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Zymographic Detection and Clinical Correlations of Cysteine Cathepsin and Matrix Metalloproteinase in Human Breast Cancer Tissue

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This is to certify that the thesis prepared by Solomon Tsegaye entitled: Zymographic detection and clinical correlations of cysteine cathepsins and matrix metalloproteinase in human breast cancer tissue in Addis Ababa public Hospitals, 2015 and Submitted in the Partial Fulfillment of the Requirements for the Degree of Master of Science (Medical Biochemistry) complies with regulation of the university and meets the accepted standards with respect to originality and quality.

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Declaration

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Abbreviations and acronym

BC	Breast cancer
BCA	Bicinchonic acid assay
CTSK	Cathepsin K
CTSL	Cathepsin L
DNA	Deoxy ribonucleic acid
ELISA	Enzyme Linked Immunosorbent Assay
FDA	Federal Drug Association
MMPs	Matrix Metalloproteinase
MRI	Magnetic Resonance Imaging
PAGE	polyacrylamide gel electrophoresis
PET	positron emission tomography
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate – polyacrylamide gel electrophoresis
SNP	Single nucleotide polymorphism
WHO	World Health Organization

List of Tables

Table 1: Breast cancer lump noticed for first time in breast cancer patients 31
Table 2: Physical finding at the time of diagnosis in breast cancer patients 32
Table 3: Subject characteristics. 33
Table 4: Mean, standard deviation and correlation of MMPs and cathepsins between normal and tumor breast tissue specimens. 36
Table 5: Significantly increased activity of cathepsin and MMPs in mothers. 38
Table 6: Non-significant activity change of cathepsin and MMPs in mothers. 39
Table 7: Increased activity of cathepsin and MMP-9 in late menarche. 39
Table 8: increased activity cathepsins and MMPs in early menarche. 40

List of Figures

Figure 1: The three-dimensional structure of cathepsin K (Novinec and Lenarčič, 2013)..... 12
Figure 2: Schematic representation of the domain structure and potential roles of MMPs..... 16
Figure 3: Age distribution of breast cancer patients.. 30
Figure 4: Cathepsin zymogram of tissue specimens in breast cancer..... 34
Figure 5: Matrix metalloproteinases zymogram in breast cancer tissue specimens.. 35
Figure 6: Analysis of densitometrically quantified cathepsin and MMPs activity within age group in breast cancer. 37
Figure 8: Pre-menopausal breast cancer patients showed increased activity cathepsin and MMPs. 41
Figure 9: Post-menopausal women with breast cancer showed increased activity of cathepsin and MMPs..... 42
Figure 10: Analysis of cathepsin and MMPs between premenopausal and post-menopausal. 43
Figure 11: Parity does not affect cathepsin and MMPs activity.. 44
Figure 12: CTSK and CTSL activity detection between normal and tumor..... 45
Figure 13: Pro- and mature MMP-9 and MMP-2 activity. 45
Figure 14: Tumor size-specific differences of cathepsin K and cathepsin L activity..... 46
Figure 15: Tumor size-specific difference of latent and mature MMP-9 and MMP-2..... 47
Figure 16: Stage-specific differences in cathepsins K and L..... 48
Figure 17: Stage-specific differences MMPs in human breast cancer patients.. 49

Abstract

Breast cancer is the most frequent cause of cancer death in women in less developed countries including Ethiopia. Cellular proteases thought to increase the likelihood of cancer invasion and metastasis by degrading components of extracellular matrix. These proteases could be used as tumor markers for early diagnosis, monitoring the prognosis or targeting therapeutics drugs.

***Objective**: To investigate zymographic detection and clinical correlations of cysteine cathepsins and matrix metalloproteinase in human breast cancer tissue*

***Methodology:** Hospital based cross-sectional study was conducted from January 2015 to June 2015. Thirty six women with breast cancer, who underwent mastectomy for first time were recruited from surgery department of Menelik II Hospital, Saint Paul's Millennium Medical College and Zewditu Memorial Hospital. Both tumor and normal tissues were harvested from the same patient within 10 minute after surgery. The tissue was processed and metastatic protease activity was evaluated by measuring functional enzymatic activity of cathepsin K, cathepsin L, and matrix metalloproteinase-2 and matrix metalloproteinase-9 using zymography and quantified by densitometry.*

***Result:** Normal and tumor tissue specimens were tested for functional cathepsins and matrix metalloproteinases activities. Mean cathepsin K activity was significantly higher in tumor tissue specimens than the activity detected in normal breast tissue specimens ($n = 36$, $p < 0.001$), mean cathepsin L activity was also significantly higher in tumor tissue than normal tissue specimens ($n = 36$, $P < 0.001$). Furthermore, mean matrix metalloproteinase-2 and -9 activities in tumor breast tissue was significantly higher in tumor tissue than normal tissue ($P < 0.05$).*

***Conclusion:** Our result showed different pattern of protease activity expression between normal and tumor tissue using zymography. It shows increased protease activity in tumor tissue compared to normal tissue sample. Therefore, tissue proteases could be used together with histopathological technique to discriminate the putative subgroup of patients within the same clinical category.*

***Key words**: Breast cancer, cathepsin K, cathepsin L, matrix metalloproteinase-9, matrix metalloproteinase-2*

Table of Contents

Acknowledgment 1
Abbreviations and acronym 3
List of Tables 4
List of Figures 5
Abstract 6
1. INTRODUCTION 1
1.2. Literature Review 3
1.2.1. Breast Cancer Epidemiology 3
1.2.2. Pathogenesis 3
1.2.3. Diagnosis of Breast Cancer 6
1.2.4. Proteases 7
1.2.5. Biomarker 17
1.2.6. Zymography 18
1.3. Statement of the Problem 20
1.4. Significance of the Study 21
1.5. Hypothesis 22
2. OBJECTIVE 23
2.1. General Objective 23
2.2. Specific Objectives 23
3. MATERIALS AND METHODS 24
3.1. Study Area and Period 24
3.2. Study Design 24
3.3. Study Population 24
3.3.1. Source Population 24
3.3.2. Sample Population 24
3.4. Sample Size 24
3.5. Sampling technique 24
3.6. Inclusion and Exclusion Criteria 25
3.7. Variable 25
3.8. Data and Sample Collection Procedures 26

3.8.1. Questionnaire 26
3.8.2. Sample collection 26
3.8.3. Data collector 27
3.9. Multiplex Zymography Protocol 27
3.10. Quantitation of Gel 28
3.11. Data quality management 28
3.12. Data Analysis and Interpretation 28
3.13. Ethical Consideration 29
3.14. Operational definition 29
3.15. Dissemination of Result 29
4. RESULT 30
4.1. Demographic Characteristics of the Study Participants 30
4.2. Clinical Characteristics of the Study Participants 31
4.3. Physical Finding during Hospital Visit 32
4.4. Clinical and pathological results 32
4.5. Cathepsin K and cathepsin L expression 34
4.6. MMP-2 and MMP-9 expression 35
4.7. Activities profile of cathepsin and MMPs between normal and tumor breast tissue specimens 36
4.8. Tumor size specific difference of cathepsins and MMPs in breast cancer 46
4.9. Stage-specific difference in cathepsins K and L in human breast cancer 47
4.10. Stage-specific difference in MMPs enzymatic activity in human breast cancer 48
5. DISCUSSION 51
6. CONCLUSION 56
7. Limitation of the Study 57
8. RECOMMENDATION 58
9. REFERENCE 59
Annex I: Material 63
Annex III: Total protein quantification (BCA assay) 67
Annex IV: consent form English version 68
Annex V: Consent form Amharic version 69
Annex V: Questionnaire 71

1. INTRODUCTION

Cancers represents group of unprecedentedly heterogeneous diseases that affect humans with high frequency and contribute in significant manner to overall morbidity and mortality. Globally the prevalence and incidence of cancer is increasing at an alarming rate at the current time. Around 14.1 million affected by cancer and 8.2 million people died as a result of cancer and cancer related complication in 2012 (Ferlay *et al.*, 2015). Cancer is a major public health problem in years to come unless we deter the prognosis by early screening and diagnosis which lead a way for prevention.

Breast cancer is the second most common cancer in the world and, by far the most frequent cancer among women with an estimated 1.67 million new cancer cases diagnosed in 2012 (25% of all cancers). A slight majority of cases occur in women in less developed regions. Incidence rates vary nearly fourfold across the world regions, with rates ranging from 27 per 100,000 in Middle Africa and Eastern Asia to 96 in Western Europe. Breast cancer ranks as the fifth cause of death from cancer overall (522,000 deaths) and while it is the most frequent cause of cancer death in women in less developed regions (324,000 deaths, 14.3% of total), it is now the second cause of cancer death in more developed regions (198,000 deaths, 15.4%) after lung cancer (Ferlay *et al.*, 2015). The range in mortality rates between world regions is less than that for incidence because of the more favorable survival from breast cancer in (high-incidence) developed regions (Ferlay *et al.*, 2010).

The lowest incidence rates are found in most African countries but here breast cancer incidence rates are also increasing. This increase incidence of breast cancer in the developing country is due to increased life expectancy, increased urbanization and adoption of western lifestyles (Shulman *et al.*, 2010). Although breast cancer is thought to be a disease of the developed world, almost 50% of breast cancer cases and 58% of deaths occur in less developed countries. Incidence rates vary greatly worldwide from 19.3 per 100,000 women in Eastern Africa to 89.7 per 100,000 women in Western Europe. In most of the developing regions the incidence rates are below 40 per 100,000 in 2008 WHO estimation (Alero Fregene and Lisa A. Newman., 2004, Ferlay *et al.*, 2010) .

Breast cancer survival rates vary greatly worldwide, ranging from 80% over in North America, Sweden and Japan to around 60% in middle-income countries and below 40% in low-income countries. The low survival rates in less developed countries can be explained mainly by the lack of screening programmes, resulting in a high proportion of women presenting with late-stage disease, as well as by the lack of adequate diagnosis and treatment facilities (Anderson *et al.*, 2006, Jemal *et al.*, 2007).

Breast cancer is the second most often occurring cancer (cervical cancer is first) among women in Ethiopia. It is estimated that around 10,000 Ethiopian women and men have breast cancer with thousands of more cases unreported as women living in rural areas often seek treatment from traditional healers before seeking help from the government health system (http://www.csrwire.com). According to the African cancer registry network the incidence of breast cancer in Addis Ababa city, Ethiopia were 33% in 2014 report (http://www.afcrn.org).

In limited resource settings like Ethiopia, where breast cancer incidence is relatively low and the majority of women are diagnosed in late stages have the option to implement early diagnosis programmes based on awareness of early signs and symptoms and prompt referral to diagnosis and treatment (Anderson *et al.*, 2006).

So far the only breast cancer screening method that has proved to be effective is mammography. Mammography screening is costly for developing country, but is cost-effective and feasible in countries with good health infrastructure that can afford a long-term organized population-based screening programmes. Low-cost screening approaches, such as clinical breast examination, could be implemented in limited resource settings when the necessary evidence from ongoing studies becomes available (Anderson *et al.*, 2006, Tfayli *et al.*, 2010a). With the increasing application of molecular markers there will be a change in the way that breast cancers are classified and it is likely that much more information about an individual tumor will be routinely reported, such as its likelihood of metastasis, prognosis and to which therapeutic agents it will be susceptible.

1.2. Literature Review

1.2.1. Breast Cancer Epidemiology

Breast cancer is becoming frequently diagnosed cancer in developing country. The burden of cancer is increasing in economically developing countries as a result of population aging, an adoption of cancer-associated lifestyle choices including smoking, physical inactivity, and ‘‘westernized’’ diets (Ahmedin Jemal *et al.*, 2011) (Omid Beiki *et al.*, 2012).

The contribution of various modifiable risk factors, example 21% of all breast cancer deaths worldwide are attributable to alcohol use, overweight and obesity, and physical inactivity. This proportion was higher in high-income countries (27%), and the most important contributor was overweight and obesity. In low- and middle-income countries, the proportion of breast cancers attributable to these risk factors was 18%, and physical inactivity was the most important determinant (10%) (Tfayli *et al.*, 2010b, Anderson *et al.*, 2006).

The differences in breast cancer incidence between developed and developing countries can partly be explained by dietary effects combined with later first childbirth, lower parity, and shorter breastfeeding (Jemal *et al.*, 2007). The increasing adoption of western life-style in low- and middle-income countries is an important determinant in the increase of breast cancer incidence in these countries.

Generally, important risk factors for breast cancer are age, gender, reproductive history, hormonal factors, and family history. Although a family history of breast and/or ovarian cancer is common in patients diagnosed with breast cancer, less than ten percent of all breast cancers are associated with germline mutations (Sweeney *et al.*, 2004).

1.2.2. Pathogenesis

Breast cancer may arise from the epithelium of the duct system anywhere from the nipple end of the major lactiferous ducts to the terminal duct unit, which is in the breast lobule. The disease may be entirely *in situ*, an increasingly common finding with the advent of breast cancer screening, or may be invasive cancer. The degree of differentiation of the tumor is usually described using three grades: well differentiated, moderately differentiated or poorly differentiated.

Commonly, a numerical grading system based on the scoring of three individual factors (nuclear pleomorphism, tubule formation and mitotic rate) is used, with grade III cancers roughly equating to the poorly differentiated group (Li *et al.*, 2003).

The invasive breast carcinomas consist of several histologic subtypes; the estimated percentages are from a contemporary population-based series of 135,157 women with breast cancer reported to the Surveillance Epidemiology and End Results (SEER) database of the National Cancer Institute between 1992 and 2001 Infiltrating ductal - 76 percent, invasive lobular - 8 percent, ductal/lobular - 7 percent, mucinous (colloid) - 2.4 percent, tubular - 1.5 percent, medullary - 1.2 percent, papillary - 1 percent. Other subtypes, including metaplastic breast cancer and invasive micropapillary breast cancer, all account for fewer than 5 percent of cases (Li *et al.*, 2005).

Infiltrating ductal carcinoma is the most common type of invasive breast cancer, accounting for 70 to 80 percent of invasive lesions. On gross pathologic evaluation, these lesions are typically hard, gray-white, gritty masses which invade the surrounding tissue in a haphazard fashion to create the characteristic irregular, stellate shape. They are characterized microscopically by cords and nests of tumor cells with varying amounts of gland formation, and cytologic features that range from bland to highly malignant. The malignant cells induce a fibrous response as they infiltrate the breast parenchyma, and this reaction is, in large part, responsible for the clinically and grossly palpable mass, the radiologic density, and solid sonographic characteristics of typical invasive carcinomas (Li *et al.*, 2003).

Infiltrating ductal carcinomas are divided into three grades based upon a combination of architectural and cytologic features, usually assessed utilizing a scoring system based on three parameters. Well-differentiated (grade 1) - have cells that infiltrate the stroma as solid nests of glands (Ferlicot *et al.*, 2004, Elston and Ellis, 1991). The nuclei are relatively uniform with little or no evidence of mitotic activity. Moderately differentiated (grade 2) - tumors have cells that infiltrate as solid nests with some glandular differentiation. There is some nuclear pleomorphism and a moderate mitotic rate. Poorly differentiated (grade 3) - are composed of solid nests of neoplastic cells without evidence of gland formation. There is marked nuclear atypia and considerable mitotic activity (Elston and Ellis, 1991).

Infiltrating lobular carcinomas are the second most common type of invasive breast cancer, accounting for about 5 to 10 percent of invasive lesions. Some infiltrating lobular carcinomas have a macroscopic appearance identical to that of infiltrating ductal cancers. However, in many cases no mass lesion is grossly evident, and the excised breast tissue may have a normal or only slightly firm consistency. Thus, the microscopic size of invasive lobular carcinoma may be significantly greater than that measured grossly (Ferlicot *et al.*, 2004).

Tubular carcinomas were relatively infrequent in the pre-mammography era, accounting for 2 percent or less of invasive breast cancers. However, in some series of mammographically screened populations the incidence is higher, accounting for 10 to 20 percent of invasive cancers. Tubular carcinoma is characterized by the presence of well-formed tubular or glandular structures infiltrating the stroma (Li *et al.*, 2003).

Mucinous carcinomas account for between 1 and 2 percent of invasive breast cancers and appear to be more common in older patients. These lesions usually have a soft gelatinous appearance on gross examination, and they tend to be well circumscribed. Mucinous carcinomas are characterized microscopically by nests of tumor cells dispersed in large pools of extracellular mucus; the cells tend to have uniform, low grade nuclei. Similar to tubular carcinomas, these lesions also represent a prognostically favorable variant of invasive breast carcinoma (Li *et al.*, 2003, Li *et al.*, 2005).

Medullary carcinomas account for anywhere from 1 to 10 percent of invasive breast cancers. However, there is considerable inter-observer variability in the diagnosis of this type of breast cancer which is, at least in part, dependent upon the classification system employed. A number of other histologic types account for the remaining invasive breast cancers. These include invasive micro papillary carcinoma, metaplastic carcinoma, adenoid cystic carcinoma, and others (Armes and Venter, 2002).

Spread of breast cancer includes local spread. It tends to involve the skin and to penetrate the pectoral muscles and even the chest wall if diagnosed late. Lymphatic metastasis occurs primarily to the axillary and the internal mammary lymph nodes. It represents not only an evolutional event in the spread of the carcinoma but is also a marker for the metastatic potential of that tumor. Involvement of supraclavicular nodes and of any contralateral lymph nodes represents advanced disease. Spread by the blood stream is a route to skeletal metastasis (Armes and Venter, 2002).

