6. Discussion

6.1. Cadherins

6.1. a. E-cadherin

The significant reduction of expression of E-cadherin in breast malignancy suggests that impaired expression of E-cadherin is a characteristic of the cells with malignant transformation, pointing to the role of E-cadherin as a tumor suppressor gene. This impairment could be due to mutations of E-cadherin gene (Behrens J, 1991), protease cleavage of cadherin peptides (Katayama M, 1994), translational disorder (Frixen UH, 1991), allelic loss of 16q (Tsuda H, 1990) in which E-cadherin gene is located or hypermethylation of the gene promoter. Our results support the earlier findings (Oka H, 1993) that the frequency of downregulation of E-cadherin expression in tumors with extensive lymph node metastasis was significantly higher when compared with their node negative counterparts. This supports the possibility that the loss of E-cadherin expression may promote tumor metastasis to lymph nodes. Epithelial to mesenchymal transition (EMT), implicated as a mechanism for tumor dissemination, is marked by loss of E-cadherin, disruption of cell adhesion, and induction of cell motility and invasion. It has been found that E-cadherin expression in metastases suggested a reversion to a more epithelial phenotype could occur at the metastatic site leading to a hypothesis that a secondary organ microenvironment could induce re-expression of E-cadherin (Chao YL, 2010).

The identification of E-cadherin in the cytoplasm and not on the membrane in most of the samples is consistent with the notion that loss of membranous E-cadherin promotes tumor disaggregation and dissemination (Pignatelli M, 1994). Because the normal role of E-cadherin is to maintain homotypic adhesion in epithelial cells, the abnormal E-cadherin expression in carcinomas is thought to contribute to the invasiveness of the cancer by permitting discohesion of cancer cells, thereby facilitating their permeation into the breast stroma. The staining patterns of E-cadherin observed in the present study suggest cytoplasmic accumulation of E-cadherin which may reflect abnormal transport to the cell surface or
abnormal reuptake of the molecule from the cell surface back to the cytoplasm (Handschuh, 1999). The detection of cytoplasmic staining in some of the benign tumors suggests that aberration in E-cadherin could be an early event, playing roles in the initiation of breast tumorigenesis. Recently, E-cadherin has been found to be downregulated by transcription factor snail and this association is found to be important in the tumor progression (Blech Schmidt K, 2007).

A number of studies are available that have attempted to resolve the prognostic potential of E-cadherin with contradicting results. The present results concur with earlier reports pointing to a correlation between loss of E-cadherin expression and poor prognosis in various cancers including breast cancer (Zhang YG, 2007; Zou W, 2002; Pederson KB, 2002; Lim S C, 2002; Yoshida R, 2001). However, some other investigators have reported that there is no correlation between loss of E-cadherin expression and survival (Soler AP, 1999; Gonzalez MA, 1999). In the present study, reduced E-cadherin expression significantly correlates with shorter overall survival in node negative breast cancer patients but did not correlate with recurrence free survival. The same result has been obtained earlier by Charpin C, 1998.

Determining the prognosis of younger breast cancer patients is extremely difficult. The current study looked at the applicability of E-cadherin as a prognostic marker to evaluate the survival of younger and older women with breast cancer. The present results indicate a significant association between E-cadherin and age of the patient and hence that an investigation of E-cadherin expression status is immensely useful in analysing the survival of younger breast cancer patients. This could probably be due to association of E-cadherin with hormone pathway markers such as ER (Mahler-Araujo B, 2007).

The biological significance of E-cadherin expression has been found to be different in high grade IDC and ILC. (Turashvili G, 2007). Recent evidence suggests that most tumors usually do not progress between grades and that groups of tumors within each grade are biologically distinct. Divergent biological behaviour of tumors within histological grades has been a problem faced by clinicians and pathologists. Hence a marker to predict the outcome of tumors within a grade is the need of the hour. The results of the present study prove that E-
E-cadherin could serve the purpose though studies with larger sample size are warranted to confirm the above hypothesis.

NPI is a widely accepted prognostic index in breast cancer which takes into account the tumor size, grade and node positivity. Hence given the prognostic utility of E-cadherin, we thought it would be useful to compare the prognostic value of E-cadherin status with NPI. The results prove conclusively that E-Cadherin and NPI are independent prognosticators in predicting the overall survival of breast cancer patients.

