exact relationship, the age at diagnosis and the number of unaffected relatives. Identification of two tumour suppressor genes BRCA1 and BRCA2 has provided new insights into the understanding of inherited predisposition to breast cancer. Women with one or more than that first degree affected relatives have an increased risk of breast cancer as compared to women without affected relative (McPherson et al, 2000).

Benign breast diseases (BBD): BBD is a heterogenous group of breast diseases classified as nonproliferative or proliferative lesion with or without atypia. Non proliferative diseases are not associated with increased risk of breast cancer, whereas proliferative disease without atypia results in a small increase in breast cancer risk (Key et al, 2001, McPherson et al, 2000).

Exposure to ionizing radiation: It increases breast cancer risk. The exposure to ionizing radiation in early life carries the greatest risk. The risk is dose dependent and decreases gradually over the years (Biglia et al, 2004).

Height: Hunter and Willett (1993) showed that within populations, a 10 cm greater height is typically associated with an increase in risk of about 10%. As height is positively correlated with energy intake during growth and early menarche, and it might be a marker for the number of susceptible breast cells.

Diet and life style: The International agency for research on cancer estimates that 25% of breast cancer cases worldwide are due to overweight/obesity and a sedentary lifestyle (IARC, 2002). A high intake of fat is weakly associated with an increased risk of breast cancer (Velie et al, 2000). Obesity has a complex relationship with breast cancer risk, which is modulated by menopausal status. The women who are physically active have lower risk of breast cancer (Thune and Furberg, 2001). Diet related factors are thought to
account for about 30% of cancers in the developed countries (Key et al, 2001). Human diet contains a great variety of natural carcinogens and anti-carcinogens (Sugimura, 2000). Doll and Peto (1981) estimated that approximately 35% of cancer can be prevented by changing dietary habits. Diet rich in fruits and vegetables, rich source of various nutrients, is associated with decrease in the breast cancer risk (Lee, 1999). These nutrients include antioxidant vitamins and flavonoids and it is hypothesized that dietary antioxidants prevent oxidative stress (Ambrosone, 2000). There is increasing evidence indicating that oxidative stress is involved in the pathogenesis of cancer, including breast cancer (Brown and Roy, 2001).

**OXIDATIVE STRESS:** The term "oxidative stress" refers to the total burden of potentially harmful reactive biochemical species that are present in the tissue as a consequence of the routine cellular oxidative metabolism of both endogenous and exogenous compounds (Nathan and Chaudhary, 1998). Oxygen, while indisputably essential for life, also participates in the destruction and/or impairs the ability of tissues to function normally. Cellular oxidants, the derivatives of oxygen, which are often called reactive oxygen species (ROS) are constantly produced in human cells as a result of mitochondrial electron transfer processes (Loft and Poulsen, 1996). ROS can also be formed as a result of metabolism of xenobiotics by cytochrome p450 enzymes or exposure to other environmental factors (Hayes and McLellan, 1999). ROS can be produced by various exogenous sources. Environmental agents including non genotoxic carcinogens directly generate or indirectly induce ROS in cells. Chlorinated compounds, radiation, metal ions, barbiturates, phorbol esters and some peroxisome proliferating compounds are among the classes of compounds that have been shown to induce oxidative stress and damage *in vivo* and *in vitro* (Klaunig and Kamendulis, 2004). Production of ROS is essential for a number of biochemical reactions involved in the synthesis of prostaglandins, hydroxylation of proline an lysine, oxidation of xanthine and other oxidative processes. Among cellular ROS, the
most aggressive entities are superoxides and hydroxyl radicals (Halliwell and Cutteridge, 1999). Numerous data demonstrate that ROS are capable of oxidizing cell constituents such as DNA, proteins, and lipids, thereby incurring oxidative damage to cell structures. Excessive oxidation leads to impairment of cell functions and development of morbid conditions (Halliwell and Cutteridge, 1999; Irshad & Chaudhuri, 2002; Klaunig and Kamendulis, 2004). Thus, ROS are powerful substances as a consequence of normal metabolism as well as due to various exogenous sources. Therefore, when there is excessive production of ROS or insufficiency of defense mechanisms oxidative stress may occur. Various studies have documented role of oxidative stress in the etiology of breast cancer (Ambrosone, 2000; Brown and Roy, 2001) and other malignancies (Hristozov et al, 2001; Manju et al, 2002).

**OXIDATIVE STRESS IN CANCER**

Oxidative stress and altered redox state cause the alterations leading to carcinogenesis, invasion and metastasis through a complex cascade of mechanisms (Figure-8). The major hypothesis explaining the importance of oxidants and imbalance of the cellular redox state is altered by pro-oxidant intracellular environment that facilitates mutations and/or inactivation of tumour suppression genes and activates oncogenes, with subsequent changes in cell growth, survival and apoptosis (Burdon, 1995; Osada and Takahashi, 2002; Sun and Oberley, 1996). Cellular redox state regulates several signaling pathways that are closely associated with cell growth and survival involving c-myc, p53 (Brunelle and Chandel, 2002; Chandel et al, 2000; Meplan et al, 2000; Vafa et al, 2002), FAS-mediated apoptosis and ras-mediated epidermal factor receptor dependent angiogenesis (Casanova et al, 2002; Kasahara et al, 1997; Suhara et al, 1998). ROS also induce CKIp21 (Cipl) gene (which cause permanent growth arrest/senescence) and inactivate PTEN, a tumour suppressor protein that cause cell death and senescence (Lee et al, 2002; Macip et al, 2002). Degradation of hypoxia-inducible factor (HIF) is strongly associated with angiogenesis. It is also regulated by prolyl hydroxylases and
asparagine hydroxylases by an oxygen-dependent mechanism (Jaakkola et al, 2001; Lando et al, 2002; Welsh et al, 2002). Thus, oxidants and cellular redox state are important determinants regulating cell growth, survival and expression of tumour suppressor genes.

**Figure-8: Oxidative stress in cancer**

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**BIOMARKERS FOR OXIDATIVE STRESS AND ANTIOXIDANT DEFENSE SYSTEM IN HUMAN BODY**

Free radicals are highly reactive and they have a very short life. Therefore, direct measurement of oxidative stress in terms of free radicals is difficult and not reliable. Hence, the oxidative damage is measured indirectly by estimating the oxidized DNA product, 8-oxo-deoxyguanosine or MDA, a major lipid peroxidation product (Valko et al, 2004). The oxidative damage can also be measured by quantifying the alterations in the antioxidant defense system. The balance between oxidative stress and antioxidant defense system plays vital role to protect the cells from detrimental effects of oxidative stress. To counteract the deleterious effects of oxidants and to protect cells from excessive oxidation, human body is endowed with an array of powerful
antioxidants. The antioxidant system comprises different types of functional components classified as first, second, third and fourth line of defenses. The antioxidants belonging to the first line of defense are enzymes like, Superoxide Dismutase (SOD), Catalase, Glutathione Reductase (GR), Glutathione-s-transferase (GST), Glutathione Peroxidase (GPx) and non-enzymatic molecules like minerals (Se, Mn, Cu, Zn) and some proteins. The second line of defense includes glutathione, vitamin-C, uric acid, albumin, bilirubin, vitamin-E, carotenoids, flavanoid and ubiquinol. Among these, β-carotene, vitamin-E and vitamin-C are important scavenging antioxidant vitamins which can not be synthesized by most mammals including humans and therefore, are obtained from diet. The third line of defense includes a group of enzymes for repair of damaged DNA, damaged protein, oxidized lipids and peroxides (Irshad and Chaudhuri, 2002). Antioxidant defenses act as a balanced and coordinated system and each relies on the action of the others. Many antioxidant defenses depend on micronutrients e.g. SOD is a Cu Zn or Mn containing enzyme and catalase, a hem enzyme (Evans and Halliwell, 2001). Therefore, such antioxidants are beneficial as biomarkers of oxidative damage or free radical damage, as alterations in the levels of antioxidants can reflect the status of oxidative damage in the body (Figure-9).

