INTRODUCTION

Breast cancer:
Breast cancer is the most common malignancy among females and breast cancer in particular in women remains one of the major cause of mortality (Parkin & Fernandez 2006). Cancer is a synonym for malignant neoplasm, a group of diseases occurring in all human and animal populations and arising in all tissues composed of potentially dividing cells (Fenton & Longo 2005). The basic characteristic of cancer is the transmissible abnormality of cells that is manifested by reduced control over growth and function leading to serious adverse effects on the host through invasive growth and metastases (Fenton & Longo 2005). The word cancer comes from the Latin for crab, probably because of the way a cancer adheres to any part that it seizes upon in an obstinate manner like the crab. Cancer arises because of alterations in DNA that result in unrestrained cellular proliferation. Most of these alterations involve actual sequence changes in the DNA i.e., mutation. They may arise as a consequence of random replication errors, exposure to carcinogens (e.g. radiation) or faulty DNA repair processes. While virtually all cancer is a genetic disease, most cancer is not inherited. Certain individuals with cancer have inherited a germ line mutation that predisposed them to the cancer, but even in that situation additional somatic mutations are required for a tumor to develop. In a truly sporadic cancer, all of the mutations responsible for the malignant phenotype arise somatically. Such a cancer is caused by genetic alterations but has no hereditary implications (Fenton & Longo 2005).

A number of DNA and RNA viruses have been shown to induce malignant transformation. Many malignancies in animals are transmissible and the etiologic agent is frequently a retrovirus, which possesses a single-stranded genome. During the life cycle of the virus, the single stranded RNA is converted to double strand DNA and is inserted at random into the host chromosome. Human T cell lymphotropic virus (HTLV) type 1 causes adult T cell lymphoma/ leukemia. HTLV-1 does not contain a growth promoting transforming oncogene. The tax protein, a 40kDa protein molecule encoded in the px region of the viral genome, induces the activation of a number of genes through interactions with rel family and CREB (cyclic AMP response element binding protein) family transcription factors (Mulloy et al. 1998). DNA tumor viruses are more commonly involved in human malignancy. Human papilloma viruses (especially types 16 & 18) cause cervical cancer and both hepatitis B and hepatitis C
viruses have been implicated in hepatocellular carcinoma (Gatza et al. 2005). In addition, the Epstein-Barr virus, a herpes virus causes endemic Burkett’s lymphoma in Africa and nasopharyngeal carcinoma & lymphomas causing immune deficiency (Andiman et al. 1983).

In 1971, it was found that oncogenes might not be unique to transforming viruses but might also be found in normal cells: indeed, it was proposed that a virus might acquire oncogenes from the genome of an infected cell. These cellular genes were called proto-oncogenes, or cellular oncogenes (c-onc), to distinguish them from their viral counterparts (v-onc). One category of proto-oncogenes and their oncogenic counterparts encodes proteins that induce cellular proliferation. Some of these proteins function as growth factors or growth factor receptors. Included among these are sis, which encodes a form of platelet-derived growth factor and fms, erbB and neu, which encode growth factor receptors. In normal cells, the expression of growth factors and their receptors is carefully regulated. Inappropriate expression of either a growth factor or its receptor can result in uncontrolled proliferation. The Src and Abl oncogenes encode tyrosine kinases (Hevezi. et al. 1993), and the ras oncogene encodes a GTP-binding protein (Puil L. et al. 1994). The products of these genes act as signal transducers. The myc, jun, and fos oncogenes encode transcription factors, over activity of any of these oncogenes may result in unregulated proliferation (Sassone-Corsi 1994).

A second category of cancer-associated genes called tumor suppressor genes, or anti-oncogenes encodes proteins that inhibit excessive cell proliferation. Inactivation of these results in unregulated proliferation. The prototype of this category of tumor suppressor gene is RB, the retinoblastoma gene (Reissmann et al. 1989).

Breast Cancer & its Pathophysiology.

Breast cancer is a malignant proliferation of epithelial cells lining the ducts or lobules of the breast. Epithelial malignancies of the breast are the most common cause of cancer in women (excluding skin cancer), accounting for one third of all cancer in women (Lippman. 2005). Human breast cancer is a clonal disease i.e. a single transformed cell, resulting either from a series of somatic (acquired) or germline mutations, is capable of expressing full malignant potential. Thus, breast cancer can exist for a long period as either a non-invasion disease or an invasive but non-metastatic disease.
Correct staging of breast cancer patients is of extraordinary importance in that, it not only permits an accurate prognosis, but in many cases, therapeutic decisions are made largely based on the TNM classification (T=primary tumor, N=regional lymph nodes, M=distant metastasis).

Table: TNM Classification of the American Joint Committee on cancer (AJCC)

<table>
<thead>
<tr>
<th>Staging Group</th>
<th>Indication</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0</td>
<td>No evidence of primary tumor</td>
</tr>
<tr>
<td>TIS</td>
<td>Carcinoma in situ</td>
</tr>
<tr>
<td>T1</td>
<td>Tumor ≤ 2cm</td>
</tr>
<tr>
<td>T2</td>
<td>Tumor &gt; 2cm but ≤ 5cm</td>
</tr>
<tr>
<td>T3</td>
<td>Tumor &gt; 5cm</td>
</tr>
<tr>
<td>T4</td>
<td>Extension of chest wall, inflammation, satellite lesions, ulcerations</td>
</tr>
<tr>
<td>Regional Lymph Nodes (N)</td>
<td>Indication</td>
</tr>
<tr>
<td>N0</td>
<td>No tumor in regional lymph nodes</td>
</tr>
<tr>
<td>N1</td>
<td>Metastasis to movable ipsilateral nodes</td>
</tr>
<tr>
<td>N2</td>
<td>Metastasis to matted or fixed ipsilateral nodes</td>
</tr>
<tr>
<td>N3</td>
<td>Metastasis to ipsilateral internal mammary nodes</td>
</tr>
<tr>
<td>Distant Metastasis (M)</td>
<td>Indication</td>
</tr>
<tr>
<td>M0</td>
<td>No distant metastasis</td>
</tr>
<tr>
<td>M1</td>
<td>Distant metastasis (includes spread to ipsilateral supraclavicular nodes)</td>
</tr>
</tbody>
</table>
Stage grouping:

<table>
<thead>
<tr>
<th>Stage</th>
<th>T (tumor)</th>
<th>N (lymph node)</th>
<th>M (metastasis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 0</td>
<td>TIS</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>Stage I</td>
<td>T1</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>Stage IIA</td>
<td>T0</td>
<td>N1</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T1</td>
<td>N1</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>Stage IIB</td>
<td>T2</td>
<td>N1</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>Stage IIIA</td>
<td>T0</td>
<td>N2</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T1, T2</td>
<td>N2</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>N1, N2</td>
<td>M0</td>
</tr>
<tr>
<td>Stage IIIB</td>
<td>T4</td>
<td>Any N</td>
<td>M0</td>
</tr>
<tr>
<td>Stage IV</td>
<td>Any T</td>
<td>Any N</td>
<td>M1</td>
</tr>
</tbody>
</table>

Different type of breast cancer.
The non-invasive breast cancer develops as a series of molecular changes in epithelial cells leading to a greater malignant behavior. The lesion falls into two groups: Ductal carcinoma in situ (DCIS) and the lobular carcinoma in situ (Lobular neoplasm).

Ductal carcinoma in-situ (DCIS).
It is the proliferation of cytogenetically malignant breast epithelial cells within the ducts. But there remains a pathologic disagreement in differentiating atypical hyperplasia from DCIS. Almost one-third of the cases of untreated DCIS progress within five years to invasive breast cancer (Figure-A)
Figure-A: Non-invasive Ductal Carcinoma In-situ (DCIS)

Breast profile: A-ducts, B-lobules, C-dilated section of duct to hold milk, D-nipple, E-fat, F-Pectoralis major muscle, G-chest wall/rib cage; Enlargement: A-normal duct cells, B- ductal cancer cells, C-basement membrane, D-lumen (center of duct).

