CHAPTER ONE
LITERATURE REVIEW

1.1 Breast Cancer Epidemiology

1.1.1 The Global burden of breast cancer

For a large number of women newly diagnosed in the world, it has been ascertain
that, breast cancer is a neglected disease in terms of other numerically more frequent
health problems. It has also been described as an orphan disease, in the sense that the
very detailed knowledge about tumor characteristics and the necessary host biology
capable of providing basic care is absent. Current international cancer policy and planning
initiatives are irrelevant to breast cancer, with the exception of nutritional
recommendation. However, progress with declines in mortality in some developed
countries has been reported (Ginsburg et al., 2011).

Breast cancer is the most prevalent cancer in the world (4.4 million survivors up to
5 years following diagnosis) and the second most common cause of cancer related
mortality in women wide world (Parkin et al., 2005). It also accounts for 23% (1.38 million)
of the total new cancer cases and 14% (458,400) of the total cancer deaths in 2008 and
ranks second most common cancer overall (10.9% of all cancers) but ranks fifth as cause
of death (Ferlay et al., 2010). 1.15 million new breast cancer cases were recorded in
2004 and over 500,000 deaths reported around the world and more than half of all cases
occurred in industrialized countries (Parkin and Fernandez, 2006). Breast cancer
incidence rates vary from 19.3 per 100,000 women in Eastern Africa to 89.7 per 100,000
women in Western Europe. They are normally high in developed regions of the world (except Japan) and low in most of the developing regions. Due to more favorable survival
of breast cancer in developed regions, the range of mortality rates is very much less,
approximately 6-19 per 100,000. Notwithstanding, it is still the most frequent cause of
cancer death in women in both developing (269 000 deaths, 12.7% of total) and
developed regions, where the estimated 189 000 deaths is almost equal to the estimated
number of deaths from lung cancer (188 000 deaths) (Ferlay et al., 2010).

For some time now, there have been some encouraging in both breast cancer
incidence and mortality trends with the incidence of new cases stabilizing as well as death
rates falling in some high income or developed countries. However, this appears to be
vice versa in developing countries (Kanavos, 2006). Notably, breast cancer incidence
rates have leveled off since 1990, with a decrease of 3.5%/year from 2001 to 2004 (Li et
al., 2003). In the same manner, breast cancer mortality rates have also declined by 24%,
with the greatest impact among young women and as well as women with estrogen receptor (ER)-positive disease (Berry et al., 2005). Also, both incidence and mortality declined in the United States; between 1999 and 2006, incidence rates decreased by 2.0% per year, and mortality decreased by 1.9% annually between 1998 and 2006 (Horner et al., 2006). The decline in breast cancer mortality has been largely attributed to the combination of early detection with screening programs and the advent of more efficacious adjuvant systemic therapy.

Breast cancer is common in women both in the developed and the developing countries, comprising 16% of all female cancers. Although it is thought to be a common cancer in the developed countries, a majority (69%) of all breast cancer deaths occurs in developing world. Indeed, increase life expectancy, increase urbanization and adoption of western lifestyles have increased the incidence of breast cancer in the developing countries (Kanavos, 2006). Eventhough it is now the most common cancer both in developed and developing regions with around 690 000 new cases estimated in each region, much of the burden of incidence, morbidity, and mortality will occur in the developing world with population ratio of 1:4 (Ferlay et al., 2010). As developing countries succeed in achieving lifestyles similar to those in advanced economies, they will also encounter much higher cancer rates, particularly cancers of the breast. This forms part of a larger epidemiological transition in which the burden of chronic, non-communicable disease once limited to industrialized nations, is now increasing in less developed countries (Kanavos, 2006).

A report by Stewart et al (Stewart and Kleigues, 2003), mentioned that many of the new cancer cases are now occurring among women from low and middle income countries, where the incidence is increasing by as much as 5% per each year and there are about three fourths of breast cancer deaths occurring worldwide. Of the 411,000 breast cancer deaths around the world in 2002, 221,000 (54%) occurred in low- and middle-income countries (LMCs). The incidence of breast cancer rose from 126,227 cases in 2002 in China (IARC: Cancer Epidemiology Database, GLOBOCAN. 2002) to over 169,000 in 2008 (IARC: Cancer Epidemiology Database, GLOBOCAN. 2008).
1.1.2 Differences in Population of Breast Cancer

Breast cancer variation among population, or the regional differences in the types have been attributed to the following: prevalence of major risk factors, availability and use of medical practices such as cancer screening, availability and quality of treatment, completeness of reporting, and age structure. However, geographic areas, and counties and parishes within countries also determine the frequency of the most commonly diagnosed cases or deaths (Garcia M et al., 2007). The highly penetrant but rare susceptibility genes, BRCA1 and BRCA2 (Fackenthal et al., 2007) and more prevalent, but lower penetrance genes, CHEK2 and FGFR (Easton et al., 2007) have been indicated to be the key inter-individual and inter-group differences in the distribution of reproductive risk factors. Countries with massive economic development over the past 50 years, such Japan, Singapore, and urban areas of China have experience an increase in breast cancer incidence (Horn-Ross et al., 2000).

Age-standardized incidence rates for breast cancer 1998–2002 were 110 (non-Hispanic Caucasians, California), 82.3 (Ontario, Canada), 41.3 (Hong Kong) and 14.7 (Jiashan, China) (Curado et al., 2007). Reports on migration studies reveal that the incidence of breast cancer changes significantly over one to two generations to more closely reflect the breast cancer risk in the adopted country (Ziegler et al., 1993), which seems to occur in parallel with dynamics in diet and certain indicators of acculturation.
(Porter, 2008). Notably, evaluation of differences in risk factors and natural history of all tumor types, would permit for comparisons based on geographical regions, socioeconomic status and levels of industrialization (Ginsburg et al, 2011).

Other differences in population of breast cancer are outlined below:

In a study by Li et al (Li et al., 2002), it was shown that the majority of breast tumours from Asian women are estrogen receptor (ER) negative. Also it has been indicated that both pre-and postmenopausal Asian women with breast cancer, are likely to have ER positive tumors as Caucasians (Uy et al., 2007). In addition, greater proportion of ER+ tumors in a Vietnamese cohort, has been found in studies on ER positivity among premenopausal breast cancer cases as compared with the comparison group of Caucasian women in Australia. (Tran and Lawson, 2004).

Considerably, variation in the gene profiles of tumors from populations of different genetic/ethnic backgrounds have also been reported. About 15% of sporadic breast cancer, which are BRCA1 origin in Caucasian women appears to have the basal phenotype. On the other hand, other studies have also suggested that breast cancer in women of African ancestry may have a higher proportion of basal phenotype (Carey et al., 2006). In similar manner among Nigerians, a high frequency of basallike tumors was observed, where 87 of 148 (59%) breast cancer cases were both ER- and HER2- (Olopade et al., 2004).
**Figure 1.2:** Incidence of female breast cancer by age in selected population, 1988-1993. (Parkin et al., 1997; Tavassoli and De Villee, 2003)

**Figure 1.3:** Female breast cancer mortality trend (Tavassoli and De Villee, 2003)
1.2 General characteristics of Breast Cancer

1.2.1 The normal Breast

There are about 15 to 20 sections called lobes in a female’s breast and each lobe is made of many smaller sections known as lobules, which in turn have groups of tiny glands or milk-producing glands that can make milk. It is also made up of ducts, tiny tubes that carry the milk from the lobules to the nipple, and stroma fatty tissue and connective tissue surrounding the ducts and lobules, blood vessels, and lymphatic vessels (American Cancer Society booklet).

1.2.2 The lymph (lymphatic) system of the breast

The lymph system is very important in breast cancer research in that, it is one way breast cancers can spread and has several parts. Lymph nodes are small, bean-shaped collections of immune system cells that are connected by lymphatic vessels. These vessels are like small veins, except that they carry a clear fluid called lymph in place of blood away from the breast. They also contain Lymph tissue fluid and waste products, in
addition to immune system cells. Breast cancer cells can enter lymphatic vessels and begin to grow in lymph nodes. Most lymphatic vessels in the breast connect to lymph nodes under the arm (axillary nodes). Some lymphatic vessels that connect to lymph nodes inside the chest are called internal lymph nodes, and those either above or below the collarbone are called supraclavicular or infraclavicular nodes (American Cancer Society booklet).

There are several types of breast cancer, but some of them are quite rare. Currently, majority of all breast cancers worldwide are the ductal and lobular subtypes. However, the ductal subtype accounting accounts for the majority of the diagnosed cases, constituting for about 40–75% (Rakha et al., 2006). In addition, several linear models of breast cancer initiation, transformation and progression, as depicted in Fig. 1.5 have been formulated. There are two models for the ductal subtype. The first ‘ductal’ model, reported by Lerwill (Lerwill, 2008) are as follow: First of all, it recognizes flat epithelial atypia (FEA), to atypical ductal hyperplasia (ADH) and then ductal carcinoma in situ (DCIS) as the non-obligate precursors of the advanced invasive and metastatic ductal carcinoma. In the second model, usual epithelial ductal hyperplasia (UDH) was proposed as an intermediate stage of progression between FEA and DCIS (Page et al., 1985). In the case of lobular subtype, atypical lobular hyperplasia (ALH) and lobular carcinoma in situ (LCIS) was also proposed as the non-obligate precursor lesions to invasive lobular carcinoma. (Boecker et al., 2002).
Figure 1.5: Models of breast cancer progression. (A) The classic model of breast cancer progression of the ductal type proposes that neoplastic evolution initiates in normal epithelium (normal), progresses to flat epithelial atypia (FEA), advances to atypical ductal hyperplasia (ADH), evolves to ductal carcinoma in situ (DCIS) and culminates as invasive ductal carcinoma (IDC). Immunohistochemical, genomic and transcriptomic data strongly support the evidence of a continuum from FEA to ADH, DCIS and IDC, indicating FEA as the potential non-obligate precursor of ADH. In contrast, the alternative model of breast ductal cancer progression (B), which was mostly based on epidemiological and morphological observations, proposes usual ductal hyperplasia (UDH) instead of FEA as the direct precursor to ADH. However, recent studies have shown that UDH has a distinct immunohistochemical and molecular profile from FEA and probably represents a biological dead end. (C) The model of lobular neoplasia proposes a multi-step progression from normal epithelium to atypical lobular hyperplasia, lobular carcinoma in situ (LCIS) and invasive lobular carcinoma (ILC). (Bombonati and Sgroi, 2011)

1.2.3 Risk factors for Breast Cancer

The aetiology of breast cancer is multifactorial and from descriptive epidemiological data it has clearly emerged that breast cancer is a disease of affluent societies which have acquired the Western lifestyle, characterized by a high-caloric diet rich in animal fat and proteins, combined with a lack of physical exercise. Regions which have featured this lifestyle for a long period of time (North America, Northern Europe, Australia) have reached a plateau of an incidence rate of 70 to 90 new cases per 100,000 population/year while countries that have more recently become industrialized and affluent show a marked increase in incidence and mortality. (ACS booklet) Various risk factors identified include age, hereditary, dietary (diet and obesity), gynecological (oral contraceptives, hormone replacing therapies, endogenous hormone levels, age of menarche and menopause, parity and mammographic density), life style (physical
activity, smoking and alcohol), oxygen reactive species, radiation and environmental pollutants. (Fig. 1.6).

With age, breast cancer incidence is known to drastically increase up to the age of 50, after which it increases slowly (Mitruen and Hirvonen, 2003). Hereditary factors are observed in about one fourth of the total cases of breast cancer, which involves two classes of genes; high and low penetrance genes. High penetrance genes with allelic variants that are relatively rare, such as BRCA1/2, tumor protein 53 gene (TP53) and ataxia telangiecttasisi mutated gene (ATM). Low penetrance genes such as the genes encoding for the enzymes involved in estrogen and carcinogen metabolism as well as in the detoxification of reactive oxygen species, for instance P450 cytochrome and Gluthioone-S-transferases (GSTs) on the other hand are more common and allelic variants confer low risk of breast cancer (Mitruen and Hirvonen, 2003).

In the case of dietary, the human diet contains variety of natural carcinogens and anticarcinogens. Uptake of fruits and vegetables which are rich in antioxidants reduces the risk (McKeown, 1999) whereas increase in polysaturated fatty acids (Bartsch et al., 1999) and meat consumption (Zheng et al., 1998) increases the risk of breast cancer. Obesity has been reported to be associated with an increase in estrogen levels and as well as a risk in postmenopausal women, who have most of their estrogen derived from the conversion of androgens, in the adipose tissue, as a result of aromatse enzyme activity (Hunter et al., 1993). However, in premenopausal women, it has been indicated that, obesity can have a protective effect, due to the higher period of frequent ovulation which reduces estrogen levels (Mannisto et al., 1996). Physical activity is a lifestyle factor which is considered as a breast cancer risk factor. It is considered protective against breast cancer because it reduces the regular ovulatory cycles and increases the level of catechol-O-methylated estrogens (Henderson et al., 1985).

Cigarette smoke is very rich in carcinogens and reactive oxygen species and may be considered as one with high risk in breast cancer (Mitruen et al., 2003). Its function is controversial, in that it can serve as a protective against cancer. It may contain anti-estrogenic effect, such as nicotine which inhibit aromatase. Further more, women who smoke tend to reach menopause earlier than nonsmokers (baron et al., 1990). Another fact to consider is alcohol. 15% of alcoholic women have the risk of developing breast cancer (Kuper et al., 2000). It has been stipulated alcoholic women have higher levels of estrogen than non-alcoholic (Reichman et al., 1993). The use of oral contraceptive increases breast cancer risk but disappears after ten years of cessation, whereas hormone replacement therapy disappears in five years. However, breast cancer cases in hormone replacement therapy (HRT) tend to be less advanced at
the time of diagnosis, and biologically less aggressive compared to women who never used such therapy (Holli et al., 1997).

Relationship between endogenous estrogen levels and breast cancer has been indicated. High estrogen levels in the serum or urine, and low levels of sex hormone binding protein (SHBG), resulting in high bioavailability of free estradiol also point for an important role for endogenous and exogenous estrogens in the risk of breast cancer (Kristensen et al., 2000).

Another strong marker of breast cancer risk is the degree of mammographic density. It has been indicated that, the risk in women with more dense breast is four to six times higher than those with less dense breast (Boyd et al., 1995). Evidence suggest that, the etiology of mammographic density may be due to the exposure to steroid hormone, since it decreases with age (Boyd et al., 2002) as well as in women on tamoxifen (Cuzick et al., 2004) and also increases in women who are on hormone replacement therapy (Rutter et al., 2001).

Environmental pollutants similar to hormones can also interfere in the control of a large family of nuclear hormone receptors, which in turn can upregulate various genes involved in the cell cycle, such as TP53, Retinoblastoma (RB) and the serine.threonine-protein kinase proto-oncogene RAF by transcriptional activation induced by ligand (Kristensen et al., 2000). These pollutants are designated xeno-estrogens, which include pesticides, dyes, food preservatives and other pollutants and can play a role in the etiology of breast cancer, since they interfere with the activity of endogenous estrogens (Garner et al., 2000). Ionizing radiation (John and Kelsey, 1993) and history of benign breast cancer (Mitruen et al., 2003) have also been established to increase breast cancer risk.
1.2.4 Breast Cancer treatment and survival

There are various treatment plans for cancer patients depending on the type and the stage at diagnosis. Surgical treatment for breast cancer has to do with breast-conserving surgery (BCS) or mastectomy and when this is done properly for localized or regional cancers, long-term survival is the same as with mastectomy (Jatoi et al., 2005). With early stage (I or II) breast cancer, 57% of women undergo BCS, 36% have mastectomy, 6% undergo no surgical treatment, and about 1% do not receive any treatment (Fig. 1.7). On the other hand, with women of late stage (III or IV) breast cancer, 13% receive BCS, 60% undergo mastectomy, 18% do not have surgery, and 7% do not receive any treatment. Most of the early stage breast cancer women who undergo BCS receive adjuvant treatment; nearly one-half undergo radiation therapy alone and one-third receive both radiation therapy and chemotherapy. Contrary, most of them diagnosed with late stage disease undergo chemotherapy in addition to surgery and other therapies. (Siegel et al., 2012).
Patients can expect to be cured or to experience at least long-term survival of more than 10 years. There has been an improvement for the overall 5-year relative survival rate for female breast cancer patients, from 75.1% between 1975 to 1977 to 90.0% for 2001 through 2007. And this is attributed to the fact that there has been an improvement in chemotherapy and hormone therapy treatment and also due to earlier diagnosis resulting from the widespread use of mammography (Siegel et al., 2012). In the case of localized breast cancer, the 5-year relative survival rate is 98.6%; which declined to 83.8% for regional stage and 23.3% for distant stage. Other factors that influence survival include tumor grade, hormone receptor status, and human epidermal growth factor receptor 2 (HER2) status.