Figure-9: Summary of antioxidant defenses in human body

![Diagram of antioxidant defenses in human body](image-url)
ENZYMATIC ANTIOXIDANTS

**SOD:** SODs are a family of metalloenzymes that are localized in the cytosol and mitochondria. Their main function is to reduce superoxide anions to hydrogen peroxide and water. SOD destroys oxiradicals by successive oxidation and reduction of the transition metal ions at the active sites with remarkably high reaction rates (Meier et al., 1998). In humans, there are three forms of SOD: cytosolic Cu/Zn-SOD, mitochondrial Mn-SOD, and extracellular SOD (EC-SOD). (Mates et al., 1999). MnSOD differs from two other isoforms in that it is produced in the cytosol and then localizes to the mitochondria due to post-translational modifications (Shimoda et al., 1996). Cu/Zn-SOD is believed to play a major role in the first line of antioxidant defense. Recently it is also suggested that MnSOD is essential for life (Li et al., 1995). This may be due to the fact that, mitochondria consume over 90% of cell’s oxygen (Borgstahl et al., 1996). SOD inhibits the nuclear factors, AP-1 and NF-kB in human breast cancer cells (Li et al., 1998; Mana et al., 1998). SOD can act as anticarcinogen and inhibitor of initiation and promotion/transformation stage in carcinogenesis (Ray and Husain, 2002).

**Catalase:** Catalase is a hemoprotein that has a predominant role in controlling the hydrogen peroxide concentration in human cells by converting it into water and oxygen. In most mammalian tissues, catalase is located in the peroxisomes. Catalase is not essential for some cell types under normal conditions. However, it plays an important role in the acquisition of tolerance to oxidative stress in the adaptive response of cells. *In vitro* studies have suggested that this antioxidant functions as an inhibitor in promotion and transformation of carcinogenesis (Mates et al., 1999).

**Phase II enzymes (GST and GR) and glutathione:** Glutathione is the most abundant low-molecular-weight thiol in mammalian cells. Thiols play a critical role in the determination of protein structure and function, regulation of enzymatic activity, control of the activity of transcription factors and
antioxidant protection. Antioxidant properties of thiol compounds depend on
different mechanisms. As a thiol compound, glutathione fulfils a very
important role of an antioxidant in the cells. In antioxidant reactions, the
reduced GSH is oxidized yielding disulfide (oxidized GSSG), that can again be
reduced to thiol with the participation of GR and NADPH. These reactions
constitute an important redox cycle in the cells (Wlodek, 2002).

GSTs are important in intracellular binding and transport of numerous
compounds, and play a central role in human detoxification processes. Their
main function is to catalyse the conjugation of GSH with a wide variety of
xenobiotics such as carcinogens and pharmacologically active agents as well
as ROS. This conjugation results in the formation of compounds with greater
water solubility but less biological activity, and these can be excreted in urine
(Stamler and slivka, 1996). Four subclasses of GST isoforms have been
recognized in humans: Alpha, Mu, Pi and Theta, each of which is subdivided
into one or more distinct isoforms with different reactivity towards specific
substrates (Raijmakers et al, 2001). Brockmoller et al (1992) reported that a
homozygous deletion of GST M1 or GST T1 genes results in a lack of GST
enzyme activity with the GST M1 null polymorphism and GST M1
polymorphism was found in approximately 50% in Caucasian population.

GR catalyses the reduction of oxidized glutathione (GSSG) to form reduced
Glutathione (GSH) in the presence of NADPH. This reaction maintains a high
GSH/GSSG ratio in the cell.

NON-ENZYMATIC ANTIOXIDANTS

**Vitamin-C:** Vitamin-C acts as a potent water soluble antioxidant in biological
fluids. It acts by scavenging physiologically relevant ROS and reactive
nitrogen species. In addition, it regenerate urate, glutathione and \( \beta \)-carotene
from their oxidation products, i.e. urate radicals, glutathiy radicals and \( \beta \)-
carotene radical (Halliwell, 1996). The antioxidative property of vitamin-C is due to its ability to donate electrons. By donating its electrons, it prevents oxidation of other compounds. However, vitamin-C itself is oxidized in the process. The species formed after the loss of one electron is a free radical, semi dehydroascorbic acid or ascorbyl radical which is relatively stable with a half-life of $10^{-5}$ seconds and is fairly unreactive as compared to other free radicals. Reduction of a reactive free radical with formation of a less reactive compound is sometimes called free radical scavenging or quenching. Ascorbate is therefore a good free radical scavenger due to its chemical properties (Padayatty et al, 2003). Vitamin-C can act as a co-antioxidant by regenerating $\alpha$-tocopherol from the $\alpha$-tocopheroxyl radical and prevents tocopherol mediated lipid peroxidation (Bowry et al, 1995; May et al, 1998). There are a wide variety of mechanisms by which ascorbate prevents and inhibits malignant growth (Gonzalez et al, 2005). It has also been reported that vitamin-C may enhance host immunological functions (Kelley and Bendich, 1996). In addition, vitamin-C may reduce carcinogenesis through the stimulation of immune systems where cytotoxic T lymphocytes, macrophages and natural killer cells can lyse tumour cells (Bendich, 1997).

**Vitamin-E ($\alpha$-tocopherol):** Vitamin-E is lipid soluble vitamin, which is derived from the diet. The major function of vitamin-E is its role as antioxidant to protect cell membrane lipids from oxidative DNA damage by ROS. When ROS attacks the fatty acid, fatty acid radicals are formed. It reacts with oxygen that recycles to form more peroxyl radicals in a chain reaction. The phenolic hydroxyl group of tocopherol reacts with organic peroxyl radicals to form the corresponding organic hydroperoxides and tocopheroxyl radicals (Ray and Husain, 2002). Vitamin-E directly scavenges ROS and protects PUFA against lipid peroxidation and thus it is thought to be the major chain breaking antioxidant, which plays an important role in various stages of carcinogenesis through immunocompetence, membrane and DNA repair and decreasing oxidative DNA damage (Kimmick et al, 1997). Vitamin-E
circulates in conjugation with the lipoproteins and chylomicrons, and a molar ratio to cholesterol is a good index of vitamin-E status (Bates, 1997). Vitamin-E protects oxidation of LDL (Traber, 1997). Vitamin-E neutralizes ROS and reduces oxidative DNA damage and genetic mutations.

**β-carotene and vitamin-A:** β-carotene is another lipid soluble antioxidant and precursors of retinol and retinoids. It also has several other functions including protection against oxidation by quenching singlet oxygen (Stahl et al. 1997). The antioxidant actions of β-carotene are based on their singlet oxygen quenching properties. It traps peroxyl radicals in low oxygen tension and prevents lipid peroxidation. (Paiva et al, 1999). However, the antioxidant activity of β-carotene can shift into a prooxidant activity depending on factors such as oxygen tension or β-carotene concentrations (Jandacek, 2000).