1.2.3. Diagnosis of Breast Cancer

Breast cancer is diagnosed and surveyed by standard imaging modalities such as ultrasound and mammography. Despite the more frequent use of increasingly sensitive imaging techniques (e.g., MRI), diagnosis and subsequent monitoring of breast cancers rely on a mass of tumor cells that can be felt or seen (usually a mass of >1 billion cells) (Robbins *et al.*, 2010). There is intense interest in identifying serum (or plasma) biomarkers that may delineate the presence, absence or extent of disease when tumors cannot be palpated or visualized. These biomarkers could be potentially useful for prognosticating (helping to determine outcome), but they are conceivably valuable in surveillance (assessing recurrent disease). This notion is being addressed by numerous proteomic researchers, and though mounting evidence suggests that a variety of serum/plasma biomarkers may be clinically useful in breast cancer (Pitteri *et al.*, 2008), there are currently no serum/plasma biomarker assays available for systematic clinical use to monitor breast cancer progression (Abbott *et al.*, 2010).

The two basic principles of treatment are to reduce the chance of local recurrence and the risk of metastatic spread. Treatment of early breast cancer will usually involve surgery with or without radiotherapy. Systemic therapy such as chemotherapy or hormone therapy is added if there are adverse prognostic factors such as lymph node involvement, indicating a high likelihood of metastatic relapse. At the other end of the spectrum, locally advanced or metastatic disease is usually treated by systemic therapy to palliate symptoms, with surgery playing a much smaller role.

1.2.4. Proteases

The invasion and metastasis of tumor cells were shown to require proteolytic activity in order to degrade components of the extracellular matrix (ECM) and basement membrane. The hydrolysis of the ECM appears to facilitate tumor cell migration contributing to the metastatic dissemination of malignant cells to other region of the body from the primary site of cancer (Bartsch *et al.*, 2003).

During the tumor invasion process, tumor cells migrate through the ECM of the tumor tissue, the basement membrane and the blood vessel wall, to enter the vasculature. Once in the circulation, tumor cells are transported to a secondary site (such as bone) where they undergo the reverse process extravasation, exiting from the vessel and migrating through the surrounding ECM before proliferating to form metastases (Duffy and McCarthy, 1998).

Metastasis of tumor from the primary to the secondary sites, tumor cells must cross a number of different ECM components such as type I collagen in the interstitium of bone and type IV collagen in the basement membrane, and the production of a major group of proteases that has been directly linked with tumor invasion and metastasis are the cysteine cathepsin (CTS) and matrix metalloproteinases (MMPs), both are a family of endopeptidases known to cleave many ECM proteins including collagen. Under physiological conditions their activity is precisely regulated in order to prevent tissue disruption. This physiological balance seems to be disrupted in cancer making tumor cells capable of invading the tissue. In breast cancer different expression levels of several MMPs and cathepsin have been found. (Dumas and Platt, 2013b, Jinga *et al.*, 2006).

Breast cancer tumor cell dissemination is an early event of malignancy, often going undetected at the time of initial diagnosis. Growth of micro metastases at distant sites can occur, with tumor cells targeting specific organs. Of such tumors, breast cancer is good example, metastasizing to the skeleton in up to 80% of advanced cases. Following detection of secondary disease median survival is around 24 months for breast cancer patients (Beiki *et al.*, 2012).

Solid tumors are composed of a heterogeneous mixture of cell types, including both malignant and non-malignant cells such as fibroblasts, adipocytes, macrophages, endothelial cells, neutrophils, and in the case of metastases in the bone, osteoclasts and osteoblasts and the precursor cells of the bone marrow are involved (Dumas and Platt, 2013b).

Proteases (also termed peptidases) are enzymes hydrolyzing peptide bonds to complete protein catabolism. Proteases can be categorized into subgroups according to their substrate specificity and mechanisms of action. Based on the catalytic active sites, they are divided into 6 groups, which are serine proteases, threonine proteases, cysteine proteases, aspartate proteases, glutamic proteases, and metalloproteases. Cysteine proteases are named according to their common features of exploiting a cysteine residue in their active site. A histidine residue serves as nucleophile and general base. Cysteine proteases are divided into different families based on their sequence homology and structural similarities (Garbett *et al.*, 1999, Woodward *et al.*, 2007). In the following section, papain-like cysteine proteases, which are known as cathepsin cysteine proteases, will be discussed.

1.2.4.1. **Cathepsin**

Cysteine cathepsins belong to the family of papain-like cysteine peptidases (Mohamed and Sloane, 2006a). Cathepsins were first identified and characterized in lysosomes but are now known to be secreted extracellularly into the local microenvironment where they participate in extracellular matrix remodeling. Initially these enzymes were regarded as lysosomal scavengers. In recent decades, however, members of this family have been shown to be involved in numerous physiological and pathological processes, both as highly effective nonspecific hydrolases and as specific processing enzymes (Tan *et al.*, 2013). Cysteine cathepsins are a family of proteases identified in cancer, atherosclerosis, osteoporosis, and a number of other disease (Chen and Platt, 2011).

Cysteine cathepsin comprises 11 members. Among the 11 cysteine cathepsins known in human, cathepsin K stands out for several reasons. First of all, it is the principal peptidase involved in bone remodeling by osteoclasts. They are produced by several human cell types as well as by tumor cells and tumor associated cells.

Cathepsins K and L can be produced either by tumor cells or by tumor-associated cells of breast and there is growing evidence for the involvement of cathepsin K in different tumors (Mohamed and Sloane, 2006b, Tan *et al.*, 2013).

Cathepsin contribute to the pathological tissue remodeling associated with cancer. Endothelial cells (Platt *et al.*, 2007), smooth muscle cells (Hansen *et al.*, 2013), macrophages (Park *et al.*, 2012), fibroblasts (Tournu *et al.*, 1998), epithelial cells (Mohamed and Sloane, 2006b), and several other cell types have all been shown to upregulate cathepsin activity under inflammatory or disease conditions which has led to great interest in inhibiting them pharmacologically as a therapy (Dumas and Platt, 2013b).

Lysosome are the classical organelles of complex, multi enzymatic degradation. It is increasingly evident that endosome conduct much more than mere transport functions. Endosomes contain significant levels of proteases like cathepsins and are sites of potent intracellular proteolysis. Further discrete classes of endosomes harbor specific cathepsins and perform selective & specific functions. Hence, extra lysosomal proteolytic machinery within the endocytic pathway enjoy spatial & temporal control over proteolytic functions (Mohamed and Sloane, 2006b, Lankelma *et al.*, 2010).

The effect of cathepsin can be regulated by many factors in tumor microenvironment. Major factor includes: temperature, pH, and oxidative potential. Including affinities and hydrolytic rates for extracellular matrix components vary by cathepsin (Barry and Platt, 2012). Cysteine cathepsins are a family of lysosomal proteases, with a physiological functions in protein turn over, that are involved in degradation of extracellular matrix, facilitating growth, invasion, and metastasis of tumor cells, in tumor angiogenesis, in apoptosis, and in events of inflammatory and immune responses. The cathepsins have emerged as key players in several tumorigenic processes. Increased cathepsin expression and activity has been linked to many malignancies including glioma, breast, prostate, and pancreatic cancer (Tan *et al.*, 2013).

In addition, despite their usual lysosomal localization, they have been shown to be secreted and associated with the cell surface of tumor cells implying an extracellular role in cancer. Based on the function of cathepsin and their specific distribution in the human body could lead us to use

them as biological markers for early detection of breast cancer to indicate the survival rate or prognosis of the disease (Dumas and Platt, 2013b).

1.2.4.1.1. Cathepsin K (CTSK)

Specifically, cathepsins K have been implicated in osteoclast activation leading to osteoporosis and in elastin degradation in cardiovascular disease and are produced and secreted by endothelial cells, macrophages, and smooth muscle cells in the arterial wall. Cathepsin K is the most potent mammalian collagenase, capable of cleaving collagens I and II both intra helically and at the telopeptide regions and for these functions, it is the major protease used by osteoclasts for bone resorption (Barry and Platt, 2012).

At the molecular level, cathepsin K is unique as it has the ability to cleave the triple helix of collagen molecules at multiple locations, an activity that is unparalleled among human collagenases (Garnero *et al.*, 1998). Moreover, cathepsin K is thus far the only papain like cysteine peptidase that has been shown to be regulated allosterically. Glycosaminoglycans (GAGs), foremost chondroitin-4-sulfate (C4S), have been characterized as allosteric cathepsin K regulators and have been shown to affect the enzyme in a highly complex manner (Novinec and Lenarčič, 2013).

Platt study showed that cathepsins K and L in tissue preparations observed as clear bands of proteolytic activity after gelatin substrate SDS-PAGE zymography in non-denaturing and non-reducing with conditions optimal for cathepsin and MMPs renaturing activity. Densitometric analysis of the zymogram provides quantitative information (Dumas and Platt, 2013a).

As the number of breast cancer patient continues to rise, so does the need for low cost, broad use quantitative assays to detect their activity and can be translated to the clinic in the hospital or in low resource settings. Multiplex cathepsin zymography is one such assay that detects sub-nanomolar levels of active cathepsin K and cathepsin L (Chen and Platt, 2011).

Collagens are the most abundant proteins in the human body, constituting about 30% of the total protein mass. Their characteristic feature is the formation of trimers that contain one or more triple-helical regions usually enriched in glycine, proline and hydroxyproline residues (McQueney *et al.*, 1997).

Collagen is the most abundant protein in the human body and the main structural element in the extracellular matrix. The study done by McQueney showed that 28 types of collagen identified and defined. They can be divided into several groups according to the structure they form. These are: fibril forming or fibrillar collagens, fibril-associated collagens and network-forming collagens. Fibrillar collagens contribute to the mechanical properties of tissues such as bone, ligaments and skin. Among them, one of the most common collagen is type IV collagen, forms basal lamina , the epithelium secreted layer of the basement membrane (McQueney *et al.*, 1997).

Type I collagen is a heterotrimer consisting of two α 1 chains and one α 2 chain, whereas type II collagen is composed of three identical α 1 chains. Each chain consists mostly of a long triple helix-forming domain of about 1000 residues and short non-helical extensions on both ends, called the N- and C-terminal telopeptides, respectively (Ratnikov *et al.*, 2002). An inherent characteristic of the triple helix of fibrillar collagen is its resistance to proteolysis by most endogenous peptidases. Human enzymes that can cleave this structure include several collagenases from the family of matrix metallopeptidases (MMP-2, and -9) (Das *et al.*, 2008, Woodward *et al.*, 2007), the serine peptidase neutrophil elastase (Jinga *et al.*, 2006) and cysteine cathepsin K and L.

While MMPs and neutrophil elastase cleave the triple helix at a single position within an unwound region (Jinga *et al.*, 2006), cathepsin K can cleave at multiple positions along the molecule. Multiple cathepsin K cleavage sites have been identified in collagen molecules from different animal sources. In type I collagen, most are located near the N-terminal end (McGrath *et al.*, 1997)of the triple helical domain and one cleavage site is located in the C-terminal part. Similarly, a cleavage site in the N-terminal part of the triple helix has also been identified in type II collagen. In contrast to the triple helical region, which constitutes the bulk of the collagen structure, the non-helical telopeptide regions are much more susceptible to proteolysis. These regions contain covalent cross-links between two telopeptides and the triple helix of an adjacent collagen molecule in the form of a pyridinoline ring formed from three lysine residues, one from each chain.

Cathepsin K has been shown to directly release cross-linked N-terminal and C-terminal telopeptides from type I collagen (Garnero *et al.*, 1998).

Cathepsin K is the cysteine protease predominantly involved in bone homeostasis. Cathepsin K cleaves type I collagen very efficiently, and its activity is retained at low pH, plays a vital role in bone resorption (Barry and Platt, 2012). Cathepsin K has the typical three-dimensional structure of a cathepsin-L like peptidase without major structural alterations in comparison to its closest homologs. Its collagenolytic activity was thus not acquired by major structural changes in the molecule but rather by subtle alterations of an existing structure (McGrath *et al.*, 1997).

The active site is located at the top of the molecule in the form of a V-shaped cleft (Figure:1) and contains the catalytic diad cysteine – histidine (shown as yellow and blue sticks, respectively). Initial substrate profiling of cathepsin K has shown that the enzyme can accept a Pro residue in the P2 position, which is correlated to the high content of Pro and hydroxyproline residues in collagen. Apart from this unusual preference, the enzyme showed specificity typical for cysteine cathepsins, with a preference for hydrophobic residues in the P2 position and loose specificity in other positions (Decock *et al.*, 2005).

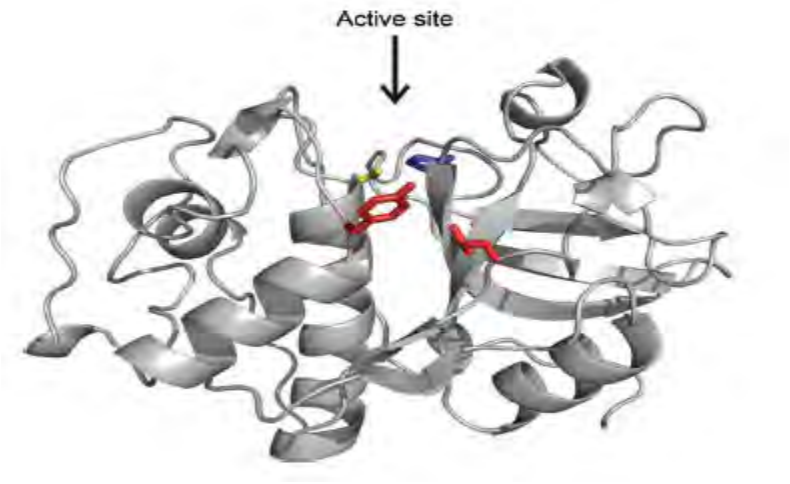

Figure 1: The three-dimensional structure of cathepsin K (Novinec and Lenarčič, 2013).

The three-dimensional structure of human cathepsin K is shown in the standard orientation. The position of the active site is marked by an arrow. The catalytic Cys 25 and His 162 residues are shown as yellow and blue sticks, respectively. Residues Tyr 67 and Leu 208 , which determine the unique substrate specificity of cathepsin K, are shown as red sticks. All residues are numbered according to the mature chain numbering (Schr o dinger, Inc., Portland, OR, USA).

1.2.4.1.2. Cathepsin L (CTSL)

Cathepsin L constitutively expressed endopeptidase in many tissue, and in addition, the enzyme has been implicated in multiple pathological processes. Cathepsin L expression is increased in atherosclerosis and cancer as well and is secreted at sites of inflammation (Binbin Chen and Manu O Platt., 2011). Expression level of cathepsin L is enhanced by several growth factor like epidermal growth factor, fibroblast growth factor, platelet driven growth factor and hormones like follicle stimulating & luteinizing hormone (Lankelma *et al.*, 2010).

Cathepsin L is an important lysosomal cysteine protease and a member of papain superfamily of enzymes. Cathepsin L functions as a very potent endosomal/lysosomal endopeptidase involved in the degradation and recycling of a wide range of intra- and extracellular protein substrates. In addition to lysosomal role as a processor of intracellular protein, cathepsin L is also a key mediator of extracellular matrix breakdown leading to tumor invasion and metastasis. This makes it an important enzyme in cancer detection and chemotherapy research. Assessment of elevated level of extracellular cathepsin has proven to be an important prognostic indicator of cancer chemotherapy outcome (Lankelma *et al.*, 2010).

1.2.4.1.3. Mode of Action of Cysteine Cathepsin

The action of cysteine cathepsins, especially the papain superfamily has been extensively characterized more than their counterparts in the asparagine protease family. Most of cysteine protease cathepsins require acidic pH for optimal activity. Since Cys 25 at the catalytic center exhibit a very low pKa value, which helps formation of thiolate +/- imidazolium ion-pair with histididne -159 required for the catalysis. Substrate bind into the active site into an extended conformation and the carbonyl carbon of the siccile bond undergo nucleophilic attack from the active site thiol, resulting in the release of amine product.

The ensuing acyl enzyme react with water to release the carboxyl product (deacetylation), resulting the generation of free enzyme. Cysteine cathepsin do not have single specific substrate, although they do differ considerably in their preferred cleavage site (Novinec and Lenarčič, 2013, Turk *et al.*, 2012).

1.2.4.2. Metalloproteases

The metalloproteinases include the matrix metalloprotease (MMPs), a disintegrin and metalloproteinases (ADAMs) and a disintegrin and metalloproteinases with thrombospondin motifs (ADAMTS) enzymes (Toth and Fridman, 2001a). Classic matrix metalloproteinase family members includes interstitial collagenases, stromelysins, gelatinases and elastases.The MMPs are about 26 multi-domain proteases that contain a zinc atom in the active site and are produced in a latent inactive form (zymogen) and are activated extracellularly by serine proteases such as plasmin and also by other MMPs (Lijnen, 2001).

Acquisition of enzymatic activity requires cleavage of the inhibitory N-terminal domain. Thus, generation of the active form usually occurs concomitantly with a decrease in molecular mass and exposure of the active site. Once activated, all the MMPs are specifically inhibited by a group of endogenous protease inhibitors known as the tissue inhibitors of metalloproteinases (TIMPs), which bind to the active site inhibiting catalytic activity. Generally, MMP activity can be regulated transcriptionally by post-translational modifications, proenzyme activation and endogenous inhibition by the naturally occurring family of tissue inhibitor of metalloproteinase (TIMP) proteins (Têtu *et al.*, 2006).