Lobular carcinoma in-situ.

Lobular carcinoma In-situ is thought to be a premalignant lesion that suggests an elevated risk of subsequent breast cancer rather than a form of malignancy itself (Figure-B)
Figure-B. Lobular Carcinoma In-situ (LCIS).
Breast profile: A-ducts, B-lobules, C-dilated section of duct to hold milk, D-nipple, E-fat, F-Pectoralis major muscle, G-chest wall/rib cage; Enlargement: A-normal lobular cells, B-lobular cancer cells, C-basement membrane.

Invasive ductal carcinoma (IDC): It accounts for about 80% of all breast cancers. It is ductal because the cancer began in the milk ducts—which are the "pipes" that bring milk from the lobules to the nipple. The invasive breast cancers break through normal breast tissue barriers and invade surrounding areas. Much more serious than non-invasive cancers, invasive cancers can spread cancer to other parts of the body through the lymphatic system (Figure-C)

Figure-C: Invasive Ductal Carcinoma (IDC).
Breast profile: A-ducts, B-lobules, C-dilated section of duct to hold milk, D-nipple, E-fat, F-Pectoralis major muscle, G-chest wall/rib cage; Enlargement: A-normal duct cells, B-ductal cancer cells breaking through the basement membrane, C-basement membrane.
Invasive lobular carcinoma (ILC): It accounts for about 10%-15% of all breast cancers. It is lobular because the cancer began in the lobules—the glands that actually make milk (Figure-D).

**Figure-D: Invasive Lobular Carcinoma (ILC).**

Breast profile: A-ducts, B-lobules, C-dilated section of duct to hold milk, D-nipple, E-fat, F-Pectoralis major muscle, G-chest wall/rib cage; Enlargement: A-normal duct cells, B-lobular cancer cells breaking through the basement membrane, C-basement membrane.

Not more than 10% of human breast cancer can be linked directly to germline mutations. Several genes have been implicated in familial cases. The Li-Fraumeni syndrome is characterized by inherited mutations in the p53 tumor suppressor gene, which lead to an increased incidence of breast cancer, osteogenic sarcomas and other malignancies. Inherited mutations in PTEN (a protein tyrosine phosphatase with homology to tensin, is a tumor-suppressor gene on chromosome 10q23) have also been reported (Lynch et al. 1997).

Another tumor-suppressor gene, BRCA-1, has been identified at the chromosomal locus 17q21; this gene encodes a zinc finger protein, and the product therefore may function as a transcriptional factor. The gene appears to be involved in gene repair. Women who inherit a mutant allele of this gene from either parent have at least a 60 to
80% lifetime chance of developing breast cancer and about a 33% chance of developing ovarian cancer. The risk is higher among women born after 1940, presumably due to promotional effects of hormonal factors. Men who carry a mutant allele of the gene have an increased incidence of prostate cancer and breast cancer. A fourth gene, termed BRCA-2 which has been localized to chromosome 13q12, is also associated with an increased incidence of breast cancer in men and women (Lippman, 2005).

BRCA-1 and BRCA-2 can now be sequenced readily and germline mutations detected; patients with these mutations can be counseled appropriately. These genes play a vital role in sporadic breast cancer. The p53 mutation is present in approximately 40% of human breast cancers as an acquired defect. Acquired mutations in PTEN occur in about 10% of the cases. BRCA-1 mutation in primary breast cancer has not been reported, however decreased expression of BRCA-1 mRNA (possibly via gene methylation) and abnormal cellular location of the BRCA-1 protein have been found in some breast cancers. Loss of heterozygosity of BRCA-1 and BRCA-2 suggests that tumor-suppressor activity may be inactivated in sporadic cases of human breast cancers (Lippman, 2005). Increased expression of a dominant oncogene plays a role in about a quarter of human breast cancer cases. The product of this gene, a member of the epidermal growth factor receptor superfamily, is called erbB2 (HER-2, neu) and is overexpressed in these breast cancers due to gene amplification; this overexpression can contribute to transformation of human breast epithelium (Lippman, 2005).

Role of hormones in breast cancer.

Breast cancer, the most frequent spontaneous malignancy diagnosed in women is a classical model of hormone dependent malignancy (Russo et al, 1998) in that women without functional ovaries who never received estrogen replacement do not develop breast cancer. Many epidemiological features of the disease suggest that endogenous hormones are of importance in the genesis of this disease. Though studies have provided etiologic clues, the understanding of the hormonal aberrations that promote the development of breast cancer remains unclear (Thomas. 1986). It has been suggested that progestins are the predominant mitogen for normal breast epithelium while estrogen assumes that function in neoplastic epithelium.

There is a substantial evidence to suggest that breast cancer risk is associated with
prolonged exposure to female hormones, since early onset of menarche, late menopause, hormone replacement therapy and post menopausal obesity are associated with greater cancer incidence (Lippman. 2005). Alkaline phosphatase, S'-nucleotidase and gammaglutamyl transferase activities were found to be elevated in breast carcinoma tissues of both pre and post menopausal groups (Ramalingam et al. 1993). Therefore the search for a hormonal marker in breast cancer has centered on estrogen and its metabolites (Jones et al. 1987) either of ovarian or extra ovarian origin, as supported by the observations that breast cancer does not develop in the absence of ovaries and ovariectomy might cause regression of established malignancies (Lippman. 2005).

Estrogen and progesterone receptor status are of prognostic significance in this context. Tumors that lack either or both receptors are more likely to recur than tumors that have them. Several measures of tumor growth rate correlate with relapse among which S-phase analysis using flow cytometry is the most accurate measure. Studies suggest that tumors with a high proportion of cells in the S-phase pose a greater risk of relapse (Lippman. 2005). Histological classification of the tumor has also been used as a prognostic factor. Tumors with poor nuclear grade have a higher degree of recurrence than tumors with a good nuclear grade (Lippman. 2005).

Molecular changes in the tumor are also useful; e.g., tumors that overexpress the epidermal growth factor receptor erbB2 or that have a mutated p53 gene have a bad prognosis. Other variables that have also been used to evaluate prognosis include proteins associated with invasiveness, viz. type IV collagenase, cathepsin D, plasminogen activator receptor and the metastasis suppressor gene nm23, none of these have been widely accepted as a prognostic variable for therapeutic decision-making.
Nitric oxide.

The discovery of nitric oxide, a molecule formed by vascular endothelial cells has opened a new area of biological research. While NO is now thought to be a messenger molecule which plays a crucial role in varied biological effects (Bredt. et al. 1994), it was demonstrated first by Furchgott and Zawadzki that vascular relaxation induced by acetylcholine (Ach) was dependent on the presence of the endothelium and provided evidence that this effect was mediated by a labile humoral factor, later known as Endothelium Derived Relaxing factor (EDRF). The endothelium dependent relaxation was subsequently demonstrated in many vascular preparations, including some veins, arteries, and microvessels to occur in response to a variety of substances, such as Ach, adenine nucleotides, thrombin, substance P, calcium ionophore A23187 and bradykinin. Furchgott and Zawadzki reported that the endothelium was obligatory for acetylcholine elicited relaxation of the isolated rabbit aortic preparations (Furchgott & Zawadzki. 1980). Acetycholine was postulated to interact with muscarinic receptors on the surface of endothelial cells to stimulate the release of EDRF that diffused into and interacted with the underlying smooth muscle cells (Furchgott. 1988). Some other agents, such as vasodilators, atrial natriuretic factor, bovine penis retractor factor, β-adrenergic agonists, and prostacyclin induced vascular relaxation by endothelium dependent mechanisms (Moncada. 1986).

Identification of Endothelium-derived relaxing factor as Nitric Oxide.