There are differences between African American women and white women on the basis of stage and survival. African American women are less likely than white women to be diagnosed with local stage breast cancer (51% vs 61%) and have lower survival rates than white women within each stage of disease. Though difficult to explain this reasons, it may be explained in large part by a combination of socioeconomic factors, less access to care among African American women, and biological differences in cancers (Siegel et al., 2012).
1.2.5 Biomarkers and chemotherapy in Node-negative breast cancer

The most prevalent form of breast cancer worldwide is node-negative breast cancer (Fig. 1.9) and in regions or countries with widespread breast cancer screening and disease awareness among women, it is likely to be rated between 65%–70% of breast cancer patients (Harbeck and Thomssen, 2011). Most patients have no or only a few (1-3) axillary lymph nodes involved and therefore have a good chance of being cured. There is no substantial difference in the underlying tumor biology between node-negative and node-positive disease, and the question that remains in adjuvant chemotherapy today is in proper patient selection. Node-negative breast cancer does not automatically suggest a good prognosis, or the lack of a need for chemotherapy.

Data from Adjuvant Online show that the mortality risk may even be higher in patients with node-negative grade 3 tumors than the risk demonstrated in some patients with node-positive disease, suggesting a high risk among these patients to indicate adjuvant chemotherapy. But then, there is still a good degree of uncertainty in determining whether patients with node-negative disease actually benefit from chemotherapy, which lead many clinicians to hesitate before indicating chemotherapy in many node-negative patients. (Harbeck and Thomssen, 2011). With node-positive disease, it is associated with an overall mortality rate of approximately 20%, and oncologists do not hesitate to prescribe chemotherapy for these patients.
Adjuvant systemic therapy in women with early stage disease is guided by prognostic and predictive factors, including stage, grade, estrogen receptor (ER) and progesterone receptor (PR) status, Ki-67 status and HER2 amplification. These parameters help physicians to select adjuvant systemic therapy. However, these remain imperfect tools, in that some patients receive systemic chemotherapy even though they can be cured by surgery alone. If these parameters are used alone in selecting treatment as recommended by 1998 and 2001 St Gallen consensus statement, up to 90% of node-negative breast cancer patients will be candidate for adjuvant chemotherapy, although only about 30% of them will relapse and thus need adjuvant chemotherapy. In terms of tumor grade, it is certainly important in that it is predictive of risk over time, but it lacks standardization. In case of undifferentiated cancers (grade 3), patients are truly at high-risk and may benefit from chemotherapy, whereas in case of well-differentiated grade 1 cancers, the risk of recurrence may be rather low. However, in the heterogeneous group of grade 2 tumors, it is essential to know for which patients the benefits of chemotherapy will outweigh its potential side effects.

Therefore, new prognostic factors are still required to optimize treatments among these patients. However, most patients are offered chemotherapy according to current guidelines, leading to over-treatment of a large proportion, since there is no means of clearly identifying those patients who will not relapse and hence do not need adjuvant chemotherapy. (Goldhirsch A et al., 2005). One of the major clinical questions is how to identify those patients who may be able to avoid adjuvant chemotherapy because of their low risk of recurrence.

Figure 1.9: Approximate percentages of patients with node-negative disease at time of diagnosis in different parts of the world. (Harbeck and Thomssen, 2011).
1.2.6 Node status and relapse Rate

Another clinical question is how to identify individuals with high risk who may benefit from adjuvant chemotherapy. One important risk factor for disease relapse is nodal stays. Most patients with node-negative breast cancer have a fairly good ten-year overall survival with loco-regional treatment alone, (Fig 1.10) however, about 30% relapse developing distant metastasis. In fact, approximately 70% of node-negative patients respond sufficiently to surgery, radiotherapy, and endocrine therapy, and do not require additional chemotherapy. The problem faced by most clinicians now face is that approximately 30% of node-negative patients will need chemotherapy because of their risk for recurrence, but there are limited tools currently available to identify this subset of patients. Data from Adjuvant! Online have shown that even patients with grade 1, estrogen receptor– negative, node-negative tumors may have a high relapse rate of almost 20% over 10 years. Indicating that the relapse rate is even higher in patients with grade 2 and grade 3 tumors (Harbeck and Thomssen, 2011).

Therefore, markers to predict individual risk who may benefit from adjuvant chemotherapy and also to identify patients not requiring aggressive adjuvant therapy are urgently needed, so as to avoid unnecessary exposure of women to the potential toxicity and side-effects of such treatment, and also to reduce the overall cost of breast cancer management as well as preventing under-treatment of node-negative breast cancer are needed.

The main cause of morbidity and mortality in patients with cancer is the formation of distant metastases, which is a multistep event involving local invasion, degradation of extracellular matrix, angiogenesis, intravasation, evasion of apoptosis and survival in circulation, extravasation and growth at secondary site. Key mediators of this process include certain proteinases such as uPA, PAI-1, MMPs and ADAMs which has caused increase attention to be drawn on these factors as potential prognostic markers for risk assessment in node-negative breast cancer. However, unlike MMPs, uPA, and PAI-1, little work has been done on ADAMs role as a prognostic factor.
Figure 1.10: Lymph node status and relapse rate in primary breast cancer (Harbeck and Thomssen, 2011).
1.3 Expression and functions of ADAMs

The ADAMs (A Disintegrin And Metalloproteases) family, are modular type I transmembrane proteins which belongs to the zinc protease superfamily. Although they are frequently referred to as a family, they are made up of a subfamily of the M10 family which, in turn, belongs to the metzincin clan of metalloproteases (Duffy MJ et al., 2009; Arribas J et al., 2006). Forty gene members have been identified so far, but then it is believed that about 21 functional in humans. Members of the family have a modular design, characterized by the presence of metalloprotease and integrin receptor-binding activities, and a cytoplasmic domain that in many family members specifies binding sites for various signal transducing proteins (Seals and Courtneidge, 2003). Based on their primary structure analysis, it has been indicated that, in addition to the disintegrin and metalloprotease, all ADAMs contain the following domains: signal peptide, prodomain, cysteine-rich, epidermal growth factor (EGF)-like, transmembrane and cytoplasmic (Fig. 1.11).

ADAMs expression profile can vary considerably and are mostly not expressed in Escherichia coli, Saccharomyces cerevisiae, or plants but are rather found in vertebrates, Caenorhabditis elegans, Drosophila, and Xenopus. The fission yeast Schizosaccharomyces pombe has what may be an early progenitor of the ADAMs family, although its properties have not been studied (Seals and Courtneidge, 2003). Transcription of ADAM mRNA is modulated both positionally and temporally. Although some ADAMs are testis specific, others are found in variety of tissues and the transcription of ADAMs 1-6 in the testis is developmentally regulated. For example, in mammals many of them (including ADAMs 2, 7, 18, 20, 21, 29, and 30) are exclusively or predominantly expressed in the testis and/or associated structures. Other members (ADAMs 8, 9, 10, 11, 12, 15, 17, 19, 22, 23, 28, and 33) show a more broad somatic distribution. Originally, ADAMs 9, 12, and 19 were cloned from myoblasts, but have been shown subsequently to be more broadly expressed. (Seals and Courtneidge, 2003)

Many ADAMs have been reported to be expressed in multiple splice form. For instance, ADAM22, ADAM29 and ADAM30 with two to three forms that vary in the lengths of their cytoplasmic tails, but then no functional differences in their isoforms have been reported. On the other hand others have produced proteins with markedly different activity as in the case of ADAM12 with two splice forms: L, which produces a membrane-bound protein, and S, diverges just upstream of the transmembrane domain, which results in a shorter form that is secreted from the cell (Seals and Courtneidge, 2003). Studies by Shi et al (Shi et al., 2000) indicated that ADAM12-S has functional IGFBP-3 and IGFBP-5 protein cleavage activity and due to its overexpression during pregnancy, it is possible
that ADAM12-S is responsible for increasing the pool of IGF in the bloodstream during pregnancy through IGFBP proteolysis.

Another protein to consider is ADAM28. It produces isoforms with different subcellular localization patterns and tissue expression. There are three isoforms in Murine ADAM28, two larger ones for encoding membrane-anchored proteins and expressed in the epididymis and lung, and smaller one predicted to encode a secreted protein with testis-specific expression. However, in human there are only two forms: the secreted form which is preferentially expressed in the spleen, and the membrane-bound form with specificity to lymph node (Seals and Courtenidge, 2003). Other ADAMs with documented evidence of alternative splicing are ADAM9, ADAM10, ADAM11 and ADAM3.

The ADAMs have been implicated in processes such as the activation of the proforms of certain growth factors and cytokines as well as the shedding of the extracellular domains of growth factor receptors and adhesion proteins, control of membrane fusion, and cell migration, as well as physiological processes such as muscle development, fertilization, neurogenesis, adipogenesis, myogenesis and cell fate determination. Another function is the activation of NOTCH signalling by Notch ligand Delta shedding from the cell surface by ADAM-10 (Rocks et al., 2008). Accumulating evidence demonstrates ADAMs as proteins that support both proteolytic activity and cell adhesion, making them candidates to mediate both the remodelling of the extracellular matrix (ECM) and the changes in cell adhesion that characterize certain pathological processes such as tumor development, bacterial infection, cardiac hypertrophy, and asthma (Murphy, 2008). Of these different diseases, it is in cancer where most research has been carried out.

Key features of malignant tumours are their abilities to invade surrounding tissues, to have access to the vascular and lymphatic systems, and to disseminate to distant organs by metastatic spreading (Butler et al., 2006). Major ADAMs shown to play a role in cancer include ADAM8, -9, -10, -12, -15, -17, -19, -28 and ADAMTS1, -4 and -5. The related group of ADAMTS are secreted soluble proteins that contain a variable number of thrombospondin-like repeats (Fig. 1.11). Consistent with a causative role in cancer, several ADAMs are emerging as potential cancer biomarkers for aiding cancer diagnosis and predicting patient outcome. Furthermore, a number of selective ADAM inhibitors, especially against ADAM10 and ADAM17, have been shown to have anti-cancer effects. Collectively these results have led to the proposal of these metalloproteases as putative targets of anti-tumor therapy.
Figure 1.11: The topography of the ADAMs and related metalloproteases. Comparison of domain structures of ADAMs, SVMP-P-II, SVMP-P-III, SVMP-IV, ADAM-TS and MMPs. ADAM protein contains an N-terminal signal peptide (S.P.), a pro-peptide domain, a metalloprotease domain, a disintegrin-like domain, a cysteine-rich region, an EGF-like domain, a transmembrane domain (TM) and a cytoplasmic domain (Cyt. Tail). (Lu et al., 2007).

Table 1.1 Potential functions of human ADAMs

<table>
<thead>
<tr>
<th>ADAM</th>
<th>Potential functions and features</th>
<th>Localization</th>
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<tbody>
<tr>
<td>Proteolytically inactive</td>
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<tr>
<td>ADAM2</td>
<td>Sperm-egg binding and fusion</td>
<td>Sperm</td>
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<td>ADAM7</td>
<td>Sperm maturation</td>
<td>Testis</td>
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<td>ADAM11</td>
<td>Integrin ligand, neural adhesion, tumor suppressor</td>
<td>Brain</td>
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<tr>
<td>ADAM18</td>
<td>Oocyte recognition</td>
<td>Testis</td>
</tr>
<tr>
<td>ADAM22</td>
<td>Adhesion</td>
<td>Brain</td>
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<tr>
<td>ADAM23</td>
<td>Tumour suppressor, Cell adhesion, neural development</td>
<td>Brain, Heart</td>
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<tr>
<td>ADAM29</td>
<td>Unknown</td>
<td>Testis</td>
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<tr>
<td><strong>Proteolytically active</strong></td>
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<tr>
<td>ADAM8</td>
<td>Shedding of adhesion molecules, leukocyte receptors, neutrophil infiltration, osteoclast stimulation</td>
<td>Macrophage, neutrophil</td>
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<tr>
<td>ADAM9</td>
<td>Alpha-secretase activity, cellular adhesion, shedding of HB-EGF, TNF-p75 receptor, cleavage of APP, digestion of fibronectin and gelatin,</td>
<td>Various tissues</td>
</tr>
<tr>
<td>ADAM10</td>
<td>Alpha-secretase activity, shedding of TNF alpha, betacellulin, HER2, Notch, and collagen IV, cellular adhesion, digestion of gelatin and myelin, basic protein, cleavage of delta, APP, L1, and CD44, shedding of HB-EGF, presence of RRKR sequence.</td>
<td>Kidney, Brain, Chondrocyte</td>
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<tr>
<td>ADAM12</td>
<td>Cellular adhesion, shedding of HB-EGF, muscle formation, presence of RRKR sequence, digestion of IGFBP-3 and 5, digestion of collagen IV, gelatin and fibronectin</td>
<td>Osteoblast, muscle, chondrocyte, placenta</td>
</tr>
<tr>
<td>ADAM15</td>
<td>Cellular adhesion, expression in arteriosclerosis, digestion of collagen IV and gelatin</td>
<td>Smooth muscle cell, chondrocyte, endothelial cell, osteoclast</td>
</tr>
<tr>
<td>ADAM17</td>
<td>Release of several growth factor ligands, e.g., TNF-alpha, TGF beta, and specific EGFR/HER ligands, cellular adhesion, shedding of TRANCE and HB-EGF, presence of RRKR sequence, cleavage of APP, Notch, L-selectin and CD44</td>
<td>Macrophage, Various tissues</td>
</tr>
<tr>
<td>ADAM19</td>
<td>Formation of neuron, digestion of neuregulin, sheddase, dendritic cell development.</td>
<td>Testis</td>
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<tr>
<td>ADAM28</td>
<td>Shedding of IGFBP3, immune surveillance, Digestion of myelin basic protein.</td>
<td>Testis, lung, lymphocyte, pancreas, uterus</td>
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<tr>
<td>ADAM33</td>
<td>Mutation in bronchial asthma patients, cleavage of APP, KL-1 and insulin B chain, involved in pathogenesis of gastric cancer via IL-18 secretion</td>
<td>Lung (fibroblast, smooth muscle)</td>
</tr>
</tbody>
</table>

(Duffy et al., 2011)

**1.3.1 Subcellular location of ADAMs**

ADAMs have been proposed to probably synthesized in the rough endoplasmic reticulum and later mature in a Golgi compartment and this is done by the removal of the prodomain from the ADAM precursor protein. Studies have proved that, bulk of the protein resides in a region near the nucleus, where they colocalize with Golgi markers (Seals and Courtneidge, 2003). However, certain ADAMs such as ADAMs 9, 10, 15, 17, and 28 can also be detected on the cell surface. Hougaard et al (Hougaard et al., 2000) reported that there was a regulated transition of the alternatively spliced short form of ADAM12 (ADAM12-S) from intracellular compartments to the cell surface, whiles the large form was retained in the trans-Golgi network.

However, several ADAMs family members may be active intracellularly. For instance, it has been reported that, most of the mature form of ADAM15 is resistant to trypsinization treatment, indicating a predominantly intracellular pool. Also the metalloprotease activity of ADAMs 10, 17, and 19 can also occur within intracellular compartments. Nevertheless, the cell type, the ADAM, and the substrate involved may determine such differences in subcellular localization and activity.
1.3.2 Structure and Domain activity of ADAM Proteins

The generalised structure of an ADAM protein contains 8 distinct domains or regions. In the typical ADAM protein, these domains are a signal domain, a prodomain, a metalloproteinase domain, a disintegrin or integrin-binding domain, a cysteine rich region, an EGF (epidermal growth factor)-like domain, a transmembrane sequence and an intracellular C-terminal end (McGowan et al., 2007). Like most proteases, the ADAMs are initially synthesised as enzymatically-inactive precursor proteins.
1.3.2.1 The prodomain

At the N terminus of ADAMs resides a signal sequence that directs ADAMs into the secretory pathway and a prodomain that lead to ADAMs maturation. (Seals and Courtneidge, 2003). The metalloprotease site of ADAMs are kept inactive, through a cysteine switch by the prodomain. The conserved cysteine residue located within the prodomain preferentially coordinates the required active site zinc atom, and thereby sequesters the metalloprotease domain in an inactive conformation (Edwards et al., 2008; Becker et al., 1995). For protease activation, this prodomain is removed by a furin-like convertase or by autocatalysis, depending on the specific ADAM (Murphy, 2008). The mechanism employed is the cleavage of the prodomain from the rest of the protein by proprotein convertases (PCs) at a conserved Rx(R/K)R motif, thereby effectively releasing the prodomain and switching the zinc coordination to the metalloprotease domain, enabling it to undertake its catalytic activity. Several studies support this mechanism (Seals and Courtneidge, 2003).