Vitamin-A is a fat soluble vitamin, which is essential for growth, maintenance of visual function, reproduction and differentiation of epithelial tissues. Vitamin-A occurs mainly as retinol in plasma and circulates as a 1:1:1 complex with two hepatically synthesized proteins RBP and transthyretin. It is reported to play a vital role in suppressing carcinogenesis by increasing immune surveillance. Vitamin-A deficiency promotes carcinogenesis, but paradoxically, an excess of vitamin-A may have a similar effect. Vitamin-A and its metabolites play a crucial role in regulating the differentiation and proliferation of epithelial cells (Ray and Husain, 2002)

Thus, antioxidants work in concert with one another by a series of oxidation-reduction (redox) reactions to quench ROS (Figure-9). Redox buffering systems of the common antioxidants are all related in stepwise, sequential recycling process, which provides ongoing neutralization of free radicals. The synergistic action of vitamin-C, vitamin-E and β-carotene is probably the most important antioxidant mechanism protecting LDL against lipid peroxidation.
LIPID PEROXIDATION (LPX), LIPIDS AND LIPOPROTEINS:

Lipids are the major cell membrane constituents which are important for membrane integrity and have various biological functions including cell growth and division of tissues. ROS directly attack lipids, which lead to the peroxidation of PUFA resulting in toxic byproducts. The major products of lipid peroxidation are MDA (malondialdehyde), 4-HNE (4-hydroxynonenal) and various 2-alkanals (Devasagayam et al, 2003). MDA is a mutagenic and genotoxic agent that contributes to the development of human cancer. Lipid hydroperoxides also directly induce DNA chain breaking and produce lipid peroxy and alcoxy radicals that cause oxidation in DNA. Peroxide and hydroperoxides have also demonstrated tumour promoting activity in vivo. It can result in the formation of cyclic DNA adduct. MDA forms adducts with adenine and cytosine, which contributes to the carcinogenecity and mutagenecity in mammalian cells (Ray and Husain, 2002). The damage caused by lipid peroxidation is highly detrimental to the functioning of the cell and its survival. As a result, there is a greater utilization of lipids including cholesterol, lipoproteins and triglycerides for new membrane biogenesis.

RECENT STUDIES ON ENZYMATIC AND NON-ENZYMATIC ANTIOXIDANTS, LIPIDS, LIPOPROTEINS, THIOL, LIPID PEROXIDATION AND CANCER

The first evidence for the biological significance of ROS production came from the observation that, activated neutrophils used superoxide radical as defense mechanism against bacteria (Babior, 1978). Subsequently, various studies, suggested ROS as a key regulatory factor in a variety of diseases as well as in molecular pathways linked to tumour development. Higher oxidative stress due to higher ROS and redox imbalance led to the altered pro-oxidant intracellular environment, which ultimately altered various pathways of cellular growth, survival and apoptosis (Kinnula and Crapo 2004). Elimination of excess ROS by antioxidant defense decreases the tumourigenesis of various types of tumour cells and has opened up new areas for cancer research in the field of cancer biology. Endogenous as well as exogenous
antioxidants play a significant role in detoxification of ROS. Thus, ROS oxidative stress and antioxidants have emerged as interesting areas of cancer research. A large number of studies have shown the importance of ROS, oxidative stress and antioxidants in breast cancer.

Most of the *in vitro* studies have suggested that SOD and catalase function as promotion /transformation inhibitor in carcinogenesis (Ray and Husain, 2002). The circulatory and tissue levels of these enzymatic antioxidants have been studied in various malignancies. Significantly elevated SOD activity was found in breast cancer (Deliconstantinos et al, 1995; Koksoy et al, 1997). Increased levels of plasma Cu and Zn have been reported in breast cancer patients which further support higher Cu-Zn SOD activities in breast cancer patients (Huang et al, 1999). Khanzode et al, (2004) showed gradual increase of serum SOD levels from stage I to stage IV in breast cancer patients. Studies have also found lower levels of SOD activity in esophageal, gastric, hepatic, colorectal and pancreatic cancer (Puscas et al, 1999). Lower levels of catalase activity were also shown in lymphoma patients (Casado et al, 1995). A decreased expression of catalase m-RNA was observed in thyroid carcinoma patients (Hasegawa et al, 2002). In mesothelioma patients higher expression of catalase and MnSOD was observed (Kahlos et al, 2001). Lower catalase activity was also reported in colon tumour tissue as compared to normal tissues (Skrzydlewska et al, 2001).

Phase II enzymes catalyse the conjugation of activated xenobiotics, to an endogenous water-soluble substrate, such as GSH by GSTs. It can also catalyse nucleophilic aromatic substitutions from reactive oxidants or carcinogens. The GST enzymes conjugate reactive chemical groups, including ROS and diol-epoxide, to glutathione and thereby prevent them from binding and damaging DNA. They also detoxify other endogenous oxidation products including α-quinones formed from catacholamines and estrogen-3-4-quinones (Cavalieri et al, 1997; Yager and Liehr, 1996). The cytosolic GST comprise of
four class alpha, mu, pi and theta. Of these, GSTM1 is of particular interest because it shows a null polymorphism that results in lack of the enzyme expression in approximately 50% of the population (Brockmoller et al, 1992). Phase II detoxification enzymes mainly GST and GR are also studied from tissues as well as from plasma/serum and erythrocytes. Erythrocyte activity of GST and GR were significantly decreased in colorectal cancer patients as compared to the controls (Saygili et al, 2003). Iscan et al (1998) showed significantly higher tissue GST activity in breast cancer patients as compared to normal tissues. Elevated tissue levels of GST were observed in head and neck squamous cell carcinoma and non small cell lung carcinoma as compared to normal tissues (Ferruzzi et al, 2003). Elevated GR activity was also reported in colon tissue as compared to normal tissues (Skrzydlewska et al, 2001). Reduction in GST expression was reported in Barret’s esophagus and esophageal tissue by immunohistochemistry (IHC) (Cobbe et al, 2003).

Various studies suggest inverse association between dietary antioxidants and cancer. Non-enzymatic antioxidants are exogenous and supplemented through diet. They can be divided into two groups, aqueous phase and lipid phase antioxidants (Young and Woodside, 2001). Lipid phase chain breaking antioxidants scavenge radicals in membranes and lipoprotein particles and are crucial in preventing lipid peroxidation. It mainly includes β-carotene, vitamin-E, vitamin-A, flavanoids etc. Apart from its antioxidative action, β-carotene may act as promoter of carcinogenesis through its metabolisation to biologically active products (Wang et al, 1999). Recently it has been reported that Vitamin-E derivative, vitamin-E succinate (VES) possess the ability to induce only cancer cells to undergo apoptosis (Kline et al, 2001). Vitamin-C is an important chain breaking antioxidant of aqueous phase (Young and Woodsides, 2001). It prevents oxidation of LDL by scavenging ROS in aqueous milieu (Frei et al, 1988). Recently it has been reported that lipid hydroperoxides can react with ascorbic acid. The product thus formed could potentially damage DNA. This fact suggests that vitamin-C may enhance
mutagenesis and risk of cancer (Lee et al, 2001). Non-enzymatic antioxidants were also studied from serum as well as from plasma in variety of cancers. Ching et al (2002) reported increased levels of serum β-carotene and retinol with reduction in breast cancer risk. Serum β-carotene, ascorbic acid and α-tocopherol levels were lower in gastric cancer patients as compared to controls. Serum retinol levels were comparable in controls and cancer patients (Choi et al, 1999). It is found that lung cancer patients have significantly lower serum levels of vitamin-A, β-carotene and vitamin-E than controls. While higher serum levels of vitamin-A, β-carotene were associated with stomach cancer risk (Kumagai et al, 1998). Lower levels of serum vitamin-A, vitamin-C and carotene were reported in breast cancer and cervical cancer patients (Ramaswamy and Krishnamoorthy, 1996). Saintot et al (2002) showed association of plasma vitamin-E in breast cancer specific and disease free survival independent of tumour characteristics. Recent studies by Khaw et al (2001) showed higher plasma vitamin-C concentration has inverse relation with cancer mortality. Ladas et al (2004) has also shown role of antioxidants in cancer therapy and thus, non-enzymatic antioxidants are the major area of debate in cancer prevention.

Lipids are major cell membrane components. Cell membrane phospholipids are very sensitive to oxidation. They are the frequent targets of radical induced damage that enables them to participate in free radical chain reaction. There are numerous reports on lipids and lipoproteins in cancer. Eichholzer et al (2000) showed low cholesterol levels as the risk factors for various cancer including lung, stomach, prostate and colon. Chang et al (1995) also suggested lower cholesterol levels play a major role in lung cancer risk. Damage to lipids results in lipid peroxidation which can further cause DNA damage. The initial products formed by LPx are short lived lipid hydroperoxides. By reacting with other compounds, they generate a number of products which themselves are reactive. MDA is the major aldehyde product of lipid peroxidation. They are mutagenic as well as carcinogenic.
GSH being the only endogenous antioxidant, protect the cells from oxidative stress and functions as substrate for enzymatic antioxidants. Gonenc (2001) et al reported elevated MDA levels in lung and breast cancer. Ahmed et al (1999) reported higher lipid peroxide levels and lower glutathione levels in cervical cancer patients. Significantly higher levels of MDA were observed in laryngeal cancer patients (Taysi et al, 2003).