Early experiments suggested that EDRF might be a product of arachidonic acid lipoxygenase (Singer & Peach 1983) or of the cytochrome P-450 enzyme system (Izzo et al. 1983) or was a compound with a carbonyl group at the active site, but it did not lead to the identification of its chemical structure. Based on the pharmacological similarities between EDRF and NO (Nitric Oxide) generated from acidified NO⁻ it was Furchgott who suggested that EDRF may be NO (Furchgott & Zawadzki. 1980) or a closely related species.

Palmer and coworkers reported that EDRF released from cultured porcine aortic endothelial cells grown on microcarrier beads was NO as identified by the reaction between EDRF and ozone to yield a chemiluminescent product that was indistinguishable from the reaction product between NO and ozone.(Palmer et al. 1987). Studies from this laboratory employing two different chemical approaches
indicated that EDRF and NO were closely related chemical species (Ignarro et al. 1987a; Ignarro et al. 1987b). First, hemoglobin was found to react with EDRF released from freshly isolated aortic endothelial cells in a manner that was identical for the same reaction involving NO. Under conditions of low oxygen tension and presence of reduced hemoglobin, NO is known to react rapidly with the heme moiety of hemoglobin to yield the nitrosyl (or NO) adduct of hemoglobin. NO-hemoglobin possesses a distinct absorbance in the soret region that can be easily distinguished from the absorbances of related hemoprotein adducts such as oxyhemoglobin, carboxyhemoglobin, or methemoglobin. EDRF reacted with hemoglobin under identical conditions to those employed for NO to yield the same NO-hemoglobin reaction product. In another study, the chemical identification of both arterial and venous EDRF as NO or a nitroso compound was made by use of the principle of NO-catalysed diazonitization of sulfanilic acid at acidic pH (Ignarro et al. 1987b). EDRF released from perfused intact artery and vein was superfused over bioassay strips, collected in acidic medium, and then chemically assayed. The EDRF released from the vessels was indistinguishable, both chemically and pharmacologically, from the authentic NO superfused over the bioassay strips. The diazonitization reaction, however, is not very sensitive and cannot distinguish among NO, acidic nitrite, and labile nitroso compounds. Thus, it is conceivable that EDRF is a labile nitroso species that spontaneously generates NO.

EDRF from artery and vein possessed the same half-life as NO (3-5 seconds), both EDRF and NO elevated cyclic GMP levels in the bioassay strips, and the relaxant responses of EDRF and NO were abolished by superoxide anion and enhanced by superoxide dismutase (SOD). These and other observations provided experimental evidence that at least one EDRF released from cultured porcine aortic endothelial cells, intact bovine pulmonary artery and vein and freshly isolated bovine aortic endothelial cells is either NO or a chemically related unstable nitroso species (Ignarro et al. 1986b; Ignarro et al. 1988b). NO which was described as a potent vasodilator and inhibitor of platelet aggregation (Mellion et al. 1981), appears to occur endogenously as EDRF in mammalian blood vessels, and perhaps also in other cell types. The potent vasodilatory glyceryl trinitrate, which has been used clinically for over a century, can be regarded to have an endogenous counterpart in NO since NO is the chemical species responsible for its vasodilator action.
It was also shown that bradykinin induced vasorelaxation in culture porcine endothelial cells was mediated by the release of EDRF (Palmer et al. 1988). Furthermore the levels of NO released by the cells also accounted for the inhibition of platelet aggregation and adhesion (Mellion et al. 1981). Comparison of EDRF and NO on vascular strips (Hutchinson et al. 1987) and on platelets (Radomski et al. 1987b) showed that the two compounds were indistinguishable (Ignarro et al. 1988a) and had identical chemical stability under artificial conditions as determined by the rate of decay during transit in polypropylene tubes. Both inhibited aggregation of platelet aggregation and caused disaggregation of aggregated platelets (Mellion et al. 1981) and inhibited platelet adhesion to monolayers of bovine endothelial cells in culture (Radomski et al. 1987a). Moreover their biological half lives as inhibitors of platelet aggregation were similar. The actions of EDRF and NO on vascular strips and platelets were potentiated by superoxide dismutase and cytochrome c and inhibited by Ferrous ion and some redox compounds (Ignarro et al. 1988a). In addition the inhibitory action of Hb on EDRF can be explained by the fact that this substance binds avidly to NO (Gruetter et al. 1981). Moreover EDRF and NO act on platelets and smooth muscle cells through the stimulation of soluble guanylate cyclase and elevation of cyclic GMP (Katsuki et al. 1977; Craven et al. 1978; Arnold et al. 1977). Furthermore, perfusion of segments of pulmonary artery or pulmonary vein with Acetylcholine or bradykinin caused relaxant responses and elevation of vascular cyclic GMP levels in the bioassay tissues that could be matched with the effects of NO (Ignarro et al. 1984b; Ignarro et al. 1986a; Cherry et al. 1982). Later, the use of a spectrophotometric assay, based on the reaction between NO and Hb, also demonstrated the release of NO from vascular endothelial cells (Mellion et al. 1983).

Formation and release of nitric oxide:
NO is a chemically unstable substance with a very short half-life; the assumption might be made that Endothelial derived nitric oxide (EDNO) elicits its local action immediately after synthesis. The chemical properties of NO are highly conductive to rapid diffusion of NO to nearby smooth muscle cells and platelets adhering to the intimal surface. The requirement of calcium and oxygen for endothelium-dependent relaxation might then be attributed to their necessity for NO formation and/or coupling of the extracellular relaxant-receptor interaction to NO formation.
Although several different biological metabolic pathways for NO formation are known, none is yet known to occur in mammalian cells despite the findings that NO has been identified to exist endogenously in mammalian vascular endothelial cells (Palmer et al. 1987; Ignarro et al. 1987a; Ignarro et al. 1987b). Possible pathways for NO formation might include enzymatic (nitrite reductase) or nonenzymatic (thiols and/or acidification) reduction of inorganic nitrite to NO. Sources of nitrite might include organic nitrate esters that can be enzymatically (nitrate reductase) or nonenzymatically (thiols) monodenitrated to nitrite. Another pathway could be the oxidation of certain amino groups derived from basic amino acids such as L-arginine or related basic polypeptides containing L-arginine or L-lysine. The basic nitrogens may be oxidized to NO or to nitrite followed by conversion to NO. Another possibility is that a basic amino function could first be converted to ammonia, which could then be oxidized to NO.

Evidence indicates that the inhibitory factor, possessing vasodilator properties, extracted from bovine retractor penis muscle is inorganic nitrite and that acidification results in formation of NO (Furchgott 1988; Martin et al. 1988). The extract is inactive until acidified, and the acid-activated form possesses the chemical and biological properties of NO. The extract may contain a NO-stabilizing substance, such as a thiol or sugar that favors conversion of nitrite to NO (Martin et al. 1988).

Mammalian cells are capable of synthesizing nitrite and nitrate in the absence of any anaerobic microorganisms. Stuehr, Marletta and coworkers have shown that murine macrophages synthesize nitrite and nitrate in response to activation by lipopolysaccharides, Calmetta-Guerin bacilus (BCG) infection, lymphokines, and related factors and that T-lymphocytes enhance this synthesis. Nitrite and nitrate may be involved in the cytotoxic actions of activated macrophages, perhaps by stimulating intracellular nitrosation reactions with the resulting N-nitroso compounds causing the cytotoxicity. Nitrite and nitrate are synthesized in the constant ratio of 3:2 (Stuehr & Marletta. 1987) but most of the nitrate formed could originally be derived from oxidation of nitrite by oxyhemoglobin (Miwa et al. 1987). Nitric oxide is formed and oxidized to both nitrite and nitrate by oxyhemoglobin and myoglobin. The nitrogen of nitrite and nitrate are derived exclusively from the terminal guanido nitrogens of L-arginine, which appears to be the precursor to nitrite/nitrate in activated mammalian macrophages (Iyengar et al. 1987).