Logistic regression analysis shows that E-cadherin could be used as a predictive marker of node positivity in breast cancer patients, which suggests that changes in E-cadherin are associated with lymph node metastasis. This is highly significant in view of many breast cancer patients presenting with micro metastasis in the nodes that are clinically undetectable at the time of diagnosis. Hence the use of E-cadherin as a marker of nodal metastasis could aid the clinicians in deciding the correct therapeutic regimen and in predicting the outcome of patients. This result has earlier been published by us (Madhavan M, 2001). The results of the Cox regression analysis too highlight the independent prognostic potential of E-cadherin in predicting the outcome of breast cancer patients.

Deranged expression and/or function of cadherin have been implicated in malignant tumor development and prognosis. The current study underscores the fact that reduction or lack of expression of E-cadherin is an early alteration promoting initiation and progression of breast carcinogenesis which makes E-cadherin an ideal candidate for anticancer gene therapy. Loss of E-cadherin expression showed a highly significant association with the overall survival that is significant also as an independent prognostic factor. Moreover, E-cadherin could be a novel marker for nodal metastasis in breast cancers. To sum up, the use of E-cadherin as a prognostic marker along with the accepted standard prognostic indicators could provide valuable additional information regarding the metastatic potential and treatment outcome in breast cancer patients.
One of the aims of the present study was to evaluate the expression of the cell adhesion molecule P-cadherin in a series of breast carcinomas, using immunohistochemistry, and correlate this with other clinicopathologic parameters like tumor histology, nodal status, recurrence status, grade of tumor, invasiveness etc. In breast cancers, P-cadherin expression is most common in high grade, ER-negative tumors and has been shown to be an independent marker of poor prognosis in some but not all studies. Recent data have shown that P-cadherin expression is significantly more frequent in BRCA1-related breast cancers than in either BRCA2 or non-BRCA1/2 breast cancer. (Arnes JB, 2005).

P-cadherin null mice develop mammary gland hyperplasia, dysplasia, and abnormal lymphoid infiltration, (Radice GL, 1997) demonstrating that loss of normal P-cadherin expression leads to cellular and glandular abnormalities. Hence we think it is logical to interpret that the loss of P-cadherin expression as an early event prior to invasion, as can be evidenced in the present study from the significant underexpression of P-cadherin in invasive breast cancers compared to pre-invasive lesions and a similar trend is also observed in node positive cancers compared to node negative cancers. This implies that aberration in P-cadherin expression occurs somewhere early in the metastatic cascade, and that may be why the same alteration is seen in cancers spreading to lymph node and those which become invasive. A similar observation has been beautifully presented by RG Hardy et al, (2002) who suggested that aberrant P-cadherin expression occurs in the earliest morphologically identifiable stage of colonocyte transformation and that these changes occur prior to alterations in E-cadherin, catenin or APC. The loss of P-cadherin expression probably comes after P-cadherin cytoplasmic relocalisation. Therefore, in the initial phase of tumor growth, the high expression of P-cadherin may be crucial in the formation of a tumor mass which is ready to progress and metastasize (Yasui W, 1993). Cytoplasmic relocalization or loss of P-cadherin expression may be responsible, together with other known/unknown upregulated oncogenes and downregulated tumor suppressor genes, for the later stages of tumor progression, such as invasive growth and metastasis. To date, no specific mechanism has been elucidated for the downregulation of P-cadherin expression in cancers, though mechanisms...
such as epigenetic silencing and mutations have been proposed for the downregulation of E-cadherin in cancers. We are not sure whether the same genetic mechanism operates for the reduced expression of E-cadherin and P-cadherin. Subsequent challenges will be to dissect the molecular mechanisms leading to loss of P-cadherin expression and to elicit their downstream effects. In this manner manipulation of such molecules may become possible, leading to the prospect of therapies designed to reverse or prevent breast cancer invasion and progression.

However, P-cadherin, as distinct from E-cadherin did not have significance in predicting the overall/recurrence free survival of breast cancer patients. Similar results have been published by Potemski P, 2007 while several others have found P-cadherin to be a significant indicator of poor prognosis in human breast cancers. (Paredes J, 2007). Another recent report by Fanelli M A (2008) describes that the disease-free survival and overall survival were significantly shorter for patients expressing P-cadherin. We propose here, for the first time that P-cadherin expression is an indicator of increased overall survival in node negative patients. Hence detection of P-cadherin could be used as a prognostic tool for assessing the survival of node negative breast cancer patients.