There are various reports on enzymatic and non-enzymatic antioxidants, lipids lipid profile and oxidative stress related markers from India. Saroja et al (1999) reported higher GST levels in oral tumour tissues as compared to normal tissues. Elevated levels of serum GST and GR were reported from our laboratory (Patel et al, 2002). Kumaraguruparan et al (2002) observed increased lipid peroxidation and concomitant decrease in circulating GST, GR, SOD, catalase and non-enzymatic antioxidants vitamin-E and vitamin-C in breast cancer patients as compared to patients with fibroadenoma. The authors also found lower levels in fibroadenoma patients as compared to controls. They also observed higher lipid peroxidation accompanying with higher GST, SOD, Catalase and GSH in breast tumour tissues as compared to uninvolved normal tissues (Kumaraguruparan et al, 2002). Serum SOD and MDA levels were increased in breast cancer patients as compared to the controls whereas, plasma ascorbic acid levels were lower in breast cancer patients as compared to the controls (Khanzode et al, 2004). Ray and Husain (2001) reported that levels of serum triglycerides, total cholesterol and LDL were higher and HDL levels were lower in breast cancer patients as compared to the controls. Kolanjiappan et al (2003) showed decreased lipid peroxidation levels and increased total cholesterol levels in oral tumour tissues as compared to the normal tissues. Levels of plasma total cholesterol, LDL and TG were found to be elevated with decreased HDL levels in breast cancer patients as compared to the controls. The investigators also observed lower levels of vitamin-E and vitamin-C in breast cancer patients as compared to the controls (Ray and Husain, 2001). Elevated serum cholesterol and LDL levels
were observed in breast cancer patients as compared to controls (Hasija and Bagga, 2005).

In addition to enzymatic and non-enzymatic antioxidants to counter harmful effects of oxidative stress, there is a range of cytoplasmic “antioxidants” the cytoplasmic redox chaperones e.g. small heat shock proteins and hsp70. They protect the target proteins by covering their sensitive sites. It also captures denatured proteins and hold them until they are refolded and degraded. Thus antioxidant mechanisms of enzymatic, non-enzymatic antioxidants and hsps are interrelated in many ways (Papp et al, 2003).

HEAT SHOCK PROTEINS (hsps):
The heat shock response was discovered in by Ritossa (1962), who observed a pattern of Drosophila salivary gland chromosome puffs that were induced in response to transient exposures to elevated temperatures. Since then, efforts from a large number of investigators have shown that, the heat shock response is ubiquitous and highly conserved in all organisms from bacteria to plants and animals—as an essential defense mechanism to protect cells from a wide range of harmful conditions, including heat shock, alcohol, inhibitors of energy metabolism, heavy metals, oxidative stress, fever, inflammation etc. (Jolly and Morimoto, 2000).
Table-2: Heat Shock Protein Families And Their Functions

<table>
<thead>
<tr>
<th>Heat shock proteins</th>
<th>Main functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsp100</td>
<td>Thermotolerance.</td>
</tr>
<tr>
<td>hsp90</td>
<td>Regulation of cytoskeleton dynamics, cell shape and mobility. Prevention of aggregation of denatured proteins. Regulating function by interacting with several protein kinases.</td>
</tr>
<tr>
<td>hsp70</td>
<td>Folding of newly synthesized proteins. Protein transport and sorting between compartments. Regulation of stress response. Thermotolerance and resistance to apoptosis inducing agents.</td>
</tr>
<tr>
<td>hsp60</td>
<td>Mitochondrial chaperone involved in protein import and folding. Have role in apoptosis regulation.</td>
</tr>
<tr>
<td>Small hsps (10-28kDa)</td>
<td>Signal transduction to actin microfilaments. Thermotolerance and resistance to apoptosis inducing agents.</td>
</tr>
<tr>
<td>Ubiquitin</td>
<td>Ubiquitination of proteins for degradation, activation of nuclear factors.</td>
</tr>
</tbody>
</table>

Stress-inducing agents often affect the redox state and hydration of cells, which, in turn, causes increased levels of misfolded proteins that are deleterious by virtue of their altered biological activities. The cellular response to stress is represented at the molecular levels by the induced synthesis of hsps. hsps have been classified into six major families according to their molecular size: hsp100, hsp90, hsp70, hsp60, and small heat shock proteins (Table-2). Within each gene family there are members that are constitutively expressed, inducibly regulated, and/or targeted to different compartments. This superfamily of proteins is also referred to as 'molecular chaperones' due to their ability to stabilize proteins and polypeptides, thereby minimizing protein misfolding and aggregation within the cell (Creagh et al, 2000)

**Heat Shock Protein 70 (hsp70):**
The 70kDa heat shock proteins comprise a family of highly conserved molecular chaperone that regulate a wide variety of cellular processes during normal and stress conditions (Table-2).
Molecular structure of hsp70:
Members of the hsp70 family exhibit complex patterns of growth regulated and stress-induced gene expression and are targeted to different subcellular compartments. hsc70 (heat shock constitutive 70) and hsp70 proteins are cytosolic and nuclear, whereas Grp78 (glucose-regulated protein 78) is localized to the endoplasmic reticulum and mhsp70 (mitochondrial hsp70)/Grp74 (glucose-regulated protein 74) is a mitochondrial localized protein. Among all these proteins hsp70 is one of the most abundant of hsps, accounting for 1-2% of total cellular proteins. In humans there are at least 11 distinct genes that code for hsp 70 isoforms, which are located on several different chromosomes. Using DNA recombinant technology two major domains have been characterized (Figure-10). The N-terminal ATPase domain is a 44 kDa fragment that binds to and hydrolyses ATP. Whereas, 18 kDa c-terminal binds peptides for final packaging, degradation or repair.

Figure – 10: Domain structure of human hsp70

hsp70 interacts with co-chaperones through the N-terminal ATPase domain and with substrates at the C-terminal substrate binding domain. Binding of unfolded or partially unfolded proteins is regulated by ATP-hydrolysis-induced conformational changes in the ATPase domain of hsp70, which is stimulated by the co-chaperone hsp40 (Hdj-1). Release of substrates requires the binding of ATP to hsp70, after which the substrates either enter a new cycle of binding and release or fold into their native conformation. Co-chaperone interactions can influence the hsp70-substrate binding-and-release cycle by stimulating, inhibiting or altering the trafficking of hsp70-interacting substrates. Hip binds to the ATPase domain and increases the chaperone activity of hsp70 by stabilizing the ADP state, which is the substrate-bound...
Introduction

state of hsp70. Bag1, by contrast, inhibits the chaperone activity of hsp70 in part by accelerating nucleotide exchange, which affects the premature release of the unfolded substrate. Hip and Bag1 bind to hsp70 at the same site on the ATPase domain and directly compete to influence hsp70 chaperone activity. (Nollen and Morimoto, 2002)

REGULATION AND FUNCTIONS OF hsp70:
The expression of hsp is regulated by specific transcription factors (HSFs) which binds to heat shock element (HSE), the promoter region of hsp gene which regulate transcription with synergistic action by crosstalk of various HSFs. In humans there are two HSFs, HSF1 and HSF2. In unstressed cells HSF1 is present in inactive form. Following stress, denatured proteins bind hsp90, causing dissociation of hsp90 and HSF1. This will lead to phosphorylation resulting into HSF activation. The active HSF form trimers and bind to the promoter of the hsp70 gene to stimulate transcription (Creagh et al, 2000). hsp70 family members protect cells and tissues from lethal heating as well as several other environmental and pathological stress conditions. hsp70 increases resistance to cell death induced by tumour necrosis factor (TNF), monocytes, oxidative stress, ceramide, terminal differentiation, UV radiation, caspase-3 over expression, ischemic injury, hydrogen peroxide toxicity, apoptosis etc. (Jattella, 1999; Witzmann et al, 1996). hsp70 and co-chaperones also work together to regulate the function of p53 tumour suppressor protein (Zylicz et al, 2001). Cells or tissues from a wide range of tumours have been shown to express atypical levels of one or more hsps (Jolly and Morimoto, 2000).