Activated rat peritoneal neutrophils release an unstable chemical substance that relaxes...
isolated endothelium-denuded preparations of rat aorta (Rimele et al. 1988). The properties of this neutrophil-derived relaxing factor were very similar to those of endothelium derived NO in that relaxant responses were enhanced by SOD and low oxygen tension, inhibited by methylene blue, and unaltered by indomethacin. Further studies revealed that the neutrophil-derived relaxing factor stimulated cyclic GMP accumulation in vascular smooth muscle (Lee et al. 1988) relaxed vascular as well as nonvascular smooth muscle, and was most likely NO derived from inorganic nitrite (Stuehr & Marletta 1985).

Evidence was provided that L-arginine can serve as a precursor for endothelial derived NO (EDNO) in cultured porcine aortic endothelial cells. This conclusion was based on direct observation that endothelial cells utilized radio labelled L-arginine ($^{15}$N in guanidino group) to generate $^{15}$NO, as assessed by mass spectroscopy (Palmer et al. 1988) and on indirect observations that structural analogues of arginine such as N°-monomethyl-L-arginine and L-canavanine antagonize endothelium dependent relaxation (Schmidt et al. 1988). A high concentration (4mM) of L-canavanine preincubated with vascular tissue for 3 hours was found to be necessary for moderate inhibition of endothelium dependent relaxation. A lower concentration (0.1mM) of N°-monomethyl-L-arginine inhibited endothelium-dependent relaxation after only 5 minutes of preincubation with vascular tissue. Addition of 0.3mM L-arginine caused a rapid and marked reversal of inhibition. Neither of inhibitors altered the relaxant responses to NO. Additional bioassay experiments revealed that perfusion of intact pulmonary artery with either L-canavanine or N°-monomethyl-L-arginine inhibited the further formation of EDNO. These findings support the hypothesis that endogenous endothelium derived L-arginine could serve as a natural precursor to EDNO (Palmer et al. 1988).

Although it appears that NO can be formed from L-arginine, NO may be formed directly from one of the basic amino nitrogens of L-arginine, or NO may be formed from an intermediate generated from L-arginine such as an organic nitroso compound, ammonia, or nitrite. Moreover, larger molecules containing arginine or other basic amino acids could serve as precursors for EDNO. Even basic polyamino acids including poly-arginine, poly-lysine, and poly-ornithine cause endothelium-dependent arterial (Thomas et al. 1986) and venous relaxation and cyclic GMP accumulation via the formation and release of EDNO (Ignarro et al. 1989). The mechanism of this
relaxation is unknown, but one possibility is that the basic nitrogen atoms of such polyamino acids are converted to NO or intermediate nitroso compounds.

**Metabolism and termination of action of nitric oxide.**

Chemical instability endows EDNO with one of the most important requisite properties of a local modulator or autocoid, namely, rapid termination of action so that the biological effect remains localized. The very short half-life of NO of only several seconds makes it unnecessary to demand other mechanisms of termination of action such as enzymatic degradation or reuptake mechanisms. In the spontaneous conversion of NO to nitrite and nitrate, it is conceivable that nitrite and nitrate could be transported back into endothelial cells for conversion to NO. Another mechanism of inactivation of NO might be the concomitant generation and release of superoxide anion from vascular endothelial cells, smooth muscle cells, and phagocytic cells. This reaction is very well known to occur in activated macrophages and neutrophils and evidence exists that vascular endothelium and smooth muscle (Heinecke et al. 1986) can generate superoxide anion.

**Chemistry of Nitric oxide.**

NO can act both as a Lewis acid and Lewis base which accounts for its numerous reactions. As a free radical, NO readily undergoes addition, substitution, redox and chain-terminating reactions, which serve as the molecular basis for its biologic effects. NO possesses an intermediate oxidation state for which it acts both as oxidizing and reducing agents. NO is a colorless gas and exist as a free radical (paramagnetic nature). But it differs from other molecules with odd electron number, in that it is colorless, exists in the gaseous form, lacks dimerization in the gaseous state (or solution) and has relatively low chemistry reactivity.

The electronic structure of NO has been represented as both:

1) $\cdot\hat{N}=\hat{O}$; 2) $\cdot\hat{N}=\hat{O}$;

Structure 2 is more favorable (resonance) because the dimerization of NO (observed in structure 1) is not observed (Ragsdale, 1973). NO gas is only slightly soluble in water at standard temperature and pressure, yielding a saturated solution of 1-3 nM.
In lab reactions NO can be formed in large quantities non-enzymatically as in the following reactions wherein NO exceeds its soluble limit in aqueous media and evolves as a gas.

i) \[ \text{Fe}^{2+} + \text{NO}_2^- + 2\text{H}^+ \rightarrow \text{Fe}^{3+} + \text{NO} + \text{H}_2\text{O} \]

ii) \[ 2\text{NO}_2^- + 2\Gamma^- + 4\text{H}^+ \rightarrow 2\text{NO}^- + \text{I}_2 + 2\text{H}_2\text{O} \]

NO in biological tissues are generated by enzymatic chemical reactions. Such reactions involve azide anion (N$_3^-$), hydroxylamine (NH$_2$OH), hydrazine (NH$_2$NH$_2$) and L-arginine.

Some of the important chemical reactions involving NO which occur under biological conditions are discussed below:

i. Reactivity with oxygen to yield NO$_2$ (gas) or NO$_2^-$ (in solution):
\[ 2\text{NO} + \text{O}_2 \rightarrow 2\text{NO}_2^- \]

ii. Reactivity of superoxide anion (O$_2^-$) yielding an unstable intermediate product (peroxonitrite anion), which rearranges to form NO$_3^-$
\[ \text{NO}^+ + \text{O}_2^- \rightarrow \text{OONO}^- \rightarrow \text{NO}_3^- \]

iii) Reactivity with ozone to yield an activated NO$_2$ which can be readily detected by chemiluminescence (Zafiriou & McFarland. 1980) useful in the assay of Nitric oxide (Palmer. 1987).

iv) Reactivity with oxyhemoglobin yielding methemoglobin and inorganic nitrate.
Molecular oxygen binds as superoxide anion to the heme iron atom of hemoglobin (Wallace, et al. 1974) and this oxyhemoglobin readily isomerizes to NO$_3$ (Blough. et al. 1985). This principle of nitric oxide reacting with oxyhemoglobin to yield methemoglobin is used to assay NO spectrophotometrically (Kelm. et al. 1988)

\[ \text{Hb}^{3+} + \text{O}_2^- + \text{NO} \rightarrow \text{met Hb} + \text{OONO}.......(i) \]
\[ \text{OONO}^- \rightarrow \text{NO}_3^- .................(ii) \]
\[ \text{HbO}_2 + \text{NO} \rightarrow \text{met Hb} + \text{NO}_3^- ...............(final reaction) \]

V. Reactivity with thiols (R-SH) yielding S-nitrosothiols (R-SNO):
R-SH + NO —> R-SNO + H^+ ..........................................................(i)
R-SH + NO_2^- (in acidic aqueous solution) + H^+ —> R-SNO + H_2O... (ii)

vi) Reactivity with heme iron to yield nitrosyl-heme adducts:
Reduced iron (Fe^{2+}) complexed with protoporphyrin IX forming heme has a high binding affinity for nitric oxide. This is especially true for hemoproteins (proteins with heme moiety). The binding affinity of hemoglobin for nitric oxide far exceeds its affinity for CO. So hemoproteins viz. hemoglobin, myoglobin, cytochrome-c, soluble guanylate cyclase readily reacts with NO forming the corresponding nitrosyl-heme adduct (paramagnetic in nature). Solutions of reducing and oxidizing hemoproteins are dark red and brown respectively, whereas, NO hemoproteins are bright pink-red. This chemistry is the basis for the development of the spectrophotometric assay of nitric oxide (Ignarro. 1987a).

vi) Other reactions:
- Synthesis of N-nitrosamines by cytotoxic activated macrophages.
- Murine macrophages activated by lipopolysaccharides and γ-interferon synthesizes N-nitrosomorpholine from morpholine.
- NO reacts with sulfanilic acid at low pH and the product couples with N-(1-naphthyl) ethylenediamine to yield an intense chromophore.