6.1. c. Imbalance in the co-expression of cadherins

Reduced E-cadherin expression in breast carcinoma is found to be accompanied by aberrant P-cadherin expression (Palacios J, 1995) which suggest interdependence of expression of E- and P-cadherin in some circumstances. Since both E-cadherin and P-cadherin serve the purpose of cell adhesion and both of them are localized on the chromosome 16, we thought it would be useful to look at the anomalies in the co-expression status of E- and P-cadherin in breast cancer. Also, the prognostic impact of the co-expression of E-cadherin and P-cadherin in breast cancer has to be investigated.

In the present study, it was found that the group of patients who expressed both the cadherins had the highest overall survival while the lowest survival was shown by those who did not express either of the cadherins. Thus, we hypothesise that looking at the co-expression pattern of both E- and P-cadherins could be very fruitful in predicting the overall survival of
breast cancer patients. A recent publication shows that the establishment of E- and P-cadherin-dependent cell adhesion leads to regulation of a common gene expression program, and modulation of particular genes that may potentially underlie their anti-invasive effect. In addition, they can also specifically modify a subset of genes, thus suggesting that they could mediate both common and specific biological functions in invasive breast carcinomas (Sarrio, D, 2009).

To our knowledge, this is the first report highlighting the prognostic significance of the co-expression of E-cadherin and P-cadherin in breast cancer patients. In the present study, when all the patients were grouped together, only E-cadherin showed promise as a prognostic marker to predict the overall survival while P-cadherin did not show any significance. Thus even if alterations in one of the cadherins may be missed out in a particular breast cancer patient who belongs to any specific group, prediction of overall survival will be almost completely successful by analyzing their co-expression pattern of both the cadherins.

6.2. Signaling molecules

62.a. Beta catenin

Loss of E-cadherin- β-catenin adhesion is an important step in the progression of many epithelial malignancies. Hence while studying the abnormalities in the expression of E-cadherin system in the development of breast cancer; it is nonetheless important to investigate the expression of β-catenin in the pathogenesis of breast cancer. The immunohistochemical expression pattern of β-catenin was looked into the study sample, and its significance in relation to nodal and distant metastasis and its prognostic importance in breast cancer was also examined.

In the present study, the analysis was focused on the cytoplasmic expression of β-catenin and an important requirement for the signalling competence of β-catenin is the existence of a cytoplasmic pool of β-catenin that is not associated with E-cadherin or any other protein that could prevent association with a member of lymphocyte enhancing factor/T-cell factor family. In short, the significant downregulation of β-catenin expression in
malignant tumors of the breast in comparison with benign tumors and in node positive tumors in comparison with node negative breast cancers implies an abrogation of the catenin signaling pathway during the malignant progression in breast cancer. This is in concurrence with a recent report by Zhang YG (2007) who found that reduced junctional β-catenin is associated with positive lymph node status in invasive breast cancers. Zou W (2002) have reported that the lower expression of β-catenin could be used as an important marker of metastasis and prognosis in breast cancer. But in gastric adenocarcinoma a study by Joo M et al (2003) have found that the expression of β-catenin is significantly associated with the histological type but not with tumor progression and lymph node metastasis by which they have confirmed that the expression of cadherin-catenin complex is more probably related to tumor morphology than to tumor progression. Apart from the interaction with cadherins, a novel interaction between BRCA1 and β-catenin has been illustrated recently by Li H (2010). They found that loss of wild type BRCA1 leads to impaired expression of the nuclear form of β-catenin, which may contribute to the pathogenesis of breast cancer.

In Hepatocellular carcinoma, β-catenin expression has been shown to be an indicator of poor prognosis particularly in poorly differentiated tumors (Inagawa S et al, 2002). In another study, decreased β-catenin immunoreactivity was associated with poor outcome in bladder cancer (Shimazui T, 1996). A study by Dolled-Filhart M et al (2006) shows that loss of β-catenin membranous expression may be useful as a prognostic marker in breast cancer. Considerable data are available pointing to a significant role for β-catenin in predicting the post-operative survival and recurrence free survival of breast cancer patients. In the present study, a Kaplan-Meier analysis could not bring out any significant difference in the overall survival or recurrence free survival of breast cancer patients on the basis of β-catenin expression status though there was a marginal increase in both the overall and recurrence free survival of breast cancer patients expressing β-catenin.