RECENT STUDIES ON hsp70 AND CANCER
Heat shock proteins occupy a central role in intracellular homeostasis. Differential expression of individual hsp occurs in a broad range of neoplastic processes. The cell synthesis of heat shock proteins is increased by a variety of stress factors including oxidative stress. The 70 kDa heat shock protein
family (hsp70 family) constitutively expresses hsc70 and heat inducible hsp70. The family members of hsp70 are often associated with cell cycle related proteins including p53, cdk4, c-myc, pRb and p27 (Helmbrecht et al, 2000). hsp70 chaperone activity influences tumourigenesis by regulating these cell cycle regulatory proteins. hsp70 negatively regulates various stages of the p53-dependent or independent apoptotic pathways (Gabai et al, 2000, Li et al, 2000). It is also observed that higher expression of hsp70 is associated with inhibition of apoptosis (Jattella, 1999; Samali and Orenius, 1998; Vayssier and Polla, 1998). hsp70 also function at multiple points in the apoptotic signaling pathways, which suggests that constant titration occurs between these pathways. Beere et al (2000) showed that high levels of hsp70 prevents stress induced apoptosis mainly by preventing the recruitment of procaspase 9 and 3 to the apoptosome complex. The balance between these pathways can determine the fate of the stressed cells.

hsp70 confers different immunological functions depending upon the subcellular localization. Elevated cytoplasmic hsp70 levels play an important role in protection against cell damage induced by exogenous stress. These include, heat shock, tumour necrosis factor, oxidative stress, cytoplasmic drugs and radiation (Jaattella et al, 1998; Li and Lindquist 2000; Ricci et al 2001; Vargas-Roig et al, 1998;). Plasma membrane bound hsp70 has been determined as tumour selective target structure for the cytolytic attack mediated by natural killer (NK) cells (Multhoff et al, 1997). hsp70 protein, as well as the immunostimulatory peptide “TKDNNLLGRFELSG” residing in the c-terminal domain of hsp70 activates the cytolytic activity of NK cells against hsp70 (Multhoff et al, 2001; Multhoff et al, 1995). Tamura et al (1997) have also shown that hsp70 elicit a potent anticancer immune response. They act as carrier molecule for tumour derived immunogenic peptides that are taken up by antigen presenting cells and represented to cytotoxic T lymphocytes (Schild et al, 1999; Tamura et al, 1997). hsp70 also functions as epitope for cytolytic attack (Asea et al, 2000; Dressel et al, 2000).
Over expression of cytoplasmic hsp70 is seen in various malignancies. In colorectal cancer tissues, hsp70 was overexpressed as compared to normal tissues (Kanazawa et al, 2003). Over expression of hsp70 was found in breast tumour tissues (Yano et al, 1996). Vargas-Roig et al (1997) showed that breast tumour that over expressed hsp70, had a high rate of proliferation. Lazaris et al, (1997) showed association of over expression of hsp70 and advanced tumour grade in lymph node positive breast cancer patients. hsp70 over expression reported to be associated with short term disease free survival, metastasis and poor prognosis among breast cancer patients treated with chemotherapy, radiotherapy and hyperthermia (Liu et al, 1996; Vargas-Roig et al, 1998). It has been also reported that, inducible hsp70 protects cells against cytotoxic effects of hyperthermia (Barnes et al, 2001). In cervical cancer, hsp70 plays an important role in tumour cell proliferation and cervical intra epithelial neoplasia (CIN) (Kim et al, 1998). In bladder cancer, hsp70 was observed as an independent prognostic factor (Nambu et al, 1998). Kaur and Ralhan (1995) have shown a wide variation in hsp70 expression in normal, pre-malignant and malignant oral lesions. They have suggested that hsp70 is differentially expressed during oral carcinogenesis and may be implicated in tumour development. Further, among the oral tumours assessed, a significantly positive association was observed between hsp70 expression and differentiation of oral squamous cell carcinomas. Kaur et al (1998) also observed significantly higher cell surface expression of hsp70 in clinically advanced stage tumours as compared with early stage tumours.

**MATRIX METALLOPROTEINASES (MMPs)**

After neoplastic transformation, tumour-host interactions promote coordinated molecular and cellular processes underlying a continuum of steps. These steps are sequential and interrelated ultimately leading to metastasis. The sequential steps in the metastatic process are explained in the figure-11. It includes: (i) escape of cells from primary tumour, with the disruption of the basement membrane with subsequent invasion of malignant cells into the
host stroma, (ii) intravasation (entry of cells into the lymphatic or blood circulation), (iii) survival and transportation in the circulation, (iv) arrest in distant organs, (v) extravasation (escape of the cells from circulation) and (vi) growth of cells to form secondary tumours in the new organ environment (Chambers and Matrisian, 1997). This complex cascade of events involves the organized breakdown of extracellular matrix (ECM), aided by array of proteolytic enzymes.

**Figure-11: Role of MMPs in growth, invasion and metastasis**

ECM, including the basement membrane is a specialized matrix composed of type IV collagen, laminin, entactin, proteoglycans and glycosaminoglycans. The matrix metalloproteinases (MMPs), as their name implies are associated with the degradation of ECM (Nelson et al, 2000). These MMPs are regulated by the inhibitory action of tissue inhibitor of metalloproteinases (TIMPs), which play a vital role in inhibiting tumourigenesis and subsequent malignant progression (Jiang et al, 2002).
DEFINITION: The MMPs are a family of zinc dependent neutral endopeptidases that are collectively capable of degrading essentially all the components of the ECM (Hidalgo and Eckhardt, 2001).

CLASSIFICATION AND NOMENCLATURE: MMPs were first demonstrated by Gross and Lapiere in 1962, when they showed that diffusible enzymes produced by fragments of involuting tadpole could degrade gels made of native fibrillar collagen. Since then, a family of related enzymes has been identified in species from hydra to humans and collectively called MMPs. The MMPs have been grouped into the 'metazincin' zinc endopeptidase super family, due to an identical catalytic Zn environment, a characteristic methionine containing tight turn placed below the catalytic zinc ion, and a unique topology of the catalytic domain (Stocker et al, 1995). In humans, the MMP gene family comprise of 23 homologous proteinases, 20 of which have been well characterised (Overall, 2002). These 23 proteinases are divided into six groups according to the organization of MMP domains, together with their substrate specificity and sequence similarity are shown in table-3. Most of the MMPs are secreted; however, membrane type MMP (MT-MMP) display transmembrane domains and are expressed as cell surface enzymes.
### Table- 3: Members of the MMP Family