Pharmacology of nitric oxide.
The earlier evidence that nitric oxide elicits important biological actions were the finding that nitric oxide and S-nitrosothiols could activate cytoplasmic or cytosolic guanylate cyclase and stimulate cyclic GMP formation in mammalian tissues (Arnold, et al. 1977). To test whether cyclic GMP could mediate the vascular smooth muscle relaxant effect of nitroglycerin & nitroprusside, the vasodilatory effect of NO was studied. Nitric oxide caused a marked and potent but transient relaxation of isolated strips of bovine coronary artery, which was associated with guanylate cyclase activity. NO stimulated cyclic GMP formation had a similar effect on veins but the bovine intrapulmonary vein was more sensitive than the corresponding underlying artery to the effects of NO, nitroso compounds and nitroglycerin.
NO elicited potent and marked inhibitory effects on platelet aggregation (Mellion. et al.
The novel action of NO was mimicked by nitroprusside, S-nitrosoguanidines, nitric oxide, S'-nitrosothiols and related chemical agents that generate NO activate cytosolic guanylate cyclase by heme-dependent mechanisms (Ignarro et al. 1984a). The requirement of heme is attributed to the formation of the para-magnetic species, NO-heme that is responsible for the enzyme activation (Craven et al. 1979). In addition to the cytosolic or cytotoxic role of NO generated by macrophages, neutrophils, Kuppfer cells and other scavenger cells, the NO released from these cells may elicit local effects on the microcirculation and platelet function, causing vasodilation of arterioles and venules and inhibition of platelet adhesion to vascular endothelial surfaces and platelet aggregation.

**Nitric oxide synthase:**

Neurotransmitters like Ach, glutamate and glycine have long been known to be associated with elevated cGMP levels in the brain (Garthwaite 1990). Addition of L-arginine to rat synaptosomal cytosol in the presence of NADPH resulted in the formation of NO and citrulline and was accompanied by stimulation of soluble guanylate cyclase (Knowles 1989; Palmer et al. 1989). Both of these processes were inhibited by hemoglobin and L-NMMA, L-NIO and L-NA. These also show that rat brain also possess NO synthase. This enzyme was dependent on the free Ca\(^{2+}\) concentration in synaptosomes (approx 80nM) whereas it was fully active at concentrations of 400nM. As in the vascular endothelium and the platelet, the NO synthase could be stimulated for NO production by increasing Ca\(^{2+}\) concentration. Bovine brain cytosol has also been shown to contain the NOS (Schmidt et al. 1988b). This enzyme was first purified from rat cerebellum and shown to be calmodulin dependent (Bredt et al. 1990). The purified enzyme migrates as a single 150 kDa band on SDS-PAGE and appears to be a monomer (Bredt & Snyder 1990). Furthermore, apart from endothelial cells and platelets the presence of NOS has been demonstrated in adrenal medulla and the retina, where it may be involved in regulation of catecholamine release. NO synthase thus found in brain, kidney, retina, endothelial cells and platelets was c-NOS which was also found in macrophages that had been activated by lipopolysaccharide alone or in combination with IFN-γ (Stuehr et al. 1987). This c-NOS required protein synthesis for its expression. There was a lag phase of approximately 8h before NO\(_2\) and NO\(_3\) synthesis was detected. The synthesis of these
products continued either until no more substrate was available or until the death of the cell. NOS of this form would be stimulated by L-homoarginine, L-arginine methyl ester, L-arginyl L-aspartate which could also substitute for l-arginine (Hibbs et al. 1987) and could be inhibited both by L-NMMA and L-cancanavine. This enzyme required NADPH and its activity was enhanced by Mg$^{2+}$ although this cation was not indispensable for NO generation, but was dependent on tetrahydobiopterin. It could also incorporate oxygen into citrulline and was thus a dioxygenase. All these indicated that the NOS present in macrophages was inducible in nature.

The presence of similar inducible NOS in neutrophils has recently been confirmed which has same characteristics as that of being cytosolic and Ca$^{2+}$ independent. Later it was thought that the NOS in the vessel were the constitutive Ca$^{2+}$ dependent enzyme in the endothelium.

The porcine endothelial cells in culture have shown to express a Ca$^{2+}$ independent NO synthase following activation in vitro with lipopolysaccharides and IFN-$\gamma$. The induction of NOS in the vascular endothelium and the vascular smooth muscle layer was time dependent and inhibited by cycloheximide. Polymoxin B, a lipopolysaccharides agonist would prevent the induction of this enzyme (Rees et al. 1990).

Endothelial cells that have been depleted of L-arginine are able to synthesize this amino acid from endogenous sources, indicating that under normal circumstances the availability of l-arginine is well regulated. In case of inducible NOS, once activated they continue producing NO which might lead to the death of cell itself. Therefore, a decrease in levels of l-arginine may be associated with NOS activity and consequently of NO production which might systemically cause hypertension, atherosclerosis and other vascular diseases.

Two distinct isoforms have been identified of which two are named after the cell types from which they have were first cloned:
Neuronal NOS (nNOS, NOS 1 gene product), which produces NO in neuronal tissue in both the central and peripheral nervous system. Neuronal NOS also performs a role in cell communication and is associated with plasma membranes (Grozdanovic & Baumgarten. 1999).

Inducible NOS (iNOS, NOS 2 gene product), present in monocytes or macrophages, smooth muscle cells, microvascular endothelial cells, fibroblasts, cardiomyocytes, hepatocytes and magakaryocytes, which can be found in the immune system but is also found in the cardiovascular system. (Flesch et al. 1994).

Endothelial NOS (eNOS, NOS 3 gene product or Constitutive / cNOS); generates NO in blood vessels and is involved with regulating vascular function. A constitutive Ca2+ dependent NOS provides a basal release of NO (Awolesi et al. 1994). eNOS is also associated with membranes of Golgi bodies within cells.
Comparison between the 2 forms of NOS:

<table>
<thead>
<tr>
<th>Constitutive</th>
<th>Inducible</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADPH dependent</td>
<td>NADPH dependent</td>
</tr>
<tr>
<td>Dioxygenase</td>
<td>Dioxygenase</td>
</tr>
<tr>
<td>Inhibited by l-arginine analogues</td>
<td>Inhibited by l-arginine analogues</td>
</tr>
<tr>
<td>Ca2+/calmodulin dependent</td>
<td>Ca2+/calmodulin independent</td>
</tr>
<tr>
<td>Picomoles of NO released</td>
<td>Nanomoles of NO released</td>
</tr>
<tr>
<td>Short-lasting release</td>
<td>Long lasting release</td>
</tr>
<tr>
<td>Unaffected by glucocorticoids</td>
<td>Induction inhibited by glucocorticoids eg-dexamethasone.</td>
</tr>
</tbody>
</table>

**Insulin activated nitric oxide synthase (IANOS).**

It has been demonstrated for the first time from our laboratory, that insulin is capable of activating a membrane bound NOS in a wide variety of cells such as platelets, leukocytes, endothelial cells, liver, kidney, skeletal muscle cells. NO thus formed produces insulin like effect in the system in the absence of the hormone itself (Kahn et al. 2000). This form of NOS could be activated by insulin in vivo and in vitro leading to the stimulation of NO synthesis.