Aside this mechanisms, there are cases in which ADAMs may undergo autocatalytic activation. Example is when, activity-blocking mutations in the metalloprotease domains of ADAM8 and ADAM28, produces only the precursor form of the protein in transfected cells (Schlomann et al., 2002). Another functional aspect of the prodomain is to chaperone the proper folding of the metalloprotease domain of ADAMs. It has been suggested by studies that the removal of the prodomain of ADAM17 generates a protease-inactive protein (Milla et al., 1999). Similarly, an ADAM10 construct lacking its prodomain is catalytically inactive in vivo. Hence, it can be concluded from this evidence that, the prodomain appears to be necessary in assisting in the proper folding of ADAMs, in the structuring of the catalytic active site, and in the proper transit of ADAMs throughout the secretory pathway (Seals and Courtneidge, 2003).

1.3.2.2 The metalloprotease domain

This domains are well conserved, but then, only 25 out of a known total of 40 members of the family (ADAMs 1, 8-10, 12, 13, 15, 16, 17, 19-21, 24-26, 28, 30, and 33-40), have the zinc binding catalytic site consensus sequence of HEXXHXXGXXHD (single letter amino acid code) where X is stands for any other amino acid; the three H residues bind zinc, the G allows a turn and E constitutes the catalytic residue, comprised of a water molecule tetrahedrally coordinated to the zinc, and the E residue acting as a catalytic base (Lu et al., 2007).
Metalloprotease domain is located next to prodomain and only about 60% of ADAMs exhibit nonprotease activity, although they all posses this domain. On the basis of their structural definition, of the 21 human ADAMs identified, only 13 are proteolytically active. ADAMs shown to exhibit protease activity include ADAM8, 9, 10, 12, 15, 17, 19, 28 and 33 (Kawaguchi et al., 2002). The mechanism of the proteolytic activity, which is the best defined function of ADAMs currently, has been more accurately evaluated through crystallization of the metalloprotease domain. At the active site is zinc and water atoms necessary for the hydrolytic processing of protein substrates, and which are coordinated by three conserved histidine residues and a downstream methionine. The methionine lies in a Met-turn motif that loops around to face the consensus HExxHxxGxxH site. Although individual proteins among the various metzincins exhibit certain distinguishable structural features that may impart specificity for substrates and protease inhibitors, there exist remarkable conservation within this catalytic site (Seals and Courtneidge, 2003).

1.3.2.3 The disintegrin domain

The disintegrin-like domain located at the downstream of the metalloprotease domain, consists of 60 to 90 amino acid long with 6 to 15 Cys residues with sequence similarity to that of the snake venom disintegrins (Marcinkiewicz C, 2005). This sequence is found in all ADAMs and they binds to integrins, which are a group of adhesion proteins implicated in cell adhesion, migration and cell signalling (Stupack, 2007). Snake venom disintegrins, which confers the ability of these molecules to interact with integrins in different cell systems have been characterized as potent inhibitors of the function of various integrins. The disulfide bridge pattern of RGD-containing disintegrins, which may be important to their biological activity, especially their potency and selectivity has been determined by means of chemical methods, NMR spectroscopy and crystallography. Not all ADAMs have the RGD sequence and in place of that, others contain sequences such as KGD, MVD, MLD, VGD, ECD, or MDG (single letter amino acid code). (Lu et al., 2007)

ADAMs distegrin derived its name for its presence in the snake venom metalloproteases (SVMPs), involved in binding of platelet integrin receptors. Consequently, the binding to this receptors, prevents the association of platelets with their natural ligands such as fibrinogen, and results in a block in platelet aggregation at the wound site. This disintegrin-mediated interaction of SVMPs along with the breakdown of basement membrane components by their metalloprotease activity leads to the severe hemorrhaging caused by bites from snakes harboring these toxins (Seals and Courtneidge, 2003). Although, the disintegrin domain has been widely described as being
able to interact with integrin molecules and therefore mediating cell-cell and cell-matrix interactions (Reiss et al., 2006), it has been shown not to be available for protein binding due to protein folding (Takeda et al., 2006). It may be therefore considered as a structural feature rather than an integrin ligand.

1.3.2.4 The cysteine-rich and EGF-like domains

The Cys-rich and EGF-like domains, though not much is known about them may play very important role for interactions of ADAMs with other proteins such as chaperones involved in biosynthesis and or with other partners on the cell surface. Structurally, the two domain consist of about 160 amino acid with 10 to 14 Cys residues and about 40 amino acid with 6 Cys residues, respectively. EGF domains have been indicated to be found in proteins that are either completely secreted or have transmembrane regions that tether the protein to the cell surface (Lu et al., 2007). On the other hand, the Cys-rich domain is located at the carboxy terminal end of ADAMs, which has been considered likely to complements the binding capacity of the disintegrin-like domain and imparts specificity to disintegrin domain-mediated interactions (Emi et al., 1993). The Cys-rich domain of TACE/ADAM-17 may play a role in the release of the pro-domain and may be required for the shedding of interleukin 1 receptor type II as well aid in as well the recruitment of accessory proteins involved in targeting TACE to some substrates (Reddy et al., 2000)

Several other fuctions have been reported for Cystein-rich domain as follows: It has been indicated to be involved in cell-cell fusion (Huovila et al., 1996), regulating protease activity and controlling substrate specificity (Reiss et al., 2006), function as a ligand for the cell-adhesion molecule syndecan, especially syndecan-4 (Iba et al., 1999, 2000).

1.3.2.5 Transmembrane domain

Mostly, ADAMs belong to the type I membrane proteins, anchored through a transmembrane (TM) domain near the C-terminus and also have an alternatively spliced form that diverges before the TM domain, leading to the production of a soluble, secreted form. All of the ADAMTSs lack a TM domain and are therefore termed as secreted proteases. Examples of ADAMs with transmembrane domain include 11, 12, 17, and 28 (Cerretti et al., 1999; Lu et al., 2007).

The ability to posses both soluble and membrane-anchored forms, allows ADAMs to regulate events not only on or near the cell surface, but also at a distance from cells.
However, very little is known as to whether all the membrane-anchored ADAMs have a soluble counterpart generated through either alternative splicing or shedding from the cell surface. (Cerretti et al., 1999; Lu et al., 2007).

1.3.2.6 The cytoplasmic tail

ADAMs have unusual cytoplasm tail, with many rich in proline, serine, glutamic acid and or lysine. They are highly variable both in length (between 40 to 250 amino acid) and in sequence and contains a phosphorylation sites and SH3 binding domains. This domain contains noticeable and specialized motifs that have been postulated to be involved in the inside-out regulation of metalloprotease activity, the outside-in regulation of cell signaling, and or the control of maturation and subcellular localization (Lu et al., 2007; Seals and Courtneidge, 2003). PxxP binding sites for SH3 domain-containing proteins are the most notable motifs which can be found in human ADAMs 7, 8, 9, 10, 12, 15, 17, 19, 22, 29, and 33. Several ADAMs also have potential phosphorylation sites for serine-threonine and or tyrosine kinases. Not only might this regulate ADAM function directly, but the resulting phosphotyrosine residues could also provide ligands for SH2 domain-containing proteins (Seals and Courtneidge, 2003).

In a study, ADAM9 binds to endophilin I and SH3PX1 (Howard et al., 1999). Given the potential function of endophilin I and SH3PX1 in vesicle sorting, it is speculated that these interactions may play a part in the regulation of ADAM maturation and or subcellular localization. Also it was reported that the membrane proximal region of the tail of ADAM9 associates with the catalytic domain of protein kinase C-alpha (PKC-alpha) (Izumi et al., 1998). The tail of ADAM9 can be phosphorylated by PKC in vitro at one or more serine and threonine residues. It therefore speculated that PKC-alpha helps to recruit ADAM9 to specific sites on the plasma membrane and upon phosphorylation or activation of ADAM9, shedding of HB-EGF occurs (Seals and Courtneidge, 2003).

1.3.3 Mechanisms by which ADAMs play a role in cancer

There are several different mechanisms through which ADAMs promote cancer formation and progression. Some of these processes includes Cell proliferation, Angiogenesis and Apoptosis.
1.3.3.1 Cell Proliferation

Cell proliferation by ADAMs can occur through the following processes:

1.3.3.1.1 Activation of positively-stimulating pathways

Several proteolytically active ADAMs regulate cell proliferation by cleaving growth factors or cell surface proteins or by activation of positively-stimulating growth factors. Many of these growth factors are first and foremost synthesised as inactive transmembrane precursor proteins that require ectodomain shedding for activation in order to function at its exert maximum capacity. Ligands for several growth factor receptors are processed by ADAM family members and amongst the best-studied growth-stimulating factors that are activated by ADAMs are the EGFR/HER family of ligands (EGF receptor ligands (heparin-binding EGF (HB-EGF), amphiregulin, betacellulin, epiregulin) with primary conversion mediated by either ADAM10 or ADAM17 (Duffy et al., 2011). However, other ADAMs such as ADAM8, 9, 12, 17 and 19 can also activate one or more of these ligands (Horiuchi et al., 2007).

What actually happens is that, the shed form of the ligands binds to one or more of the EGFR/HER family of receptors. Upon homo- or heterodimerisation, several different pathways including the mitogenactivated protein kinase (MAPK) pathway, the phosphatidylinositol 3-kinase (PI3K) pathway and janus kinase/signal transducer and activator of transcriptional (JAK/STAT) pathway, are activated by downstream signalling which can results in some of the classical hall markers of malignancy such as enhanced cell proliferation, increased cell motility and increased cell survival (Peeters et al., 2009; Duffy et al., 2011).
Figure 1.13: Mode of action of ADAMs in the activation of EGFR/HER receptor signalling

ADAMs are involved in proteolytic ectodomain shedding of membrane bound ligands. The released ligands (EGF, HB-EGF, TGFα, heregulins) are free to bind to and activate EGFR, HER3 and HER4. Following receptor dimerisation, downstream signalling through many pathways is activated, including MAPK, PI3K and JAK/STAT. (Duffy MJ et al., 2011).

In a work reported by Singh et al it was showed that UV irradiation of skin cancer cells activated ADAM9 and 17 which was followed by amphiregulin shedding, EGFR transactivation and increased cell proliferation. ADAM-10 also contributes to cell proliferation by modulating b-catenin signalling through E-cadherin shedding and increasing gene cyclin D1 levels (Shtutman et al., 1999).

1.3.3.1.2 Inactivation of growth-inhibitory pathways

Inactivation of growth-inhibitory pathways has been indicated in TGFb which signals via TGFbR1 and TGFbR. It has been proposed that, in normal and early malignant cells, TGFb inhibits proliferation where as in progressive malignancy, TGFb promotes proliferation (Ikushima and Miyazono, 2010 ; Duffy et al., 2011). ADAM17 was reported to mediate shedding of the type 1 TGFb receptor, thereby decreasing TGFb signalling which led to decreased growth inhibition and the reduction in growth inhibition complements the growth stimulation, resulting from increased release of the EGFR/HER ligands (Liu et al., 2009; Duffy et al., 2011).
1.3.3.1.3. Shedding of adhesion proteins

One of the means of increased cell proliferation is by means of shedding of adhesion proteins by ADAMs. For example ADAM10 appears to be the major sheddase for the release of EGF and betacellulin (Sahin et al., 2004, 2007) and also contributes to E-Cadherin shaddase (Ito et al., 1999). The sunsequent release of soluble E-cadherin in the extracellular milieu leads to the abrogation of cell-cell contacts, thereby facilitating cell migration. ADAM-10 also contributes to cell proliferation by modulating b-catenin signalling through E-cadherin shedding and increasing gene cyclin D1 levels (Shtutman et al., 1999) Najy et al also reported that ADAM15-mediated shed form of cadherin E bound to and activated HER2 in breast cancer cells. The increased proliferation and migration observed was attributed to the fact, the shed form of cadherin E formed a complex with HER2 and HER3, resulting in enhanced ERK signalling.

Apart from increased cell proliferation, this shedding might also be expected to weaken cell:cell interaction and thus allow dissociation of potential invasive and metastatic cells in the primary cancer which could potentially place a malignant cell or group of cells on their pathway to metastasis. Shedding of other adhesion proteins such as L-selectin, ICAM-1 or VCAM, on the other hand, might be expected to modulate binding of tumour cells to the vasculature wall and thus play a role in the intravasation (Duffy et al., 2011)

1.3.3.2 Angiogenesis

Cancer growth and metastasis can be mediated by angiogenesis which consists of the formation of new blood vessels devoted to vascularise the tumour tissue. This process is essential for tumours to grow beyond approximately 2 mm in diameter. (Duffy et al., 2011). Angiogenesis process is under the dependence of a balance of pro- and antiangiogenic factors (Bajou et al., 2004). Proteinases in general, have been initially considered as positive regulators of angiogenesis but recent studies have evidenced complex and sometimes opposite roles of MMPs, ADAMs and ADAMTSs in regulating tumoral angiogenesis (Handsley and Edwards, 2005). However, several evidences have also proved that ADAMs may promote cancer growth and metastasis through this process. Some of these evidences are: Pulmonary hypovascularisation in mice expressed catalytically inactive ADAM17 (Zhao et al., 2001), and the deletion of ADAM17 resulted in pathological neovascularisation and reduced growth of injected tumour cells in a mouse model (Weskamp et al., 2010).
Some studies carried out has indicated the recombinant disintegrin domain (RDD) of ADAM-15 as a potent inhibitor of angiogenesis. ADAM-15 RDD induces a reduction of MDA-MB-231 tumour growth associated with less tumour vascularization in vivo. Transgenic B16F10 melanoma cells form less metastasis in mouse lungs after turning on RDD expression (Trochon-Joseph et al., 2004). It has been proposed that mechanisms implicating ADAM-15 in the regulation of angiogenesis could be related to the presence of Arg-Gly-Asp (RGD) sequence in the disintegrin domain which binds integrins. In addition, certain ADAM proteinases have been indicated to control cell apoptosis. In a mammary cancer model induced by the expression of polyoma middle T oncoprotein, ADAM-12 has been shown to increase stromal cell apoptosis and decrease tumour cell apoptosis (Kveiborg et al, 2005). ADAM-10 knock-out (KO) embryos suffer from cell growth arrest and apoptosis associated with an overexpression of full-length E-cadherin (Maretzky et al., 2005).

1.3.4 Evidence of a Role for ADAMs in Cancer and their potential use as Biomarkers

Based on the ability of ADAMs to release ligands which is capable of stimulating cell proliferation as well as migration, several studies from cell lines grown in culture, animal models and human malignancies suggest that a number of ADAMs in cancer formation. The most established ones include ADAM9, ADAM10, ADAM12, ADAM15 and ADAM17 are involved in cancer formation and/or progression of which the strongest evidence for a role in malignancy exists for ADAM17 (Duffy et al., 2009).

Several studies have shown that increased expression of certain ADAMs enhanced in vitro invasion, proliferation and promoted tumour formation in vivo (McGowan et al., 2007; Borrell-Pages et al., 2003), while decreased expression reduced these processes. It has been shown that deficiency of specific ADAMs such as ADAM9, 15 and 17 resulted in decreased growth of heterotopically injected tumour cells in mice models (Guaiquil et al., 2009). Biomarkers are potentially useful in cancer detection, prognosis assessment, and predicting therapy outcome or likely resistance to therapy as well as monitoring ongoing therapy.
Five different pathways may be involved in ADAM mediated cancer cell proliferation and progression. (1) ProADAMs are activated by furin or matrix metalloproteinases (MMPs). (2) Sheddase activity of ADAMs is stimulated by external factors (e.g. 12-O-tetradecanoylphorbol-13-acetate [TPA]), leading to shedding of cell surface ligands such as heparinbinding-epidermal growth factor (HP-EGF) and transforming growth factor (TGF)-α. This process perhaps involves protein kinase C (PKC) and mitogenactivated protein kinase (MAPK) pathways. Then, soluble growth factors such as HP-EGF activate epidermal growth factor receptor on the cells in autocrine and paracrine manners. (3) The interaction of the disintegrin and cysteine-rich domains of ADAM with integrins or syndecans on the cells may help them to cleave the substrates (e.g. extracellular matrix [ECM]). (4) ADAMs modulate extracellular matrix-integrin interactions, and thus they can indirectly promote proliferation signals through integrins. (5) ADAM may process other undetermined membrane-anchored molecules such as chemokines, cytokines and their receptors, which are related to cancer cell proliferation and progression. CR, cysteine-rich domain; CT, cytosolic tail; Dis, disintegrin domain; E, epidermal growth factor-like domain; MP, metalloproteinase domain; Pro, propeptide domain; TM, transmembrane domain.