6.2. b. Focal Adhesion Kinase (FAK)

Although FAK is interesting from a basic science perspective, it appears that its relevance in the clinical arena is limited. However, a latest study shows promise for FAK as a
potential target for drug discovery (Han EK, 2007). Studies using western blotting show that FAK is over expressed in tumors of colon, breast, thyroid and prostate and that FAK overexpression is a marker for invasive and metastatic tumor. (Owens LV, 1995). These studies were however limited by the fact that FAK levels were examined in homogenates of tumor tissue and hence it would be impossible to detect changes in FAK expression that might occur in a small population of cells located within a tumor. Hence immunohistochemical approaches would be more appropriate. Even though in tumors of other tissues such as colon, oral cavity, ovary etc., FAK was over expressed when compared with normal tissue, Glukhova M etal(1995) have established that there is no elevation of FAK expression in breast tumors.

FAK is expressed in most tissues examined so far such as the developing mouse embryo, developing vasculature, brain and osteoclasts (Polte TR, 1994, Andre E, 1993, Berry V, 1994). An analysis of 49 human tumors, including breast and colon carcinomas, showed increased FAK mRNA in 17 of 20 invasive tumors, 15 metastatic tumors and 1 of 8 normal adenomatous tissues (Weiner TM, 1993). Western blot analysis showed increased FAK levels in epithelial and mesenchymal tumor tissue compared with normal tissue from the same patient (Owens LV, 1995). In contrast, FAK did not seem to be elevated in breast tumor tissue sections that were immunohistochemically stained with anti-FAK antibodies (Glukhova M, 1995).

The present study revealed a significant increase in expression of FAK in cancers compared with benign tumors. Moreover, the percentage of cases showing intense positivity of FAK was observed to be very high among cancer cases (21.9%) again underlining the fact that overexpression of FAK is sufficient for acquisition of transformed phenotype. Increased FAK expression and activity in breast cancer specimens are frequently associated with indicators of poor prognosis (Lark A L, 2005) and correlate with progression to metastasis. Currently there is no evidence to propose that FAK acts as a classic oncogene and thus initiates cell transformation. Our results suggest that FAK can behave like an oncogene and alterations in this oncogene can lead to initial stages of malignant transformation, probably disturbing the cell signalling of adhesion. Hence we think that more studies should definitely be directed towards dissecting the oncogenic role of FAK.
To date, most of the evidence suggests that overexpression of FAK is a marker for invasive and metastatic tumors. Cells which overexpress FAK may have a propensity to invade surrounding tissues and metastasize in vivo. Focal adhesion kinase would have a dual effect in this regard. This would be due to the ability of FAK to (1) confer resistance to apoptosis under anchorage independent conditions and (2) increase cell migration. From a biochemical standpoint, one could predict that cells over expressing FAK would have a high potential for invasion and metastasis. Increased FAK expression may lead to a growth advantage for select tumor cells by inhibiting apoptosis under anchorage independent conditions (Illic D, 1995). Additionally, FAK is involved in cell migration. Thus FAK over expressing cancer cells may have a growth advantage in addition to being highly motile. Current literature shows that phosphorylation of FAK is involved in the extravasation of breast cancer cells. (Earley S, 2008). These properties would greatly facilitate invasion and metastasis. Thus it is reasonable to speculate that overexpression of FAK can contribute to the development of invasive and metastatic phenotype which is highly dependent on cell migration. But in the present study, no such significant difference was observed between groups differing in invasive and metastatic status.

Identification of new markers for predicting overall and recurrence free survival of cancer patients is always a thrust area of clinical cancer research. Hence we probed the potential of FAK to predict overall and recurrence free survival of breast cancer patients. But none of the analyses including those in which the patients were categorized as node negative and node positive, revealed any statistical significance. However there are studies available pointing to a significantly lower survival for FAK positive patients than those without FAK expression. (Garcia S, 2007; Miyazaki T etal, 2003). It was also found that patients with FAK gene amplification by FISH in breast cancer had significantly shorter overall survival. Similarly certain other studies like those by Han NM, 1997 reveal that FAK overexpression is not a significant prognostic factor in survival. Attempts to reveal the prognostic potential of FAK for predicting the survival, recurrence free survival, node positivity, recurrence status etc. by different types of regression methods also proved futile.
6.3. **Proteolytic Enzymes**

6.3. a. **MMP-9 and TIMP-1**

The role of MMPs and TIMPs in tumor metastasis has been firmly established based on numerous previously published experimental and clinical studies. The present study describes the expression pattern of MMP-9 and its inhibitor TIMP-1 in benign and malignant breast tumors with special reference to various clinical subgroups like invasive, node positive and metastatic tumors. The association of these proteins with the prognosis of breast cancer patients has also been looked into.