Although the presence of NOS in the soluble cytosolic fraction is well-known, cytosolic NOS could not be stimulated by insulin, and the biochemical characteristics of the membrane bound insulin activable NOS are different from those of the cytosolic NOS. e.g.- For the synthesis of NO from L-arginine by the insulin activated NOS, unlike the soluble cytosolic NOS, did not require the addition of ATP, NADPH in the reaction mixture, although the addition of 1.5mM Ca^{2+} stimulated the formation of NO. The IANOS, a constitutive membrane bound enzyme was found to be a regulatory enzyme in the maintenance of homeostasis of plasma NO level. One of the interesting properties of IANOS was that the enzyme could be activated by NO (the reaction product), representing a rather unusual case of feedback activation. The effect of NO on the IANOS however was biphasic in nature. At low concentrations of NO (0.1-0.4 μM) IANOS was activated, but in the presence of higher concentrations of NO (>0.4 μM),
the degree of stimulation was gradually decreased.

The purified IANOS showed some properties similar to those of the insulin receptor itself (Bhattacharya. et al. 2001) in that not only could the insulin dependent activation of IANOS be blocked by anti-insulin receptor antibody but the characteristics of the Scatchard plot of the hormone binding to the purified enzyme (Kahn & Sinha.1990) and molecular weights of IANOS and its subunits were similar to those of the purified insulin receptor (Massague. et al. 1980). The above studies strongly suggested that the purified insulin activated nitric oxide synthase is the insulin receptor itself. Insulin activated nitric oxide synthase has a very important role in the path physiological conditions where there is severe impairment of nitric oxide production (Sinha et al. 2002; Sinha et al. 1999).

Physiological actions of nitric oxide:

NO is implicated in a wide variety of physiologic effects. The reaction of NO with the heme group of guanyl cyclase leading to the activation of guanyl cyclase is the principal effector reaction of NO in the cardiovascular system. Agonist-mediated increase in the endothelial cell calcium activates eNOS. Also, flow mediated vasodilation with exercise depends in part on the flow sensitive increase in the endothelial cell calcium, which leads to increased eNOS activity (Upchurch.et al. 1997).

Nitric oxide relaxes gastrointestinal smooth muscle and leads to reduced motility, relaxation of the sphincter of Oddi and relaxation of the lower esophageal sphincter. Relaxation of bronchial smooth muscle can be provoked by inhaled nitric oxide and endogenously produced nitric oxide may contribute to the maintenance of basal bronchial and basal pulmonary arterial tone (Loscalzo. 1992). Some other effects of eNOS include maintenance of vascular integrity, impairment of leukocyte adhesion to the endothelium, inhibition of smooth muscle migration and proliferation. Endothelial nitric oxide plays a critical role in hemostasis wherein the basal production of nitric oxide by eNOS inhibits both adhesion and aggregation of platelets in vasculature. Endothelium derived nitric oxide is an important determinant of cerebral blood flow. The nNOS in cerebral and glial cells contribute to the regulation of cerebrovascular tone, memory and learning through its involvement in long-term potentiation in the CNS. Nitric oxide is a likely transmitter of non-adrenergic, non-cholinergic neurons
and may thereby have a role in the regulation of myocardial contractility, heart rate, gastrointestinal motility, bronchial tone and penile erection.

The iNOS produced NO in macrophages, lymphocytes and neutrophils is an important determinant of immune and inflammatory responses. The bactericidal, fungicidal, viricidal, parasiticidal and tumoricidal activities of macrophages are partly determined by the robust elaboration of nitric oxide by iNOS (Loscalzo. 1995). Nitric oxide also limits lymphocyte proliferation and attenuates the allogenic immune response. The iNOS being expressed by a broad range of non-immune cell types, it is thought that NO produced by this isoform is involved in non-specific immunity, especially in the liver and lungs. By a similar mechanism, nitric oxide produced by iNOS may also be involved in apoptotic responses in a variety of cell types.

The effects of nitric oxide are modulated by both direct and indirect interactions that can be dose-dependent and cell-type specific. Understanding the regulatory mechanisms of nitric oxide in apoptosis and carcinogenesis provides important clues in diagnosis and treatment of tissue damage and cancer (Kim. et al. 2001).

The second messenger role of nitric oxide:

It has been reported that nitric oxide as the second messenger of insulin for its diverse effects in various physiologic and pathologic events (Kahn et al. 2000). Insulin plays an important role in carbohydrate metabolism, fat and protein metabolism (Czech. 1977). The hormone also has its effects in atherosclerosis, thrombosis and neuropathological events. The effects of the hormone are the result of the binding of the protein molecules to the target tissue. The interaction of insulin with specific cell surface receptors results in the activation of the insulin receptor kinase (Weiss. 1993), which is thought to modify the biological activity of various proteins through downstream phosphorylation by the activated enzyme. It has been recently showed that the administration of physiologic concentrations of insulin in mice resulted in the decrease of blood glucose content with a simultaneous increase in plasma methemoglobin (Sinha et al. 1999). The formation of methemoglobin suggested the formation of NO in the system. It has been demonstrated that physiologic concentrations of insulin specifically activates a membrane bound constitutive NOS. The NO formation catalysed by the NOS, produced insulin like effects on carbohydrate metabolism both in vivo and in vitro.
Role of nitric oxide in carbohydrate metabolism.

NO, the product formed from the activation of NOS (through the binding of the hormone to its receptors) was capable of mimicking one of the most important effects of insulin i.e. stimulation of carbohydrate metabolism both in vitro and in vivo. The stimulatory effect of insulin in glucose transport activity and glucose oxidation of L-NAME to the incubation mixture which blocked the insulin induced synthesis of NO in the tissues also completely blocked the insulin induced synthesis of NO in the tissues also completely blocked the stimulatory effects of insulin, but the presence of L-NAME had no effect on the insulin mimetic effect of NO which indicated that the NO synthesis was necessary for the stimulation of glucose metabolism by insulin and that the effects of insulin might be mediated through the NO formation by the hormone in the tissues. (Kahn et al. 2000).

Pathophysiological effects of nitric oxide.

Both a deficiency and excess of NO are believed to be involved in several pathological states. During the past two decades, nitric oxide has been recognized as one of the most versatile players in the immune system, it is involved in the pathogenesis and control of infectious diseases, tumors, autoimmune processes and chronic degenerative diseases. (Bogdan. 2001) Because of its variety of reaction partners (DNA, proteins, low molecular weight thiols, prosthetic groups, reactive oxygen intermediates), its wide spread production (different varieties of NO synthases) and the fact that its activity is strongly influenced by its concentration, NO continues to surprise and perplex immunologists. Protective and toxic effects of NO are frequently seen in parallel. NO is a critical determinant of basal vascular tone and a deficiency of NO is associated with hypertension. Common disorders that promote atherosclerosis, viz. hypertension, hyperlipidemia, smoking and diabetes are all associated with abnormal endothelial function, one manifestation of which is a comparative deficiency of bioactive NO (Sherman et al. 1997).

A deficiency of NO producing neurons in the gastrointestinal tract is responsible for certain abnormalities in Gastrointestinal motility viz. Hirschsprung's disease, achalasia and chronic intestinal pseudo-obstruction (Loscalzo. 1995b). NO plays an important role in gastric cytoprotection by increasing the mucosal blood flow and modulation of gastric epithelial function. In addition, NO produced by hepatocyte iNOS plays a role
in protection of these cells against a variety of hepatic toxins (including ethanol and acetaminophen). Impairment of NO production by endothelial cells in disorders of endothelial dysfunction or following percutaneous angioplasty may help to facilitate smooth muscle proliferative responses in these settings. The cytoprotective and cytotoxic effects of NO are important.