Matrix metalloprotease shows different characteristics: firstly, MMPs are calcium dependent zinc containing endopeptidases. Collectively, these enzymes are capable of degrading most components of the ECM proteins at 7 pH. They play a significant role in tissue remodeling in normal physiological as well as pathological processes. Many of which were first identified by their overexpression in tumor cells (Toth and Fridman, 2001a). Secondly, this family is defined by a highly conserved catalytic domain containing three histidine residues that coordinate zinc atom-binding. In the latent state the pro-enzyme domain has a second highly conserved sequence that

contains an unpaired cysteine residue which can occupy the fourth co-ordination position of the zinc atom. Disruption of this sulfur–zinc coordination activates the enzyme by a mechanism termed the ‘cysteine switch hypothesis.

A third defining characteristic of the MMPs is susceptibility to a class of endogenous inhibitors, the tissue inhibitors of metalloproteinases or TIMPs (Hofmann *et al.*, 2005).

Gelatinase A (MMP-2) and gelatinase B (MMP-9) differ from other MMPs in that they have three tandem fibronectin type II repeats within the amino terminus of the catalytic module that mediates gelatin binding. Matrix metalloproteinases, in particular the gelatinases MMP-2 and MMP-9, have received great attention in recent years as putative tumor markers for clinical applications (Toth and Fridman, 2001a). Moreover, these two enzymes interact with physiological inhibitors (respectively, TIMP-2 and TIMP-1). Traditionally, MMP-2 and MMP-9 have been correlated with the invasive stage of carcinomas, because of their ability to degrade type IV collagen, a major constituent of basement membranes (Das *et al.*, 2008).

More recent evidence suggests that MMP-2 and MMP-9 may also be involved in breast cancer initiation and growth through complex interactions with the main oncogenes and tumor-suppressor genes involved in the early stage of tumorigenesis (Das *et al.*, 2008). For example, transfection of MCF- 10A breast cancer cells with either c-erbB-2 or c-ras resulted in increased expression of MMP-2 (Duffy and McCarthy, 1998), whereas transfection of MCF-7 cells with the gene PEA-3 led to increased production of MMP-9. Due to the key role of MMPs in tumorigenesis, several authors have proposed MMP-2 and or MMP-9 as useful prognostic markers (Duffy and McCarthy, 1998). Recent work on breast cancer patients has suggested that MMP-2 negativity may be linked with a favorable prognosis in node-negative breast carcinoma (Hirvonen et al, 2003) and that high activity levels of plasma MMP-9 in breast cancer patients are associated with a worst overall survival rate (Bartsch *et al.*, 2003).

MMP-2 and MMP-9 were originally described as type IV collagenases because of their ability to promote the hydrolysis of collagen IV, a major component of basement membranes and a major structural barrier for tumor cell invasion. Both enzymes can also cleave a variety of ECM proteins but they are extremely efficient in hydrolyzing denatured collagen I (gelatin) and consequently they are referred to as gelatinases (d'Avila-Levy *et al.*, 2012). The ability of MMP-2 and MMP-9

to degrade denatured collagen IV was developed into a relatively easy yet powerful technique to detect their presence in biological samples. This technique, known as gelatin zymography, identifies gelatinolytic activity in biological samples using sodium dodecyl sulfate (SDS)-polyacrylamide gels impregnated (copolymerized) with gelatin (Heussen and Dowdle, 1980).

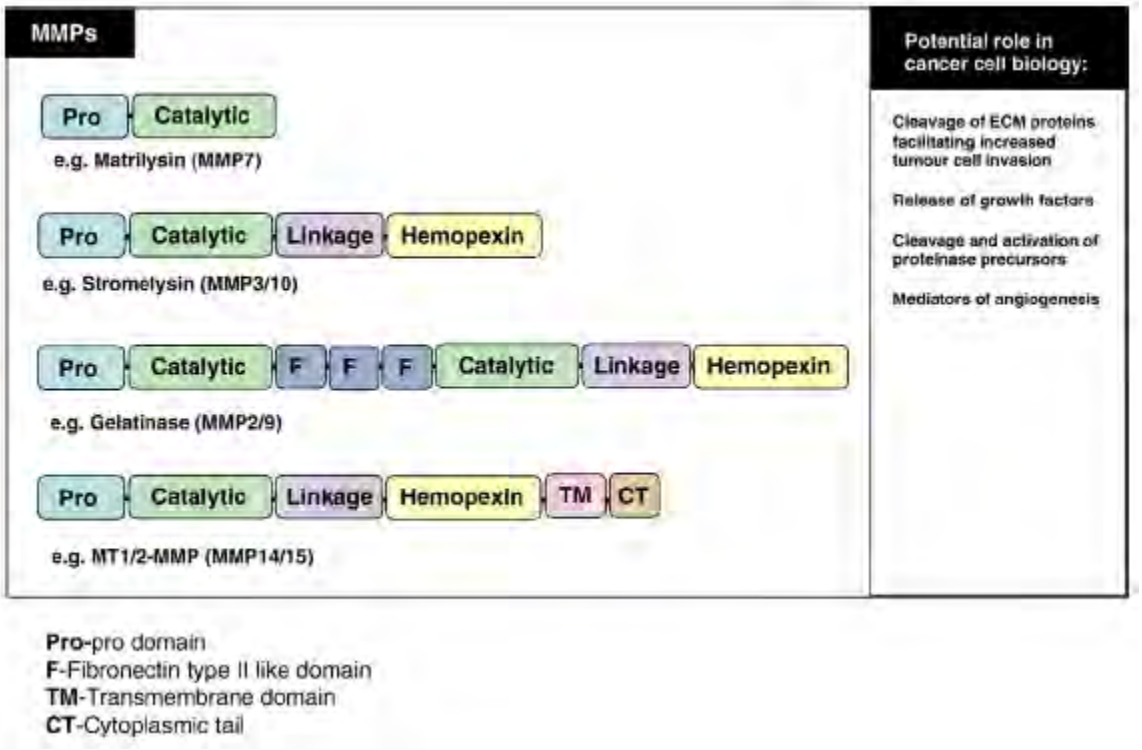

Figure 2: Schematic representation of the domain structure and potential roles of MMPs and MT-MMPs in tumor biology (Woodward *et al.*, 2007).

Many studies have assessed MMP expression at the protein or nucleic acid level and correlated this with invasive ability or metastatic potential of the tumor. The enhanced expression of many MMP family members has been correlated with the invasive behavior of human cancer (Stetler-Stevenson *et al.*, 1996).

It is now recognized that these enzymes are in no way tumor specific, but are also synthesized and secreted by normal cells under conditions associated with physiological tissue remodeling. The differential protease production under physiological conditions versus production by neoplastic cells may be that in tumor cells these proteases may be constitutively expressed at a high level or induced by autocrine growth factors. Tumor cell expression of MMP activity may be unresponsive to signals from host cells and matrix which would down-regulate MMP expression in normal cells (Sheu *et al.*, 2001).

The role of the MMPs in human cancer has been investigated by examining their expression in tumor tissues by immunohistochemistry and by in-situ hybridization (Scorilas *et al.*, 2001).

A direct correlation between MMP expression and the invasive phenotype of human tumor cells has been observed in lung, prostate, stomach, colon, breast, ovary, thyroid and oral squamous cell cancers, where many MMPs are expressed at high levels. Although gelatinase A is expressed in all tumor types examined, no single MMP is consistently over expressed in every single tumor of a given histopathologic classification (Têtu *et al.*, 2006). This probably reflects tumor cell heterogeneity, possible variations in MMP expression with tumor progression and differential expression in response to the changing extracellular matrices encountered during tumor progression (Sheu *et al.*, 2001).

1.2.5. Biomarker

According to the definition developed by the National Institute of Health (NIH), a biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.

Biomarkers may be certain proteins present on the tumor or released by the tumor in the blood, which indicate the recurrence of the disease after a curative surgical intervention, a single nucleotide polymorphism (SNP) haplotype correlated to the risk that patients will develop a certain drug-related toxicity, the expression level of mRNA or the presence of a gene mutation targeted by a drug, but even the metabolic activity of the tumor, measured by the standard uptake value (SUV) of images obtained during positron emission tomography (PET) examination, or the

number of circulating tumor cells may as well considered biomarkers (Dugeshwar Karley *et al.*, 2011).

By another definition biological markers (biomarkers) have been defined as cellular, biochemical or molecular alterations that are measurable in biological media such as human tissues, cells, or fluids.

1.2.5.1. Properties of Biomarkers

The ideal biomarker discriminate the events between exposure and disease, perform establishment of dose response, identifies early events in the natural history, identifies mechanisms by which exposure and disease are related, reduces misclassification of exposures or risk factors and disease, established variability and effect modification, enhanced individual and group risk assessments (Dugeshwar Karley *et al.*, 2011).

Biomarkers are characterized biological properties that can be detected and measured in parts of the body like the blood or tissue. According to Food and Drug Administration (FDA) an ideal biomarker should be specific, sensitive, predictive, robust, simple, accurate, and inexpensive. It should be used in standard biological sources such as serum and urine as the basis of measurement (Karley *et al.*, 2011). More generally a biomarker is anything that can be used as an indicator of a particular disease state or some other physiological state of an organism.

1.2.6. Zymography

Zymography offers several features which render it particularly useful with respect to alternative methods such as enzyme linked immunosorbent assay (ELISA): No expensive materials are routinely required (e.g., antibodies) and several proteases showing activity on the same substrate can be detected and quantified on a single gel. MMPs are released from cells in a proteolytically inactive pro-form (zymogen) which is about 10 kDa larger than the activated form. Since the pro-form becomes activated during the process of renaturation after gel electrophoresis, both forms can be detected on zymograms.

In addition, MMPs in solution are often associated with their corresponding tissue inhibitors of metalloproteases (TIMPs). During electrophoresis the inhibitors dissociate from the MMP and do not interfere with detection of the enzymatic activity (La Rocca *et al.*, 2004).

Gelatin zymography, however, is a useful qualitative tool for the detection and analysis of the level and type of the gelatinases expressed in different cell types/tissues at any given time and/or after different treatments. For example, it is possible to determine which gelatinases are expressed in tumor cells with various degrees of invasive potential whether they are derived from established cancer cell lines or from tumor biopsies. Furthermore, the regulation of gelatinase expression invitro in response to a variety of factors can be studied. It should be pointed out, however, that owing to the enzymatic nature of the method and the many variables involved, zymography is too crude to be used as a quantitative technique (Toth and Fridman, 2001b).

1.3. Statement of the Problem

Developing countries, like Ethiopia accounts for the majority of breast cancer deaths. Diagnostic and treatment services are limited and most patients are first seen when the disease is advanced. In sub-Saharan Africa only 32% of women are still alive five years after diagnosis, compared with 81% in the US (Morse *et al.*, 2014).

Unlike the Western world, where women diagnosed early and have a good chance of survival, women in Ethiopia usually present late and are expected to have a very limited life span (Tfayli *et al.*, 2010b).

Breast cancer diagnosed with imaging techniques like ultra sound, magnetic resonance imaging (MRI) and mammography. Mammography is also used as screening tool in developed country, while it is not affordable in developing country. Confirmatory diagnosis and staging usually done by histopathology.

A new era in cancer research was initiated with the development of methods to measure the expression of thousands of genes in tumors and normal tissues. Among these new methods, the determination of RNA levels by microarray analysis has found wide application. Currently, this method can measure RNA expression from virtually all known genes. The expression profiles obtained from DNA microarray analysis are known as *gene expression signatures or molecular profiles*. The application of this technique to the study of breast cancers has been particularly rewarding. It was recently found that there are breast cancer subtypes that can be identified by their molecular profiles and that the molecular signatures of some of these subtypes can help predict the course of the disease (Robbins *et al.*, 2010). This technique is on the way of development and thought to be expensive.

Finding a new accessible and cheap way of diagnosing tool for diagnosis of breast cancer could decrease the prevalence of breast cancer. Lysosomal and plasma biomarker are important for early identification as well as to follow the prognosis of the disease. Cysteine cathepsin and matrix metalloproteinase considered to be a potential for biomarker.

1.4. Significance of the Study

The use of chemical tools to evaluate biological systems is a powerful means to investigate cellular processes, signaling networks, and molecular mechanisms.

We, therefore, evaluated protease expression pattern to elucidate the presumed mechanism for cancer metastasis. In addition, our study gives knowledge on the use of protease activity profiles using for molecular breast cancer subtyping, which render different therapeutic opportunities for the patients.

The method is cheap and can be used as valuable laboratory based investigation. The result collectively motivate diagnostic and therapeutic investigation for cysteine cathepsin and MMPs inhibitors in human cancers. Besides, the information generated from the study may lay a ground for further related studies.

1.5. Hypothesis

Laboratory testing of functional protease activity level in human breast tissue using gelatin zymography could be used to discriminate subgroups across different stages and shows breast cancer invasiveness.

2. OBJECTIVE

2.1.General Objective

- To investigate zymographic detection and clinical correlations of cysteine cathepsins and MMPs in human breast cancer tissue.

2.2.Specific Objectives

- ☞ To detect and quantify the activity of cathepsin K and L using gelatin zymography technique
- ☞ To detect and quantify the activity of pro-MMP-2, MMP-2, pro-MMP-9 and MMP-9 using gelatin zymography technique
- ☞ To compare cathepsin and MMPs activities between normal and tissue specimens.
- ☞ To compare the relationship of cathepsins and MMPs activity with histopathological variables
- ☞ To compare the relationship of cathepsin and MMPs activity with clinical variables

3. MATERIALS AND METHODS

3.1.Study Area and Period

The study was conducted at surgery department of Minilik II Hospital, St Paul's Hospital Millennium Medical College and Zewditu Memorial hospital, in Addis Ababa, Ethiopia. These hospitals provide mastectomy service for breast cancer patients for those seeking treatment from Addis Ababa city and from all over Ethiopia. The study period was from January – June 2015 G.C.

3.2. Study Design

Hospital based comparative cross sectional study design were implemented. The study were encompass two groups, these were control and study group.

3.3.Study Population

3.3.1. Source Population

Women with breast tumor attending Menelik II Hospital, Saint Paul Hospital Millennium Medical College and Zewditu Memorial Hospital.

3.3.2. Sample Population

All women with histologically verified breast cancer (American Joint Committee on Cancer, AJCC) and who had breast excisional surgery during the study period.

3.5 Sample Size

A total of 36 breast cancer patients recruited for the study. Both tumor and normal tissue specimens were harvested from the same patient. Totally 72 tissue samples were run for zymography and duplicated.

3.4. Sampling technique

We used purposive sampling technique to select the health facility and convenient sampling technique to get the calculated number of study participants in the study period.

3.5. Inclusion and Exclusion Criteria

Inclusion criteria

- ✓ Histologically confirmed breast cancer patients
- ✓ Female patients
- ✓ Patient who undergo their first breast surgery (mastectomy)
- ✓ All age group
- ✓ Volunteers participants

Exclusion criteria

- ✓ Female patients who undergo surgery for more than one times
- ✓ Patient who doesn't undergo breast surgery
- ✓ Medically confirmed chronic disease like HIV, TB
- ✓ Male breast cancer patients
- ✓ Benign breast tumors
- ✓ Patients on chemotherapy and radiation treatment
- ✓ In volunteers participants

3.6. Variable

Independent variables

Independent variables of the study are the following:

- Sociodemographic factors
- Reproductive history
- Histopathological history

Dependent variable

- Cysteine cathepsin activities
- Matrix metalloproteinase activities

3.7.Data and Sample Collection Procedures

3.7.1. Questionnaire

Data was collected by using structured closed ended as well as open ended self-administered questionnaire. The questionnaire has two parts. In the first part, sociodemographic characteristics were addressed. Clinico-pathological variables were considered in the second part. The questionnaire were approved by Department of Biochemistry.

Information on tumor size (T) and nodal status (N) was used to derive stage by the American Joint Committee on Cancer staging system AJCC (six edition, (Singletary and Connolly, 2006)): stage 1 (TxN0); stage 2 (T0N1, T1N1, T2N0, T2N1 or T3N0) and stage 3 (TxN2, T3N1, T4Nx or TxN3). T-stage was assessed according to the 2009 TNM classification. For N-stage, the information on involved lymph nodes (LNs) from the pathologist was used, given that an adequate number of LNs was examined (≥ double the number of involved nodes for N1 and any number for N2 or N3). This information was used to derive the stage (UICC) as a potential prognostic factor (Sobin *et al.*, 2011).

3.7.2. Sample collection

From eligible participants, breast tissue specimens both normal and tumor were collected within 10 minutes after surgery. The samples were soaked and preserved with phosphate buffered saline (PBS) pH 7 and capped in bottle then transported in cold chain to Tikur Anbessa Spcialized Hospital and freezed in -80 ^{o}C Binder refrigerator.

All samples were prepared adequately to maintain the function of the enzymes and used immediately after collection or stored frozen at -80°C. During the time of laboratory analysis, the tissue of interest was minced (~50 mg) into small pieces with scalpel in sterile Petri dish. We removed any visible fat by scalpel. Cold lysis buffer of 250 µl with protease inhibitor (leupeptin) freshly added on the top of minced tissue specimens. Then, we homogenized the tissue with the lysis buffer (20 nM Tris–HCl at pH 7.5, 5 mM EGTA, 150 mM NaCl, 20 mM β-glycerol-phosphate, 10 mM NaF, 1 mM sodium orthovanadate, 1% Triton X-100, 0.1% Tween-20, 0.1 mM leupeptin freshly added) with a stainless steel spatula, then we collected the supernatant aliquot, and incubated on ice for at least 5 minutes. The aliquot were Vortex-mixed and centrifuged at

10,000 rpm for 10 min at 4°C in a micro centrifuge. We collected the supernatant and measured protein concentration. Therefore we adjust the protein concentration to 1 μg per μL, with bicinchonic acid (BCA) total protein assay, and vortexed with 5X sample buffer. Then, we loaded onto freshly prepared gel equal amounts of protein per lane

3.7.3. Data collector

Demographic, reproductive and histopathologic data were collected by trained clinical nurses, who were working in the department of surgery. The data collector had similar experience in data collection from previous other studies. The tissue specimen were harvested by duty surgeon and the sample transported by principal investigator.