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>MMP</th>
<th>Name</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Collagenase</td>
<td>MMP-1</td>
<td>Collagenase -1</td>
<td>Col I, II, III, VII, X, gelatin</td>
</tr>
<tr>
<td></td>
<td>MMP-8</td>
<td>Collagenase -2</td>
<td>Col I, II, III, VII, VIII, X, aggrecan, gelatin</td>
</tr>
<tr>
<td></td>
<td>MMP-13</td>
<td>Collagenase -3</td>
<td>Col I, II, III, IV, IX, X XIV, gelatin</td>
</tr>
<tr>
<td></td>
<td>MMP-9</td>
<td>Gelatinase B</td>
<td>Gelatin, Col IV, V</td>
</tr>
<tr>
<td>3. Stromelysins</td>
<td>MMP-3</td>
<td>Stromelysin-1</td>
<td>Col II, IV, IX, X, XI, gelatin</td>
</tr>
<tr>
<td></td>
<td>MMP-10</td>
<td>Stromelysin-2</td>
<td>Col IV, Laminin, Fibronectin, elastin</td>
</tr>
<tr>
<td></td>
<td>MMP-11</td>
<td>Stromelysin-3</td>
<td>Col IV, Fibronectin, Laminin, aggrecan</td>
</tr>
<tr>
<td>4. Matrilysins</td>
<td>MMP-7</td>
<td>Matrilysin-1</td>
<td>Fibronectin, laminin, Col IV, gelatin</td>
</tr>
<tr>
<td></td>
<td>MMP-26</td>
<td>Matrilysin-2</td>
<td>Fibrinogen, Fibronectin, Gelatin</td>
</tr>
<tr>
<td>5. MT-MMP</td>
<td>MMP-14</td>
<td>MT1-MMP</td>
<td>Gelatin, Fibronectin, Laminin</td>
</tr>
<tr>
<td></td>
<td>MMP-15</td>
<td>MT2-MMP</td>
<td>Gelatin, Fibronectin, Laminin</td>
</tr>
<tr>
<td></td>
<td>MMP-16</td>
<td>MT3-MMP</td>
<td>Gelatin, Fibronectin, Laminin</td>
</tr>
<tr>
<td></td>
<td>MMP-17</td>
<td>MT4-MMP</td>
<td>Fibrinogen, fibrin</td>
</tr>
<tr>
<td></td>
<td>MMP-24</td>
<td>MT5-MMP</td>
<td>Gelatin, Fibronectin, Laminin</td>
</tr>
<tr>
<td></td>
<td>MMP-25</td>
<td>MT6-MMP</td>
<td>Gelatin</td>
</tr>
<tr>
<td>6. Others</td>
<td>MMP-12</td>
<td>Macrophage metalloelastase</td>
<td>Elastin, Fibronectin, Col IV</td>
</tr>
<tr>
<td></td>
<td>MMP-19</td>
<td></td>
<td>Aggrecan, Elastin, Fibrin, Col IV, Gelatinise</td>
</tr>
<tr>
<td></td>
<td>MMP-20</td>
<td>Enamelysin</td>
<td>Aggrecan</td>
</tr>
<tr>
<td></td>
<td>MMP-21</td>
<td>XMMP</td>
<td>Aggrecan</td>
</tr>
<tr>
<td></td>
<td>MMP-23</td>
<td></td>
<td>Gelatin, Casein, fibronectin</td>
</tr>
<tr>
<td></td>
<td>MMP-27</td>
<td>CMMP</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>MMP-28</td>
<td>Epilysin</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

Col=Collagen

**DOMAIN STRUCTURE OF MMPS:** Each of the MMPs consists of a specific domain sequence with several domain motifs. The minimal domain structure of MMPs comprise of signal peptide, the prodomain and a catalytic domain (Figure-12) (Rundhaug, 2003). All MMPs are synthesized with a N-terminal signal peptide ("pre" domain), which is removed upon insertion of MMPs into the endoplasmic reticulum yielding the latent proenzymes. The propeptide (prodomain) consists of amino acid sequence PRCGVPDV that maintains the
Introduction

enzyme latency. The catalytic domain contains a conserved cystein residue consisting of amino acid sequence HEXGHXXGXXHS (the “cystein switch”) that coordinates with the catalytic zinc to maintain inactivity of MMPs. The catalytic domain dictates the cleavage site specificity.

**Figure-12: Domain structure of MMPs**

Matrilysins (MMP-7 and MMP-26) are the simplest MMPs, which consists of the minimal domain. The secreted MMPs include collagenases and stromelysins, which have an additional hemopexin like domain connected by a hinge region to the catalytic domain. While, the gelatinases consist “inserts” i.e. gelatin binding site (that resembles the collagen-binding type II repeats of fibronectin) within their catalytic domain in addition to the simple hemopexin domain structure. The MT-MMPs are membrane bound and all have trans-membrane domain and short cytoplasmic tail.
REGULATION of MMPs: MMPs are regulated by (i) Activation: transcriptional activation by growth factors, inflammatory cytokines, oncogenes, post-transcriptional activation by alterations in mRNA stability and by activation of latent form, and (ii) Inhibition: endogenous inhibition by TIMPS.

Activation: Transcription factor AP-1 plays an important role in regulation of several MMPs. Transcriptional activation of MMP gene is mediated by induction or suppression of promoter region of MMP gene (Fini et al, 1998). MMPs can be activated by inducers like interleukin (IL)-1α, IL-1β, IL-8, transforming growth factor (TGF)β-1, and tumour necrosis factor, basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), and vascular endothelial cell growth factor (VEGF). The cytokines and growth factors may act in conjunction to regulate both MMPs and their inhibitors to create the environment necessary for either physiological or pathological processes (Kanno et al, 1998; Lamoreaux et al, 1998; Parsons et al, 1997).

Except MT-MMPs, all MMPs are secreted in latent form. The activation of latent zymogen is necessary in order to degrade matrix components. In the peptide domains of MMPs, a cystein residue (cys^{73}) is present which forms a bond with Zn^{+2} in catalytic domain. The presence of this bond is important for the latency of MMPs. Due to disruption of this cystine switch, a water molecule binds to the Zn^{+2} ion and replaces the cystine residue after dissociation. The non catalytic Zn is then switched to a catalytic one, which results in an intermediate active enzyme. Additionally the prodomain of MMP is removed by autolytic cleavage or by other proteases. This cleavage causes a reduction in molecular mass and results in fully active enzyme (Patricia et al, 2005). MMPs, once activated, are also capable of activating themselves. For example, MMP-1 is activated by several proteases, e.g. plasmin, MMP-3 and MMP-10 and it can activate MMP-2. MMP-11 and MT1-MMP are activated
prior to their secretion by Golgi-associated furin-like proteases (Nagase, 1997).

**Inhibition:** MMP activity is tightly controlled by endogenous inhibitors. The main inhibitor in the tissue fluids is $\alpha$2-macroglobulin which binds to MMPs. The MMP-$\alpha$2-macroglobulin complex then binds to "scavenger receptors", a class of receptors that 'scavenge' cellular debris and is irreversibly cleared by endocytosis (Egeblad and Werb, 2002). The class of inhibitor is TIMPs. As compared to $\alpha$2-macroglobulin, TIMPs are small molecules. They are expressed in various tissues and fluids. There are four members of mammalian TIMP family, TIMP-1, TIMP-2, TIMP-3, TIMP-4 (Gomez et al, 1997). The amino terminal domain present in all TIMP molecules is responsible for MMP inhibitory activity. TIMPs form high affinity, non covalent complexes with all active MMPs in 1:1 stoichiometry ratio. Individual TIMPs differ in their ability to inhibit various MMPs (Wossener and Nagase, 2000). TIMP-1 and TIMP-2 can preferentially inhibit the proforms of MMP-9 and MMP-2 respectively. TIMP-1 is a 28.5 kDa glycoprotein and it has wide variety of functions like growth factor activity, stimulation of cell morphology changes and inhibition of angiogenesis. TIMP-2 is a non glycosylated 21 kDa protein. In contrast to TIMP-1 and TIMP-2 which are secreted, TIMP-3 is associated with matrix. TIMP-4 is a 24 kD protein. (Gomez et al, 1997). All the TIMPs differ in terms of their gene regulation and tissue specific pattern of gene expression (Edwards, 2001). Independent of their inhibitory activity, they also exert growth-promoting activity. Therefore the balance between protease and inhibitor is critical in determining the net proteolytic activity.