The significant alteration of MMP-9 in tumors with metastatic potential noted in this study indicates that deregulation of MMP-9 is a late event in breast carcinogenesis, playing a role in the regulation of invasion and distant metastasis. In contrast to MMP-9, alteration of TIMP-1 expression occurs mainly between benign and malignant breast tumors which highlights the theory that TIMP-1 deregulation is an early event occurring somewhere in the malignant transformation of benign breast tumors. The significant correlation between MMP-9 expression and tumor grade noted in the present study reflects a role for MMP-9 in predicting the aggressive behavior of breast cancer. A similar positive association between active MMP-9 expression and increasing tumor grade has been reported by Di Nezza L A (2002) in endometrial carcinoma. In oral cancer, higher expression of MMP-9 has been found in high grade tumors (Barros SS, 2011). Possible mechanisms by which MMPs contribute to tumor growth include promotion of angiogenesis, activation of stimulating growth factors or their receptors and inactivation of inhibiting growth factors. It is interesting to note that, although clear evidence exists that MMPs potentiate angiogenesis, they also have the potential to inhibit this process. For example, MMP-9 can degrade plasminogen generating the angiogenesis inhibitor angiostatin (Stetler-Stevenson, 1999).

Although early studies have shown TIMPs to have anti-tumor or antimetastatic effects, more recent reports indicate a dual function with positive correlation between increased TIMP levels and poor outcome in some human malignancies (Mushashige M, 1996; Ree AH, 1997).
Increased TIMP expression has been associated with decreased tumor growth, invasiveness and metastasis in cancer cell lines of the breast (Alonso DF, 1998). We observed a similar trend in our study with invasive tumors as well as node positive tumors showing a downregulation of TIMP expression that support the anti-tumor effects of the protein. However in tumors with distant metastasis, the results are contrary with an upregulation which is more in line with recent evidence documenting a multifunctional complex role for TIMPs. The mechanisms supporting the paradoxical positive effect of TIMP in tumor progression are not completely understood and are the subject of intense investigation. This tumor promoting activity may be attributable to either proteolytic degradation of extracellular matrix or direct influence on cell survival and growth. Some TIMPs can directly affect cell growth/survival independent of their action on MMPs. Stimulation of cell growth by TIMPs is thought to be mediated by cAMP dependent activation of protein kinase A (Corcoran ML, 1995) and tyrosine phosphorylation (Yamashita K, 1996). Several factors regulate the activity of TIMP towards tumor suppression or tumor promotion such as local TIMP concentration, cellular distribution, association with pro-MMPs and presence of a putative “TIMP receptor” (Hayakawa T, 1994).

Numerous investigators used various techniques and reported the prognostic significance of increased MMP expression in several human malignancies. A significant correlation between increased MMP and poor prognosis, including shortened patient survival has been documented in carcinomas of the esophagus (Murray GI, 1998), stomach (Sier CF, 1996), breast (Talven saari-Mattila A, 1998), prostate (Stearns ME, 1996) and lung (Kawano N, 1997). Similarly numerous reports are available pointing to the prognostic impact of TIMP in various cancers (Kallakury BV, 2001).

Under physiological conditions the expression of MMPs and TIMPs is highly coordinated at the level of gene expression and this balanced expression guarantees normal tissue structure and organ function and prevents excessive ECM degradation. Some factors in malignant tumors contributes to the overexpression of MMPs without matched TIMP expression thus breaking the balance and thereby leading to excessive ECM degradation and cancer metastasis. Hence we hypothesized that it is probably the overall balance between the concentrations of each form of proteinase and inhibitor that will determine whether matrix
degradation occurs at each stage of tumour invasion and metastasis in vivo. For the same reason we sought to analyze the influence of the co-expression pattern of MMP-9 and TIMP-1 on the prognosis of breast cancers in addition to analyzing the prognostic value of MMP-9 and TIMP-1 separately. This concept has earlier been well established for proteases that have specific inhibitors.