(Mochizuki S and Okada Y, 2007)
1.3.5 Contribution of ADAMs in different types of cancer as diagnostic marker

1.3.5.1 Lung cancer

It has been proved in several studies that dysregulation of the production of several ADAMs leads to lung cancer. Some of the have been indicated here. Primary bronchial epithelial cells and bronchial cell lines exposed to smoke components showed an increased proliferation rate associated with EGFR phosphorylation with possible mediation by ADAM-17 which can activate several EGFR ligands (Lemjabbar et al., 2003). Also ADAM17 was reported to be upregulated in non-small cell lung carcinoma (NSCLC), with possible heregulin3 (HER3) signalling (Zhou et al., 2006). Another example is ADAM-8, which was strongly expressed in NSCLC by tissue microarray analysis, and correlates with clinical stage of the disease (Ishikawa et al., 2004). Also, ADAM-9 mRNA and protein expression levels are enhanced in EBC-1 lung cancer cell line displaying a tropism for brain metastasis as compared to parent EBC-1 or EBC-1 cell line with a tropism for bone tissue (Shintani et al., 2004).

ADAM-12 mRNA and protein levels was reported to increase in NSCLC when compared to non-cancerous tissues (Rocks et al., 2006) ADAM-15 was reported to be expressed in both small cell lung carcinoma (SCLC) and NSCLC cell lines with higher expression in tumoral cells than in normal epithelial cells of pulmonary tumours (Schutz et al., 2005).

ADAM28 was one of the first ADAMs shown to be elevated in serum from patients with cancer (Kuroda et al., 2010). ADAM-28, cleaved insulin-like growth factor binding protein-3 (IGFBP-3), and was found to be about 16-fold over-expressed in NSCLC (Mochizuki et al., 2007).

1.3.5.2 Brain tumours

ADAM-22, restricted to the brain is implicated in cell-cell and cell-matrix interactions through their binding to integrins and extracellular matrix and may be involved in neural development (Sagane et al., 1998). Cytoplasmic variants of ADAM-22 have been indicated to have been expressed differently in normal human brain tissue and gliomas (Harada et al., 2000), which gives evidence of ADAM genes expression in brain tumours. Brain tumour cell lines cultured under hypoxic conditions demonstrated an upregulation of ADAM-17 expression levels, and its activity correlated with increased tumour cell invasion (Zheng et al., 2007). Also, ADAM-8 and ADAM-19 mRNA are upregulated in primary brain tumours and their expression and activity are correlated with
invasiveness of glioma cells (Wildeboer et al., 2006). There was an overexpression of the membrane-bound ADAM-12 variant in glioblastomas (Kodama et al., 2004). However, treatment of cultured glioblastoma cells with an ADAM-12 inhibitor decreased the production of the mature HB-EGF indicating its role in HB-EGF signalling pathway in those cells.

1.3.5.3 Prostate cancer

There is a possibility of the existence of both androgen-independent and androgen-dependent cases in prostate cancer though early stages involve androgen-dependent (Bertram et al., 2006). Androgen-dependency has been suggested to interfere with ADAM-related regulation processes since the mRNA expression of several ADAMs is regulated by androgens (McCulloch et al., 2000). Androgen or serum starvation enhances ADAM-9 protein expression in androgen receptor-positive prostate cancer cells (Shigemura et al., 2007). Peduto et al. reported that well-differentiated prostate cancers developed in ADAM-9-deficient mice compared with poorly differentiated tumors in control mice expressing ADAM-9. They also showed that overexpression of ADAM-9 in mouse prostate epithelial cells gave rise to epithelial hyperplasia and prostate intraepithelial neoplasia, a putative precursor lesion for prostate cancer because of its ability to cleave EGFR ligands and the receptor for fibroblast growth factor (Peduto et al., 2005).

In studies on prostate cancer cell lines in culture, overexpression of ADAM-9 was found to be associated with the conversion of LNCaP cells to an androgen-independent and metastatic state (Sung et al., 2008). In prostate carcinoma, ADAM-9 levels were significantly associated with prostate-specific antigen (PSA) relapse-free survival (Fritzsche et al., 2008). ADAM-8 protein expression has been demonstrated to be significantly associated with higher cancer stages including positive nodal status, and higher Gleason scores (Fritzsche et al., 2006). Najy et al. also found that down-regulation of ADAM-15 in the prostate cancer cell line PC3 decreased migration and adhesion to specific extracellular protein matrix proteins, such as fibronectin, vitronectin, and laminin.

1.3.5.4 Liver cancer

In activated hepatic stellate cells, TGF-b1 induces ADAM-12 expression which might also participate in tumour progression (Le Pabic et al., 2005). This suggest that ADAM-12 might contribute to growth inhibitory signalling in normal epithelial cells which is lost during tumour progression. Several studies have indicated ADAM-17 as a
contributing factor to EGFR-ligand release and induction of cell proliferation and invasion (Rocks et al., 2008). In a study, it was reported that ADAM-17 mRNA levels were higher in hepatocellular carcinomas than in paired non-cancerous liver tissues suggesting that this proteinase might be implicated in tumour invasiveness by either activating EGFR by amphiregulin (Lemjabbar et al., 2003) or TGFalpha (Borrell-Pages et al., 2003). ADAM-9 in one study has been reported to promote invasiveness of liver metastatic carcinoma cells by degrading basement membrane components such as laminin-1 (Mazzocca et al., 2005).

1.3.5.5 Breast cancer

There has been several reports of ADAMs implications in breast cancer patients. One of the first ADAMs shown to have diagnostic potential was ADAM12 in breast cancer. ADAM-12, an apoptosis-modulating gene accelerated the development of tumour by delaying tumour cell apoptosis by the overexpression of its soluble form lacking the cytoplasmic tail (secreted splice variant of ADAM-12) (Kveiborg et al., 2005; Rocks et al., 2008). In urine, using Western blotting, ADAM-12 levels were enhanced in breast cancer patients vis-à-vis a healthy control group which suggest a potentially important non-invasive biomarker in breast cancer (Roy et al., 2004).

Using logistic regression analysis, the authors calculated that the predictive probability of the presence of breast cancer was ≥ 80%, when levels of ADAM12 exceeded 40 arbitrary units (Roy et al., 2004). In a follow-up study to above, Pories et al found that urinary ADAM12 levels were also increased in women with putative premalignant lesions of invasive breast cancer such as atypical hyperplasia and lobular carcinoma in situ, compared to levels in healthy controls. This finding, if confirmed, suggests that measurement of ADAM12 in urine could identify women at increase risk of developing breast cancer. In the PyMT mouse model, overexpression of ADAM-12 was found to promote breast cancer progression (Kveiborg et al., 2005). Also, expression of both ADAM12 isoforms was found to be significantly elevated in human malignant breast tissue overexpression which resulted in increased tumor take, tumor size, and metastasis in vivo. Of the two isoforms, only the secreted isoform, ADAM12-S, enhanced the ability of tumor cells to migrate and invade in vitro and resulted in a higher incidence of local and distant metastasis in vivo (Roy et al., 2004).

In vitro studies have shown that overexpression of ADAM-17 in breast cancer cells increases invasion and proliferation (McGowan et al., 2007) and targeting it, reverts the malignant phenotype by preventing shedding of TGF-a and amphiregulin (Kenny and Bissell, 2007). Active ADAM-28 was found to be overexpressed in breast carcinoma cells, contributing to the regulation of cell proliferation through IGFBP-3 cleavage, enhancing...
the bioavailability of IGF-I (Mitsui et al., 2006). Alternative splicing could also be an important tool used by cancer cells to acquire an invasive phenotype. For example, different isoforms of ADAM-9 proteins and ADAM-15 mRNA have been detected in breast cancer cells (Ortiz et al., 2004) which calls for attention to set up a powerful diagnostic tool by studying the differential production of ADAM-9 or -15 domains. In breast cancer, ADAM-9 expression was significantly higher in node-positive than node-negative primary cancers whereas the active form of ADAM-17 was increased in high-grade versus low-grade tumors (McGowan et al., 2007).

In vivo, loss of ADAM-15 decreased metastasis to bone and using breast cancer cell lines, it was reported that ADAM-15 cleaved cadherin E after growth factor deprivation (Najy et al., 2008). In another study, it was shown that the human breast cancer cell lines, MCF-7 and MDA-MB453, strongly express ADAM 9, 12, and 17, whereas ADAM 10 and 15 were expressed at a lower level, indicating a putative pathophysiological role of these ADAMs in breast cancer biology (Lendeckel et al., 2005). ADAM17 processed through major histocompatibility complex (MHC) class I molecules was showned to be expressed in breast, ovarian and prostate cancer, making it a potential immunotherapeutic target in these cancers. (Sinnathamby et al., 2011).

**Figure 1.15 Prospective role of ADAMs in breast carcinoma cell proliferation.**

ADAM28 is overexpressed as active forms in breast carcinoma cells. ADAM28 cleaves insulin-like growth factor binding protein-3 (IGFBP-3) and releases insulin-like growth factor-I (IGF-I) through the IGF-I–IGFBP-3 complex. IGF-I induces cell proliferation through phosphorylation of the IGF type I receptor (IGF-IR) and extracellular signal-regulated kinase 1/2 (ERK1/2). IGFBP-3 cleavage can be inhibited by treatment with anti-ADAM28 antibody or an ADAM inhibitor, KB-R7785, as well as ADAM28 small interfering RNA (siRNA) (Mochizuki S and Okada Y, 2007)
1.3.5.6 Gastric and colon carcinoma

In vivo, ADAM-10 and -17 are overexpressed in antral mucosa during H. pylori infection and it has been established that, ADAM-9, -10, -12, -15, and -17 are increased in gastric tumours (Carl-McGrath et al., 2005). ADAM-10, which is found to be overexpressed in vitro after gastric cell infection, could establish a link between Helicobacter pylori-induced inflammation and carcinogenesis in stomach and it acts through EGFR ligand shedding leading to gastric cell proliferation (Joy et al., 2005). In colon carcinomas, ADAM-17 is overexpressed independently of tumour stage or grade and is involved in tumour growth and angiogenesis possibly via an autocrine/paracrine pathway implicating EGFR (Blanchot-Jossic et al., 2005). ADAM-9 is also overexpressed in a colon cell line and is co-localized with E-cadherin suggesting a potential role in E-Cadherin-mediated metastasis. It has been showed that a soluble form of ADAM-9 secreted by hepatic stellate cells promoted colon cancer cell invasion in vitro. (Mazzocca et al., 2005).

1.3.5.7 Kidney, bladder carcinoma

Inhibition of ADAM-17 by a dominant negative ADAM-17 mutant prevents pro-HB-EGF cleavage, EGFR activation and cell proliferation in kidney carcinoma cells, indicating the importance of EGFR signalling in the development of kidney cancer since (Schafer et al., 2004). ADAM-12 mRNA was found to be overexpressed in bladder cancer and its levels correlated with disease stage. In another study, the levels of ADAM-12 was also found to be higher in the urine from patients with bladder cancer compared with healthy control subjects (Frohlich et al., 2006) and concentrations tended to be higher in those with the largest invasive tumours. In some cases, urinary ADAM12 levels decreased following surgical removal of the bladder cancer but increased again with recurrent disease (Fröhlich et al., 2006) which suggesting the propability of using the measurement of urinary ADAM12 for monitoring patients with bladder cancer.

1.3.5.8 Pancreatic carcinoma

Some of the ADAMs implicated in pancreatic carcinoma are ADAM-9, -10 and -17 but are restricted to specific compartments. Analysis of mRNA expression levels in microdissected cancer samples shows an overexpression of ADAM-9 and -15 proteinases in pancreatic tumour cells (Rocks et al., 2008). ADAM-17 was once again overexpressed in all pancreatic ductal adenocarcinoma (PDAC) and pancreatic cancer cell lines. (Yamada et al., 2007).
1.3.5.9 Squamous Cell Carcinoma

This G-protein which are known to activate ADAMs can also transactivate epidermal growth factor receptor (EGFR) (Ohtsu et al., 2006). For example, ultraviolet (UV) radiation of skin cancer cells activates ADAMs and induces EGFR ligand shedding and EGFR transactivation (Singh et al., 2009). One mechanism for this process is the likelihood that, the UV irradiation induced reactive oxygen species (ROS) generation, which in turn activated ADAM9 and ADAM17, and finally cleaving EGFR ligands, particularly AR. Skin cancer proliferation can be induced by the binding of the soluble form of AR to EGFR. Overexpression of protein kinase ε (PKCε) in mouse epidermis which resulted in the rapid development of papilloma independent metastatic SCCs via the two-stage model of carcinogenesis have also been reported (Wheeler et al., 2003). PKCε transgenic mice have elevated serum TNF-α levels during skin tumor promotion by 12- O-tetradecanoylphorbol-13-acetate (TPA).

Since TNF-α is linked to skin tumor promotion by TPA, this increase may be linked to the development of metastatic SCC. TPA stimulated shedding of TNF-α could be completely prevented in PKCε transgenic mice and isolated keratinocytes by an ADAM17 inhibitor, TAPI-1. These results indicate that PKCε signal transduction pathways to TPA-stimulated TNF-α ectodomain shedding are mediated by ADAM17. Injection of a TNF-α synthesis inhibitor during skin tumor promotion completely prevented the development of metastatic squamous cell carcinoma in PKCε transgenic mice (Wheeler et al., 2003).

1.3.5.10 Basal Cell Carcinoma

The most common type of skin tumor is Basal Cell Carcinoma which rarely metastasizes but is locally invasive and highly destructive. ASAMs implicated in this disease are ADAM10, 12, and 17. Compared with central areas of basal cell carcinoma tumor cell nests, these ADAMs increased at the peripheral tumor margin. Expression of ADAM10 and ADAM12 is increased in the deep margin of invading tumor cell nests. In contrast, ADAM17 is increased in superficial basal cell carcinoma. All the three ADAMs showed different expression patterns in basal cell carcinoma histologic subtypes, indicating their different roles in the pathogenesis of BCC (Oh et al., 2009).

1.3.5.11 Malignant Melanoma

ADAM9, ADAM10, ADAM12, ADAM15, ADAM17, HB-EGF and TGF-α are overexpressed in more than 1 malignant melanoma (MM) cell lines which can induced the migration of MM cells. (Singh et al., 2009). In ADAM10, the expression was
significantly elevated in melanoma metastases compared with primary melanomas (Lee et al., 2010). Expression of several components of the Notch pathway, which can be cleaved by ADAM10, were also upregulated in malignant melanoma compared with common melanocytic nevi (Massi et al., 2006). Down-regulation of ADAM10 with specific siRNA resulted in the suppression of cell growth and the reduced migration of MM cells.

ADAM10 has also been implicated in constitutive CD44 cleavage from malignant melanoma cells, and its expression can impair tumor cell proliferation (Anderegg et al., 2009). In malignant melanoma cells, both ADAM10 and ADAM17 are significantly expressed in histological sections but only ADAM10 is involved in the constitutive shedding of native CD44 from this cells. (Ahrens et al., 2001), ADAM9 is detected in malignant melanoma cells and in peritumoral stromal fibroblasts, while it is absent in fibroblasts distal to the tumor site. In contrast, in nevi, ADAM9 expression is absent both in nevus cells and in stromal cells close to nevus cell nests (Zigrino et al., 2005).

1.3.6 ADAMs as prognostic markers

With increased ADAMs involvement in cancer proliferation and progression, several studies have investigated for their potential prognostic impact in patients with cancer and it has been indicated that, there exist a correlations between levels of specific ADAMs and parameters of tumour progression (eg. tumour size, grade, metastasis to local lymph nodes and patient outcome) in human cancers (Valkovskaya et al., 2007). These markers are important in the management of patients with cancer as they help avoid the overtreatment of indolent disease and undertreatment of aggressive cases, especially in the cases of breast and prostate cancer. It has been indicated that, none of the available serum markers for breast cancer are increased in patients with early disease and are thus of little value in identifying women at increased risk of developing this malignancy (Duffy, 2006). In breast cancer cases, prognostic markers may help identify those patients whose prognosis is so good that they are unlikely to benefit from receiving adjuvant chemotherapy whiles at the same time identifying patients with aggressive disease that may derive benefit from receiving such therapy.