**Role of nitric oxide in neoplastic conditions.**

NO, a biological messenger is reported to possess tumoricidal properties and to induce programmed cell death (apoptosis) and differentiation in neoplastic cells (Jun et al. 1996). It has also been reported that the tumoricidal effects of lipopolysaccharides and γ-interferon are mediated through the production of NO by the macrophages (Deunas-Gonzalez et al. 1997). Krick et al observed that the exposure of rat pulmonary artery smooth muscle cells to NO, derived from NO donor S-nitroso-N-acetyl-pencillamine increased the number of cells undergoing apoptosis. Hence, they concluded that NO induced apoptosis might be related to the mitochondrial membrane depolarization in pulmonary artery smooth muscle cells (Krick et al, 2002). It was also proposed that NO produced as a result of exaggerated response to inflammatory mediators may result in inducing cell apoptosis. Though the exact mechanism by which NO causes apoptosis is unknown, the Fas (CD95) and Fas ligand (Fas L)(CD 95L) have been thought to be responsible. Nitric oxide induced apoptosis in rat and human pulmonary artery smooth muscle cells is mediated, at least partly, through the Fas-FasL pathway with cAMP increasing the expression of Fas and FasL (Hayden et al, 2001).

**Role of nitric oxide in breast cancer.**

The presence of inducible nitric oxide synthase in breast cancer cell lines and endothelial nitric oxide synthase in human breast tumors have been reported previously (Martin et al. 1999). It has also been reported that loss of NOS expression is associated with the progression of breast cancer (Martin et al. 2000). The effects of the phytoestrogen biochanin A on the growth of the MCF-7 human breast cancer cell line are linked to the decreased levels of iNOS by biochanin A this inhibiting the production of NO, a known second messenger and inducer of apoptosis and thereby affecting the overall cell protein pattern (Hsu et al. 2000). Because the role of nitric oxide in apoptosis is controversial, the NO production, iNOS
expression and enzyme activity in relation to TNF-α induced apoptotic cell death were investigated in the human breast cancer cell line MCF-7 and other malignant cell lines (Binder et al. 1999), it has been observed that iNOS induction plays an essential role in TNF-α induced apoptosis of the investigated human breast cancer cell line MCF-7. Breast cancer is characterised by its ability to metastasize rapidly. One of the factors that might facilitate this metastatic potential includes tumor vascularity. Nitric oxide is thought to be a major regulator not only of physiologic vascular tone but also of the abnormal vascularity associated with many tumors. (Duenas-Gonzalez. et al. 1997).

**Neutrophils-Morphology and Function.**

Leukocytes are the major cellular components of inflammatory and immune responses and include neutrophils, T and B lymphocytes, natural killer cells, monocytes, eosinophils, and basophils. Neutrophils account for 70% of all leukocytes. Neutrophils are produced in the bone marrow, released into blood, circulate briefly, and migrate into tissue spaces or on to epithelial surfaces such as those in the respiratory, digestive, or urogenital tracts. Production is continuous in order to provide for the continual demand for neutrophils in the tissues and maintain the circulating pool in the blood. Transit time for generation of neutrophils in marrow is approximately 4 - 6 days and the marrow maintains a five-day supply of mature neutrophils in storage.

Neutrophils evolve from pluripotent stem cells under the influence of cytokines and colony stimulating factors. The myeloblast is the first precursor cell and is followed by the promyelocyte. The promyelocyte evolves when the classic lysosomal granules, called the primary or azurophil granules are produced. The primary granules contain hydrolases, elastase, myeloperoxidase, cationic proteins, and bactericidal/permeability-increasing (BPI) protein important for killing gram-negative bacteria. Azurophil granules also contain defensins, a family of cysteine-rich polypeptides with broad antimicrobial activity against bacteria, fungi, and certain envelopes viruses. The promyelocyte divides to produce the myelocyte, a cell responsible for the synthesis of the specific or secondary granules. During the final stages of maturation there is no cell division, and the cell passes through the metamyelocyte stage and then to the band neutrophil with a sausage shaped nucleus. As the band cell matures, the nucleus assumes a lobulated configuration. The nucleus of neutrophils normally contains up to four segments.
Neutrophils serve as the primary defense against invasion of tissues by microorganisms. They accumulate at sites of inflammation or bacterial infection by a process of directional migration or chemotaxis. The cellular and molecular mediators of inflammation generate chemotactic substances and promote migration and adhesion of neutrophils to vascular endothelium at sites of inflammation. Neutrophils leave the blood stream and enter the tissues by transmigration between endothelial cells. At the site of inflammation, neutrophils are capable of phagocytosis and microbicidal activity. Fusion of lysosomal granules with the phagocytic vesicle releases lytic enzymes and chemicals capable of killing bacteria. Neutrophils phagocytose pathogenic materials that have been properly opsonized by IgG and the complement product. Concomitant with phagocytosis there is a burst of oxygen consumption and activation of the hexosemonophosphate shunt. A membrane-associated NADPH oxidase, consisting of membrane and cytosolic components, is assembled and catalyzes the reduction of oxygen to superoxide anion, which is then converted to hydrogen peroxide and other toxic oxygen products. Hydrogen peroxide + chloride + neutrophil myeloperoxidase provide a particularly toxic system that generates hypochlorous acid (bleach), hypochlorite, and chlorine. These products oxidize and halogenate microorganisms and tumor cells. Strongly cationic proteins and defensins also participate in microbial killing. After 1 to 4 days in tissues neutrophils die.

It has also been shown that neutrophils produce NO, which plays many roles in the immune system (Del-Maschi et al. 1989; Salvemini et al. 1989). In few of the
interesting studies it has been shown that neutrophils have an important role in controlling tumor growth, one study shows the cytokine IL-8 elevated the neutrophils and macrophages at the site of tumor, which was able to control the tumor compared to control (Lee et al. 2000), other study using heat treatment of tumors in mice increased granulocytic infiltrate at the tumor site as determined using immunohistochemical analysis (Ostberg et al. 2005). As described below we hypothesized that neutrophils have an important role in producing anti-tumor protein maspin and with initial studies we found marked impairment of maspin production in the neutrophils of breast cancer volunteers in control to normal volunteers.

Maspin and breast cancer.
Gene: maps to 18q21.3 (Schneider S.S. et al., 1995). The PAI-2 gene maps to the same region. Ets, AP-1 and hormonal-responsive elements (HRE) have been found in the maspin promoter (Zhang M. et al. 1997a)

mRNA: size: 3.0 kb, maspin protein: 375 amino acids.

Maspin (Mammary serpin proteinase inhibitor) a novel serine protease inhibitor, was first isolated and identified to be a 42kDa protein by subtractive hybridisation and differential display analysis comparing the differences in gene expression in normal mammary epithelium and invasive mammary carcinoma cells (Zou et al. 1994), and shown to inhibit cell invasion, promote apoptosis and inhibit angiogenesis (Sheng. 1994; Zhang et al. 2000; Jiang et al. 2002).

Maspin is localized in the cell surface, and in the extra-cellular matrix (Pemberton et al. 1997, Zou et al. 1994) is the cytoplasmic maspin and other kind is the nuclear maspin confined to nucleus (Maass. et al.2002; Reis-Filho. et al. 2002).

While maspin mRNA and the protein are expressed abundantly in normal mammary epithelial cells, the expression of maspin decreased with the progression of the condition and with its malignancy grade (Maass et al. 2001; Hojo et al. 2001). Several lines of evidence demonstrated that maspin is a tumor suppressor gene product (Zou et al. 1994; Hendrix et al. 2000), in that the transfection of maspin cDNA in nude mice with mammary carcinoma (MDA-MB435 cells) resulted in reduced rate of tumor growth and metastasis (Zou et al. 1994; Ngamkitidechakul et al. 2003). Furthermore
exogenous administration of maspin is reported to block tumor cell mobility and invasion (Zou et al. 1994; Sheng et al. 1994; Liu et al. 1999) in contrast these anti-tumor effects of maspin could be reversed by using the antiprotease inhibitor antibody (Sheng et al. 1994; Liu et al. 1999; Sternlicht et al. 1997).

