ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

PhD Dissertation

Breast Cancer Subtypes, Associated Biomarkers, and the Involvement of Human Papilloma Virus in Ethiopian Population

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A Thesis Submitted to the School of Graduate Studies of Addis Ababa University in Fulfillment of the Requirement of the Degree of Doctor of Philosophy in Microbial, Cellular, and Molecular Biology

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December 22, 2023

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WORK DECLARATION

I hereby declare that the content of this dissertation is entirely my work and that all data and information used in its preparation have been properly cited. This dissertation was submitted to Addis Ababa University's College of Natural and Computational Sciences, Department of Microbial, Cellular, and Molecular Biology, to fulfill the requirement for the Doctor of Philosophy in Microbial, Cellular, and Molecular Biology. I hereby certify that this dissertation will not be submitted to another school to get an academic degree.

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CONFIRMATION

This dissertation has been submitted for examination with my approval as an advisor.

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ABSTRACT

Breast cancer is the most common type of cancer in the world as well as in Ethiopia. Although research on breast cancer in Ethiopia has been conducted, none of them have evaluated breast cancer in multiple regions of the country, which is important considering Ethiopia's enormous ethnic and genetic diversity. Hence, this study was carried out to evaluate the distribution of breast cancer subtypes and associated immune cell biomarkers, hormone receptors, matrix metalloproteinases (MMPs), and HPV genotypes in selected Ethiopian regions.

A total of 227, 81, 58, and 120 formalin-fixed paraffin-embedded (FFPE) tissue blocks were collected for breast cancer subtyping, immune cell biomarkers analysis, MMP expression, and HPV genotyping, respectively. Immunohistochemistry (IHC) staining was performed for breast cancer subtyping based on estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER-2), and Ki-67 proliferation markers, and for additional immune cell biomarker expression. RNA was extracted and quantitative reverse-transcription PCR was performed for MMP expression analysis. DNA was extracted from archived FFPE breast tissue specimens and target genes were amplified using PCR for HPV genotyping. SPSS Version 25 was used to enter and analyze data. For immune cell biomarkers and MMP results, GraphPad Prism version 8.0.0 was used for statistical analysis.

A large percentage of breast cancers were found to have advanced clinical and pathologic features, such as substantial lymph node involvement, large tumor size, and high histological grade. The percentage of ER and PR-negative tumors were 48.3% and 53.2%, respectively. The IHC subtype distribution was 33.1% triple-negative (ER-, PR-, HER-2-) breast cancer, 27.6% luminal B ((ER+, PR+, HER-2- and Ki-67 ≥ 20%) or (ER+, PR+, and HER-2+)), 25.2% luminal A (ER+, PR+, HER-2- and Ki-67< 20%), and 14.1% HER-2-enriched (ER-, PR-, HER-2+). In multiple logistic regression analysis, grade III and HER-2 positivity were associated with larger tumor size, and tumor size was also higher in samples from Southwestern Ethiopia (Jimma) as compared to Northern Ethiopia (Mekele).

The MMP-11 expression levels were significantly higher in breast cancer cases than in benign breast tumors (P=0.012).

The non-luminal (triple-negative and HER-2-enriched) breast cancer subtype had a higher percentage of stromal CD20+, intratumoral CD3+ tumor-infiltrating lymphocytes, and CD68+ tumor-associated macrophages than the luminal (Luminal A and Luminal B) subtype. The stromal programmed cell death ligand 1 (PD-L1) +, intratumoral CD3+ tumor-infiltrating lymphocytes, CD163+ tumor-associated macrophages, and PD-L1+ were also more commonly found in grade III breast cancer than in grade I and II breast cancer, respectively. Human papillomavirus was found in 20.6% of breast cancer patients and 29.6% of non-malignant breast tumors. Human papillomavirus infection was nearly 10-fold more common in ER-positive than ER-negative breast cancer.

A considerably high prevalence of triple-negative breast cancer was reported in our study, demanding additional research that includes identifying genetic predisposition factors. A significant association was found between the breast cancer subtype and stromal CD20+, intratumoral CD3+ tumor-infiltrating lymphocytes, and CD68+ tumor-associated macrophages. The stromal PD-L1+, intratumoral CD3+ tumor-infiltrating lymphocytes, CD163+ tumor-associated macrophages, and PD-L1+ were also associated with tumor grade. Our findings suggest an important impact of MMPs in breast cancer pathophysiology, particularly MMP-11. This study also showed no proof of a link between HPV infection and breast cancer; however, the finding that HPV was more prevalent in breast tumors that were ER-positive than ER-negative warrants further attention.