**GELATINASES**

This subgroup of MMP family has two members; gelatinase A (MMP-2, 72 kDa type IV collagenase) and gelatinase B (MMP-9, 92 kDa type IV collagenase). MMP-2 is expressed by various cell types like fibroblasts, keratinocytes, endothelial cells, chondrocytes, osteoblasts, monocytes and by different types
Introduction

of transformed cells (Birkedal et al, 1993). MMP-9 is produced by normal alveolar macrophages, polymorphonuclear leukocytes, osteoclasts and keratinocytes, invading trophoblasts and by several types of transformed cells (Birkedal et al, 1993). Gelatinases are complex MMPs. They have an additional fibronectin like domain. This consists of three fibronectin type II repeats, which enable binding to the substrates. MMP-9 has an additional type IV collagen like domain (Opdenakker et al, 2001). Collier et al (1988) reported the complete sequence of the human MMP-2 enzyme except for the signal peptide. The sequence includes a triple repeat of fibronectin type II domain, which contribute to the binding of the enzyme to gelatin substrate. This makes MMP-2 a one of the longest of the MMPs. There is a difference between MMP-2 and MMP-9 that, MMP-2 is associated with TIMP-2, whereas MMP-9 is associated with TIMP-1. MMP-2 is constitutively expressed whereas MMP-9 is inducible. Primary mechanism by which MMPs promote cancer spread is by degradation of ECM, which consists of two main components, basement membrane and interstitial connective tissues. Though there are other proteins such as laminin, proteoglycans, entactin and osteonectin are also present in this structure, Type IV collagen is the main component of the basement membrane which is degraded mostly by gelatinases MMP-2 and MMP-9. Therefore, these MMPs play a critical role in invasion and metastasis (Duffy et al, 2000).

ROLE OF MMPs IN PHYSIOLOGY AND PATHOLOGY: MMPs participate in many normal biological processes like embryonal development, blastocytic implantation, organ morphogenesis, nerve growth, ovulation, cervical dilation, postpartum uterine involution, endometrial cycling, hair follicle cycling, bone remodeling, wound healing, apoptosis, angiogenesis etc. MMPs are also involved in various pathological processes including cancer, arthritis, cardiovascular disease, nephritis, neurological disease, breakdown of blood brain barrier, periodontal disease, skin ulceration, gastric ulcer, corneal
ulceration, liver fibrosis, emphysema, fibrotic lung disease etc. (Parks and Mecham, 1998)

**ROLE OF MMPs IN TUMOUR GROWTH, INVASION AND METASTASIS**

The role of MMPs in cancer was explicated by Liotta et al in the early 1980s, when they identified proteolysis as one of the three essential steps of tumour invasion and identified a type IV collagenase as being involved in melanoma invasion and metastasis (Liotta et al, 1980). Shortly after the cloning of the first MMPs (Goldberg et al, 1986; Matrisian et al, 1985), it became clear that this activity was likely to be attributed to gelatinase A and/or gelatinase B. Although, initially it was assumed that the tumour cells were the source of the MMPs, there was growing evidence that host stromal cells respond to tumour cells by the induction of MMPs (Nabeshima et al, 1991).

**Figure-13: The steps of metastatic cascade**

1. Localised tumour  
2. Breakthrough  
3. Invasion  
4. Transport  
5. Lodgement  
6. Extravasation  
7. Metastasis
Wyke (2000) described the mechanism involved in tumour growth, invasion and metastasis process (Figure-13). The first stage of this process is growth of the primary tumour at a localized site. Cell multiplication, allied to the genetic instability of tumour cells favours the selection of the variants able to complete some or all the steps required for metastasis (Poste and Fidler, 1980). The primary tumour cannot grow beyond 1-2 mm size without blood supply and this could limit the scope for selected variation. Endothelial growth and migration into the tumour are, however stimulated by hypoxia. Hypoxia induces production of growth factors such as the vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) by the tumour and some infiltrating cells. Angiogenesis is also assisted by (ECM) breakdown and it is documented that MMPs are expressed early in tumour growth.

The next two stages are, breaking through the tumour basement membrane and entrance in to blood or lymphatic vessels. Both the stages require degradation of the ECM. The ECM provides cell attachment, ligands for cellular receptors and it protects and sequesters inactive forms of growth factors and proteases. Of the proteases, MMPs are produced by stromal and endothelial cells, in response to factors produced by tumours. In addition, MMPs are also produced by tumours themselves. MMPs are regulated in balance with TIMPs and they are associated with early tumour growth and angiogenesis.

The defining characteristic of the invasion stage is cellular movement. Cellular movement may be a passive translocation in response to mechanical pressure or an active migration. The later requires, in addition to local ECM degradation changes in cell adhesive properties and cellular locomotion. In many epithelial tumours, adherens junction component, E-cadherin, is lost or reduced, leading to decreased cell-cell adhesion, thus enabling tumour cells to detach from their neighbours.
The next stage in metastasis is transport of tumour cells in the blood or lymphatics to distant sites. Few tumour cells survive this hazard of being destroyed by either: mechanical trauma, immune attack by NK cells or cytotoxic ‘T’ lymphocytes or anoikis- a programmed cell death due to lack of attachment to ECM. Tumour cells can avoid destruction by clumping with each other, or with immune cells and platelets, thus reducing trauma, possibly preventing anoikis and enhancing the next stage of metastasis i.e. lodgement. Lodgement and extravasation are the next two stages of metastasis. Anatomical and haemodynamic factors play a significant role, as may be selective chemotaxis to soluble or fixed attractants. It is also likely to be selective cell adhesion; exposed endothelial basement membrane would be expected to offer better attachment and there could be more keen interactions between tumour cell integrins and fibronectins and other ECM molecules at the favored site of lodgment. Extravasation displays the same requirement as intravasation, but most tumours thereafter apoptose or remain dormant and fail to complete the final stage of metastasis, growth at new site. This failure may be due to the lack of appropriate stimulatory growth factor and ECM or it may reflect the prevalence of inhibitory interactions. All these steps require sufficient proteolytic activity. Thus MMPs and TIMPs play an important role in providing the microenvironment needed for successful metastasis of the tumour.