3.8.Multiplex Zymography Protocol

Cathepsin zymography was performed as described previously (Chen and Platt, 2011). Briefly, non-reducing loading buffer (5X—0.05 % bromophenol blue, 10 % SDS, 1.5 M Tris, 50 % glycerol) was added to all samples prior to loading. Protogel, an optimized mix of acrylamide and bisacrylamide solution, was added with appropriate buffers, SDS, gelatin solution (5 mg/mL), and ammonium persulfate (APS) (a radical initiator). TEMED, a catalyst of the formation of free radicals produced with APS, was added and the solution was mixed. Subsequently, acrylamide and bis-acrylamide were polymerized between glass plates to form a gel matrix, incorporating 3.3 % gelatin, which was used for sieving proteins. Running buffer comprising of Tris-base, glycine, and SDS was added to the gel during the electrophoresis process as a majority of the current is carried by buffer ions, controlling the conductivity of the gel. Mini gels were prepared from shortened glass plates, and a mini-Protean 3 multi-casting chamber (Bio-Rad) was used to prepare standard size gels approximately 92 * 68 mm^2 (width * height). Standard zymograms were rinsed three times for 10 min each in renaturing buffer (20 % glycerol, 65 mM Tris buffer, pH 7.4), equilibrated in assay buffer (phosphate buffer, pH 6, 2 mM DTT, and 1 mM EDTA) for 30 min prior to overnight incubation at 37 ^{0}C in fresh assay buffer. Mini-zymo gels were washed three times for 5 min each in renaturing buffer, equilibrated in assay buffer, prior to assay buffer incubation to activate enzymes.

MMP zymography was carried out similarly except the enzymes were renatured in 2.5% Triton-X 100 and incubated in 50 mM TrisHCl pH 7.4, 10 mM calcium chloride, 50 mM sodium chloride, 0.05% Triton-X 100 assay buffer overnight.

3.9.Quantitation of Gel

Gels were quantitated using ImageJ-NIH software. Gels were scanned using 8 MP mobile camera. The image of the gel was inverted to reveal white bands on a dark background. The molecular weight, area and optical density of each band were determined.

3.10. Data quality management

Standard operational procedures and manufacturer instructions were strictly followed throughout the procedures. The questionnaire were translated to the study participant's mother tongue by the data collector. Training for data collectors were given to maintain the quality of the data collection process. Data were checked daily after sample collection and necessary correction was taken. Data cleaning and cross validation was done prior to subsequent analysis. All reagents for each experiment were stored in appropriate storage conditions as recommended by the manufacturer. Zymography was duplicated for each samples to maintain its reproducibility then, the average result were taken for the statistical analysis.

3.11. Data Analysis and Interpretation

For each group of subjects, data derived from zymographic quantification of activity of CTSK, CTSL and latent and mature MMP-2 and MMP-9 were plotted using MS Excel software for total protein assay. Statistical analyses were performed using IBM-SPSS version 21 software for student unpaired Student's t-test, one-way ANOVA with Post Hoc and Pearson correlation analysis. Fitness of data to normal distribution was assessed using the method of Kolmogorov and Smirnov. This normality test quantifies the discrepancy between the examined distributions of data and an ideal Gaussian distribution; the test returns a P-value which is considered acceptable for values >0.05. For all the distributions examined the test confirmed their fitness with a normal distribution after log transformation. In order to estimate the significance of differences between cancer patients and control subjects, unpaired Student's T-test and one-way ANOVA was applied.

Correlation of CTSK, CTSL and latent and mature MMP-2 and MMP-9 activity levels with clinico-pathological variables for breast cancer patients was performed using the Pearson correlation test. In all cases, data were considered significant for values of $P < 0.05$.

3.12. Ethical Consideration

Ethical clearance for this study was obtained from the research and ethical committee of biochemistry department of Addis Ababa University, medical faculty with protocol number of M.Sc. Thesis 12/14 and reference number of SOM/BCHM/129/2006. Permission for data collection were obtained from respective ethical committee of Menelik II Hospital, St. Paul's Hospital Medical Millennium College and Zewditu Memorial Hospitals.

Written informed consent were obtained from all study participants. The objective of the study were verified for patients in their mother tongue. The participants were told any of their personal information will not be disclosed to third party. All participants were told they have the right to respond, deny or respond partly.

3.13. Operational definition

Densitometry: zymography image that were scanned with 360 dpi mobile camera were analyzed with ImageJ-NIH software to quantify its pixel density. The pixel density were used as relative concentration of enzymatic activity.

3.14. Dissemination of Result

The final report of this study will be presented to college of health science department of biochemistry as partial fulfillment of master's degree in Biochemistry. It will be disseminated through publication & presentation in scientific conferences & workshops.

4. RESULT

4.1.Demographic Characteristics of the Study Participants

The age of the women in this study ranges from 24 to 83 years of age. Median age of the study participants were 39 years. The peak age distribution of study participants found between 30 to 39 years (n=10, 27.8%). The distribution of women's age among different age group were depicted in figure 3.

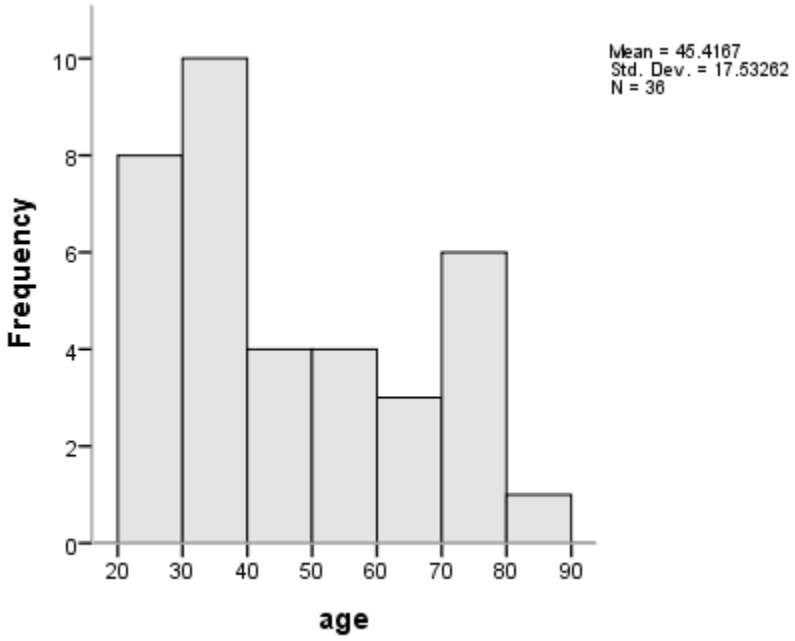

Figure 3: Age distribution of breast cancer patients. Values were expressed in frequency and age were in years.

Among the 36 study participants 17 were (47.22%) from Addis Ababa city; 30.56 % from Oromia region; 11.11 % from Amhara and 11. 11% from south nation nationality and people region (SNNPR). From the total study participants 61.1% (n=22) came from urban regions of Ethiopia. The rest 38.9% (n = 14) came from rural part of Ethiopia. From the total patients involved in this study 8 (22.2%) were incapable of paying their hospital expenses.

Educational status of the study participants showed that 36.1% (n=13) did not get formal education and 55.6% (n=20) were at high school level and the remaining 8.3% (n=3) attended college at the time of data collection.

Marital status of participants were as follows: 72.2% (n=26) were married, 16.7% (n = 6) were widowed and 11.1% (n = 4) were single at the time of data collection.

The participant who came for visit to health facility to get treatment after onset of the symptom to the day of surgery were in the range of between 2 – 48 months (median 5.5 months). All the study participants had no history of alcohol consumption and cigarette smoking in their life time. However, among the study participants (n=11, 30.6%) of them took oral contraceptive pills (OCP) and the remaining (n=25, 69.4%) had no any experience of OCP in their life time.

4.2.Clinical Characteristics of the Study Participants

Patients with first degree family history of histologically confirmed breast cancer were two (n=2, 5.6%), similarly patients with first degree ovarian cancer were two (n=2, 5.6%). Our study participants didn't have second or third degree family history either breast or breast cancer. For the first time patients noticed the breast lump accidentally by themselves before visiting health facility were (n=31, 86.1%). Patients who underwent mammographic screening were two patients only from total study participants. The rest of the data are presented in the following table.

Table 1: Frequency of breast cancer lump noticed for first time in breast cancer patients

Variables	Frequency	Proportion (%)
Self accidentally	31	86.1
Self as regular physical examination	1	2.8
Routine physical examination	2	5.6
Mammographic screening	2	5.6

4.3. Physical Finding during Hospital Visit

Breast cancer patients, who visited hospital revealed the following physical finding at the time of diagnosis. The most common symptoms reported by physician were peau d'orange and nipple retraction. The rest of the data are presented in table 2.

Table 2: Physical finding at the time of diagnosis in breast cancer patients

Variables	Yes (n)	Proportion (%)	No (n)	Proportion (%)
Nipple retraction	26	72.22	10	27.78
Bloody discharge	10	27.78	26	72.22
Palpable axillary lump	8	22.2	28	77.8
Fixation to overlying skin	18	50.0	18	50.0
Fixation to underlying muscle	7	19.4	29	80.6
Erythema of the skin	11	30.6	25	69.4
Peau d'orange	29	80.6	7	19.4
Satellite nodule	1	2.8	35	97.2

"n" is number of study participants.

4.4. Clinical and pathological results

From the total study participants (n=27, 75.0%) of them node positive. Histopathological result showed that 34 (94.4%) had infiltrative ductal carcinoma. Women who had had a child were all breast fed (n=26). From the clinical and mammography result we found increased number of patients with upper outer quadrant (UOQ) of the breast region were detected with breast tumor. That is 20 (55.6%) patients were present with UOQ breast lump. Among the 36 breast cancer patients 19 patients had histologically confirmed right breast cancer. The rest part of the information described in the table 3.

Table 3: Subject characteristics. n=36

Characteristics	Number	Proportion %
Total population	36	100.0%
Nodal status		
Positive	27	75.0
Negative	9	25.0
Histological type		
Infiltrative ductal	34	94.4
Lobular	2	5.6
Histological grade		
Well differentiated (I)		
Moderately differentiated (II)		
Poorly differentiated (III)		
Breast feeding		
Breast fed	26	72.2
Non breast fed	10	27.8
Location of breast cancer		
Upper outer quadrant	20	55.6
Upper inner quadrant	5	13.9
Lower outer quadrant	6	16.7
Lower inner quadrant	2	5.6
Central	3	8.3
Breast cancer site		
Right	19	52.8
Left	16	44.4
Both	1	2.8
Tumor size (mean ± SD)		
Length	6.49 cm ± 3.90 cm	
Width	6.30 cm ± 3.57 cm	

4.5. Cathepsin K and Cathepsin L expression

Cathepsin zymography detects mature cathepsin K and L activity after loading equal amount of sample 20 ul (20 µg/µL) of sample aliquot to each gel wells.

Cathepsin K and L appeared as zymographically active bands at distinct molecular weights from breast cancer samples (Figure 4); mature cathepsin K band appeared near the 37 kDa size and cathepsin L at 21 kDa. Multiplex zymography does not show clear visible white bands for normal tissue specimen even after 40 µg/µL sample loaded to each gel wells. Representative zymogram is not shown for control in this report.

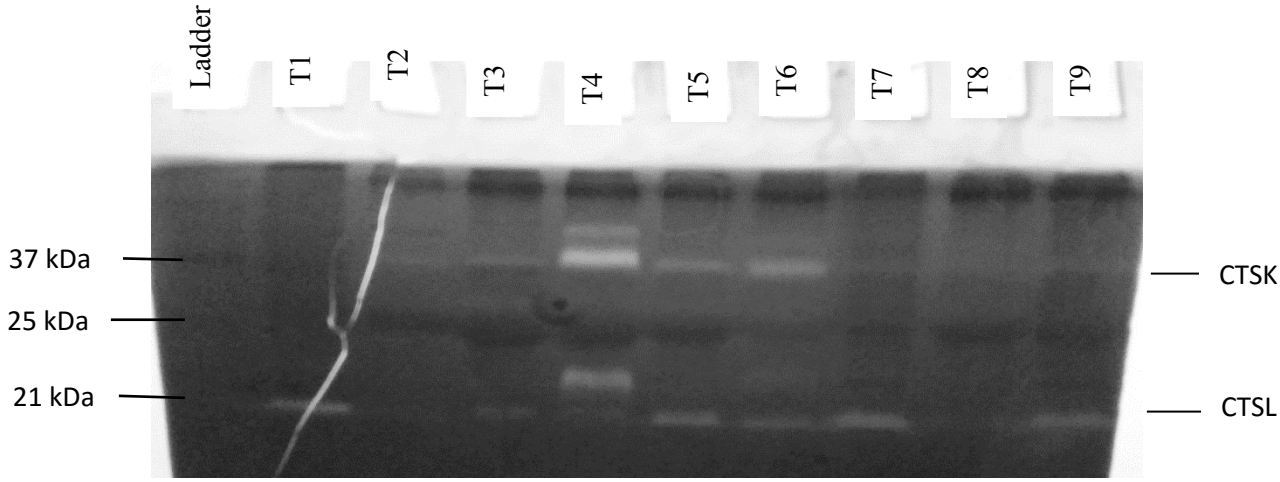

Figure 4: Cathepsin zymogram of tissue specimens in breast cancer. Molecular weight standards are shown on the left. Shows 20 µg tumor tissue specimens from patients with breast cancer biopsies were loaded for zymography. Cathepsin K band were visible at 37 kDa and cathepsin L at 21 kDa.

4.6. MMP-2 and MMP-9 expression

Gelatin zymography identifies and separates the gelatinases MMP-2 and MMP-9 in both latent and active forms due to differences in their molecular mass. In breast cancer and normal tissue samples the following four lysis bands were observed in the samples: 92 kDa corresponding to latent MMP-9; 84 kDa active MMP-9; 72 kDa latent MMP-2, and finally 68 kDa active MMP-2.

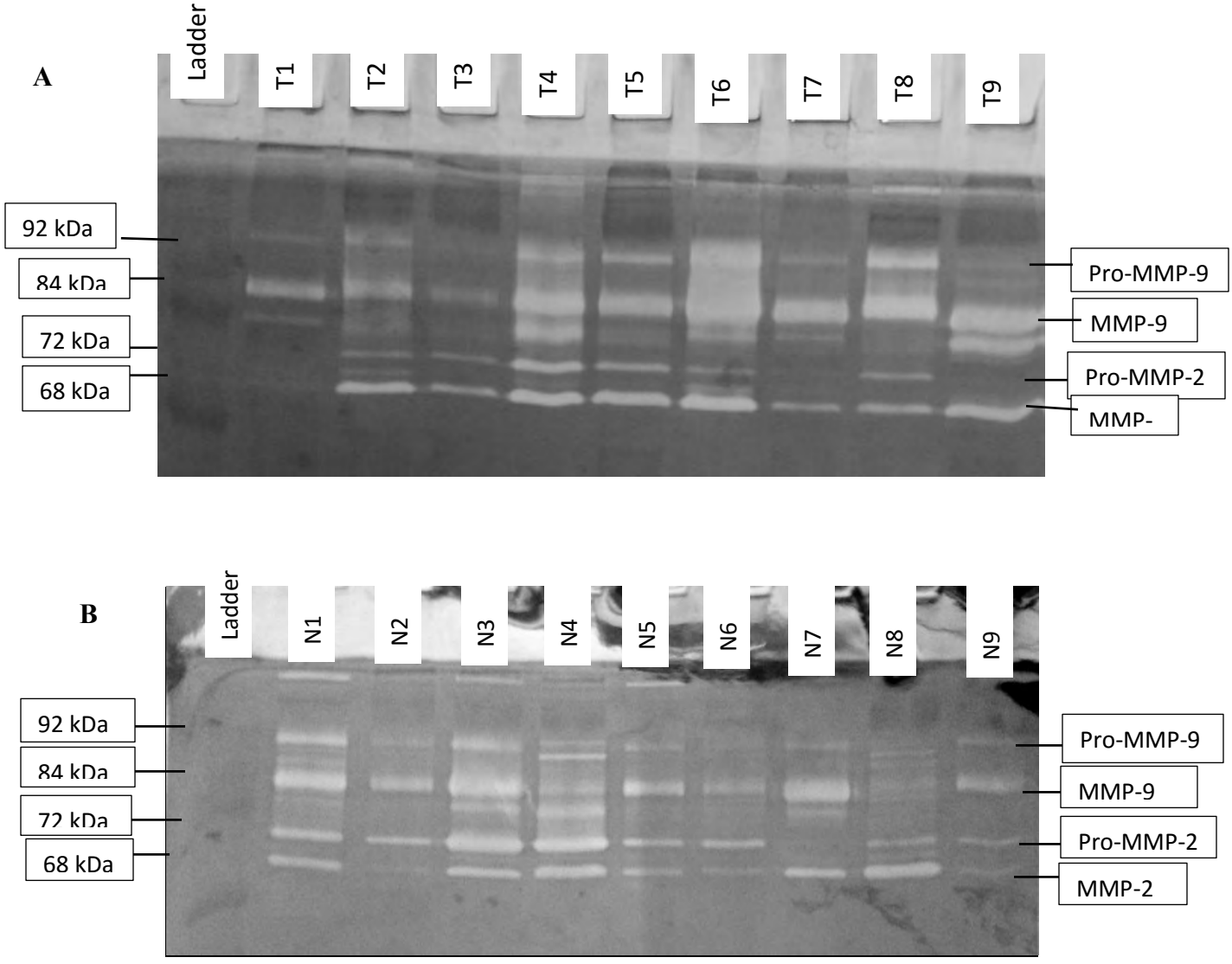

Figure 5: Matrix metalloproteinases zymogram in breast cancer tissue specimens. 20 µg of tumor (A) and normal (B) breast tissue from breast cancer patient biopsies were loaded for zymography. Pro-MMP-9 band is visible at 92 kDa, MMP-9 at 84 kDa, pro-MMP-2 at 72 kDa and MMP-2 at 68 kDa. Representative zymogram is shown.