One of the best validated ADAMs for predicting patient outcome is ADAM17 in breast cancer with the active form being more associated with breast cancer progression than the precursor form (McGowan et al., 2007). It has been showed that patients with breast cancers expressing high levels of ADAM17 protein had significantly shorter overall survival compared to those with low expression of the protein (McGowan et al., 2008) and the prognostic impact was independent of tumour size, grade and lymph node status.
Also high expression of ADAM17 mRNA was also found to predict adverse outcome in patients with breast cancer (Kenny et al., 2007). For the first time it was reported that the increased expression of ADAM17 in gastric cancer is associated significantly with aggressive progression and poor prognosis which suggested ADAM17 as an important molecular marker for predicting the carcinogenesis, progression, and prognosis of gastric cancer. (Zhang et al., 2011).

Another prognostic marker for breast cancer is ADAM15 which have four isoforms or variants (ADAM15-A, ADAM15-B, ADAM15-C and ADAM15-D) with different effects in vitro (Zhong et al., 2008). In this study, ADAM15-A was found to increase cell invasion, migration and adhesion, while ADAM15-B was shown to decrease adhesion. However, high expression of both predicted shortened relapse-free survival in lymph node-negative breast cancer patients. ADAM15-C, on the other hand, correlated with improved relapse-free survival in lymph node-positive but not in lymph node-negative patients. In another study, it was suggest that ADAM15 is generally overexpressed in adenocarcinoma and is highly associated with metastatic progression of prostate and breast cancers. (Kuefer et al., 2006). Fritzche et al, showed that increased expression of ADAM9 in prostate cancer was significantly associated with shortened relapse-free survival as measured by increasing serum PSA levels and the prognostic impact was independent of the conventionally used factors for this malignancy such as tumour size, Gleason grade and preoperative PSA level.

This independent prognostic impact of ADAM9 was found in both the total population of patients investigated as well as in those treated with anti-androgens (Fritzsche et al., 2007). ADAM9 has also been shown to have prognostic value in renal (Roemer et al., 2004) and pancreatic cancers (Grützmann et al., 2004). Also, an increased expression of ADAM9 in hepatocellular carcinoma was indicated to be an independent prognostic marker of overall survival following heptectomy with a very have poor outcome (Tao et al., 2010).

Furthermore, Using publicly available gene expression data for patients with lymph node-negative breast tumors who did not receive systemic treatment, it was shown that ADAM12L is the only ADAM whose expression level is significantly associated with decreased distant metastasis-free survival times (Hui Li et al., 2012).
1.3.7 ADAMs as therapy predictive markers

Some ADAMs could be relevant markers of therapeutic response. Predictive markers are factors that are associated with upfront response or resistance to a particular therapy (Duffy et al., 2005). They are important in the management of cancer patients as tumours of the same histological type or tissue of origin vary widely in their response to most available systemic therapies. Few studies have been carried out on ADAMs as therapy predictive biomarkers, and in one of them, it was reported that high levels of mRNA for ADAM9 and 11 but not for ADAM10 or ADAM12 were associated with increased benefit from tamoxifen in patients with recurrent breast cancer (Sieuwerts et al., 2005).

This finding was especially true for patients whose primary tumour contained large amounts of stroma. In this same study, ADAM9 but not ADAM11 provided independent predictive information over estrogen receptors, progesterone receptors, menopausal status and dominant site of relapse. ADAM-9 and ADAM11 mRNA levels in tumours are indeed associated with better response to tamoxifen therapy and ADAM-9 protein production is an indicator of poor prognosis (O'Shea et al., 2003; Sieuwerts et al., 2005).

1.3.8 ADAMs inhibitors as Therapeutic Targets for the Treatment of Cancer

In many types of cancers, ADAMs are upregulated and it might be expected that inhibition of these proteases could be used to treat cancer. Several recent studies have therefore highlighted the potential of targeting ADAMs family members has therefore been highlighted as a new approach for antitumor therapy (Kataoka, 2009). Inhibitors of ADAMs metalloprotease activity fall into four broad classes: those that inhibit by denaturation; those that inhibit by Zn-chelation; small molecule inhibitors of catalysis; and proteinaceous inhibitors called TIMPs.

The first two categories represent nonselective inhibitors such as reducing agents or zinc chelating agents. The third class arose from efforts to develop inhibitors of both MMPs and ADAMs, and comprise hydroxamate-based inhibitors that bind competitively to the active site. These have proved to be useful tools for studying ADAMs (Moss et al., 2001) and have resulted in the development of at least four potential approaches exist to block ADAM protease activity. These include use of low molecular weight synthetic inhibitors (table 2), purified or synthetic forms of ADAM prodomains, modified TIMPs and monoclonal antibodies (Lendeckel et al., 2005; Rapti et al., 2008).
Of these potential approaches, only the use of low molecular weight synthetic inhibitors has been subjected to detailed investigation. Most of the low molecular weight ADAM inhibitors use hydroxamate as the zinc binding group and were designed to interact or bind to the prime subsites of the MMP-like catalytic site (Moss et al., 2008). Although the majority of those described, inhibited a number of MMPs as well as some ADAMs, some are relatively selective for specific ADAMs especially ADAM10 and or ADAM17 (Zhou et al., 2006). Some of these molecule inhibitors despite their potency, are not always selective for MMPs, but are equally potent inhibitors of ADAMs (Moss et al., 2001). For example Batimastat and Ro-31-9790 inhibit ADAM17 better than several MMPs (Barlaam et al., 1999). TIMP-3 has been reported to also inhibits ADAM17 and ADAM12 where as ADAM10 is inhibited by both TIMP-1 and TIMP-3 (Amour et al, 2000).

Of the ADAMs inhibited compounds indicated, the most widely investigated for anticancer activity are INCB3619 and INCB7839 (Incyte Corporation, Wilmington, DE) (Witters et al., 2008). INCB3619 an orally active low molecular weight compound that selectively inhibits ADAM-10 and ADAM-17 with half maximal inhibitory concentration (IC50) values of 14 and 22 nM/L, respectively (Incyte Corporation; Zhou et al., 2006). In contrast, the IC50 for ADAM-8, ADAM-9, and ADAM-33 were 1,000, >5,000, and 1,036 nM/L. INCB3619, a selective inhibitor of a subset of ADAM proteinases, blocks the shedding of ErbB ligands, reduces ErbB ligand shedding in vivo and inhibits ErbB pathway signalling, tumour cell proliferation and survival.

In an early study, using non small cell lung cancer (NSCLC) cells in culture, INCB3619 was reported to block release of the HER3 ligand, heregulin, rendering these cells sensitive to the EGFR inhibitor, gefitinib (Zhou et al., 2006). Also, using NSCLC cell, INCB3619 increased apoptosis and reduced the apoptotic threshold for response to paclitaxel (Zhou et al., 2006). In another study, the addition of INCB3619 to MCF-7 breast cancer cells in vitro resulted in minimal growth inhibition. However when combined with the dual EGFR/HER2 tyrosine kinase inhibitor, GW2974 (Sigma Aldrich), synergistic growth inhibition was observed (Fridman et al., 2007). The combination of INCB3619 and GW2974 also gave rise to decreased phosphorylation of ERK and AKT.

Furthermore, using lung cancer, mice bearing non–small cell lung cancer xenografts were administered gefitinib, INCB3619, or a combination of the two agents. When used singularly, neither gefitinib nor INCB3619 were effective but the combination of INCB3619 and gefitinib significantly reduced tumor growth (Zhou et al., 2006). In preclinical models, INCB3619 has also been shown to synergerize with cisplatin in reducing the growth of head and neck cancers, and with paclitaxel in inhibiting the growth of breast cancer in a xenograft model (Zhou et al., 2006).
An inhibitor related to INCB3619 is INCB7839 (Incyte Corporation; Fridman et al., 2007). When this drug combined with lapatinib in a study, they completely prevented growth of human breast cancer xenografts in mice (Witters et al., 2008). Another selective ADAM inhibitor is known as WAY-022 (Wyeth-Aherst). WAY-022, which is a selective inhibitor of ADAM-17, was found to decrease DNA replication and cell growth in colorectal cancer cells (Merchant et al., 2008). Furthermore, the combination of suboptimal concentrations of WAY-022 with an EGFR monoclonal antibody or a selective EGFR kinase inhibitor resulted in cooperative growth inhibition (Merchant et al., 2008). Consistent with its ability to inhibit ADAM-10 and ADAM-17 at low levels, INCB3619 blocked the release of TGF-alpha, HB-EGF, amphiregulin, and heregulin at nanomolar concentrations (Fridman et al., 2007).

Table 1.2 Selective ADAMs inhibitors

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<th>Company</th>
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<tr>
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<tr>
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<td>17</td>
<td>Pfizer</td>
</tr>
<tr>
<td>KB-R7785</td>
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</table>

(Duffy MJ et al., 2011)
1.4 Characteristics and specific functions of ADAM17

1.4.1 Structure of ADAM17

Like other ADAMs, ADAM17, a modular type I protein, contains in addition to the signal peptide, four functionally distinct extracellular domains followed by a transmembrane region and a cytoplasmic tail (Fig. 1.15) (Arribas and Esselens, 2009; Duffy et al., 2009) as previously described for all ADAMs. Its involvement in major contemporary pathologies like cancer, inflammatory and vascular diseases seem to be connected to its cleavage abilities and this is due to the large variety of substrates it is able to cut. Certain growth factors and receptors can be activated and inactivated respectively by ADAM17 by shedding their extracellular domain from the cell membrane and in the same way, it can detach cells by cleaving cell adhesion molecules. These proteolytic activities normally form part of cleavage cascades referred to as Regulated Intramembrane Proteolysis, leading to intracellular signaling.

ADAM17 (also named as tumor necrosis factor-alpha-converting enzyme, TACE) is a member of the ADAM family and a transmembrane metalloprotease and primary sheddase for multiple EGFR pro-ligands, including transforming growth factor-alpha (TGF-a), heparin-binding epidermal growth factor (HB-EGF), and amphiregulin and all these ligands have been implicated in cancer development and progression (Xu P et al., 2010). Of all ADAM members, the most extensively investigated and the strongest evidence for a role in malignancy exists for ADAM17, which has been demonstrated to be overexpressed in the breast, ovary, kidney, colon, and pancreas cancer tissues and regulates important biological phenomena in these cancers through EGFR/PI3K/AKT pathway.
1.4.2 The degradome of ADAM17

Currently, there are over fifty known substrates of ADAM17 which are functionally and structurally heterogeneous and include membrane anchored growth factors and cytokines, receptors, cell adhesion molecules and ectoenzymes. Mostly, ADAM17 cleavage has been reported to control the function of a given substrate leading to activation, inactivation or modulation of the activity. (Arribas and Esselens, 2009)

1.4.2.1 Membrane-Anchored Ligands

Sheddase of the extracellular domain of many soluble growth factors and cytokines by ADAM17 gives an idea of how certain signaling pathways are controlled by metalloproteinases. This regulatory mechanism enable swift environmental responses, rapidly switching a particular pathway either on or off, independently from the slower transcription-translation system (Arribas and Esselens, 2009).
This can be seen in the following instances: The release of TGF-alpha, constituted a straightforward activation process, due to the inactive state of the membrane-bound proTGF-alpha (Borrell-Pages et al., 2003). Also it was indicated that the cleavage of TNF-alpha by ADAM17 resulted in a transition between two active forms with different properties (Aggarwal, 2003). In confirmation to the above study, the human carcinoma cell line Colo205, is resistant to the soluble TNF-alpha, whereas is sensitive to membrane-associated proTNF-alpha (Grell et al., 1995).

In the same manner, a ligand called neuregulins, were fully active even when membrane bound and its cleavage by ADAM17 converted from physically restricted growth factor into a diffusible isoform (Montero et al., 2000).

1.4.2.2 Receptors

The ADAM17 degradome includes many receptors, aside ligands. Receptors cleavage offers another way to regulate the response of the cell to growth factors and cytokines. In this case, shed receptor in addition to being unable to transmit signals to the cell, can also act as a ligand-sequestring scavenger (Arribas and Esselens, 2009). This is shown in a study by Aggarwal, where the shedding of the receptors for TNF-a by ADAM17 and the subsequent sequestering of TNF-alpha by the soluble receptors, prevented the binding of the cytokine to the full-length receptors (Aggarwal, 2003).

ADAM17, can also be an activator of certain receptors. Typical example is a receptor that determines the developmental fates of mainly neuronal and hematopoietic cell populations, called Notch receptor. ADAM17 carry this process out by first releasing the extracellular domain of Notch following ligand binding, while the “stub”, (transmembrane and cytoplasmic domain), stays behind in the membrane. The Notch intracellular domain released, then translocate to the nucleus in order to regulate its target genes expression (Selkoe and Kopan, 2003). ADAM17 has been indicated to mediate the cleavage of ErbB4, N-cadherin and CD44 in the same manner as indicated above. (Arribas and Esselens, 2009).
1.4.2.3 Cell Adhesion Molecules

Shedding of cell adhesion molecules by ADAM17 results in the weakening of cell-cell interactions (Arribas et al., 2006), which is very important in signal transduction. For instance, the shedding of CD44 by ADAM17 is followed by a Regulated Intramembrane Proteolysis (RIP) event, that releasing the intracellular domain (ICD) of CD44. The released ICD in turn is translocated to the nucleus, where it finally regulates the transcription of target genes (Nagano and Saya, 2004).

1.4.2 ADAM17 Dysfunction and cancer

Despite the remarkably large number of ADAM17 substrates identified, most of the proposed pathological roles in cancer of the metalloproteinase are related to just a few, namely, TNF-α and ligands for HER in general. Upon ligand binding, the receptors undergo homo- or heterooligomerization and activation. Activated HER receptors transduce signals through a plethora of intracellular factors that, ultimately, regulate the expression of groups of genes that control cellular proliferation, migration, adhesion, differentiation and apoptosis (Yarden et al., 2001). Different components of the EGFR pathway, such as HER2, are overactivated and play a causal role in breast cancer progression (Baselga, 2001). ADAM17 is frequently overexpressed in breast cancers (Borrell-Pages et al., 2003) and its overexpression is associated with tumor progression and metastasis (McGowan et al., 2007).

1.5.1 Release/Shedding of TNF-α by ADAM-17.

ADAM-17 was originally identified by its ability to release membrane-bound TNF-α from its precursor and its purification was based on its ability to hydrolyze both peptide and full-length TNF-α–based substrates at their physiologic cleavage site (Moss et al., 1997). TNF-α is a pluripotent peptide with multiple activities potentially important in tumor formation and/or progression.

Apart from TNF-α, ADAM-17 has been shown to shed several membrane-bound proteins, including E-selectin, p75 TNF receptor, transforming growth factor-α, amphiregulin, heparin-binding EGF, and epiregulin. The shedding of a number of these molecules is potentially important in cancer progression. ADAM-17 implication in TNF-α release was documented in vivo where T cells derived from ADAM-17–deficient mice (TACEDZn/ZnD) lost almost all of their ability to shed TNF-α (Black et al., 1997).
TNF-a role in malignancy include its ability to up-regulate MMP expression, induce angiogenic factors, enhance cell migration, promote the epithelial-to-mesenchy, induce expression of the transcription factor nuclear factor nB, induce reactive oxygen species that damage DNA, and induction of matrix degrading proteases and release of cytokines and chemokines (Pilarsky et al., 2004; Babbar et al., 2006). Suganuma et al. showed that a deficiency of this cytokine rendered mice resistant to chemically induced skin carcinogenesis (Suganuma et al., 1999). Because ADAM-17 catalyzes the formation of active TNF-a, it might also be expected to play a role in malignancy via the generation of this cytokine.

1.5.2 Shedding of HER ligands by ADAM-17 implicated in breast cancer

The HER (also known as ErbB) proteins belong to the subclass I of the superfamily of receptor kinases. There are four members of the family: EGFR/ErbB1/HER1, HER2/ErbB2, HER3/ErbB3, and HER4/ErbB4 with an extracellular domain, a transmembrane domain and an intracellular domain. Two members of this family (EGFR and HER2) mediate cell growth, cell survival, cell migration, angiogenesis, and invasion (Olayioye et al., 2000). Because of their key role in these processes, altered expression of EGFR and HER2 has been implicated in the formation/progression of multiple cancer types and these two proteins are currently among the best-validated targets for cancer treatment, especially HER2 in breast cancer (Hynes et al., 2006).