**RECENT STUDIES ON MMPs AND TIMPs IN CANCER**

Although considerable amount of efforts has been directed in determining the etiology of cancer, the clue regarding the metastatic potentials of tumours is still to be elucidated. Metastatic spread of cancer continues to be the greatest barrier to cancer cure and the majority of basic cancer research has been focused on unraveling molecular mechanisms of tumour formation and metastasis. Recently, Egeblad and Werb (2002) showed that MMPs are involved in various steps of cancer development. MMPs follow all six fundamental alteration in cell physiology underlying cancer progression: (i)
self-sufficiency in growth signals, (ii) insensitivity to growth-inhibitory (antigrowth) signals, (iii) evasion of programmed cell death (apoptosis), (iv) limitless replicative potential, (v) sustained angiogenesis, and (vi) tissue invasion and metastasis. MMPs promote growth of cancer cells by cleaving insulin-growth-factor-binding protein (IGF-BP), thereby liberating IGF (Manes et al, 1999). They also promote growth by shedding transmembrane precursors of growth factors including transforming growth factor-α (TGF-α) (Peschon et al, 1998). MMPs also regulate the ECM, which promotes growth indirectly through interactions between ECM molecules and integrins (Agrez et al, 1994). MMPs also promote survival of cancer cells by liberating IGF and by cleavage of FAS ligand (FASL), a ligand for the death receptor FAS (Powell et al, 1999). MMPs also promote apoptosis, probably indirectly by changing the ECM composition, which influences integrin signalling. MMPs promote angiogenesis by increasing the bioavailability of the pro-angiogenic growth factors VEGF, FGF-2, and TGF-β (Fang et al, 2000). These factors stimulate proliferation and migration of endothelial cells. In addition, MMPs promote invasion of endothelial cells by cleaving structural components of the ECM, such as collagen types I (Col-I) and IV (Col-IV) and fibrin. The MMPs regulate invasion by degrading structural ECM components. Specifically, the MMPs promote invasion and migration by cleaving laminin 5 (Lam-5) (Giannelli et al, 1997). They also promote invasion by cleavage of the adhesion molecules CD44 and E-cadherin (E-cad). The released part of E-cad might then bind and inhibit the function of other uncleaved E-cad molecules (Noe et al, 2001). MMPs also promote the epithelial-to-mesenchymal transition (EMT) — a transition that is associated with malignant behavior — by cleaving the cell-adhesion molecule E-cad and by liberating TGF-β (Sternlicht et al, 1999). MMPs also promote differentiation. The mechanism for that is unknown, but it might involve changing the ECM composition and influencing integrin signalling. Thus, apart from its role in invasion and metastasis, they function before invasion in the development of cancer.
Animal studies have shown that MMPs are active contributors of cancer progression. In transplantation assay when MMP expression is unregulated, benign cancer cells acquire malignant properties. Conversely, highly malignant cells become less aggressive when MMP expression or activity is reduced (Coussens and Werb, 1996). Numerous study showed that, higher the MMP expression in the tumour, more aggressive the cancer. The expression and activity of MMPs are reported to be increased in various malignancies. The higher levels have been found to correlate with advanced stage, increased invasion and metastasis and shortened survival. The most frequently encountered MMPs are MMP-2, MMP-3, MMP-7, MMP-9, MMP-11, MMP-13 and MMP-14 (Hoekstra et al, 2001). Among these, MMP-2 and MMP-9 are the major proteinases, involved in ECM degradation and are extensively studied. High levels of MMP-2 and MMP-9 were obtained in Hodgkin’s and Non Hodgkin’s lymphoma patients and emerged as independent prognostic markers (Hazar et al, 2004). Higher post-operative MMP-2 value predict DFS and over all survival of breast cancer patients (Leppa et al, 2004). MMP-2 and MMP-9 are reported to have role in diagnostic or prognostication of disease. Serum, plasma or cerebrospinal fluid (CSF) levels of MMPs may predict tumour stage, metastasis and recurrence. Higher serum MMP-2 levels are reported in patients with prostate cancer as compared to healthy controls or patients with benign prostate hyperplasia. In gastric cancer patients serum levels of both pro enzymes of MMP-2 and MMP-9 were higher as compared to healthy volunteers (Endo et al, 1997; Gohji et al, 1998). Role of MMP-2 and MMP-9 in angiogenesis has been explored in variety of studies on cancer. It has been suggested that MMP-9 plays a regulatory role in angiogenesis (Vu et al, 1998). In ovarian cancer cells, MMP-9 is found to play an important role in the release of biologically active VEGF (Belotti et al, 2003).

As MMPs are involved in most of the phases of carcinogenesis, inhibition of these proteases can be helpful for the prevention of cancer development and for inhibition of dissemination. Therefore, increased MMPs in malignant
tumours would be accompanied by decreased TIMP expression. However, in majority of the tumours when MMPs are elevated, the levels of TIMPs were also found to be elevated. This may reflect an attempt to control the degradative potential of MMPs or it may indicate that TIMPs are multifunctional molecules (Jiang et al, 2002). Recent studies have indicated that some TIMPs interact with cell surface proteins and modulate intracellular signal transduction pathways independent of their MMP inhibitory functions (Seo et al, 2003, Fernandez et al, 2003). TIMP-2 has antiproliferative and angiogenic activities (Seo et al, 2003). This antiproliferative/angiogenic activity of TIMP-2 was mapped to loop 6 of TIMP-2 protein. This domain is dispensable for MMP inhibition, which demonstrates a novel TIMP-2 activity independent of its MMP inhibition (Fernandez et al, 2003). TIMP-1 inhibits intrinsic apoptosis in many cell types including hepatic stellate cells, erythroid cells, Burkitt's lymphoma cell lines, human breast epithelial cells and mammary epithelial cells in transgenic mice either in MMP dependent or independent manner (Alexander et al, 1996; Gudez et al, 1998; Gudez et al, 1998; Li et al, 1999; Murphy et al, 2002; Liu et al, 2003; Yoshiji et al, 2002). Liu et al (2005) have shown that TIMP-1 may exert oncogenic activity in breast cancer through inhibition of both intrinsic and extrinsic apoptosis. In breast cancer, TIMP-1 expression is found to be associated with poor prognosis (McCarthy et al, 1999). TIMP-1 expression is also shown to be induced at the early stages of carcinogenesis and confined to aggressive tumours. Several studies have showed that higher TIMP-1 and TIMP-2 levels were correlated with poor prognosis. Over expression of TIMP-1 is associated with adverse prognosis in NSCLC patients (Aljada et al, 2004). Higher tumour tissue levels of TIMP-1 are also associated with poor prognosis in patients with primary breast cancer (Schrohl et al, 2004). Nair et al (2003) reported that cervical tumour tissue samples exhibited increased expression of MMP-2, MMP-9, TIMP-1 and TIMP-2 with the progression of cervical cancer from low grade squamous intraepithelial lesions (SIL) to high grade SIL to squamous cell carcinoma. Recent report from our laboratory (Patel et al, 2005)
documented higher MMP-2 and MMP-9 levels in malignant tissues as compared to normal tissues of oral cancer patients.

**SCOPE OF PRESENT STUDY:**
Stress, a common phenomenon in today's society, plays a major role in the development of various diseases including cancer. There are certain endogenous as well as exogenous factors responsible for development of oxidative stress. Inefficiency to counteract the oxidative stressors by antioxidant defense system leads to direct or indirect impact on the cells causing damage to DNA, lipids and proteins. It elicits a stress response to recover the native configuration of cell components, which is partially lost due to oxidative stress. There is certain threshold level, above which normal cells cannot cope with oxidative stress. The persistant oxidative stress results in redox imbalance, and a cascade of mechanisms leading to carcinogenesis and ultimately invasion and metastasis. Inspite of the recent advances in diagnosis and treatment, breast cancer is still a leading female malignancy. Invasion and metastasis to local or distant sites are very common in breast cancer. Therefore, the current study was undertaken considering the importance of oxidative stress in breast carcinogenesis, invasion and metastasis. The major thrust of the study was to endeavor the role of enzymatic antioxidants; GST, GR, SOD and catalase and non-enzymatic antioxidants; β-carotene, vitamin-A, vitamin-E and vitamin-C, lipid profile parameters; cholesterol, HDL, LDL, VLDL and triglycerides, oxidative stress related markers; thiol and lipid peroxidation levels, cytoplasmic antioxidant; hsp70, invasion and metastasis markers; MMP-2, MMP-9, TIMP-1 and TIMP-2 levels in identification of high risk group, early diagnosis, prognostication and treatment monitoring of breast cancer patients. The major objectives were as follows.

1. Evaluation of circulating levels (plasma and erythrocyte) of enzymatic and plasma non-enzymatic antioxidants, lipid profile parameters, as
1. Analysis of enzymatic and non-enzymatic antioxidants, lipid profile levels and oxidative stress related markers in controls, patients with BBD and breast cancer patients.

2. Odds ratio (OR) analysis of enzymatic and non-enzymatic antioxidants, lipid profile levels and oxidative stress related markers to evaluate their role in risk of breast cancer.

3. To assess the association between enzymatic and non-enzymatic antioxidants and oxidative stress related markers in breast cancer patients and association of the alterations of these markers with clinicopathological features in breast cancer patients.

4. Analysis of "cytoplasmic antioxidant", hsp70 in tumour and adjacent normal tissues of breast cancer patients and their association with clinicopathological features.

5. Evaluation of enzymatic and non-enzymatic antioxidants and oxidative stress related markers in breast cancer patients at various intervals during anticancer therapy.

6. Analysis of MMP-2 and MMP-9 from tumour and adjacent normal tissues as well as from positive and negative lymph node tissues of breast cancer patients and their association with clinicopathological features.

7. Analysis of plasma levels of MMP-2, MMP-9 and their inhibitors, TIMP-1, and TIMP-2 in controls, patients with BBD and breast cancer patients at diagnosis, and after anticancer therapy.