4.7. Activities profile of cathepsin and MMPs between normal and tumor breast tissue specimens

We detected significantly higher protease activity profile in breast cancer tissue specimens compared to normal breast tissue specimens.

Table 4: Mean, standard error of mean of MMPs and cathepsins between normal and tumor breast tissue specimens. (n=36)

Variables	**Tumor**		**Normal**		**P-value**
	Mean	SEM	Mean	SEM	
proMMP9	2856	307	1225	217	0.001***
MMP9	7229	662	3911	534	0.001***
proMMP2	1338	207	1558	328	0.247
MMP2	3504	392	1393	308	0.001***
CTSK	3321	801	744	150	0.001**
CTSL	2985	439	516	80	0.001***

"***" at P<0.001, "**" P<0.01 the mean difference were significant.

The gelatinolytic activity of cathepsins and MMPs were analyzed within two age groups. The age were categorized into ≥50 and <50 years of age. The result were compared in the same age group between normal and tumor tissues. In women aged <50 years of age (n=22) figure 6.A. In women aged above and ≥50 years of age (figure 6B, n=14)

The following figure 6.B showed that a very significant increment in the activity of cathepsin L above the age group of 50 years ($P < 0.001$). In this same age group cathepsin K also shows significant increment in the enzymatic activity ($P<0.01$).

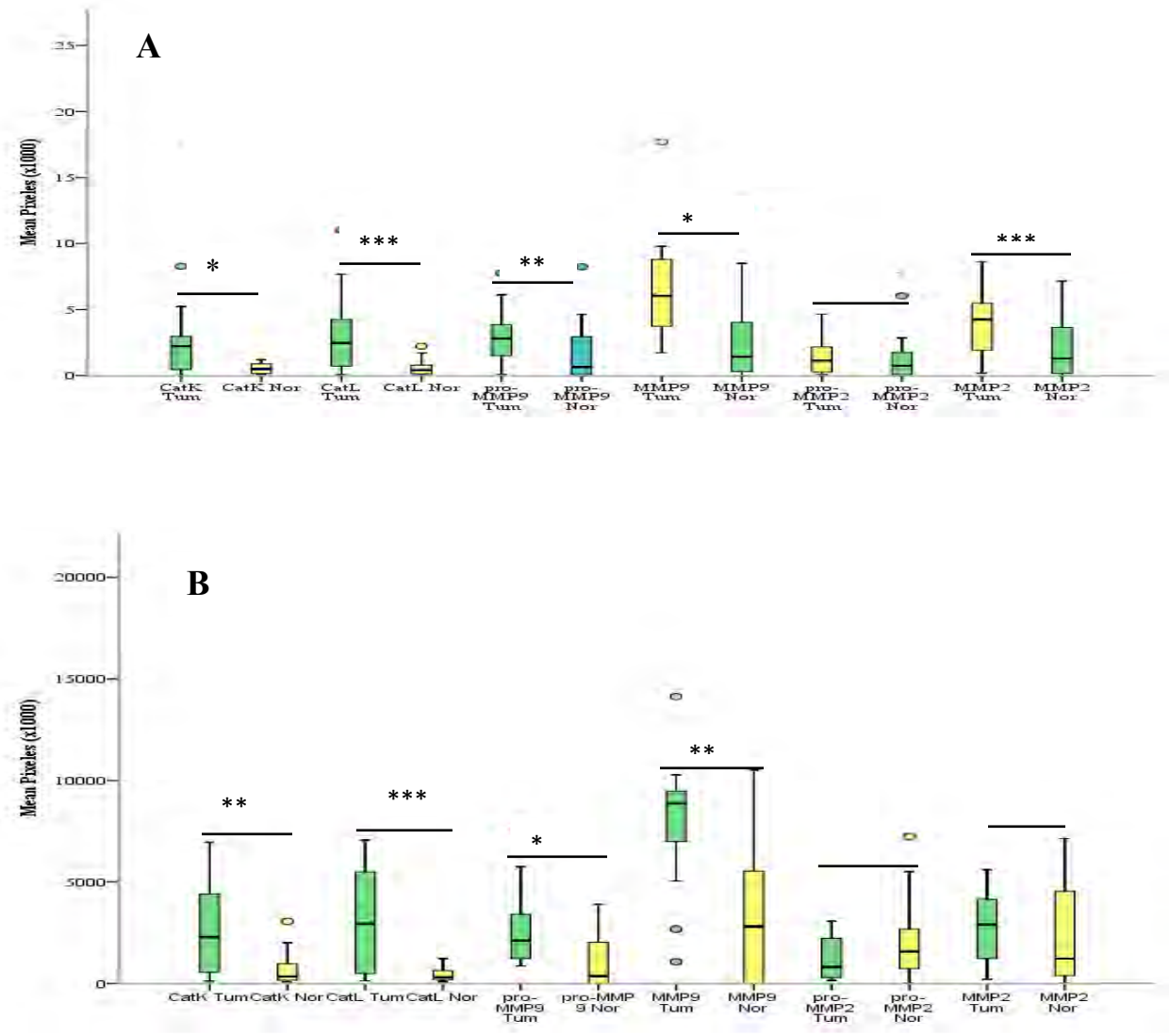

Figure 6: Analysis of densitometrically quantified cathepsin and MMPs activity within age group in breast cancer. **A)** Comparison of age <50 year with cathepsin and MMPs between tumor and normal tissue specimens in breast cancer patients (n=22). **B)** Comparison of age ≥50 years with cathepsin and MMPs between normal and tumor tissue specimen breast cancer patients (n=14). The line within the box plot corresponds to the median value, the box length to the interquartile range, and the lines emanating from the box (whiskers) extend to the smallest and largest observations. All values were expressed in range pixel count x1000 for fig 6A (*P<0.05 **P<0.01 ***P<0.001).

Our comparison result did not show significant activity of cathepsins and MMPs between tumor tissues of women aged <50 and ≥50 years of age (data were not presented in this report).

The comparison of cathepsins and MMPs gelatinolytic activity in mothers with breast cancer aged <25 years at first live birth between tumor and normal breast tissue specimen showed significantly high activity in tumor tissue. Pro-MMP-2 was not significantly upregulated. The full information were described in table 5.

Table 5: Significantly increased activity of cathepsin and MMPs in mothers age <25 during first live birth (n=20).

Variables	**n**	**Tumor**	**Normal**	**p-value**
		Mean ±SEM	Mean ±SEM	
CTSK	20	3272±928	834±217	0.015*
CTSL	20	3728±649	550±111	0.001***
Pro-MMP9	20	3387±487	1106±272	0.001***
MMP9	20	7976±844	3485±573	0.001***
Pro-MMP2	20	1315±300	1912±463	0.286
MMP2	20	3591±610	1839±468	0.028*

"***" P<0.001 "*" P<0.05, mean difference were significant

The comparison of cathepsins and MMPs gelatinolytic activity in mothers with breast cancer ≥25 years at first live birth between tumor and normal breast tissue specimen showed non-significant activity in tumor tissue. However, MMP-2 showed significant gelatinase activity. The full information were described in table 6.

Table 6: Non-significant activity change of cathepsin and MMPs in mothers age ≥25 during first live birth (n=6).

Variables	n	Tumor	Normal	p-value
		Mean ± SEM	Mean ± SEM	
CTSK	6	1870± 485	949± 500	0.216
CTSL	6	2793± 1011	361± 106	0.038
Pro-MMP9	6	2914± 290	1629± 641	0.098
MMP9	6	7451± 2354	5215± 1588	0.449
Pro-MMP2	6	1990± 445	1041± 999	0.406
MMP2	6	3953± 1030	490± 432	0.011*

"*" P<0.05, mean difference were significant

In the table 7 latent MMP-9, pro-MMP-2 and MMP-2 does not show significant activities in women who experienced their first menarche ≥ 14 when compared between tumor and normal tissue specimens (P<0.001). Cathepsin L actually showed significant upregulation in tumor tissue compared to normal breast tissue (P < 0.01). The rest of data was depicted in Table 7.

Table 7: Increased activity of cathepsin and MMP-9 in late menarche women with breast cancer. Menarche ≥14 years (n=14).

Variables	n	Tumor	Normal	p-value
		Mean ± SEM	Mean ± SEM	
CTSK	14	3248±1230	629±152	0.044*
CTSL	14	3200±891	465±95	0.005**
Pro-MMP9	14	2707±498	1496±327	0.052
MMP9	14	7485±1241	3631±679	0.011*

Pro-MMP2	14	1346±333	1926±556	0.379
MMP2	14	2615±492	1866±562	0.325

"**" P<0.01 "*" P<0.05 the mean difference were significant

In table 8 cathepsin L (CTSL), pro-MMP-9, MMP-2 showed highly significant activities in women who experienced their first menarche ≤14 years when compared between tumor and normal tissue specimens (P<0.001). The rest of the data were described in table 8.

Table 8: Increased activity cathepsins and MMPs in early menarche women with breast cancer. Menarche <14 years (n=21).

Variables	**n**	**Tumor**	**Normal**	**p-value**
		Mean ± SEM	Mean ± SEM	
CTSK	21	3479±1121	846±236	0.027*
CTSL	21	2859±482	569±121	0.001***
Pro-MMP9	21	3002±414	918±266	0.001***
MMP9	21	6977±798	3783±741	0.006**
Pro-MMP2	21	1376±281	1180±397	0.689
MMP2	21	4199±542	1082±369	0.001***

"***" P<0.001 "**" P<0.01 "*" P<0.05 the mean difference were significant

Despite pro-MMP-2, other enzyme like cathepsin K, cathepsin L, pro-MMP-9, MMP-9 and MMP-2 were significantly expressed in breast cancer tumor compared to normal breast tissue in premenopausal women. The data was presented in 7.

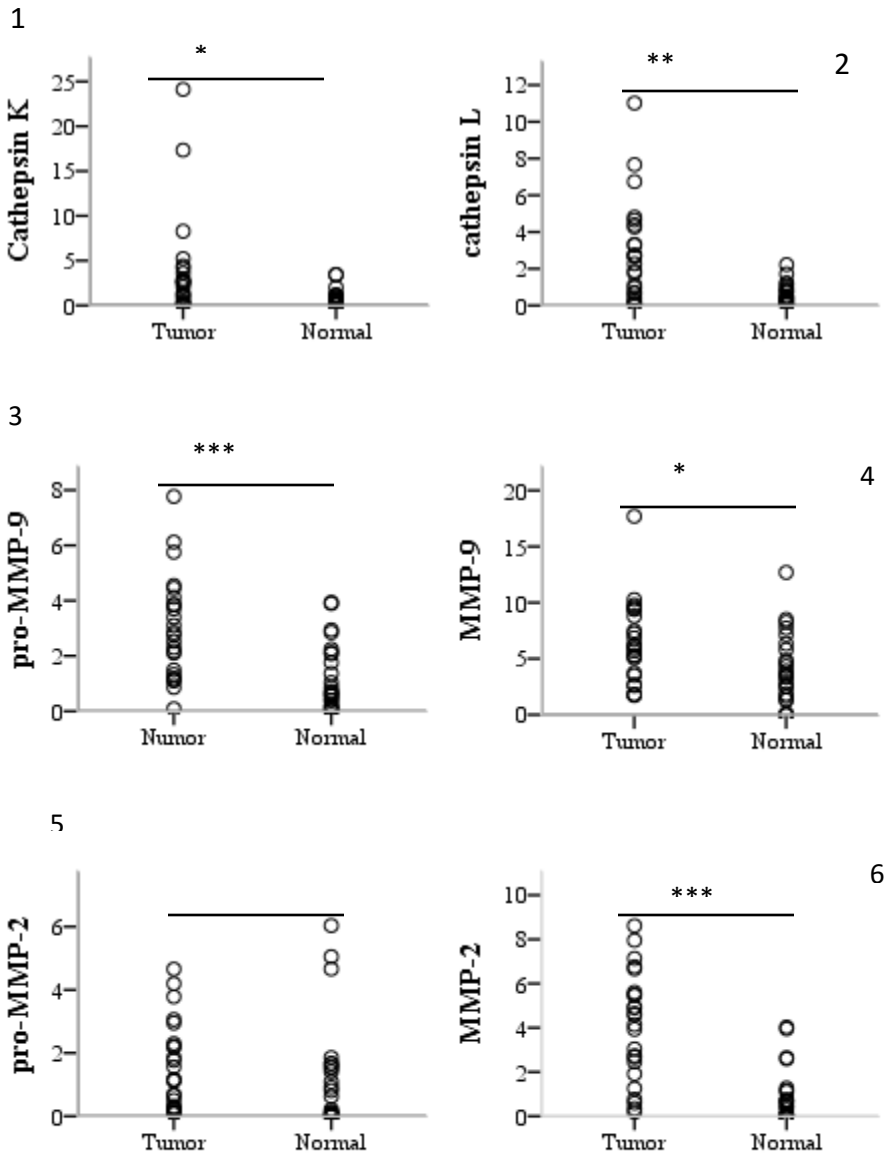

Figure 7: Pre-menopausal breast cancer patients showed increased activity cathepsin and MMPs. 1-6 showed enzyme activity between tumor and normal tissues in women age <50 years in breast cancer patients (n=24). All values are frequency of pixels X1000.

Pro-MMP-2 and pro-MMP-9 were not significantly expressed in breast tumor cell when compared to normal tissue specimen in post-menopausal women. The data is presented in figure 8

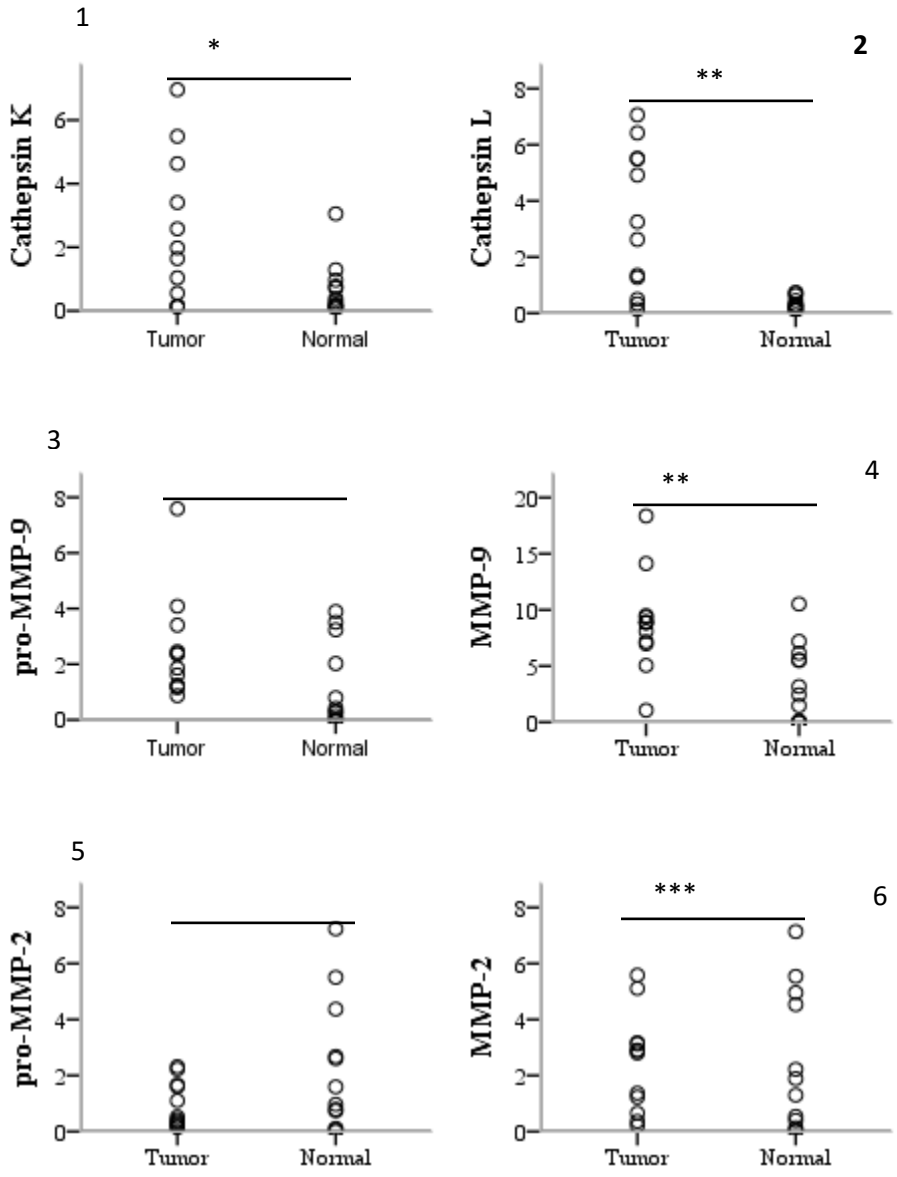

Figure 8: Post-menopausal women with breast cancer showed increased activity of cathepsin and MMPs. between tumor and normal tissues in post-menopausal breast cancer patients (n=12). All values were frequency of pixels times with 1000 (*P<0.1 **P<0.01 **P0.001).