The results of the current study show that the cellular expression of MMP or its inhibitor does not influence significantly the overall survival or recurrence free survival of breast cancer patients. However an imbalance in the co-expression of MMP-9 and TIMP-1 has a significant bearing on the overall survival of breast cancer patients. This finding together with the significant imbalance in the expression of MMP and TIMP noted in invasive breast cancers suggest that the expressive imbalance between MMP-9 and TIMP-1 is an important factor in tumor invasion and prognosis. To our knowledge, this is the first report that although MMP-9 or TIMP-1 may not serve as an indicator for patient prognosis, there is a significant association of the expressive imbalance between MMP-9 and TIMP-1 with the survival of breast cancer patients. However, such associations have been noted in gastric cancer (Zhang S, 2003) and hepatocellular carcinoma (Gianelli G, 2002). Moreover, our data stress the strong clinical relevance of assessing the prognostic value of several proteolytic enzymes jointly rather than separately.

6.3. b. Urokinase plasminogen activator (uPA)

Activation of proteolysis by plasminogen activator system has been reported in several human malignancies and is believed to contribute to tumor cell mobility and invasion. Relatively few studies have been carried out on uPA levels in human tumors, especially using immunohistochemistry. The present study examined the relationship between the expression of uPA and the clinicopathological parameters of the disease in order to elucidate the role of uPA in the invasive and metastatic processes of breast cancer. The results, however, demonstrated that positive uPA expression is not significantly associated with invasion and/or distant metastasis.

Duffy (2000) hasbeautifully reviewed the changing roles of uPA and MMP illustrating their change of role from metastasis to cell proliferation and/or angiogenesis. This
could most likely be the reason for contradictory reports linking uPA and invasion/survival of cancer patients. Newer and newer roles are being proposed for uPA. A publication indicates that uPA is involved in the modulation of cell proliferation/apoptosis ratio through the dynamic control of cell-matrix interactions (Hildenbrand R, 2008). The role of uPA in cell proliferation is evident from our results which show a significant positive correlation between uPA and mitotic index. We think that these effects could probably be mediated through c-myc, in the light of a new finding (Alfano D, 2010). They found that c-myc, besides its well accepted role as a transcriptional activator could also cause repression. Their study points out that uPA is under the repressional control of c-myc and indeed this repression of uPA leads to the pro-apoptotic and anti-migratory activity of c-myc. Hence we speculate that loss of repressional control of c-myc could enhance expression of uPA and could lead to an anti-apoptotic or pro-mitotic effect.

uPA is the first proteinase shown to have a prognostic significance in human malignancy. Berger DH (2002) has proposed that use of uPA as a serum or tumor marker could help in the accurate determination of prognosis in colon cancer. A study by Leissner P (2006) demonstrated that uPA expression does not have any prognostic value in node positive ER-positive breast cancer patients. Our investigations also indicate that uPA does not correlate significantly with established prognostic markers in breast cancer such as stage, grade, node positivity, NPI etc. Similar findings have earlier been reported by Janicke et al (1990, 1993). In the current study, uPA expression does not correlate significantly with the outcome i.e. either overall survival or recurrence free survival. But as earlier reports indicate, the present study projects uPA as a strong prognostic marker for axillary node negative breast cancer patients (Duffy, 1994; Foekens, 1992).

6.4. a. Vascular Endothelial Growth Factor (VEGF)

Angiogenesis, the complex process involved in metastasis, is known to be regulated by a number of growth factors such as bFGF, VEGF, PDGF and TGF-α. Among others, VEGF is a highly specific mitogen for vascular endothelial cells and may directly stimulate the growth of new blood vessels. VEGF expression has been documented in human cancers of
various organs including brain, breast, lung, stomach, colon and prostate (Maeda K, 1997; Abdulrauf SI, 1998).

In the present study, VEGF expression was significantly elevated in patients who later developed distant metastasis. This result agrees with most of the earlier reports (Seo et al, 2000; Takahashi Y, 1995). Elevation of VEGF expression indicates an increase in tumor vessels which enhances the chance for the entry of tumor cells into the circulation and newly formed vessels have leaky and weak basement membranes through which tumor cells can penetrate more easily than those of mature vessels, thus leading to hematogenous metastasis.

In cervical cancer, a new finding has elucidated the mechanism for VEGF promoting metastasis, by upregulation and activation of moesin protein through RhoA/ROCK-2 pathway. (He M, 2010). At the same time, we could not find any significant correlation between VEGF expression and nodal invasion. It is known that factors influencing lymphatic and hematogenous metastasis differ. VEGF mainly influences angiogenesis, which is closely related with metastatic potential. As a result, VEGF expression may not be directly related to lymphatic invasion.