The HER tyrosine kinases are activated by a number of ligands that are initially synthesized as transmembrane precursors, which are necessary for biological activity. There are three main groups: (a) EGF, TGF-a, amphiregulin, and epigen, which specifically bind to EGFR; (b) heparin-binding (HP)-EGF, epiregulin, and betacellulin, which bind to EGFR or HER4; and (c) neuregulin (also known as heregulin), which binds to HER3 and HER4 (Olayioye et al., 2000; Hynes et al., 2006). No naturally occurring ligand has yet been identified that specifically bind to HER2, but then it is preferred dimerization partner for the other three ligand-activated HER members. It has been reported that most of the HER ligands can be released from their precursor forms by specific ADAMs. For example, a knockout studies with the use of mouse embryonic cells showed that ADAM-17 was the major sheddase for TGF-a, amphiregulin, HB-EGF, and epiregulin (Sahin et al., 2004).

ADAM-mediated release of HER ligands can be activated by a number of physiologic and pharmacologic stimuli. Among the best-studied stimuli are agonists for G protein–coupled receptors. The binding of these agonists to their receptor results in
ADAM-mediated release of HER ligands and transactivation of EGFR (Ohtsu et al., 2007). Thus, in recent years, there has been a growing interest in ADAM17 as a new therapeutic target in several EGFR-dependent tumour types. Literally, it is so obvious that ADAM17 fulfills a key role in diverse processes and pathologies, making it a prime target for developing therapies.

Several reports have been documented on the evidence implicating ADAM17-mediated growth factor ligand release and EGFR. For example ADAM17, via ligand release and activation of the EGFR-PI3K-AKT pathway, enhanced in vitro breast cancer cell proliferation and invasion. (Zheng et al., 2009) Also, amphiregulin released by ADAM-17 cleavage enhances cell proliferation of cancer cells (Gschwind et al., 2003; Zhang, et al., 2006). It was also shown by Mendelson et al that treating mouse embryonic fibroblasts with platelet derived growth factor receptor beta (PDGFRb) led to activation of ADAM17, release of EGFR ligands and EGFR/ERK signalling which ultimately resulted in enhanced migration.

1.6 Blocking of ADAM17

The structure of the proteolytic domain of ADAM17 at a resolution of 1.7 Å, has enable the development of numerous inhibitors (Table 1.3) with various degrees of specificity. Some compounds block, in addition to ADAM17, closely related ADAMs and MMPs, while other seem to be highly specific. ADAM17 inhibitors are being assayed in different preclinical models as in clinical trials, as shown in the table below.

Using a three-dimensional culture model of human breast cancer progression, Kenny et al (Kenny et al., 2007) reported that either knockdown of ADAM-17 expression with the use of small interfering RNA or inhibition of protease activity with the use of a low–molecular weight inhibitor (TNF-alpha protease inhibitor-2) reversed the malignant phenotype by preventing the release of amphiregulin and TGF-alpha. In renal cancer cells, silencing of ADAM-17 was found to restore a dependence on exogenous growth factors, reduce invasion, and block in vivo tumor formation. The role of ADAM-17 in pancreatic cancer is underscored by experiments showing that inhibition of ADAM-17 gene expression, by using small interfering RNA (siRNA) technique, affects invasiveness of tumour cells (Ringel et al., 2006). Treatment of breast cancer cell lines with anti-ADAM17 antibodies leads to a decrease in cell proliferation (Lendeckel et al., 2005).
<table>
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(Arribas J and Esselens C, 2009)
1.7 Objectives of study

The general aim of this study was to determine the expression of the impact of ADAM17 in node-negative Breast Carcinoma

Specific aims were as follow:

- To evaluate the tissue-based protein expression of ADAM17 and correlate it with clinical and pathological features of breast cancer.
- To compare the expression of ADAM17 in node-negative and node-positive breast Cancer.
- To differentiate among the heterogeneous grade 2 patients to aid in grouping them into high and low risk category.
- To determine the potential prognostic effect of ADAM17 to aid in differentiating indolent node-negative Breast Cancer from aggressive node-negative Breast Cancer.
CHAPTER TWO
MATERIAL AND METHODS

2.1 Patient population

50 Cases of cancer patients who have undergone radical mastectomy with no prior treatment, with ages ranging from 24 - 89 were obtained from Saint Anthony General Hospital, Porto Portugal together with an informed consent form of participation in the project. Patients involved were pathologically confirmed of the following node-negative and node-positive breast cancer: Invasive ductal carcinoma, In situ ductal carcinoma and Invasive lobular carcinoma with histological grades 1 to 3. There were 40 node-negative cases and 10 node-positive cases with tumor size ranging from 0.6 cm to 4.7 cm. In addition to ADAM17, HER2, Ki67, Progesterone and Estrogen receptors were also determined. The ADAM17 protein expression in breast cancer tissue samples were confirmed by immunohistochemistry analysis, which was performed on formalin-fixed, paraffin-embedded, 3 micro meters thick tissue sections using the avidin-biotin-peroxidase complex method.

2.2 Sample Processing

2.2.1 Microscopic examination

The tissue samples were collected as quickly as possible after the removal of the organ from the subjects (patients) and immediately fixed in 10% formalin. This was to preserve the cells and cellular constituents in a state as close as possible to the living cells and also maintains the antigenicity to allow them to be processed without change. Each sample was stratified according to the following: Age, size, weight, anatomical location, tumor relation to surgical margin and nodal status. They were then sectioned and placed on embedding cassette for tissue processing.

2.2.3 Tissue processing

The main steps in tissue processing are dehydration and clarification. In order to remove the fixative and water from the tissues, they were sent through graded alcohols of increasing concentrations (70% - 95% - 100%). The alcohol was then replaced by a
“clearing” solution, Xylene to subsequently aid in carrying out the impregnation with paraffin wax and was left overnight, since xylene is miscible with paraffin wax.

2.2.4 Impregnation and sectioning

The processed tissues in cassettes were properly aligned and oriented in paraffin at 58-60 degree Celsius. They were then allowed to cool to form paraffin blocks. With the help of a microtome, the tissue in the paraffin block were first trimmed to exposed the tissue of interest after which it was cut into thin sections of about 3 micro meters. The cut sectioned were placed in cold water to remove any creases that may exist to enable it stretch. They were then picked with a coated glass slide and placed again in warm water about 60 degrees Celsius for adhesion and extension onto the slides. The slides with the tissues were then put in a refrigerator under room temperature for 30 minutes.

2.2.5 Immunohistochemical staining for all the antibodies using avidin-biotin peroxidase complex protocol

2.2.5.1 Dewaxing and Hydration

Slides with tissues were de-waxed (removal of wax) by placing them in xylene twice for 5 minutes each. They were then hydrated in series of alcohol in decreasing concentration (100% - 90% - 75%) for at least 1 minute each and finally washed under running water.

2.2.5.2 Antigen retrieval

To increase the antigenicity of the tissues, 50ml of 10% Dako target retrieval solution was prepared by adding 45ml of water to 5ml of Dako target retrieval solution. 50ml solution is enough for 15 slides. The solution was then poured in plastic container after which the slides were added and waited for 5 minutes for the solution to be clear. The container with the slides were then put in water bath under 96-100 degrees Celsius for 20 minutes. The water bath was put off and the slides and container were removed and placed in cold water till it reached room temperature and finally placed in humid container to prevent drying up of the slides.
2.2.5.3 Endogenous peroxidase blocking

This process was carried out to halt the endogenous peroxidase activity. After the slides were cooled enough, a hydrophobic pen was used to mark around each slide to create tension and also to prevent reagent from spreading. Quickly, 3 drops, approximately 150 microliters of Novocastra Peroxidase block were put on each slide for 5 minutes and maintained in the humid container. After that, it was washed in TBS twice for 5 minutes each.

2.2.5.4 Protein Block

Once again, marks were created around samples on the slides with hydrophobic pen and placed in humid container and 3 drops of Novocastra Protein Block were placed on each for 5 minutes. This was done to block any unspecific antigen-antibody reaction that may lead to background staining.

2.2.5.5 Incubation of Primary Antibody

The Protein block reagent was quickly drained off after 5 minutes and 3 drops of primary antibody (monoclonal mouse anti-human Ki67 and goat polyclonal anti-ADAM17) diluted in 5% BSA.TBS at optimum concentration or dilution factor were placed on each slide. ER, PR and HER2 were determined by immunocytochemistry according techniques of routine used in Hospital Santo António. They were expressed with optimum dilution factor of 1:150. With Ki67 the optimum dilution factor was 1:100 and 1:300 for ADAM17. The slides were then incubated in the humid container overnight at 4 degrees Celsius. With the controls, no primary antibody was added.

<table>
<thead>
<tr>
<th>antibody</th>
<th>supplier</th>
<th>clone</th>
<th>dilution</th>
<th>HIER</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER</td>
<td>Dako</td>
<td>ID5</td>
<td>1:150</td>
<td>Water bath</td>
</tr>
<tr>
<td>PR</td>
<td>Dako</td>
<td>PgR 636</td>
<td>1:150</td>
<td>Water bath</td>
</tr>
<tr>
<td>HER2</td>
<td>Dako</td>
<td>-</td>
<td>1:150</td>
<td>Water bath</td>
</tr>
<tr>
<td>Ki67</td>
<td>Dako</td>
<td>MIB-5</td>
<td>1:100</td>
<td>Pressure cooker</td>
</tr>
<tr>
<td>ADAM17</td>
<td>Abcam</td>
<td>-</td>
<td>1:300</td>
<td>Water bath</td>
</tr>
</tbody>
</table>
2.2.5.6 Post/Secondary Antibody

In avidin-biotin-peroxidase complex protocol, secondary antibodies are needed to conjugate to biotin and function as a link between tissue-bound primary antibody and avidin-biotin-peroxidase complex. In other words, the secondary antibody is needed in detecting the primary antibody. The slides were washed twice in TBS for 5 minutes each. 150 microliter of Novocastra post primary antibody were placed on each slide for 30 minutes and spread carefully for even distribution. They were washed in TBS twice for 5 minutes each.

2.2.5.7 Polymerization (Avidin-Biotin)

Again, 150 microliters of Novolink Polymer was put on the slides and incubated in a humid container for 30 minutes. They were then washed with TBS twice for 5 minutes each. Polymerization is necessary in that it enable the polymer or the enzyme to first react with DAB to aid in optimum visualization.

2.2.5.8 Chromogen reaction

Chromogens are electron donors which upon being oxidized becomes insoluble colored product. An example is 3,3-diaminobenzidinetetrahydrochloride (DAB). 50 microliters of DAB for each 1000 microliters of substrate buffer were prepared in 15ml falcon tube. In aiding to visualize the results, 150ml of DAB solution were placed on each slides for few minutes or till reaction occurs. Slides were quickly placed in container with distilled water and placed under running water for 15 minutes. DAB, upon being oxidized produced brown end colour which aid in the visualization of tissue on slides.

2.2.5.9 Hematoxylin staining

For counterstain, slides were inserted in Hematoxylin for several times (6-8 times) depending on the concentration of the Hematoxylin and how frequently it has been used. They were again placed under running water for 15 minutes.
2.2.5.10 Dehydration and clearing

Slides were placed in 100% alcohol twice and moved up and down for several times in each case, to remove the water after which they were placed in xylene twice and also moved several times in each case.

2.2.5.11 Mounting of slides

Slides were removed from the xylene one after the other and were mounted with entellan. Each slide was pressed carefully after placing the cover slip to get rid of any bubbles present. Quickly, they were passed through xylene and cleaned after which they were allowed to dry for microscopic examination. In each case, the non-cancerous breast cancer tissues were used as control tissues and the omission of the primary antibody served as negative control.

The HER-2/neu was considered negative when scores of 0 and +1, and positive when scores of +2 and +3 were recorded. To be considered as +2, +3, the cellular membrane should be completely stained in more than 10% of the tumor cells. In the cases where a score 2 was found by IHC, it was further studied by FISH and classified as positive or negative according FISH results. Cells without staining, or with weak staining in part of the cell membrane and in less than 10% of the tumor cells were considered negative. In the case of ER and PR they were graded by considering 1% of tumor cells stained in the nucleus as negative and more than 10% of stained tumor cells as positive. With Ki67, scores were obtained by counting at least 100 cells (excluding mitotic cells) and taken the percentage of stained cells in the nucleus. The cells were counted at places where there were high proliferation of cells.

ADAM17 levels of expression were determined by using staining intensity. The staining intensity was evaluated using four-tier grading system (0- negative; 1-weak; 2-moderate and 3-strong). To delineate between low and high levels of ADAM17 expression, the tumors with strong ADAM17 expression were grouped against those with none to moderate ADAM17 expression.
2.2.6 Statistical Analysis of Results

Statistical significance was calculated using Fisher’s exact test to assess the relationships between ADAM17 protein expression and different clinical parameters through IBM SPSS Statists 20.0 software. This is due to the smaller sample size and the fact that some of the cells had values less than five. Significance was accepted at p<0.05.
CHAPTER THREE

RESULTS

Table 3.1: Clinicopathological features of 50 node-negative and node-positive breast cancer

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Number</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 50</td>
<td>22</td>
<td>44</td>
</tr>
<tr>
<td>&gt; 50</td>
<td>28</td>
<td>56</td>
</tr>
<tr>
<td><strong>Tumor size (cm)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 2</td>
<td>30</td>
<td>60</td>
</tr>
<tr>
<td>&gt; 2</td>
<td>20</td>
<td>40</td>
</tr>
<tr>
<td><strong>Histological grade (G)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade 1</td>
<td>9</td>
<td>18</td>
</tr>
<tr>
<td>Grade 2</td>
<td>23</td>
<td>46</td>
</tr>
<tr>
<td>Grade 3</td>
<td>18</td>
<td>36</td>
</tr>
<tr>
<td><strong>Nodal status</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>40</td>
<td>80</td>
</tr>
<tr>
<td>Positive</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td><strong>ER status</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>Positive</td>
<td>44</td>
<td>88</td>
</tr>
<tr>
<td><strong>PR status</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>9</td>
<td>18</td>
</tr>
<tr>
<td>Positive</td>
<td>41</td>
<td>82</td>
</tr>
</tbody>
</table>
In addition to ADAM17, we assessed the expression of other markers and parameters and later compared their significance differences and their impact. These are ER, PR, HER2 and Ki67 all of which will be discussed in detail later. In all, there were 44 ER positive and 6 ER negative cases, 41 PR positive and 9 PR negative cases, 2 HER2 positive and 48 HER2 negative cases, 37 Ki67 cases above 10% and 13 Ki67 below 10% as well as 5 triple negative cases. It can also be inferred from the table 1 under annex that majority of patients were above 50 years with 56% as compared to those below with 46%. We also observed in this studies, larger number of patients bearing IDC with few DCIS and ILC of which most of the patients are in ages above 50 years.

Also there were low lymph node involvement and many have small tumor size and there were more grade 2 tumors as compared to 1 and 3 all of which has been clearly indicated. In the case of ADAM17, we classified them in terms of staining intensity on a scale of 0 to 3, where 0 represent no expression, 1 representing low, 2 representing moderate and 3 representing strong expression. Virtually we differentiated among low and high expression of the protein by grouping strong expression against none to moderate. Based on that, we had 31 low and 19 high expression which could determine the low and high risk node-negative breast cancer patients in our study.

<table>
<thead>
<tr>
<th>HER2/neu status</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative (0)</td>
<td>45</td>
<td>90</td>
</tr>
<tr>
<td>Negative (+1)</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Positive (+2)</td>
<td>2</td>
<td>4</td>
</tr>
</tbody>
</table>

| Ki67 status (%) | |
|-----------------|---|---|
| ≤ 10            | 13 | 26 |
| > 10            | 37 | 74 |

| Histological type | |
|-------------------|---|---|
| IDC               | 32 | 64 |
| DCIS              | 7  | 14 |
| ILC               | 11 | 22 |
3.1 Expression of ER, PR, HER2 and Ki67

Once again from the results we obtained, we can observe that out 50 cases we examined, 6 turned out to be ER negative and 44 postive, corresponding to 12% and 88% respectively. The values ranges from 5% - 100% with 100% in more than half of the cases (Table annex). The ER-negative cases were mainly grade 1 and 3 tumors with sizes ranges from 0.8 -2.9 and all these 6 negative cases except, one occured in node-negative breast cancer patients.