We investigated the relationship between zymography results of pre-menopausal and post-menopausal women in tumor tissue specimens. Functional enzymatic activity expressed in rages of pixels between pre-menopausal women and post-menopausal women (n=24 premenopausal and n=12 postmenopausal). The data presented in figure 9.

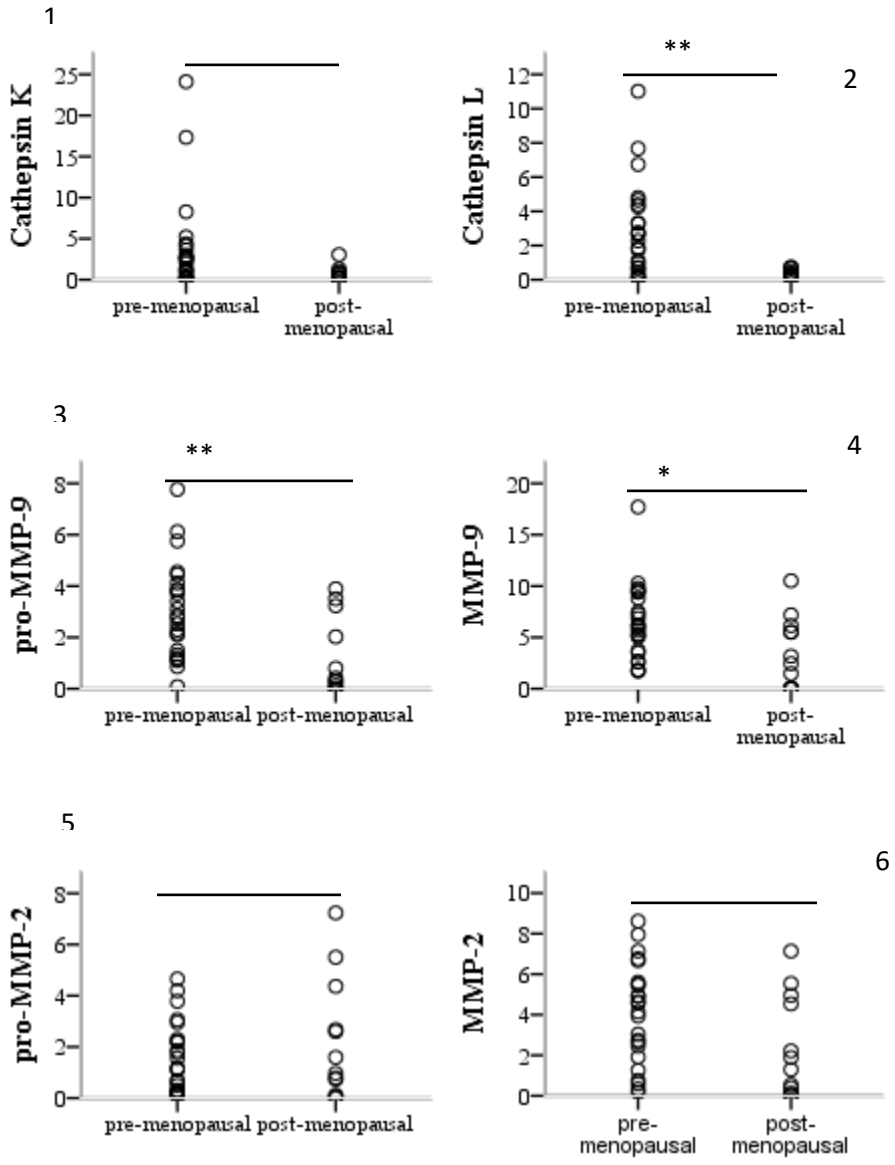

Figure 9: Analysis of cathepsin and MMPs between premenopausal and post-menopausal women in breast cancer patients. Pre-menopause (n=24) and post-menopause (n=12). All values are in frequency of pixels (*P<0.1 **P<0.001).

The activity of cathepsins and MMPs were not significantly increased between multigravida and nulliparous women. However, pro-MMP-9 was significantly expressed in tumor tissue in multi gravida women compared to nulliparous. The data were presented in the figure 10.

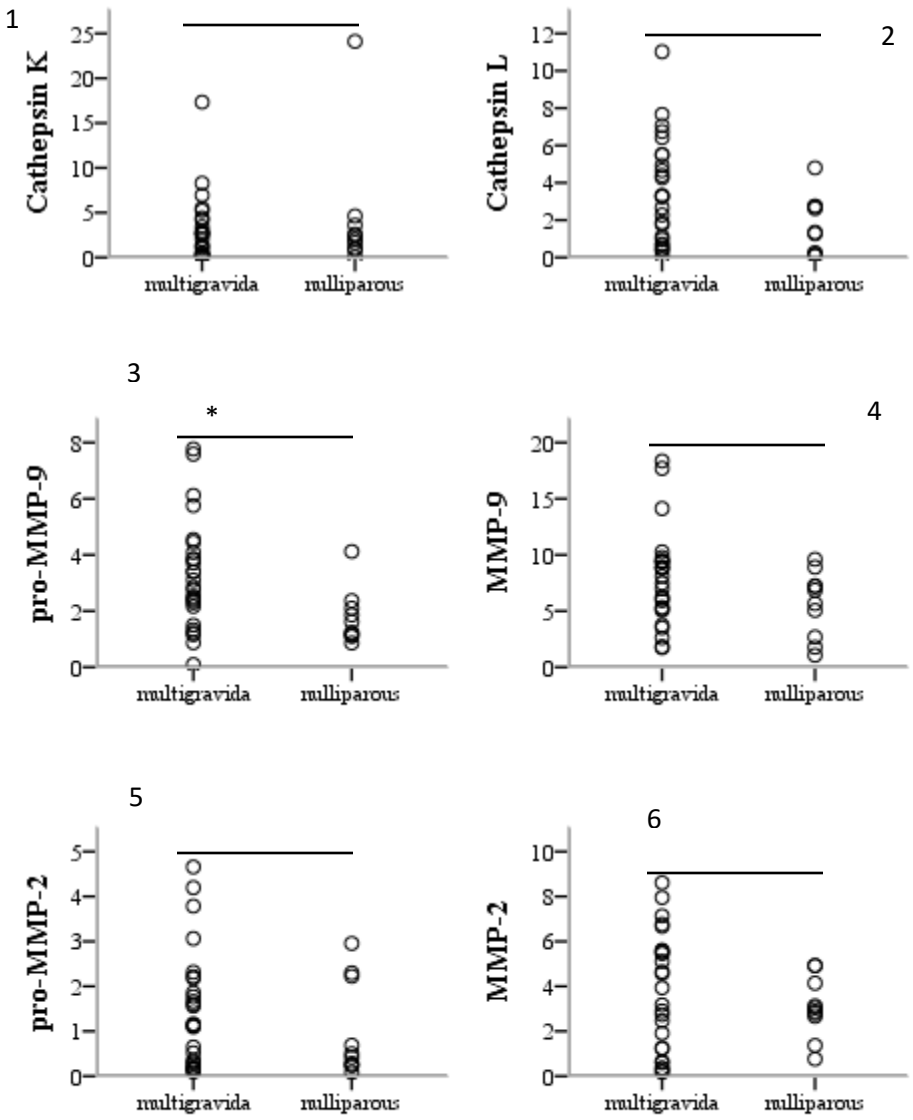

Figure 10: Parity does not change cathepsin and MMPs level of activity. 1-6 shows gelatinotinolytic activity result in multigravida and nulliparous women of breast cancer patients. Multigravida (n=26) and nulliparous (n=10). All values were in frequency pixels multiplied by 1000 (*P<0.5).

Comparison of cathepsin K and L between tumor and normal tissue showed that, both were significantly higher in tumor tissue specimens compared to normal breast tissue specimens. The data was presented in the fig 11.

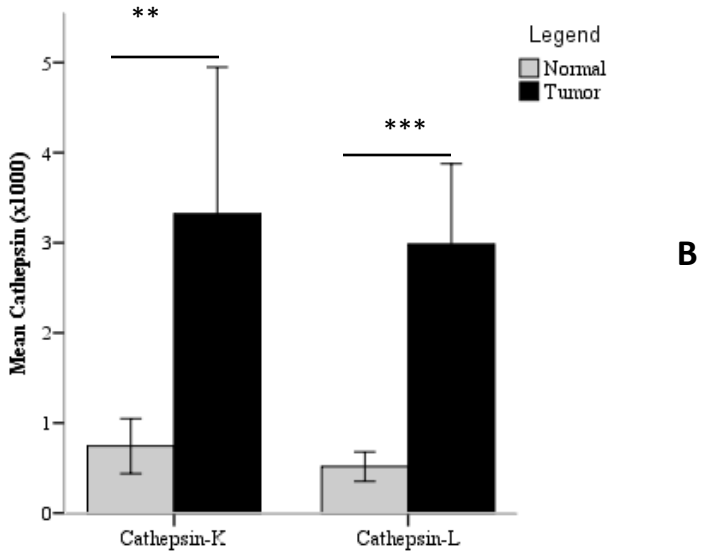

Figure 11: CTSK and CTSL activity detection between normal and tumor tissue in human breast tissue. Cathepsin activities were quantified with densitometry of each band on the gel. All values are presented in pixel Mean ± SEM of tumor compared to normal (n=36, **P < 0.01 ***P<0.001).

Tumor MMP-2 4 times increased and MMP-9 activity were 7 times higher than normal tissue. The activity of all latent and mature matrix metalloproteinase were higher in breast tissue compared to normal tissue. However pro-MMP-2 was not significantly expressed as shown in fig 12.

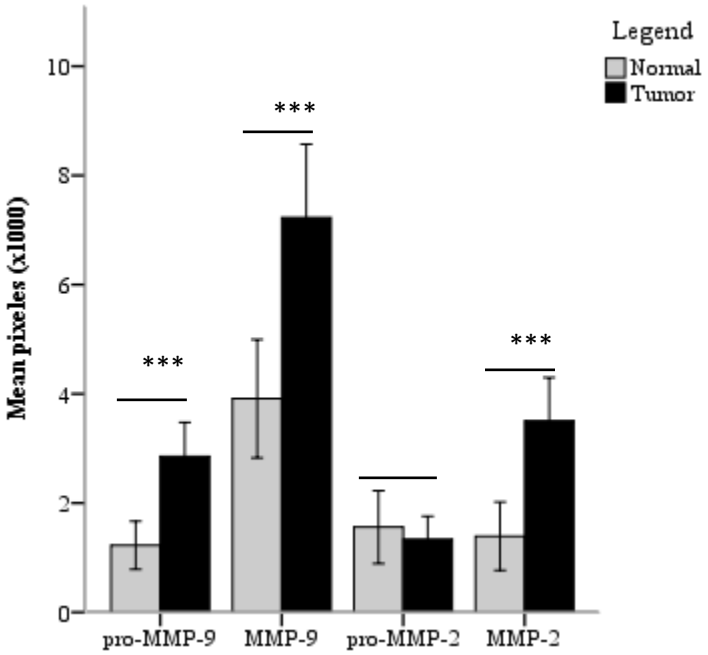

Figure 12: pro- and mature MMP-9 and MMP-2 activity detection between normal and tumor tissue in human breast tissue. Pro- and mature MMP-9 and MMP-2 activities were quantified by band densitometry. All values are presented in pixel mean ± SEM, times 1000, of tumor compared to normal (n=36, ***P < 0.001).

4.8. Tumor size specific difference of cathepsins and MMPs in breast cancer

The study indicated that there is a visible relationship between human breast cancer and functional activity of cathepsin K and cathepsin L. Both were significantly increased in tumor tissue compared to the normal tissue specimens as shown in fig 13.

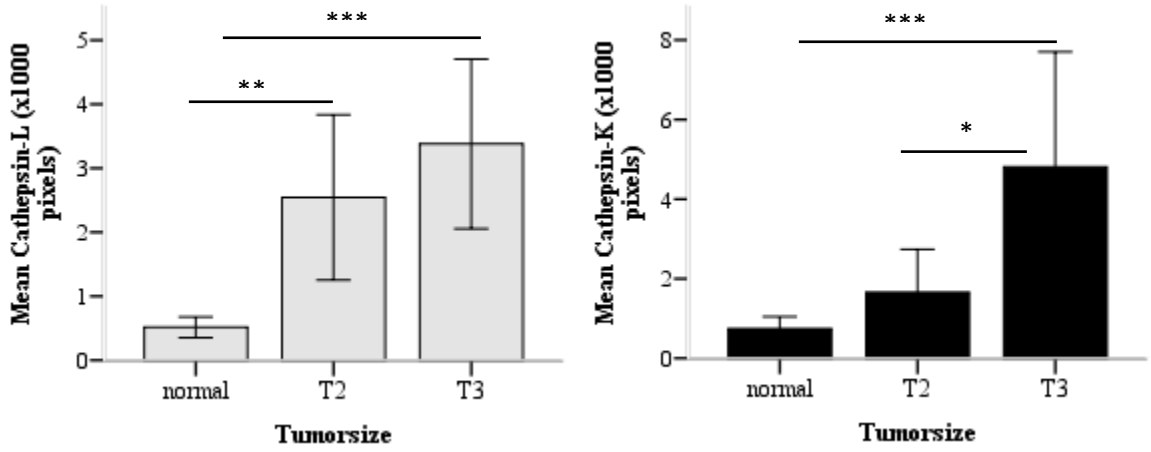

Figure 13: Tumor size-specific differences of cathepsin K and cathepsin L activity in human breast cancer. Cathepsin K and Cathepsin L were quantified by band densitometry. All values presented were in pixel mean ± SEM, times 1000 (n=36, *P<0.05 **P<0.01 and ***P<0.001)

In this study we compared MMPs enzymatic activity with tumor sizes of breast cancer. Both latent and mature MMPs enzymatic activity were analyzed and compared through the stage two and stage three. We analyzed the result of zymography band densitometry result and it revealed the following summaries. Enzymatic activity of pro-MMP9 were significantly increased between T2 tumor size and normal breast tissue specimens (n=17, ***p<0.001). As well as between T3 and normal breast tissue specimens (n=18, **P<0.01). The remaining result were depicted in figure 14.

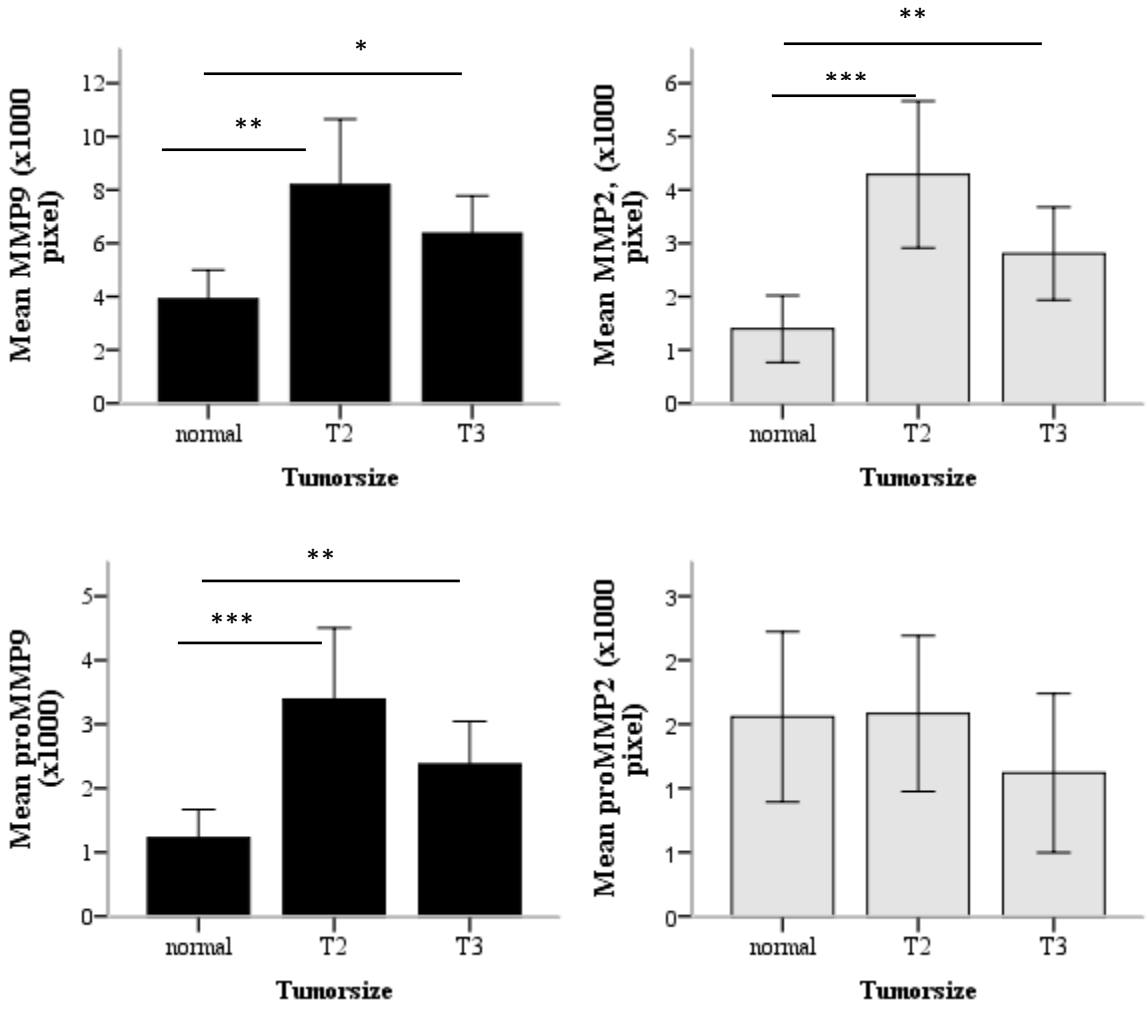

Figure 14: Tumor size-specific difference of latent and mature MMP-9 and MMP-2 in human breast cancer. Pro- and mature MMP-9 and MMP-2 band were quantified by semi-quantitative band densitometry. All values presented in pixel mean ± SEM, times 1000 (T2 (n) =17 and T3 (n) =18, *P<0.05 **P<0.01 ***P<0.001)

4.9.Stage-specific difference in cathepsins K and L in human breast cancer

We next wanted to determine any stage specific differences in breast cancer cathepsin activity using this cathepsin zymography assay. Thirty six different specimens were analyzed in different stage category.