Keywords: Breast cancer, Estrogen receptor, HER-2, HPV genotype, IHC subtype, Immunohistochemistry, MMP, Tumor-associated macrophages, Tumor-infiltrating lymphocytes, and Triple-negative

ACKNOWLEDGEMENTS

My profound and genuine gratitude and appreciation go to my advisors, Rawleigh Howe (MD, PhD), Adey Feleke (PhD), Tesfaye Sisay (DVM, PhD, Prof), Dinkisira Bekele (MD), for their unreserved support in advising me, providing valuable feedback and guidance.

I would also like to thank Eva J. Kantelhardt (MD, Prof), Marcus Bauer (MD), Tewodros Yalew (MD), Selfu Girma (MSc), Menberwork Chanyalew (PhD), Tigist Desta (MD), Menal Hassen (MD) and Bethlehem Nigussie (MD) for their critical comment, pathology laboratory work advice, and data interpretation.

I would like to express my gratitude to the Mizan Tepi University, Addis Ababa University School of Graduate Studies, College of Natural Sciences (Department of Microbial, Cellular, and Molecular Biology), and Armauer Hansen Research Institute (AHRI) for providing a suitable environment and funding for my PhD research.

I would like to express my gratitude to all pathologists and pathology experts at Hawassa Referral Hospital, Jimma University Specialized Hospital, Ayder Referral Hospital, and Hiwot Fana Specialized University Hospital for their assistance with data collection.

I acknowledged all staff of the pathology department, driver officers (Mr. Shambel and Mr. Solomon Gebre), and drivers from Armauer Hansen Research Institute for their contributions. I also acknowledged Fitsum Girma (Ph.D.) for providing a few reagents for PCR work.

I am most grateful to my wife Dareskedar Tsehay, who takes care of the house and provides devoted help with academic editing, and my children, Hasset, and Mama, who cheer me up after a demanding day at work

My special thank also extends to my family and friends, who were at my side throughout the entire study period.

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LIST OF ABBREVIATIONS

DC	Dendritic Cells
cDC	Conventional Dendritic Cell
EMT	Epithelial-Mesenchymal Transition
ER	Estrogen Receptor
FFPE	Formalin Fixed Paraffin Embedded
FISH	Fluorescence In Situ Hybridization
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
HER-2	Human Epidermal Growth Factor-2
HPV	Human Papillomavirus
IFN-γ	Interferon Gamma
IHC	Immunohistochemistry
IL	Interleukin
ILC	Innate Lymphoid Cell
MDSC	Myeloid-Derived Suppressor Cells
MMP	Matrix Metalloproteinase
NAC	Neoadjuvant Chemotherapy
NK	Natural Killer
pCR	Pathologic Complete Response
pDC	Plasmacytoid Dendritic Cell
PR	Progesterone Receptor
RT-qPCR	Reverse Transcription Quantitative Real-Time PCR
Th	T Helper
TNF	Tumor Necrosis Factor
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor

1. CHAPTER ONE: INTRODUCTION

Cancer is characterized by an uncontrolled and invasive growth of cells that spreads to other parts of the body through blood circulation and lymphatic vessels (Sudhakar, 2009). Sustained proliferation signaling, evasion of growth suppression, resistance to cell death, enablement of replicative immortality, stimulation of angiogenesis, and facilitation of invasion and metastasis are all main features of cancer (Hanahan and Weinberg, 2011).

In 2020, there were an estimated 19.3 million new cases and 9.9 million deaths from cancer and the worldwide burden of cancer has significantly increased in recent years. Breast cancer is the most common cancer type worldwide (11.7% of all cases), followed by lung (11.4%), colorectal (10.0 %), prostate (7.3%), and stomach (5.6%) cancers (Sung et al., 2021). It is one of the leading causes of mortality among women worldwide, with an estimated 2.2 million cases and 684,996 deaths in 2020, accounting for 24.5% of cancer cases and 15.5% of cancer deaths (Ginsburg et al., 2017, Bray et al., 2018). It is predicted to cause more than 3 million cases and 1 million deaths by 2040 (Arnold et al., 2022)