We also observed 9 negative and 41 positive PR cases which corresponded to 18% and 82% respectively. (Table 3.1). As in the case of ER, all the 9 negative cases occured in node-negative except one and had the same range of tumor size as ER expression. (Table annex). Unlike ER, one negative case of PR occured in grade 2 tumor. Its degree of expression were the same as ER, 5% - 100% with 100% occuring in more than half of all the cases studied. There was also high “double positive” cases for ER/PR.

In the case of HER2, we found it to be negative in most of the cases observed with very few positives, with 48 negative cases, ( 45 (0) value and 3 (+1) value) and 2 positive cases ,corresponding to 96% and 4% respectively. Both two positive cases were +2 and occured in only node-postive and Invasive Ductal Carcinoma (IDC) patients with ages below 50 years.

With Ki67, 37 cases were above 10% whiles 13 were below or equal to 10%, indicating high expression in most of the cases. ER/PR were present in almost all the cases with few negative as well in for Ki67. There was also one case which was negative for both ER/PR as well as Ki67 but postive for HER2. We also observed 5 triple negative cases, that is negative for ER/PR and HER2 but was positive for Ki67.

We have also indicated results from some of the immunohistochemical staining for Ki67 below. (Fig 3.1-3.3)
Figure 3.1: Low Ki67 expression in immunohistochemical staining in an high grade ductal carcinoma in situ. (IHC-Mayer's Hematoxylin, 200x) where IHC stands for immunohistochemistry and Mayer's Hematoxylin is the counterstain)

Figure 3.2: Immunohistochemical staining of high Ki67 expression in node-negative Invasive ductal carcinoma. (IHC-Mayer's Hematoxylin, 200x), where IHC stands for immunohistochemistry and Mayer's Hematoxylin is the counterstain)
Figure 3.3: Immunohistochemical staining of low Ki67 expression in a low grade ductal carcinoma in situ. (IHC-Mayer's Hematoxylin, 200x), where IHC stands for immunohistochemistry and Mayer's Hematoxylin is the counterstain)
3.2 Expression of ADAM17 protein

The tissue-based protein expression of ADAM 17 was investigated by immunohistochemistry using Avidin-biotin peroxidase complex protocol and it has been indicated to be expressed in the tumor cells of all patients studied. They are normally found most commonly in the cytoplasm and less commonly at the cell membrane. The anti-ADAM 17 antibodies showed none to strong immunostaining with about 19 high (strong expression) cases and 31 low expression (none to moderate expression) cases in this study by grouping strong expressions against none to moderate expression. In both strong and low expression, there were lots of node-negative cases involved as compared to node-positive. This is due to greater number of node-negative as compared to positive used in our study. (Fig. 3.4 and table annex). We have also included in the results some of the Immunohistochemical staining (Fig 3.5-3.8) as well as graphical representation of the relationship of ADAM17 expression and some of the Clinicopathological features (Fig. 3.9-3.13).

Figure 3.4: Relative amount of ADAM17 protein expression in node-negative and node-positive breast cancer
Figure 3.5: Immunohistochemical staining of negative ADAM17 expression in “normal” breast tissue (IHC-Mayer's Hematoxylin, 100x), where IHC stands for immunohistochemistry and Mayer’s Hematoxylin is the counterstain.

Figure 3.6: Immunohistochemical staining of ADAM17 expression in invasive ductal carcinoma. Strong immunoreactivity in malignant cells in contrast with weak expression in normal luminal cells (arrow). (IHC-Mayer's Hematoxylin, 200x), where IHC stands for immunohistochemistry and Mayer's Hematoxylin is the counterstain.)
Figure 3.7: Strong immunohistochemical staining of high ADAM17 expression in a node negative Invasive ductal carcinoma with papillary pattern. (IHC-Mayer's Hematoxylin, 200x), where IHC stands for immunohistochemistry and Mayer's Hematoxylin is the counterstain.

Figure 3.8: Weak immunohistochemical staining of low ADAM17 expression in a node-positive Invasive ductal carcinoma. (IHC-Mayer's Hematoxylin, 200x), where IHC stands for immunohistochemistry and Mayer's Hematoxylin is the counterstain.)
Figure 3.9: Association of relative ADAM17 expression with ER status

From this figure, the frequency of the levels of ADAM17 expressed was greater in both low and high cases. In each case, ER positive has high amount of ADAM17 expressed as compared to ER negative. ER negative has the same frequency of the expression of ADM17 in both cases.
Figure 3.10: Association of ADAM17 expression with PR status

Like ER, PR has higher frequency of ADAM17 expression of positive status compared to negative PR status. However in both high and low expression status of ADM17, high frequency was recorded for low expression.

Figure 3.11: Association of ADAM17 with tumor grade

Grade 2 from this graph indicated a high level of ADAM17 expression in both low and high expression and like the other tumor features already explained above, the highest frequency was showed in low expression for both grade 1 and 2 but not so in grade 3, where the greatest expression was recorded in high ADAM17 expression as compared to its corresponding value in low expression.
Figure 3.12: Association of ADAM17 expression with Ki67 expression

Ki67 status followed the same trend with high frequency of ADAM17 in low expression compared to high ADAM17 expression. In total, there was high Ki67 expression from the 50 cancer cases studies (Table 3.1).
**Figure 3.13: Association of ADAM17 expression with histological breast cancer types.**

Greater number of IDC were recorded in high ADAM17 expression as compared to ILC and DCIS. Both DCIS and ILC recorded higher ADAM17 levels in low expression than high expression. In the case of ILC, all the ADAM17 levels were expressed only in the low or weak category.

**Table 3.2: Association of ADAM17 protein expression with standard prognostic factors**

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>No. of cases</th>
<th>ADAM17 expression (n, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low expression</td>
<td>High expression</td>
</tr>
<tr>
<td>≤ 50</td>
<td>22</td>
<td>14 (63.63)</td>
</tr>
<tr>
<td>&gt; 50</td>
<td>28</td>
<td>17 (60.71)</td>
</tr>
<tr>
<td><strong>Tumor size (cm)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 2</td>
<td>30</td>
<td>19 (63.33)</td>
</tr>
<tr>
<td>&gt; 2</td>
<td>20</td>
<td>12 (60.00)</td>
</tr>
<tr>
<td><strong>Nodal status</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>40</td>
<td>24 (60.00)</td>
</tr>
<tr>
<td>Positive</td>
<td>10</td>
<td>7 (70.00)</td>
</tr>
<tr>
<td><strong>Histological Grade</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade 1</td>
<td>9</td>
<td>6 (66.67)</td>
</tr>
<tr>
<td>Grade 2</td>
<td>23</td>
<td>17 (73.91)</td>
</tr>
<tr>
<td>Grade 3</td>
<td>18</td>
<td>8 (44.44)</td>
</tr>
</tbody>
</table>
From table 3.2, the frequency of low (negative) expression of ADAM17 in the entire tumor characteristics were greater than higher (positive) expression, except in grade 3 and IDC, where there was higher frequency of high expression as compared to its corresponding low expression. There was also high expression of ADAM17 in patients with age above 50 years, compared to below 50 years, whereas smaller sizes below 2 cm expressed high ADAM17 as compared sizes above 2cm.

Statistical analysis was carried out between ADAM17 and Ki67; HER2; ER; tumor grade and nodal status. The results obtained showed no significance difference between any of them with P value of each greater than 0.005. However, we found significance difference between tumor grade 1, 2 and 3, and Ki67 expression (Table 3.3). Also there were significance difference between ADAM17 expression and tumor histological type (Table 3.4)
Table 3.3. Association of Ki67 expression with tumor grade

<table>
<thead>
<tr>
<th>Grade</th>
<th>Ki67 ≤ 10%</th>
<th>Ki67 &gt; 10</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>07</td>
<td>02</td>
<td>0.015</td>
</tr>
<tr>
<td>2</td>
<td>06</td>
<td>17</td>
<td>0.027</td>
</tr>
<tr>
<td>3</td>
<td>00</td>
<td>18</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Table 3.4 Association of ADAM17 expression and tumor histological type

<table>
<thead>
<tr>
<th>Tumor Histological type</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IDC VS DCIS</td>
<td>0.009</td>
</tr>
<tr>
<td>IDC VS ILC</td>
<td>0.011</td>
</tr>
<tr>
<td>ILC VS DCIS</td>
<td>1.000</td>
</tr>
</tbody>
</table>
CHAPTER FOUR

DISCUSSION AND CONCLUSION

4.1 Expression of ER, PR, HER2 and Ki67

Progress in the molecular understanding of breast cancer and the development of sophisticated diagnostic tools has led to the availability of several prognostic tools as well that aid in the determination of the outcome or the progression of individual tumors.

Breast cancer prognostic factors are essential to identify patients at risk of distant metastasis development and to decide whether adjuvant treatments are needed or not. The most validated biological marker in non-metastatic breast cancer are tumor size, histological grade, mitotic index, Ki67 rate, axillary lymph node involvement, Estrogen Receptor (ER), Progesterone Receptor (PR) and HER2 status. However, several review articles have addressed their potential uses and as well as some drawbacks. Locoregional treatment is mostly used in treating patients with lymph node-negative breast cancers, of which about 70% respond positively, however 30% relapse.

Aside these markers, many other molecules have also been assessed, including proliferation antigen Ki-67. Since its identification as a nuclear antigen associated with cell proliferation in 1983 (Gerdes et al., 1983), it has been studied with considerable enthusiasm. In breast cancer, ER/PR, HER2, and Ki-67 are important biological markers for predicting prognosis and making effective treatment decisions. Treatment decisions for breast cancer are commonly made based on the information derived from the immunohistochemistry (IHC) of these biological markers. For example, the application of chemotherapy is commonly used for patients with high Ki-67 values, endocrine therapy for ER/PR positive, and anti-HER2 (trastuzumab) therapy for HER2 positive. However, chemotherapy is once again recommended for patients with high Ki67, ER-positive and HER2 negative by St. Gallen consensus recommendation. (1999 and 2011)
4.1.1 ER/PR expression

Estrogen receptors (ER) and/or progesterone receptors (PR) presence is currently a component of routine evaluation of breast cancer specimens. ER was the first analyzed in breast cancer in the late 1950s, and was the first molecular marker evaluated for prognosis and therapy response for breast cancer. The status of ER has been shown to have significant predictive value on tumor response to hormone therapy in metastatic disease as well as for adjuvant therapy after local excision (Dowsett M et al., 2008). On the other hand, the role of PR status in predicting tumor response to therapy is still unclear, although it has shown promise. From our current study, most of the cases were positive for both ER and PR, corresponding to 88% and 82% respectively which have been confirmed with other studies.

This finding can indicate a better response to hormonal therapy in those patients with ‘double-positive’ ER/PR and clinicians will not hesitate in subjecting these patients to hormonal therapy for better response. This has been confirmed by another studies where it was reported that a ‘double-positive’ ER+/PR+ tumor responded better to hormonal therapy than ‘single-positive’ ER+/PR− or ER−/PR+ tumors (Dowsett M et al., 2008). In addition to their predictive value, as to whether they also have prognostic value has been extensively studied, but remains under debate.

Estrogens are generated by women to boost the development and maintenance of female attributes and they are necessary for the sexual organs, really important during pregnancy. Among the many parts of the women body that are the targets of the estrogens, are breasts and uterus and they bind to inner parts of the cells called estrogen receptors. In most of the cases this binding process leads to the proliferation state of the cell which is normal physiological state but can lead to breast or cancer of the uterus in most cases. We noticed that more than half of the cases were 100% ER positive even in early stage node-negative grade 1 tumors. This can be attributed to the large amount of estrogen already or naturally present in breast which binds to their receptors for normal physiological process.
4.1.2 HER2 expression

Another major prognostic marker that is currently recommended for the evaluation of primary invasive breast cancer is the human epidermal growth factor receptor 2, also known as HER2. HER2 is an oncogene belonging to the EGF receptor (EGFR) family. Gene amplification of HER2 has been shown to occur in 10–40% of primary tumors and HER2 protein overexpression is found in almost 25% of breast cancers conferring a more aggressive biology and have been shown to be associated with worse overall survival and twice the mortality rate compared with women with no HER2 expression. (Paikn et al., 1990; Van de Vijver et al., 1980). Our results obtained was not in consistent with many studies on HER2, which records higher percentage of its presence. In our study, we recorded more negative than positive expression of HER2 (98% negative and 2% positive). However, all the positive expression occured in only node-positive which could mean that they are mostly or normally expressed in advanced breast cancer cases or lymph node metastatic stages. Most of the studies carried on HER2 have been seen on advanced or distant metastasis. Also one contributing factor to higher negative result could be the small sample size of our studies.

The HER tyrosine kinases are activated by a number of ligands that are initially synthesized as transmembrane precursors and these ligands can be released from their precursor forms by specific ADAMs. Knockout studies with the use of mouse embryonic cells showed that ADAM-17 was the major sheddase for TGF-a, amphiregulin, HB-EGF, and epieregulin (Sahin et al., 2004; Borrell-Pages et al., 2003), which are all ligands for HER2. Once again, this report seem not to be in consitent with our results. It is expected that at least there would be more postive HER2 cases where there was positive or high expression of ADAM17, but we recorded only one such case.

Instead most of the high ADAM17 expression corresponded to negative HER2 expression. This could suggest a possible alternative pathway by which ADAM17 causes breast cancer initiation, progression and invasion in addtion to the already or commonly known release of ligands for EGFR/HER2. This can be confirmed by other studies reporting ADAM17 to have act on a large group of cell adhesion molecules, resulting in weakening of cell-cell interactions as well as cleavage and activation of certain receptors (Arribas et al., 2006).
4.1.3 Ki67 expression

Ki–67 was first discovered by J. Gerdes et al in 1983 suggesting its use for marking proliferating cells and it is expressed in the nucleus during cell cycle. Cancer cells are well known because of their multiplying behaviour that characterizes the disease. For this reason putting the Ki–67 antibody to work produces a reaction, obtaining the desired nuclear staining. This method is used by oncologists for diagnosing breast cancer, but it is not enough to conclude that cancer is present or not. Parallel tests have to be carried out for hormone receptors, metastasis and HER2-neu to prescribe the suitable treatment. Ki–67 is present in normal breast tissue but in a very low percentage, a figure that is notably higher when cancer is present. Furthermore, high percentage of Ki–67 is synonym of poor prognosis, which has been proven to have good response to chemotherapy treatment. For example several authors measured Ki67 in tamoxifen-treated breast cancer patients, all reporting a decrease in Ki67 (Johnston SR et al., 1994; Makris A et al., 1998).

In confirmation to this assertion, our study indicated a high expression of Ki67 in 74% of the cases which is in accordance with several research work of Ki67 expression in breast cancer. The expression was even high in most of ADAM17 negative expression. According to St Gallen consensus, patients with high Ki67 expression and ER positive are recommended to be given chemotherapy, which means if the parameters used in their recommendation is to be followed, about 74% of the patients in this study may be given chemotherapy, which may not be necessary and they can be spared form the toxic effect of adjuvant chemotherapy. We also recorded only one negative case which corresponded to high ADAM17 expression and this can be a good candidate with the treatment of ADAM17 inhibitor or chemotherapy since it was also negative for both ER and PR.

There was significance difference between Ki67 and tumor grade 1, 2 and 3 with P values 0.015, 0.027 and 0.000 respectively, showing a strong correlation between tumor grade and Ki67. Triple negative cancers are very aggressive and difficult to treat since they do not respond to HER2, ER or PR target treatment. In our study all the triple negative cases has a corresponding higher expression of Ki67 which are good candidate for chemotherapy according to St Gallen consensus recommendation. It also corresponded to both high and low ADAM17 expression which could also means that inhibitors of ADAM17 can be given for treatment, especially those who expressed high levels, aside chemotherapy.
4.2 ADAM17 protein expression

ADAM family has been demonstrated to be involved in the process of proteolytic ‘shedding’ of membrane associated proteins and hence the rapid modulation of key cell signaling pathways in the tumor microenvironment (Arribas J et al., 2006; Duffy MJ et al., 2009). Through the cytoplasmic domain, they influence downstream signalling cascade, especially EGFR through processing of EGFR-ligands. And this is of particular interest since EGFR is a well established drug target for breast cancer (Ohtsu H et al., 2006). It was established before that ADAM17, an important member of the ADAMs family, is the most extensively studied ADAM molecule. It sheds a variety of important cell surface molecules, including cytokines, growth factors, and adhesion molecules.