Maspin has sequence homology with several members of the serine protease inhibitor superfamily (serpins), including plasminogen activator inhibitors 1 and 2 (PAI-1 and PAI-2) and α1-antitrypsin, as well as the non-inhibitor serpin proteins such as ovalbumin (Sager et al. 1996; Sheng et al. 1994).

There is no gross structural alterations of maspin gene of the breast tumor cell line and normal breast epithelial cells, which have been proved by Southern blot analysis of XbaI- restricted DNA from normal and tumor cells (Schneider et al. 1995). Maspin mRNA was expressed in normal mammary epithelial strains, but not in MCF-7, MDA-MB-157, 231, 435, 468, T-47D, ZR-75-1, BT-549, and Hs578T breast cancer cell lines (BCC) in breast fibroblasts (Sabbatini et al. 2000, Merrie et al. 1999). Differential expression of maspin in normal and carcinoma-derived mammary epithelial cells was found to be regulated at the transcriptional level. Ets and Ap1 have been identified in the maspin promoters that are active in regulating maspin expression in normal mammary epithelial cells but inactive in tumor cells. The Ets site alone was sufficient to activate transcription in a heterologous promoter, whereas the Ap1 site cooperated with Ets in activation. The enhancing function by Ets and Ap1 elements was decreased in primary tumor cells (21NT) and was abolished in invasive tumor cells (MDA-MB-231). Thus, loss of maspin expression during tumor progression results at least in part from the absence of transactivation through the Ets and Ap1 sites (Zhang M et al. 1997b).

It was found that single-chain tissue plasminogen activator (sctPA) specifically interacts with the maspin reactive site loop and forms a stable complex with recombinant maspin. Major effects of r-maspin were observed on plasminogen activation by sctPA. First, r-maspin activated free sctPA, second, it inhibited sct PA preactivated by poly-D-lysine. Third, recombinant maspin (r-maspin) exerted a biphasic effect on the activity of sctPA preactivated by fibrinogen/gelatin, acting as a competitive inhibitor at low concentrations (<0.5 μM) and as a stimulator at higher concentrations (Sheng et al. 1998). Fourth, 38 kDa C-terminal truncated r-maspin further stimulated fibrinogen/gelatin-associated sctPA. r-maspin did not inhibit
urokinase-type plasminogen activator, plasmin, chymotrypsin, trypsin, or elastase. The kinetic data was quantitatively consistent with a model in which two segregated domains of maspin interact with the catalytic and activating domains of sctPA. These complex interactions between maspin and sctPA in vitro suggest a mechanism by which maspin regulates plasminogen activation by sctPA bound to the epithelial cells surface (Sheng. et al. 1998).

Treatment of MDA-MB-435 breast cancer cell lines with r-maspin was shown to result in the inhibition of breast cancer cell line invasion in vitro, in the selective adhesion of BCC to a fibronectin matrix, and in the conversion from a fibroblastic to a more epithelial-like phenotype. The inhibition of the invasive process could be abrogated by blocking antibody to the alpha5beta1 integrin (Seftor. et al. 1998).

Cultured normal human mammary epithelial cells (HMECs) were compared to 9 cultured human breast cancer cell (BCC) lines. HMECs expressed maspin mRNA and displayed a completely non-methylated maspin gene promoter with an open chromatin structure. In contrast, 7 of 9 breast cancer lines had no detectable maspin expression and 6 of these 7 maspin- negative breast cancer cell lines also displayed an aberrant pattern of cytosine methylation of the maspin promoter. Interestingly, the maspin promoter was completely methylated in maspin- negative normal peripheral blood lymphocytes. This indicates that the maspin promoter is not a functional CpG island and that cytosine methylation of this region may contribute to normal tissue-restricted gene expression. Chromatin accessibility studies with MCF-7 cells, which lack maspin expression and have a methylated promoter, showed a closed chromatin structure compared with HMECs. Moreover, maspin gene expression could be re-activated in MCF-7 cells by treatment with 5-aza-2-deoxycytidine, a DNA demethylation agent. Thus, aberrant cytosine methylation and heterochromatinization of the maspin promoter may silence maspin gene expression, thereby contributing to the progression of human mammary cancer (Domann. et al. 2000). Tumor Analysis of breast cancer specimens demonstrated that loss of maspin expression occurred most frequently in advanced cancer. This supports the hypothesis that maspin functions as a tumor suppressor (Zou et al. 1994).
Maspin inhibits the invasiveness and motility of mammary carcinoma cells.

It has been seen in the membrane invasion culture system (MICS) assay maspin inhibiting the invasiveness and motility of mammary carcinoma cells as well as prostatic cancer cells (Sheng et al. 1996), so maspin is found to play a key role in the control of invasiveness and motility in the breast cancer and prostate cancer.

Nitric oxide and maspin.

Although these studies indicated that maspin might play a critical role in the pathogenesis of breast cancer. However it has been reported that the synthesis of NO formation catalyzed by endothelial NOS or inducible NOS is reported to be involved in the expression of maspin in mammary epithelial cells in tissue culture (Khalkhali-Ellis et al. 2003), it is not known whether similar mechanism is involved in the expression of maspin in the pathophysiological setting of human breast cancer. Furthermore the regulatory control of synthesis of NO itself catalyzed by these enzymes in breast cancer however remains obscure due to the fact that NO can act both as a procarcinogenic (Iwarkiri et al. 2002) and as an anticarcinogenic agent (Kilbourn et al. 1990) depending upon the amounts of available NO at cellular level (Chakraborthy et al. 2004).

Till date, the role of Nitric oxide synthase in breast cancer remains obscure. As described above, since insulin receptor activity has been related to the production of NO, the role of insulin receptor is considered to be a very critical in the prognosis of breast cancer through the activation of IANOS leading to NO synthesis in the setting of the malignant condition. It has been reported from this laboratory that plasma NO was significantly decreased in various neoplastic conditions due to the impairment of IANOS in the erythrocyte membrane and the restoration of plasma NO level to normal ranges through the reactivation of erythrocyte IANOS resulted in favorable modification of various cancers including regression of solid tumors. (Sinha et al. 2002).

In this context, experiments were performed to understand and delineate the role of insulin receptor in the development and prevention of breast cancer, through insulin stimulated nitric oxide and maspin production in nonmalignant neutrophils of breast cancer patients in comparison to normal volunteer neutrophils and also in breast cancer tissue.
## Summary of maspin function and regulation.

<table>
<thead>
<tr>
<th>Cellular process</th>
<th>Pathway</th>
<th>Effect</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metastasis</td>
<td>Integrin profile altered by maspin UPA/uPAR activity reduced by maspin Rac1 activity inhibited by maspin PI3K/ERK pathways stimulated</td>
<td>Increased cell adhesion to fibronectin Reduced active uPA and reduced levels of cell surface uPAR lead to a reduction in active plasmin Reduced cell motility Increased cell adhesion through increased focal adhesions and stress fiber formation</td>
<td>Seftor et al. (1998) Biliran and Sheng (2001); Amir et al. (2005) Odero-Marah et al. (2003) Odero-Marah et al. (2003)</td>
</tr>
<tr>
<td>Angiogenesis</td>
<td>Unknown</td>
<td>Endothelial cell migration is blocked, anti-angiogenic Reduced microvessel density associated with reduced neovascularization</td>
<td>Zhang et al. (2000) Zhang et al. (2000)</td>
</tr>
</tbody>
</table>

### Maspin gene regulation

- **p53**: p53 induces the maspin gene through a non-consensus p53 binding site in the maspin promoter Zou et al (2000)
- **Tamoxifen**: Tamoxifen treatment results in maspin re-expression Khalkhali-Ellis et al (2004)
- **MnSOD**: Overexpression of MnSOD upregulates maspin expression in breast cancer cells Li et al. (1998).
- **Gene silencing**: maspin promoter methylation causes maspin gene silencing Domann et al. (2000).