Samples of each stages II and III breast tumor tissue (as determined by the TNM staging system according to AJCC Staging Manual) and normal tissues were collected and loaded for cathepsin zymography.

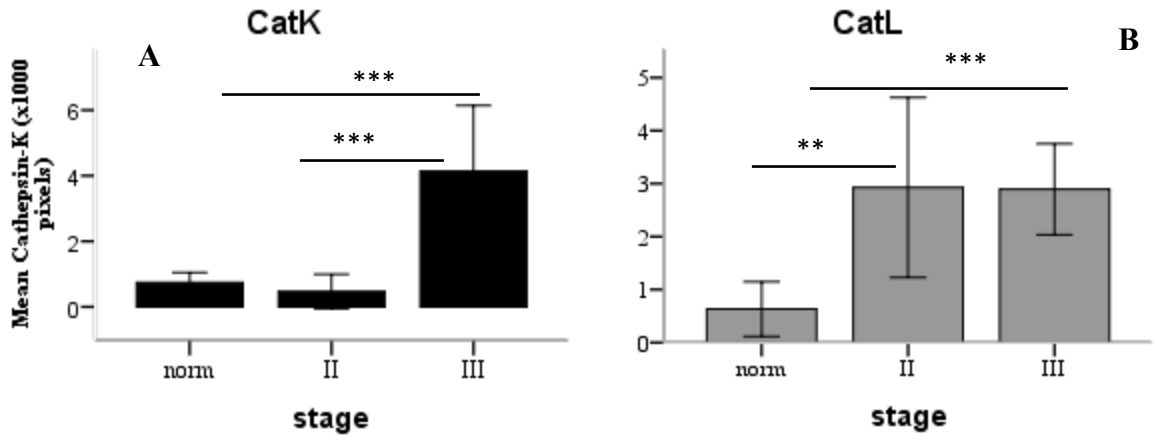

Figure 15: Stage-specific differences in cathepsins K and L human breast cancer. Cathepsins K and L activities were quantified by band densitometry. All values are presented in pixel mean ± SEM (stage II (n) =8 stage III (n) = 28, **P<0.01 ***P<0.001)

Our study participant confirmed pathological report from Tikur Anbessa Specialized Hospital showed with stage II and III breast cancer. Cathepsin K activity highly significant at stage III and stage II (Figure 15A, n = 36, *p < 0.05, **p < 0.01, ***p < 0.001). Cathepsin L activity was significantly higher than normal at stages II and III (Figure 16B, n= 36, **P < .01, ***P<0.001).

4.10. Stage-specific difference in MMPs enzymatic activity in human breast cancer

In this study we wanted to determine any stage specific differences in breast cancer MMPs activity using MMPs zymography assay. Thirty six different specimens were analyzed in different stage category. Samples of each stage II and III breast tumor tissue (as determined by the TNM staging system according to AJCC Staging Manual) and normal tissues were collected from the same patient and loaded for MMPs zymography.

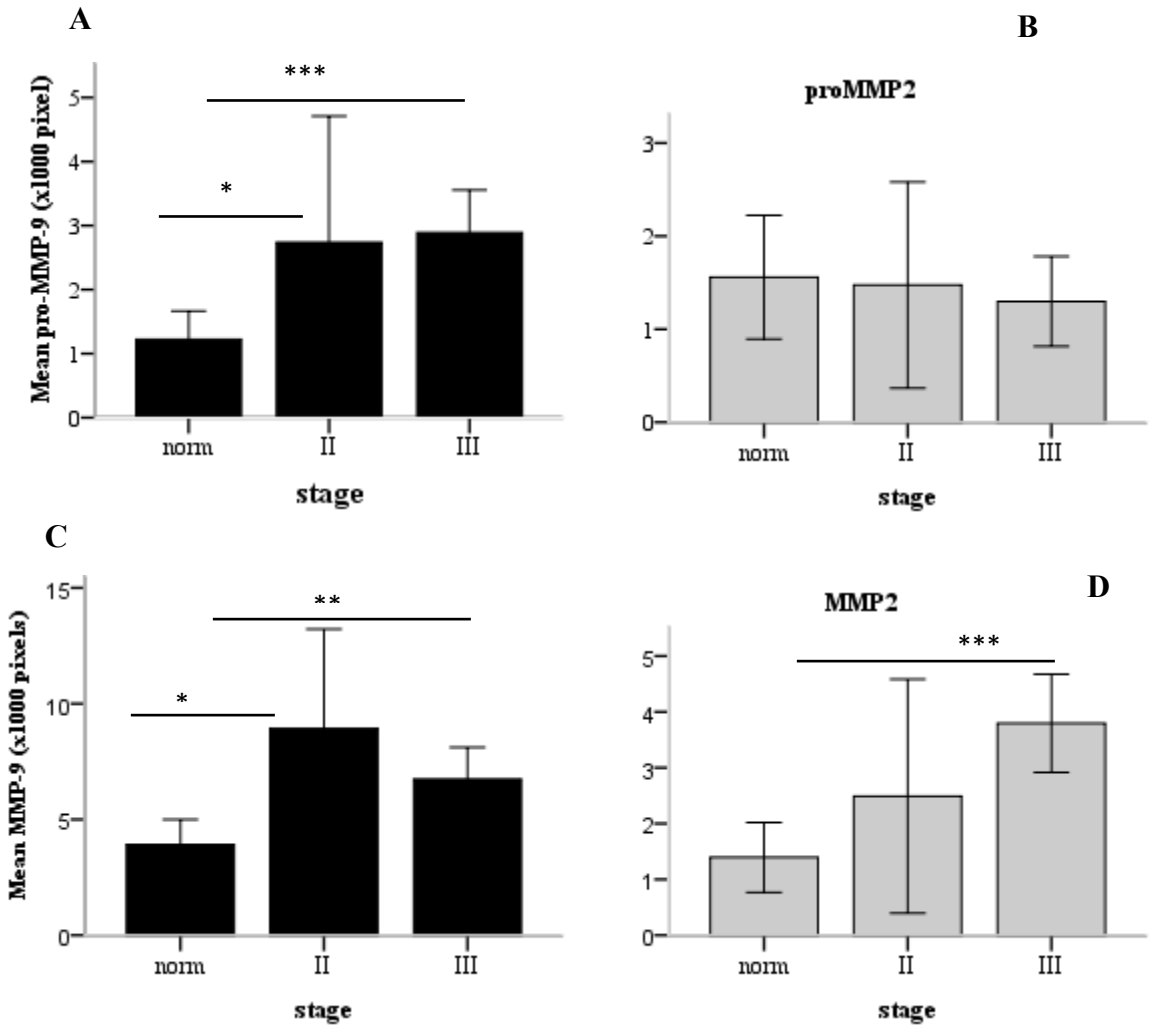

Figure 16: Stage-specific differences MMPs in human breast cancer patients. A) Pro- MMP-9 significantly higher in stage II and III compared to normal tissue. B) pro-MMP-2 doesn't show any significant change across the stages. C). MMP-9 significantly increased at stage III and II compared to normal tissue. D) MMP-2 significantly increased between stage II and normal tissue. All values are presented in pixel mean ± SEM, times 1000 (n=36, *P<0.05 ***P<0.001).

Pro-MMP-9 activity significantly increased at stage III and stage II compared to normal tissue (Figure 16A, *P<0.05, ***P<0.001). It is important to note that for pro-MMP-2 cathepsin tumor activity at all stages tested in these samples was not significantly increased compared to the normal breast tissue.

The stage-specific difference of MMP-2 and MMP-9 tested for these enzymes in this study indicates that there is a significant increment in the activity of MMP-9 between stage three and normal (n=36, **P<0.01) as well as between stage two and normal breast tissues. (n=36, *P<0.05). However there is no significant increment detected between stage two and stage three of the activity of MMP9. On the other hand MMP2 were significantly higher between stage three and normal breast tissue specimens (n=36, ***P<0.001).

In these study were observed that there is no positive or negative correlation between cathepsin K and L as well as between MMP-9 and MMP-2, also between cathepsin and MMPs. Similarly, CTS and MMPs doesn't show significant difference through histological grade between normal and tumor tissue.

5. DISCUSSION

Female breast cancer incidence is strongly related to age, with the highest incidence rate being in older women in western country, supporting a link with hormonal status. In contrary of this study our investigation showed that the incidence were higher in the age group of 30 to 39 years of age. In the UK between 2010 and 2012, an average of 80% of breast cancer cases were diagnosed over 50s, and around a quarter (24%) were diagnosed in women aged 75 and over (Akin-Odanye *et al.*, 2011).

The age distribution of breast cancer cases largely reflects the age groups eligible for breast screening in the UK. However, breast cancer is becoming the most common cancer in women aged under 40. Among women aged 35-34 in the UK, around 1,300 cases of breast cancer are diagnosed each year. Similar to this our study showed that breast cancer were diagnosed in early pre-menopausal women (Akin-Odanye *et al.*, 2011).

Zymography has great potential to impact clinical diagnoses together with histopathology due to its many benefits: (1) it does not require antibodies making it relatively inexpensive and species-independent, (2) separation of proteins by non-reducing electrophoretic migration through polyacrylamide gels visually confirms cathepsin and MMPs identity after staining of the gel, (3) densitometry can be used for quantitative analysis, and (4) multiplexed detection allows distinction of active cathepsins K, and L as well as MMPs in one cell or tissue extract (Dumas and Platt, 2013b).

Multiplex zymography utility as a supplemental screening tool of pathological specimens was effectively showed by Platt study to profile cathepsin K, L, and S activities in breast, lung, and cervical tissue at three different stages of tumor progression. According to their study, zymography information captured after clinical grading of the biopsied tissue indicating that quantitative comparisons with cathepsin zymography can supplement the gold standard histological methods of determining whether biopsied tissue is cancerous or not (Chen and Platt, 2011).

We investigated distribution of cysteine cathepsin and MMPs in normal and malignant breast tissue specimens. Our study has examined proteolysis, one of the important processes involved in tumor cell invasion and metastasis. Under normal conditions, proteolytic enzymes are tightly controlled by specific proteinase inhibitors; the MMPs are regulated by TIMPs. After zymography, the number of samples expressing each cathepsin and MMP lysis band were determined by running the relevant tumor tissue sample and normal tissue sample in separate gel in similar order down the substrate zymogram. The greatest difference in proteinase expression was observed after gelatin impregnated SDS-PAGE gel electrophoresis.

Our semi-quantitative analysis of the zymograms showed that the majority of the 36 tissue samples from breast cancer patients displayed sharp lysis bands of cathepsin and mature MMP2, MMP9 corresponding to the proenzyme forms of MMP-2 and MMP-9, respectively, in contrast to the 36 control samples which showed less pronounced lytic bands for cathepsin and MMPs.

Cathepsin and MMPs zymography revealed statistically non-significant, but shows negative correlation with the patient's age, however, CTSL has non-significant, but show positive correlation. This phenomena may be because of breast cancer in younger age more aggressive (triple negative) compared to older age (Miller *et al.*, 2002). Aggressiveness of breast cancer account by its ability of tissue invasiveness and metastasis to distant secondary organs, to do that tumor cell and the surrounding stromal cell express increased level of cathepsin and MMPs to degrade the collagen which is component of basement membrane for intravasation and cell-cell adhesion molecule and cell-ECM molecule for metastasis.

Upregulation of cathepsin and MMPs in breast cancer women who had menarche in less than 14 years may be because breast cancer in this group 1) hormone receptor negative 2) at this time breast cell extremely sensitive, thus pathogenesis of breast leads to overexpression of cathepsin and MMP (www.roswellpark.org). So, tumor cell and surrounding stromal cell express high level of these enzyme for tumor metastasis.

Up to our knowledge there is no literature that link cathepsin and MMPs with breast cancer looking parity as confounding variable. Our result doesn't show significant difference between nulliparous and multi-gravida breast cancer patients. However, there is significant increased expression of cathepsin and MMPs in breast cancer tissue compared to normal breast tissue in women who had

first live birth below twenty five years of age on contrary to, groups who had first live birth at the age of twenty five and above, these may be because the sample is small.

In these 36, age matched, breast cancer patients tissue specimens tested, tumor MMP-2 4 times increased and MMP-9 activities were 7 times higher than normal tissue (Figure 12, $n = 36$, $P < 0.001$); however, cathepsin K activity was 3-fold higher than the activity in normal breast tissue ($n = 36$, $p < 0.001$), cathepsin L was 4-fold higher (Figure 11, $n = 36$, $p < 0.001$). This because breast increased activity of cathepsin and MMPs in tumor micro-environment degrade the extracellular matrix. Our result is agrees with Platt finding (Dumas and Platt, 2013b).

The increased level of these proteolytic enzyme in tumor cell is may be because these enzyme secreted not only by tumor cell but also by stromal cell of breast tissue. Cancer cell extract energy through glycolysis even though there is enough oxygen, which create acidic tumor microenvironment that initiate downstream signaling which leads to expression of cathepsin K and cathepsin L. Other factors proposed are over expression of ErbB2, auto activation of CTS-K, truncated nucleo-cytosolic translocation of CTS-L. Cathepsin and MMPs were proteolytically degrade the extracellular matrix to facilitate tumor invasion and metastasis (Bartsch *et al.*, 2003).

From the two cathepsins studied, cathepsin L in breast tissue was especially unique in that its activity was very significantly high in cancerous tissue compared to in normal tissue. In contrary to the study done by Manu O. Platt, which showed that cathepsin K were significantly higher at all stages (Chen and Platt, 2011).

Matrix metalloproteinases (MMPs) are another family of proteases that are metal dependent endopeptidases implicated in cancer development and metastasis. MMP-2 and -9 are among the most studied members and gelatin zymography identifies their activity (Das *et al.*, 2008), similar to our study.

MMP-9 is known to play an important role in the context of tumorigenesis and metastasis because it degrades collagen IV and weakens the basement membrane (Duffy and McCarthy, 1998). Degradation products of extracellular matrix, including fragments of collagen IV, can act as signaling substances regulating cell motility (Jinga *et al.*, 2006, Quaranta *et al.*, 2007).

Compared with normal breast tissues, malignant breast tumors have increased MMP-9 activity and there is a trend towards increasing production and activation of MMP-9 in later stages of breast cancer. Nevertheless, MMP-9 expression has also been described as a positive prognostic marker in node-negative breast cancer (Scorilas *et al.*, 2001). In another study, higher MMP-9 expression in tumor cells was associated with less lympho-vascular invasion and lower tumor grade. Thus, MMP-9 expression is associated with both inhibition and stimulation of tumor growth and progression, probably because not all of these studies have measured actual MMP-9 activity (Reich *et al.*, 2014).

Zymographic study done using serum sample for control group by Scorilas et al., indicated that the mean levels of activity of circulating MMP-2 and MMP-9 in breast cancer patients were significantly higher than control serum ($P < 0.001$), and suggest that tissue samples measures of MMP-2 and MMP-9 activity may have suggestive diagnostic value for discriminating subgroups of breast cancer patients (Scorilas *et al.*, 2001).

Our study revealed that the relationship between the tumor size and enzymatic activity. Cathepsin and MMPs is significantly high in small size tumor than in larger size tumor. This may be because small size tumor easily detach from primary site as compared to larger tumor size, which is fixed at primary site (Pellikainen *et al.*, 2004). Therefore, small size tumor express these proteolytic enzyme from tumor cell as well the surrounding stromal cell. Plus through upregulation of integrin α3β1 (Têtu *et al.*, 2006) cancer tissue and hypoxia as result of fast growth in small tissue promote downstream signaling leading to increased expression of cathepsin and MMPs.

In these study we observed that upregulation of cathepsin and MMPs in late stage breast cancer as opposed to Platt study (Dumas and Platt, 2013b). This may be because late stage breast cancer are engaged in local as well as distant metastasis. Therefore these enzymes highly expressed proteolytic enzyme in later stages to degrade cell-cell adhesion molecules and cell-ECM adhesion molecules that ease the tumor metastasis.