Breast cancer mortality is high in Africa, with the highest mortality rates being recorded in sub-Saharan Africa (Azubuike et al., 2018, Jedy-Agba et al., 2016). In 2020, there were 85,700 deaths in Africa, and by 2040 it is predicted to be doubled (Sung et al., 2021). The high number of deaths in Africa may be due to inadequate access to early detection, diagnosis, and treatment and lack/shortage of public health care facilities and professionals for following and managing such cases. However, it may also related to cultural differences in lifestyle behaviors, socioeconomic factors, and differences in the biological characteristics of breast cancer (Brewster et al., 2014). Breast cancer has also been shown to occur at an earlier age in African countries than in others, with a median age of approximately 45 years (Ginsburg et al., 2017). Additionally, the survival rate for breast cancer patients in the continent is lower than the global average (Jedy-Agba et al., 2016). Reports indicate that African women have a disproportionately high incidence of breast cancer with poor prognosis, such as estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, triple-negative breast cancer (Brinton et al., 2014).

In Ethiopia, the number of cancer cases and deaths were estimated to be 77,352 and 51,865, respectively, in 2020. Breast cancer is the most common type of cancer in Ethiopia accounting for 31.9% of total cancer cases in women; 16,133 new cases and 9,061 deaths in 2020 (Sung et al., 2021). Breast cancer was shown to mainly affect young women in the Ethiopian population and patients commonly present with advanced stage at diagnosis (Gemta et al., 2019). Studies have shown the incidence of breast cancer is also rising in Ethiopia (Hailu et al., 2020).

In the past 20 years, molecular classification based on the expression of ER, PR, human epidermal growth factor-2 (HER-2), and Ki-67 proliferation index has provided prognostic, predictive, and diagnostic information. The main subtypes that have been discovered are HER-2-enriched (ER-, PR-, HER-2+), luminal A (ER+, PR+, HER-2- and Ki-67< 20%), luminal B ((ER+, PR+, HER-2- and Ki-67 ≥ 20%) or (ER+, PR+, and HER-2+)) and triple-negative breast cancer (ER-, PR-, HER-2-). These molecular subtypes are linked to distinct biological features, treatment responses, and disease-specific outcomes (Hu et al., 2006, Sorlie et al., 2001), and show significant differences in the prediction of overall and disease-free survival (Malhotra et al., 2010).

Tumor-infiltrating lymphocytes, which contribute to the anti-tumor response, are low-cost biomarkers with prognostic and predictive potential (Valenza et al., 2023). Understanding tumor, microenvironment, and host variables is critical for immunotherapy effectiveness in breast cancer (Adams et al., 2019). The expression of tumor-infiltrating lymphocytes has been shown to vary across different breast cancer subtypes, clinical characteristics, and pathological features (Duong et al., 2023). A high level of tumor-infiltrating lymphocytes is linked to a better prognosis, particularly for patients with HER-2-enriched and triple-negative breast cancer subtypes (Hong et al., 2021, Gao et al., 2020, Zgura et al., 2018, He et al., 2020, Loi et al., 2014, de Jong et al., 2022). A 10% increase in tumor-infiltrating lymphocytes was related to a considerably longer overall and disease-free survival of HER2-positive and triple-negative breast cancer (Luen et al., 2017, Denkert et al., 2018).

Tumor-associated macrophages (CD68 and CD163), which are involved in the development of breast cancer via angiogenesis, migration, metastasis, and immune evasion, were a potential therapeutic target for breast cancer (Huang et al., 2022, Williams et al., 2016, Qiu et al., 2018). A high level of tumor-associated macrophage is associated with poor prognosis (Allison et al., 2023). Another study found that a poor prognosis was only associated with high CD163-positive tumor-associated macrophage expression (Chohan et al., 2023). The distribution of tumor-infiltrating lymphocytes in distinct breast cancer subtypes revealed geographical heterogeneity (Bauer et al., 2023).

Programmed cell death ligand 1 (PD-L1) is an effective immunotherapeutic target for patients with metastatic triple-negative breast cancer (Alkaabi et al., 2023, Zhang et al., 2023). Currently, Pembrolizumab and atezolizumab, PD-L1 inhibitors, have been used in combination with chemotherapy to treat PD-L1 positive metastatic triple-negative breast cancer, and this has increased overall breast cancer survival (Emens and Loi, 2023).