Earlier reports point out the ability of VEGF to predict tumor recurrence and metastasis. But in the current study, by regression analysis, the correlation of VEGF expression with recurrence/metastasis did not reach statistical significance. These results also highlight the hypothesis that if a primary tumor has a high angiogenic index and yet does not express other factors necessary for metastasis like adhesion molecules, motility factors, growth factor receptors etc. the likelihood of metastasis occurring will be low (Cheng SY, 1996). Also, as described by Park JE (1994), tumors that do not produce significant amounts of VEGF may use VEGF related molecules such as placental growth factor, VEGF-B, VEGF-C, VEGF-D etc. to stimulate angiogenesis. Although they are less potent than VEGF, VEGF related molecules have the capacity to directly activate VEGF receptors and potentiate the activity of VEGF when this factor is produced at low levels.

The prognostic independent influence of neovascularisation has been depicted in several types of human solid cancers (Weidner, 1996). Jain L (2009) has proposed that looking at polymorphisms of VEGF could be useful in determining the prognosis of cancer.
patients. The prognostic significance of VEGF has also been well documented with patients having high VEGF expression showing a worse prognosis compared with those having low VEGF expression (Ghosh S, 2008; Shen GH, 2000; Mineta H, 2000). A recent report shows that VEGF is an independent prognostic factor in colon cancer (Liang J, 2010). Therefore, we sought to determine whether VEGF expression could be used to predict patient survival. Our results did not reveal any significance for VEGF as a prognostic marker in breast cancer patients. Hence, it can be speculated that it is unlikely that any one factor is the causal angiogenic agent for any tumor type. It is more likely that each tumor has a specific angiogenic index, with a unique profile of endogenous angiogenic and anti-angiogenic agents. Thus we hypothesise that VEGF may not be the dominant angiogenic factor in breast cancer as it appears to be in pancreatic cancer (Ellis LM, 1998) as well as colon cancer (Takahashi Y, 1995).

6.5. Endothelial adhesion molecules

Expression of ICAM-1 has previously been examined by immunohistochemistry in a variety of human neoplasms including cancer of the breast, lung, pancreas, stomach, ovary, prostate etc. (Johnson et al, 1989; Vogetseder et al, 1989; Natali et al, 1990; Koyama et al, 1992; Ogawa et al, 1998). Expression of ICAM-1 and its counter receptors lymphocyte function associated antigen-1 (LFA-1) and complement receptor type-3 (CR-3) is known to be essential for most lymphocyte-lymphocyte, lymphocyte-phagocyte and leukocyte-endothelial cell interactions in the immune response. Although the mechanism of ICAM-1 expression on tumor cells remains unclear, ICAM-1 on tumor cells was found to be inducible or augmentable by cytokines (Rothlein et al, 1988; Mortarini et al, 1990). Thus it is conceivable that cytokines produced by infiltrating mononuclear cells might influence the expression of ICAM-1 on tumor cells.

In the present study, a reduced expression of ICAM-1 was seen in breast cancer samples compared to the benign lesions. Such a relationship was observed in ovarian adenocarcinoma by Arnold JM et al, 2001. On the similar grounds, an elevation of circulating ICAM-1 was noted in head and neck cancer by Liu CM et al, 1999 which has been evaluated as a marker for endothelial damage after activation by cytokines (Gearing and Newman, 1993). These two facts together point to shedding of adhesion molecules by activated
endothelial cells and tumor cells which might not only block their counterligands on immunocompetent cells, but also allow the tumor cells to escape from surveillance by toxic T-cells and natural killer cells, thereby promoting metastasis. These shedding fusion molecules might also prevent tumor cells from adhering to endothelial cells during invasion. We have earlier reported that the shedding of endothelial adhesion molecules helps the tumor cells to escape from host anti-tumor defense mechanism (Madhavan M, 2002).

Some studies have found a positive correlation between ICAM-1 expression and metastatic disease in malignant melanoma and gastric cancer (Koyama et al., 1992). In contrast, other investigators have reported a negative correlation between ICAM-1 expression and tumor growth and metastasis in colorectal cancer and breast cancer and found that patients with ICAM-1 positive tumors had a better prognosis than those whose tumors did not (Ogawa et al., 1998). JM Arnold et al. (2001) have found that patients whose tumors expressed ICAM-1 had improved survival in ovarian adenocarcinoma. In our study, patients with ICAM-1 positive breast cancers had an increased overall survival but decreased recurrence free survival even though the results were non-significant.