Among those genes that are up-regulated in breast cancer, the epidermal growth factor receptor genes have achieved considerable attention. While gene amplification rarely exceeds eight-fold, mRNA and protein expression can be up to 120-fold higher than normal levels. They play key roles in tumor angiogenesis, invasion, and metastatic potential as well as resistance to anti-endocrine, chemo- and radiotherapy. ADAM17 is involved in cell proliferation and invasion by releasing several ligands and overexpressed in cancers of the breast, ovary, kidney, colon, and prostate. Because of its ability to release biologically important ligands such as TNF-a, transforming growth factor-a, amphiregulin, heparin-binding EGF, and epiregulin, for these receptors, it might be expected to play an important role in cancer progression.

In our study, there was expression of ADAM17 protein in both node-negative and positive breast cancer, (Figure 3.4) which was verified by immunohistochemistry analysis. The expression was clearly upregulated or high in some of the node-negative as well as node-positive breast cancer cases and as well showed low expression in both. The evidence of low expression in some of the node-negative cases through to high expression in some of the node-positive cases may suggest that, ADAM17 expression in breast cancer may play an important role in the process of tumor initiation and dissemination of tumor cells to lymph nodes and distant organs. In general, there was higher number of low expression as compared to higher expression of ADAM17 and also higher expression occurred in more node-negative than node-positive. The high frequency of low expression in general and also in node-positive contradict with other studies where almost all the breast cancer cases under study show significant upregulation. (McGowan Pm et al., 2008). This could be due to the fact that, our study was primarily aimed at node-negative or early breast cancer cases and not advanced, and we included lot of node-negative as compared to node-positive, just for comparing their differential expression.
Most of the reports we have read on ADAM17 expression in breast cancer has to do with metastatic stage or advanced stage.

The number of low expression was more than its corresponding high expression in each of the clinicopathological features examined, except in the case of IDC and grade 3 tumors where it was otherwise. More than half of the cases with high expression occurred in grade 3 tumors. The low and high expression observed in all the clinico-pathological features, which could indicate high and low risk individual gave an indication that, those parameters alone will not help in selecting individuals for adjuvant chemotherapy. We also observed high or upregulated expression in tumor cells than their adjacent non-cancerous cells which is in consistent with several work carried on ADAM17 in breast and other cancers. It was established before that ADAM family members could be differentially expressed between mammal and pathological mammary gland, but their pattern of expression and intimate mechanism of action are not yet precisely established (McGowan et al., 2008; Roy R et al., 2004).

For instance in a study performed by Lendeckel U et al., (Lendeckel U et al., 2005) on 24 breast cancer specimen and corresponding non-cancerous tissue, mRNA expression of ADAMs 9,12 and 17 were increased in cancerous tissue compared to normal adjacent tissues. Again, it was reported that, in a study on ADAM expression in breast cancer tissue, there was an elevated expression ADAM 17 (Borrell-Pages et al. 2003) in breast carcinomas compared with non-neoplastic or adjacent healthy tissue, which could be confirmed by this study. Once again, another study on ADAM17 expression pattern, observed in gastric normal mucosa and cancers, reported significantly higher expression in cancer than in normal tissue (Yoshimura T et al., 2002).

The high expression we observed are also in accordance with many other published reports, (McGowan PM et al., 2008; Lendeckel U et al., 2005). In human prostate cancer cells, ectopic overexpression of ADAM17 resulted in increased cell proliferation, and ADAM17 promoted G1- to S-phase transition concomitantly with upregulation of cyclin E, CDK2, and downregulation of p21 and p27 proteins (Lin P et al., 2011). It has been shown that ADAM17-mediated EGFR ligand cleavage enhances the proliferation and survival of squamous cell carcinoma cells as well as lung cancer cells (Baumgart A et al., 2010). In addition, ADAM17 promotes the breast cancer cell’s malignant phenotype by increased proliferation, invasion, and angiogenesis. It contributes to breast cancer progression through the activation of the EGFRPI3K-AKT signal pathway (McGowan PM et al., 2007).
We found significantly higher concentration of ADAM17 expressed in grade 3 than 1 and 2 as well as higher concentration in ductal compared to lobular. (figure 3.11 and 3.13). In the case of high ADAM17 expression, the grades were expressed in increasing order, with grade 1 having the lowest, followed by grade 2 and grade 3. This wasn't so in the low expression of ADAM17, where grade 2 had the highest expression, followed by 1 and the 3. This confirms ADAM17 expression having correlation with tumor grade. It could also mean that, though a its expression occurs at the earliest state of a disease, normally expressed at the later or high stage. In More expression in ductal than lobular may suggest that ADAM17 may have a different role in the formation of two most frequently observed histological type of breast cancer, with its expression more associated with the most common breast cancer type. This finding was in accordance with published study by McGwan PM et al (McGwan PM et al., 2008). The high expression of ADAM17 in Invasive ductal carcinoma and in grade 3 tumors, may indicate that, it is normally expressed in advance breast cancer cases and also indicate or confirm its involvement in tumor initiation and progression.

There was also high expression in ER, PR, positive and Ki67 high expression cases as compared to their corresponding negative and positive cases respectively, but was higher in negative HER2 expression. The high expression of ADAM17 in the positive cases in these markers could confirm the aggressiveness of patients diagnosed positive or having high expression of these markers. (Figure 3.9, 3.10 and 3.12). Also in figure 3.12, we observed that, some of the high Ki67 expression corresponded to low levels of ADAM17. Since Ki67 is well known in its determination of cell proliferation, it could be due to the fact that, ADAM17 uses other alternative pathway in tumor initiation and progression as explained in the case of HER2 apart from cell proliferation. Notwithstanding, there was high frequency of high Ki67 expression that corresponded to high ADAM17 expression, confirming the ki67 as a proliferative marker. But then, according to St Gallen consensus, chemotherapy is recommended for ER/PR postive and high Ki67 expression. If we are to follow this recommendation, more than half of the our cases will have to be given adjuvant chemotherapy. From our data almost all of the patients that showed these features had low ADAM17 expression also, which could mean that not all of them may necessarily need chemotherapy since they may be classified as low risk due to their low expression of ADAM17. On the other hand, some of the patients with low Ki67 and negative ER/PR actually expressed high protein of ADAM17, these people may also be classified as high risk due to their high expression of ADAM17 and they may benefit from adjuvant chemotherapy.
In analyzing the relationship between ADAM17 expression and clinicopathological parameters of breast cancer, our results obtained in agreement to study published by McGowan et al (McGowan et al., 2008) showed that expression level was independent of all the conventional prognostic factors, that is age, tumor size, ER, PR, Ki67, HER2 and grade nodal status. However there was a significance difference between ADAM17 and histological type. This suggest ADAM17 may be an independent prognostic factor.

A number of studies have indicated that high expression of ADAM17 was associated with poor outcome. For example using both ELISA and western blotting, McGowan et al, Kenny and Bissell, showed that elevated levels of ADAM-17 predict poor outcome in patients with breast cancer. Also patients with high expression had a significantly shorter survival compared with low expression. Evidence of ADAM17 as a potential prognostic marker as well as predictive of therapy, confirming the results of our studies and its importance have been shown in chapter one in various cancers.

As well as predicting patient outcome, ADAM-17 is also a potential marker of therapy outcome and a target for anticancer therapy. As a therapy predictive marker, ADAM-17 may have a role in identifying patients likely to be resistant to therapies directed against EGFR and HER-2, (tyrosine kinase inhibitors and trastuzumab respectively, due to excess formation of ligands that activate EGFR and promote heterodimerization with HER-2. Thus, in a recent study in an animal model, trastuzumab-resistant human breast cancer cells were found to express a number of EGRF ligands such as TGF-a, HB-EGF and EGF with specific reference to TGF-a (Hynes NE and Lane HA, 2005). Similarly, excess production of TGF-a as well as amphiregulin, has been shown to confer resistance to gefitinib in lung cancer (Kakiuchi S et al., 2004,2005).

ADAM-17 may also be a target for anticancer treatment. Recently, a number of relatively selective inhibitors have been described for this enzyme (Moss MI and Bartsch JW, 2004). One of these, known as INCB3619 (Incyte, Wilmington, MA), blocked tumor cell proliferation and was synergistic with existing drugs in inhibiting tumor growth in model system. An ADAM inhibitor related to INCB3619, (INCB7839) is currently in clinical trials against a number of cancers, including breast cancer. In this context, it is worth noting that etanercept, a recombinant human soluble p75 TNF receptor that inhibits TNF-a biological activity, has been evaluated in clinical trials for the treatment of advanced breast and ovarian cancer (Madhusudan S et al., 2004;2005).

ADAM17 levels were also found to be significantly associated with those of uPA. uPA is a serine protease causally involved in invasion and metastasis and is one of the most potent biological prognostic factors thus far described for breast cancer (Duffy MJ,
Recently, its prognostic effect in lymph node–negative breast cancer patients was validated in both a prospective randomized trial and a pooled analysis. The correlation between ADAM-17 levels and uPA in the breast cancers could result from ADAM-17–mediated shedding of ligands that induce expression of uPA such as amphiregulin and TNF-a (Yang WL et al., 2004). This could confirm ADAM17 as a potential prognostic marker.

4.3 OVERALL DISCUSSION

In this study, our main focus was on the expression of ADAM17 in node-negative breast cancer and its ability to identify individuals who may be classified as high or low risk of breast cancer. We correlated its impact on other clinicopathological features of breast cancer and in that essence we engaged other conventional prognostic markers such ER, PR, HER2 and Ki67 expression.

From our study population, most of the patients have smaller size of tumor with ages above 50 years. Grade 2 patients appeared to outnumber both grade 1 and 3, which call for proper attention on those patients. For instance, using grade as a marker, it is very easy to classify grade 1 as low stage of the disease and grade 3 as high and clinicians will find it quiet easy selecting patients for treatment. The most difficult population will be grade 2 since they are heterogenous which need other markers to differentiate this population into low and high risk, which was one of the motivation behind this study.

In dealing with grade 2 cancer patients for instance, other markers that are able to differentiate them are urgently needed and some of these markers that has been validated include Ki67 rate, axillary lymph node involvement, Estrogen Receptor (ER), Progesterone Receptor (PR) and HER2 status. These markers have also been employed based on the St Gallen consensus in delivering adjuvant chemotherapy in node-negative patients. However, about only 30% of node-negative patients relapse after loco-regional treatment and may need adjuvant chemotherapy. Several review articles have addressed the potential uses of these markers as well as their drawbacks. This assertion forms the bases of our present study. We were able to differentiate among grade 2 patients into high and low ADAM17 expression which could help in allocating them into low and high risk.
We recorded 6 high cases and 17 low expressions in grade 2 patients. Also from the results we obtained, high and positive expression of ER and PR were seen in most of the cases. These patients can be said to respond to hormone target therapy whiles the high cases of HER2 negative may mean those patients will not benefit from EGFR target therapy.

Considering the main focus of our study, impact of ADAM17 as a potential prognostic factor, all the results obtained was confirmed with other established studies. We recorded higher number of low expression than high expression which could be attributed to the fact that most of the cases we involved were node-negative breast cancer which were in the early stage of the disease process. Both low and high expressions were found in node-negative and node-positive and moreover a great number of established studies have also indicated upregulation or overexpression of ADAM17 in breast cancer in advanced cases. This established result confirms that ADAM17 is involved or contributes in the initiation, progression and invasion in breast cancer. We also recorded high expression in grade 3 than 2 and 1 and also in IDC as compared to ILC and DCIS, indicating its expression at later stage of breast cancer and commonly expressed in the most common histological type.

From our results, grouping those with strong expression levels of ADAM17 against those with none to moderate (low expression), we obtained two groups of breast cancer patients, low and high ADAM17 patients. We had 19 high expression and 31 low expression, which could lead to high and low risk due to ADAM17 involvement in breast cancer initiation, progression and distant metastasis. We saw that almost 80% of the cases will be recommended for adjuvant chemotherapy based on St Gallen recommendation on treatment selection. A large number of patients with ER/PR positive and high Ki67 expression corresponded to low expression of ADAM17, which means they may be spared from the toxic effect of adjuvant chemotherapy. On the other hand, some patients with negative ER/PR and low Ki67 also expressed high levels of ADAM17, and these patients could be among the 30% of the people that may actually benefit from chemotherapy.

Some of the positive expression of ADAM17 corresponded to negative HER2 status, which could be that, those tumors were not EGFR signalling. This is because, since ADAM17 has been reported of shedding ligands for HER2, we were actually expecting to see positive expression of ADAM17 corresponding to positive HER2 status. It could use other alternative way such as activation of certain receptors and shedding adhesion molecules.
Statistical analysis carried on ADAM17 with conventional prognostic markers did not correlate, except in the case of histological type, suggesting that ADAM17 may be an independent prognostic marker. ADAM17 has been found highly expressed in several cancers such as gastric cancer, prostate cancer, ovarian cancer, breast cancer indicating that its expression may be a common event in human malignant. With its involvement in signalling cascades, ADAM17, its a good candidate in designing target therapy against cancers involving EGFR/HER2 signalings. Selective inhibition of ADAM-17 could prevent the release of multiple ligands potentially important in promoting tumor growth. In recent years, a number of selective inhibitors of ADAM-17 have been described some of which have been shown to reduce tumor growth in model systems.

From our study, ADAM17 prognostic effect was determined by the low and high expression levels using the staining intensity obtained by immunohistochemical staining. This enables us to suggest those with low expression as low risk individuals and high expression as high risk individual, considering the results of other studies giving evidence of ADAM17 upregulations in breast cancer and its high expression corresponding to poor disease outcome as well as poor less patient survival and vise versa.

4.4 CONCLUSION AND FUTURE DIRECTIONS

In summary, our study provides further evidence that ADAM17 protein is involved or upregulated in human breast cancer initiation, progression and distant metastasis, indicating a putative pathophysiological role in breast cancer biology. It may directly influence downstream signalling cascades of the EGFR through processing of EGFR-ligands. This is of particular interest, as the EGFR is a well-established drug target for breast cancer. Though we were not able to carry our further investigation from our current study to prove ADAM17 prognostic impact on individual patients, relating our results with a study where its levels of expression were significantly associated with uPA, and also with other various published works with its upregulation in other tumors, as well as with poor prognosis in high expression cases, it could be confirmed that, ADAM17 may be a risk assessment marker, capable of differentiating aggressive node-negative breast cancer patients from indolent ones. Thus we suggested that those with high expression may have high risk of the disease and vise versa.
Based on that assertion and from our results, we were able to obtain two groups of patients based on their levels of expression. That is individuals with high levels of ADAM17 who may be at high risk and may also benefit from adjuvant chemotherapy as well as those at low risk who may be spared from the toxic effect of adjuvant chemotherapy, and as well as differentiated among grade 2, (a heterogenous group) patient. As a result, we had 31 cases with low expression of which we classified them as low risk and 19 high expression as high risk patients. If our results are confirmed further using in vivo experiments with humans, ADAM-17 could be a new target for the treatment of breast cancer and as well a very potent prognostic marker capable of differentiating aggressive node-negative from indolent ones. Finally, our results provide an impetus for further work on ADAM-17 inhibitors for the treatment of cancer.

Like some reports on prognostic markers in cancer, this study has a number of limitations. These include its retrospective nature and the fact that the samples investigated were very small. Other limitations in our study include our inability to expressed ADAM17 quantitatively and also in its two forms (soluble and secreted forms). Therefore in future, it would be necessary to increase sample size to enable stronger associations to be observed. This will also help in carrying multivariate analysis instead of doing only univariate, as we did. Due to the retrospective nature of our study, we could not carry out survival analysis and it is our aim to carry on an prospective studies where we can actually assess the impact of ADAM17 on patients survival after treatment. Also in future studies, we aim at expressing all ADAMs involved in breast cancer and correlate their impact and also assess ADAM17 expression as a potential biomarker for predictive therapy outcome. Once again, we also seek to set a reference point of ADAM17 expression by which we can confidently group patients into high risk or low risk by expressing it quantitatively.

Nevertheless, this is one of the few works already established to assess the prognostic impact of ADAM17 expression, specifically in node-negative breast cancer and also able to differentiate among the heterogenous group grade 2 patients as well. Our results further confirm the importance of this kind of studies and the need for more of such studies to help in treatment selection especially among node-negative breast cancer patients.
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Lee SB, Schramme A, Doberstein K et al. (2010): ADAM10 is upregulated in melanoma metastasis compared with primary melanoma. *Journal of Investigative Dermatology.* 130, 763–773.


## ANNEX

Detail description of tumor characteristics and expression of ER, PR, HER2, Ki67 and ADAM17

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<th>Node status</th>
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<th>PR/%</th>
<th>HER2 status</th>
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