Matrix metalloproteinases (MMPs) play essential roles in physiological processes such as organogenesis, cell repair, remodeling of tissues, apoptosis, and motility (Xie et al., 2017). The MMPs are also involved in pathological processes like cancer development, tumor neovascularization, angiogenesis, invasion, and metastasis (Egeblad and Werb, 2002, Quintero-Fabián et al., 2019). The expression and activity of MMPs are increased in advanced tumor stages and metastasized disease (Egeblad and Werb, 2002). Several studies also investigated the association between clinicopathological features of breast cancer with MMP-2, MMP-9, and matrix metallopeptidases -11 (MMP-11) expression. An inverse correlation between the expression of MMP-2 and MMP-9 in breast cancer has been reported (Benson et al., 2013, Ren et al., 2015, Li et al., 2017b, Egeblad and Werb, 2002, Joseph et al., 2020). Further, studies have demonstrated a positive correlation between the expression of MMP-2, MMP-9, and MMP-11 and breast cancer prognosis (Cheng et al., 2010, Zeng et al., 2013, Min et al., 2014, Yang et al., 2018). However, an earlier study by Chenard and colleagues revealed that MMP-11 levels showed no correlation with breast tumor size, axillary node status, and tumor grade (Chenard et al., 1996).

A considerable percentage of the world's burden of cancer may be linked to viruses, which are thought to be responsible for 15% of all human cancers globally. It has been demonstrated that human cancer can be caused by both DNA and RNA viruses (Liao, 2006). Viruses such as human papillomavirus (HPV), Epstein Barr virus, mouse mammary tumor virus, and bovine leukemia virus show potential roles in breast cancer development, but the epidemiological and proposed oncogenic mechanisms have not yet provided strong conclusive evidence (Afzal et al., 2022, Naushad et al., 2017). There is considerable debate and skepticism about the notion that HPV infection is the etiological factor contributing to the development of breast cancer (Kudela et al., 2019). The presence of HPV in malignant breast tumors has been reported, indicating a potential role of HPV in the early stage of breast cancer and breast carcinogenesis (Maldonado-Rodríguez et al., 2022, Cavalcante et al., 2018, Khodabandehlou et al., 2019, Salman et al., 2017, Ngan et al., 2015). In contrast, a lack of etiological linkage between HPV and breast cancer has been documented in different countries (Bønløkke et al., 2018, Gannon et al., 2015).

1.1. Statement of the Problem

Breast cancer mortality is high in Africa, with the highest incidence rates being recorded in sub-Saharan Africa (Azubuike et al., 2018). In Ethiopia, breast cancer is the most common type of cancer constitutes 31.9% of all cancer cases in women with 16,133 new cases and 9061 deaths in 2020 (Sung et al., 2021). In Ethiopia, breast cancer mainly affects young women and shows an advanced stage at diagnosis(Gemta et al., 2019), and the incidence is also rising (Hailu et al., 2020), but the exact figure is still not known. The lack of appropriate data has hampered an accurate national estimate of the breast cancer burden, a condition that is also observed in southern and southwestern Ethiopia; hence, an objective of this study was to address the gap in epidemiological data on breast cancer in southern and southwestern Ethiopia.

In recent years, several biomarkers that provide diagnostic, predictive, and prognostic information have been identified (Nicolini et al., 2017). The expression of ER, PR, HER-2, and Ki-67 proliferation biomarkers is critically important for the subtyping of breast

cancer and for providing appropriate prognostic, diagnostic, and predictive information. Triple-negative breast cancer is high in most sub-Saharan African countries, complicating the empiric therapeutic use of ER/PR antagonists. An initial study in Ethiopia reported a low number of triple-negative breast cancer subtypes (Hadgu et al., 2018). Although different studies have been conducted on the incidence and molecular subtypes of breast cancer in Addis Ababa City, few studies have been done in other regions of the country and none has assessed several regions in the context of the extensive ethnic and genetic diversity due to substantial genetic and cultural diversity found in Ethiopia (López et al., 2021, Pagani et al., 2012). Therefore, this study aimed to assess the distribution of immunohistochemistry-breast cancer subtypes in several regions of Ethiopia.