In the present study, we could not find any significant alteration in the expression levels of VCAM-1 between different study groups. But the mean values of VCAM-1 were increased in node positive tumors and breast cancers which later developed recurrence or metastasis. This finding supports the theory that VCAM-1 gene acts mainly as virulence gene that may allow tumors to aggressively invade, colonize and grow in the distant organs without markedly contributing to primary tumor growth (Minn A J, 2005).

The level of soluble E-selectin is higher in breast, ovarian and gastrointestinal tumors (Banks et al., 1993) and the increase has been attributed to the shedding of endotheliallly bound E-selectin in the tumor tissues. This could be the reason for the decrease of tissue bound E-selectin in breast cancers found in the present study in comparison with the benign tumors. The decrease in expression of the protein is more significantly pronounced in the node positive breast tumors in comparison with the node negative tumors whereas no such significant difference is noted in those tumors with distant metastasis. This implies the
Sedling of selectin molecules may be involved in an early stage of tumor progression, concerned mainly with lymphatic rather than hematogenous metastasis.

There are a number of papers that point to the prognostic significance of E-selectin (Gunev N, 2008). We noticed in the present study that, as with other members of the immunoglobulin superfamily of adhesion molecules, the overall survival was increased in patients with tumors expressing E-selectin while it was reduced in node positive breast cancer patients. But the recurrence free survival was reduced in patients with E-selectin positive breast tumors and the same trend was seen among node negative and node positive patients. Regression analysis proved that E-selectin has a significant association with node positivity in breast cancer patients and that E-selectin positivity could successfully be used for predicting the chances of nodal metastasis in a breast cancer patient. Thus the study substantiates that E-selectin could be used as a prognostic marker of recurrence free survival in node positive breast cancer patients. The node positive breast cancer patients are considered clinically the worst prognostic group and prediction of overall survival and recurrence free survival in this group is very vital.

6.6. Alterations in Cadherin promoter methylation and beta catenin exon 3 mutations

None of the metastatic proteins studied showed a significant alteration in their mean value due to E-cadherin promoter methylation. The mean value comparison indicated that E-selectin expression was under regulated in breast cancers showing beta catenin mutation. Thus, probably, beta catenin is involved in the signaling of E-selectin. There was a trend towards cytoplasmic localization of beta catenin in patients in which E-cadherin was methylated. This indicates that alterations in the methylation status of E-cadherin promoter could be involved in the regulation of beta catenin. No such correlations have been pointed out in the current scientific literature. It was also observed that neither E-cadherin methylation nor beta catenin exon 3 mutation had any prognostic significance in predicting the survival of breast cancer patients.

To conclude, E-cadherin promoter methylation or mutations in beta catenin exon 3 do not play a significant role in node metastasis or distant metastasis in breast cancer. Our results are probably due to the small sample size analysed and hence we cannot arrive at a
definitive conclusion from the study. A lot of investigations have pointed to the prognostic significance of E-cadherin aberrant methylation (Yi T Z, 2011) and beta catenin mutation in many cancers, including breast cancer. A study by Feng W (2010) indicates that E-cadherin was hypermethylated significantly in the primary tumor and the lymph node metastasis. Similarly studies are available which could not establish any prognostic significance for E-cadherin methylation or beta catenin mutation. More studies involving larger sample size are needed to prove the significance of these alterations in breast cancer.

6.7. Newly identified prognostic Markers

One of the primary aims of this study was to discern new prognostic markers in breast cancer which would be of assistance to the clinicians in deciding the best therapeutic strategy of the patients. This acquires particular significance in solving the dilemma in differentiating between the node negative and node positive breast cancer patients. Hence using Kaplan-Meier analysis, we attempted to identify whether any of the metastatic proteins included in the study have prognostic significance using univariate analysis. Those proteins which were found to have significance in the univariate analysis were included in the multivariate analysis by Cox regression analysis to prove beyond doubt that the prognostic impact of the protein is independent of the other parameters and hence the particular protein could be suggested as an independent prognostic marker in breast cancer. Since the biology of node negative and node positive breast cancers is drastically different, we pursued to identify the discrete set of prognostic markers in node negative and node positive breast cancers separately.