At present, the strongest predictors of breast cancer metastasis are lymph node involvement and histological grading. These parameters are not enough selective to discriminate the putative subgroup of patients within the same clinical category. In fact, it is well documented that breast cancer patients with the same stage of disease can have markedly different outcome and therapy responses. Therefore, searching for new molecular markers is an open area of interest.

In particular, the cysteine cathepsin and matrix metalloproteases have since long attracted the interest of investigators, due to their possible use as molecular markers and therapeutic targets. The majority of MMPs are secreted as latent proenzyme forms and are subjected to regulated activation at the cell–matrix boundary. Their proenzyme forms are also secreted in body fluids where they can be easily detected.

6. CONCLUSION

The present study revealed that cathepsin and MMPs upregulated in breast cancer tissue compared to normal breast tissue. This finding shows that cysteine cathepsin and MMPs can be used to discriminate breast cancer patient from normal individuals. Increased functional activity of these enzyme also captured in breast cancer patients who had menarche less than 14 years. Therefore cathepsin and MMPs has a potential to be used in breast cancer subtyping.

Our finding shows that cathepsin and MMPs were upregulation in lymph node positive compared to lymph node negative breast cancer patients which indicates that these enzymes could be used as metastasis marker. Identification of these enzymes in breast cancer tissue could show the stages of breast cancer and to follow breast cancer patients.

Cathepsin and MMPs significantly higher in breast cancer women with small tumor size and late stage of breast cancer. Therefore, understanding and detecting cathepsin and MMPs mediated tissue remodeling is important for not only for basic science research, but also for clinical purposes, like biomarker.

7. Limitation of the Study

- MMPs and cathepsin standard were unavailable to us

8. RECOMMENDATION

- The functional activity of MMPs and cathepsin has to be studied in benign as well as in cancerous breast tumors to discriminate the change, if any.
- Survival analysis of breast cancer patients with the activity of cathepsins and MMPs has to be studied to see the role of these enzyme in tumor progression and to see the relationship with overall prognosis impact in breast cancer patients
- Gene analysis for cathepsins and MMPs hast to studied to know whether there is genetic polymorphism.

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Annex I: Material

Material for gel preparation

1. Reusable glass gel casting cassettes, 0.75 mm thick, 10 × 10 cm. were used. Mini tetran gel electrophoresis was used
2. Protogel – 37.1 to 1 acrylamide and bis-acrylamide mix.
3. 5X Separating Buffer: (pH 8.9), for volume of 100 mL we mixed 22.66 g Tris Base 100 mL of dH20. We stored at room temperature
4. 5X Stacking buffer: (pH 6.7), for volume of 100 mL we mixed 3.8 g Tris Base and 100 mL dH20. We stored at room temperature
5. 5 mg/ml Gelatin: the gelatin were dissolved in Tris buffer, pH 8.2 and the solution were heated at 37°C in a water bath for about 20 min; it was completely dissolved and mixed well. The gelatin solution were cooled down at room temperature before use.
6. 10% w/v SDS: 10% SDS in dH_2O. We stored at room temperature
7. 1.5% w/v Ammonium persulfate (APS): 10% APS in dH_2O. Stored at 4°C.
8. *N*, *N*, *N*′, *N*′-tetramethylethylenediamine (TEMED): it were stored it in a dark bottle at room temperature.
9. SDS-PAGE 10X running buffer: (pH 8.3) later we diluted to 1X by dH_2O. We prepared 1 L of this solution by mixing 30 g Tris base, 144 g of glycine and 10 g of SDS. The pH should be correct without adjusting. We stored it at room temperature.
10. SDS-PAGE 1X running buffer: pH 8.3 we prepared 1 L at a time by mixing 100 mL of 10X running buffer stock with 900 mL of dH_2O
11. Non-reducing sample buffer (5X): we prepared 10 mL of this solution by mixing 5 mg Bromophenol Blue, 1 g SDS, 2.08 mL 1.5 M Tris Base, pH 6.8, 5 mL Glycerol and 2.5 mL dH_2O (or to fill). We stored it at −20°C in 0.5-mL aliquots. Before we used it, warm it up to dissolve the SDS.

12. Cathepsin renaturing buffer: we prepared 500 mL at a time. We mixed 3.94 g Tris-base, pH 7.4, 400ml of dH_2O and 100 mL of glycerol.

13. MMPs renaturing buffer: we prepared 100 mL solution and we mixed 2.5% v/v Triton X-100 in dH_2O. Stored it at room temperature.

14. Cathepsin developing (assay) buffer: we prepared 200 mL of a solution with 0.1 M sodium phosphate buffer (100 mL), pH 6.0, 100 mL dH_2O, 2mM DTT (400 ul of 0.5 M DTT) and 1 mM EDTA (400 ul of 0.5 M EDTA).

15. MMPs developing (assay) buffer: for 100 mL we mixed 50 mM Tris-HCL, pH 7.4 (0.788 g), 10 mM CaCl2 (0.148 g), 50 mM NaCl (0.292 g) and 0.05% v/v Triton X-100 (25 uL).

16. Coumassie Staining solution – Vol. 500 mL 325 mL dH20 1L Acetic Acid 125 mL Isopropanol 225 mg Coumassie Blue R-250. Filtered with filter paper and stored at room temperature

17. Destaining solution: Prepare 1 L of 10% methanol, 5% acetic acid in dH_2O. Store it at room temperature for months.

18. Phosphate-buffered saline (PBS): PBS stock (1 sachet) were mixed with dH_2O. pH 7.1 without pH adjustment

19. Zymogram lysis buffer: Were donated by Manu O. Platt. It were a solution of (Tris-HCl (pH 7.5), EGTA 5mM, NaCl 150 mM, glycerol phosphate 20 mM, NaF 10 mM, sodium orthovanadate 1mM, triton X-100 1%, tween 20 0.1%, 1ug/ml leupeptin). Leupeptin were added fresh to lysis buffer just before adding to tissue.

20. Sodium phosphate buffer: pH 6.0, we prepared 200 mL, 0.2 M w/v $NaH_2PO_4.H_2O$ (4.8 g) mixed in 200 mL dH_2O and 0.2 M w/v Na_2HPO_4. $2H_2O$ (5.68 g) mixed in 200 mL dH_2O. Then we mixed at a ratio of 87.7 mL to 12.3 mL respectively.

21. Mobile camera: 8 MP Samsung mobile camera to take digital JPEG picture for later densitometry analysis

22. BCA total protein quantification kit. Thermo-scientific BCA kit were obtained

Annex II: Gel preparation protocol (in detail)

The following protocol is for the preparation of 2 gels of 0.75 mm thick for cathepsin zymography. Were developed from published data by Manu O. Platt (Chen and Platt, 2011)

1. Separating gel: we mixed 860 ul of dH_2O, 3.33 ml protogel, 1.6 ml of 5x separating buffer, 1.72 mL of 5mg/ml gelatin, 400 ul 1.5% APS. 80 µl 10% SDS for (12.5% gel for cathepsin zymography). Degas the solution for approximately 2 min.
2. Separating gel: we mixed 1.53 mL of dH_2O, 2.67 ml protogel, 1.6 ml of 5x separating buffer, 1.72 mL of 5mg/ml gelatin, 400 ul 1.5% APS for (10% gel for MMPs zymography). Degas the solution for approximately 2 min
3. Stacking gel: we mixed 1.9 mL of dH_2O, 1 mL of protogel, 1 mL of stacking buffer, 1 mL of 1.5% APS, 50 ul of 10% SDS. Degas the solution for approximately 2 min.
4. Add 7.5 µL of TEMED to the separating gel solution to initiate polymerization. Swirl the solution rapidly without causing bubble formation or aeration.
5. Immediately, pipette 6.2 mL of separating gel solution into each cassette avoiding the formation of bubbles.
6. Carefully overlay the separating gel solution with dH_2O up to the top of the cassette using a pipette. Do not disturb the surface of the separating gel solution.
7. Let the gel polymerize for at least 40 minute at room temperature. Polymerization is complete when a discrete line of separation can be noted between the gel and the water overlay.
8. Decant the overlay water from the separating gel.
9. Immediately, add 4 µL of TEMED to the stacking gel solution, swirl rapidly, and pipet the solution on top of the polymerized separating gels until it reaches the top of the front plate.
10. Rapidly, we inserted 10 well comb into the liquid stacking gel, making sure that no bubbles remain trapped under the comb. Let the stacking gel polymerize at room temperature for 30 minute
11. Gently pull the comb out from the stacking gel.
12. We place the gel into the Mini-Tetran Cell, ensuring that the smaller side of the cassette faces inwards. Lock into place with the Gel Wedge. Repositioned the gel.

13. We filled the lower chamber with 1X running buffer.
14. We load 20 µL of protein molecular marker (ladder) in one well.
15. We mixed a 1:4 ratio of gel-loading buffer and of sample then loaded into the wells of the gel using gel-loading tips (changed between each sample).
16. We loaded the samples and run the gel at constant voltage (200 V). These running conditions will prevent overheating of the gel. To maintain enzymatic activity, the samples are electrophoresed under non-reducing conditions
17. We checked the formation of small bubbles on the wire of the lower chamber, indicating current circulation.
18. We monitored progress of the migration every 15 minutes, using the bromophenol blue included in the loading buffer as an indicator. We let the gel run until the indicator dye reaches the bottom of the gel.
19. We carefully removed the gel from the cassette and place it in plastic tray containing 100 mL of renaturing buffer. Incubate the gel for 3 x 10 min each (for cathepsin) or 2 x 15 min each (for MMPs) at room temperature with gentle agitation.
20. We incubated the gel at room temperature for an additional 30 min in 100 mL of developing buffer with gentle agitation.
21. We decanted the developing buffer and replace it with 100 mL of fresh developing buffer. We Incubated the gel at 37°C for approximately 20 h (overnight) in a closed tray
22. We decanted the developing solution and rinse the gel at least once with 300 mL of dH_2O.
23. We removed the dH_2O and stain the gel in staining solution for at least 1 h or until the gel is uniformly dark blue. The staining solution were collected and used again. In the following staining we took a longer staining time.
24. We destained the gel with destaining solution until areas of gelatinolytic activity appear as clear sharp bands over the blue background

Annex III: Total protein quantification (BCA assay)

The total sample protein concentration of aliquot were determined using thermos-scientific BCA kit. The protocol were developed by Platt Lab. (Manu O. Platt), and we didn't find the citation for it. We used Pierce BCA standard 2 mg/mL. We duplicated the standard as well as all the samples in 96 well plate.

- We prepared diluted lysis buffer by mixing lysis buffer with dH_2O (v/v 1:10 with dH_2O) and dH_2O and
- We then diluted the BSA standard 13 µL stock with 507 µL dH_2O. following that we set-up the standard in 96 well plate,
- We set up two wells for the blank we mixed with uniform diluted lysis buffer of 10 µL with diluted BSA starting with 0 for the blank and (4,8,16 … 60 all in µL) which increased by factor of eight. The two would be summed up and the remaining would be dH_2O to brought the total volume of 100 µL.
- The protein concentration for standard were in µg/mL 0, 2, 4, 8, 12, 16, 20, 25 and 30. Generally, the sample aliquot were diluted with 100, 500 and 1000 dilution factor and incubated for about 1 hour at 37 ºC in the incubator then measure at 562 nm with LT-4000 ELISA microplate reader.
- The result were graphed, absorbance in Y axis and concentration on X-axis to obtain linear curve using MS-excel and the unknown concentration were determined from the curve

Annex IV: consent form English version

Addis Ababa University, Medical Faculty, Department of Biochemistry Graduate Study Program

Consent Form for participation as a volunteer in the research undertaking

Code number_____

Name of study subject_____

I have been informed about a study that plans to investigate the zymographic detection and clinical correlations of cysteine cathepsins and matrix metalloproteinases in human breast cancer tissue. The aims of the study were explained to me. I am also informed that all the information contained within the questionnaire is to be kept confidential. Moreover, I have also been well informed of my right to keep hold of information, decline to cooperate and make myself withdraw from the study.

It is, therefore, with full understanding of the situation that I gave the informed consent voluntarily to the researcher to use the breast tissue and blood taken from me for the investigation. Moreover, I have had the opportunity to ask questions about it and received clarification to my satisfaction. I have also been informed that the nature of the questionnaire is private.

Signature _____ Signature _____

_____

(Participant) (Investigator) Date

If you have any questions about the study, please contact

Solomon Tsegaye Tel. 0911002814, E-mail= stsegaye45@yahoo.com

Institutional Review board, Tel. 0115538743, E-mail= aaumfirb@yahoo.com

Annex V: Consent form Amharic version

አዲስ አበባ ዩኒቨርሲቲ ፣ ሜዲካል ፋኩልቲ ፣ የባዮኬሚስትሪ ትምህርት ክፍል የድህረ ምረቃ መርሀ ግብር

የፈቃደኝነት መግለጫ ቅጽ

የሚስጥር ቁጥር ----------

የተሳታፊው ሙሉ ሥም ----------

እኔ ስሜ ከላይ የተጠቀሰዉ ውል ተቀባይ በጡት ካንሰር ሕመምተኞች ዕጢ ውስጥ የሚታይ ረቂቅ ለዉጥ ምክንያትን

ለማወቅ (zymographic detection and clinical correlations of cysteine cathepsins and matrix metalloproteinases in human breast cancer tissue) ሊደረግ ስለታሰበው ጥናት መረጃ አግኝቻለሁ :: ለዚህ ይረዳ ዘንድ የእኔን ዕጢ ለመጠቀም እንደሚፈለግ ተረድቻለሁ::

ስለጥናቱ አላማ ፤ እንዲሁም ናሙና ሲወሰድ በኔ ላይ መጠነኛ የህመም ስሜት ሊያስከትል እንደሚችል ከውል ሰጪው ገለፃ ተረድቻለሁ:: በተጨማሪም መጠይቁ ዉስጥ በተካተቱት ጥያቄዎች መሰረት የምሰጣቸው መረጃዎች በጠቅላላ በሚስጥር እንደሚጠበቁ ተገልጾልኛል ::

እንዲሁም እኔን በተመለከተ የምጠየቀዉን መረጃ ያለመስጠት ፤ በጥናቱ ያለመተባበርና ከጥናቱ በማናቸዉም ጊዜ ራሴን የማግለል መብቴ የተጠበቀ መሆኑ ተገልጾልኛል:: ስለዚህ ለውል ሰጪው መረጃና የስምምነት ቃሌን የሰጠሁት በአጠቃላይ ሁኔታውን በመረዳትና በፍፁም ፈቃደኝነት ነው:: ከኔ የሚወሰደው ናሙና ለምርምር እንደሚውል ተረድቻለሁ:: በተጨማሪም ጥያቄ ለመጠየቅ ተፈቅዶልኝ ለማወቅ የፈለኩትን ያህል ማብራሪያ አግኝቻለሁ::

የውል ተቀባይ ፊርማ ---------- የውል ሰጪ ፊርማ ---------- ቀን ----------

ጥ ናቱን በተመለከተ ማንኛዉም አይነት ጥያቄ ቢኖርዎት በሚቀጥለው አድራሻ በነጻነት መጠየቅ ይችላሉ::

ሰሎሞን ፀጋዬ, ስልክ. 0911002814, e-mail= Solomon_ts16@yahoo.com

ኢንስቲትዩቲሽናል ሪቪው ቦርድ, ስልክ. 0115538743, e-mail= aaumfirb@yahoo.com

Annex V: **Questionnaire**

Code NO_____

1. Age_____ 1.2. Sex _____ 1.3. Residence/specific/

_____

2. Height_____ weight_____ BMI_____

3. Clinical feature

3.1. Breast lump/mass

3.2. Size

3.3. Breast pain

3.4. Nipple discharge

3.5. Nipple retraction

3.6. Nipple itching

3.7. Fungated ulcer

3.8. Peau d'orange skin

3.9. Lesion fixed to skin

3.10. Dimpled skin

3.11. Axillary LNS

3.12. Supra clavicle LNS

3.13. Others

4. Duration of symptom in month _____

5. Duration of illness _____

6. History

6.1. Self-history

6.2. Family history

6.2.1. Family history of breast cancer Yes No

If yes, 1st degree_____2nd degree_____3rd degree_____

6.2.2. 1.Family history of ovarian cancer_____

Yes _____ No _____

If yes, 1st degree_____2nd degree_____3rd degree_____

Family history of other type of cancer_____

Yes No

•If yes, 1st degree_____2nd degree_____3rd degree_____

6.3. Herbal medication history

6.4. Current/recent oral HRT use (combined estrogen and progesterone) _____

6.5. History of benign breast disease _____

6.6. Prior mammographic screening _____

6.7. Alcohol consumption_____ Smoking Status _____

6.8. Oral contraceptive use_____

6.9. Reproductive history

6.9.1. Age of menarche

6.9.2. Age of menopause

6.9.3. Child birth

6.9.3.1. Never

6.9.3.2. Have _____ Number_____

6.9.3.2.1. Age at first birth

- ≤25
- 26- 29
- ≥30

6.10. Ever been breast feeding any child: Yes_____ No _____

7. Clinical stage _____

8. Tumor size

8.1. T1 < 2cm

8.2. T2 2-5 cm

8.3. T3 > 5cm

9. Lymph node involvement

9.1. No lymph node involvement

9.2. N11-3

9.3. N24-9

9.4. N3> 9

9.5. Which LN?

9.5.1. Axillary

9.5.2. Supraclavicular

10. Location

10.1. Upper Outer quadrant

10.2. Upper inner quadrant

10.3. Lower outer quadrant

10.4. Lower inner quadrant

10.5. Central

11. Stage

11.1. Early

11.2. Advance

12. Site

12.1. Right_____ 12.2. Left _____