The tumor-infiltrating lymphocytes, tumor-associated macrophages, and PD-L1 are promising predictive and prognostic biomarkers in breast cancer, notably in triple-negative breast cancer and HER-2 positive patients (Gao et al., 2020, Zgura et al., 2018, He et al., 2020, Loi et al., 2014, Dieci et al., 2020, de Jong et al., 2022, Ochi et al., 2019, Allison et al., 2023, Emens and Loi, 2023). A 10% increase in stromal tumor-infiltrating lymphocytes was related to the considerably longer overall survival of HER-2-positive and triple-negative breast cancer patients (Luen et al., 2017, Denkert et al., 2018). The distribution of tumor-infiltrating lymphocytes in distinct breast cancer subtypes revealed geographical heterogeneity (Bauer et al., 2023). There have been few studies on the immunological landscape of breast cancer in Africa. As a result, the purpose of this study is to evaluate the distribution of tumor-infiltrating lymphocytes, tumor-associated macrophages, and PD-L1 within the intratumoral and stromal parts of the tumor, as well as the relationship between tumor-infiltrating lymphocytes, tumor-associated macrophages, and PD-L1 among breast cancer subtypes and other clinicopathological characteristics.

The MMPs biomarkers' prognostic and predictive role in breast cancer has been indicated in several studies (Ren et al., 2015, Radisky and Radisky, 2015). Studies have reported an inverse correlation between the expression of MMP-2, MMP-9, and MMP-11 in breast cancer tissues and prognosis (Benson et al., 2013, Ren et al., 2015, Li et al., 2017b, Egeblad and Werb, 2002, Joseph et al., 2020, Cheng et al., 2010, Min et al., 2014). Further, studies

have demonstrated a positive correlation (Cheng et al., 2010, Zeng et al., 2013, Min et al., 2014, Yang et al., 2018). Despite these inconsistent results, there is no study conducted on the expression levels of MMPs in breast cancer cases from Ethiopia. Therefore, this study aims to explore the association between MMP-2, MMP-9, and MMP-11 expression with clinicopathologic features among breast cancer patients in Ethiopia.

Viruses have been linked to several cancer cases, accounting for 15% of all human cancers (Liao, 2006). There is considerable disagreement and debate regarding the hypothesis that HPV infection plays an etiological role in the development of breast cancer (Kudela et al., 2019). Human papillomavirus has been found in malignant breast tumors, showing that HPV may play a role in the early stages of breast cancer and carcinogenesis (Maldonado-Rodríguez et al., 2022, Cavalcante et al., 2018, Khodabandehlou et al., 2019, Salman et al., 2017, Ngan et al., 2015). In contrast, other researchers have found no etiological link between HPV and breast cancer, as well as low HPV prevalence in breast cancer (Bønløkke et al., 2018, Gannon et al., 2015).

1.2. Significance of the Study

The results of this study could provide baseline countrywide data, which assists clinicians in making more accurate decisions about therapy regimens and prognosis for breast cancer. It also provides information for the responsible stakeholders to revisit the national breast cancer policy by considering these therapeutic and prognostic breast cancer markers. This could also assist the government and other stakeholders in implementing IHC throughout the country. The obtained information may also serve as a wake-up call for researchers, pathologists, oncologists, and biochemists to conduct further research on the role of current and future promising breast cancer biomarkers in the diagnosis, treatment, and monitoring of breast cancer patients.

1.3. Objective of the Study

1.3.1. General objective

- To investigate the distribution of breast cancer subtypes in Ethiopia, and their association with immune cell biomarkers, hormone receptors, matrix metalloproteinases, and the involvement of HPV using immunohistochemical and molecular approaches.

1.3.2. Specific objectives

- To assess clinicopathological features of invasive breast cancer in eastern, northern, southern, and south-western Ethiopia.
- To investigate the distributions of immunohistochemistry-defined subtypes of breast cancer distribution in four regions of Ethiopia.
- To investigate the surface marker expression patterns on tumor-infiltrating lymphocytes, tumor-associated macrophages, and PD-L1 in breast cancer patients.
- To investigate the expression of matrix metalloproteinases in Ethiopian breast cancer patients.
- To examine the frequency and genotypes of intralesional HPV among patients with breast cancer and benign breast tumors in Ethiopia.

2. CHAPTER TWO: LITERATURE REVIEW

2.1. Cancer

Cancer is characterized by uncontrolled and invasive growth of cells (Sudhakar, 2009). There were an estimated 19.3 million new cases and 9.9 million deaths reported from cancer in 2020. A high percentage of breast cancer cases (11.7%), followed by lung (11.4%), colorectal (10.0 %), prostate (7.3%), and stomach (5.6%) cancers were reported. Lung cancer remained the leading cause of cancer death, with an estimated 1.8 million deaths (18%), followed by colorectal (9.4%), liver (8.3%), stomach (7.7%), and female breast (6.9%) cancers, but breast cancer deaths are high among the females (15.5%) (Sung et al., 2021) (**Figure 1**).

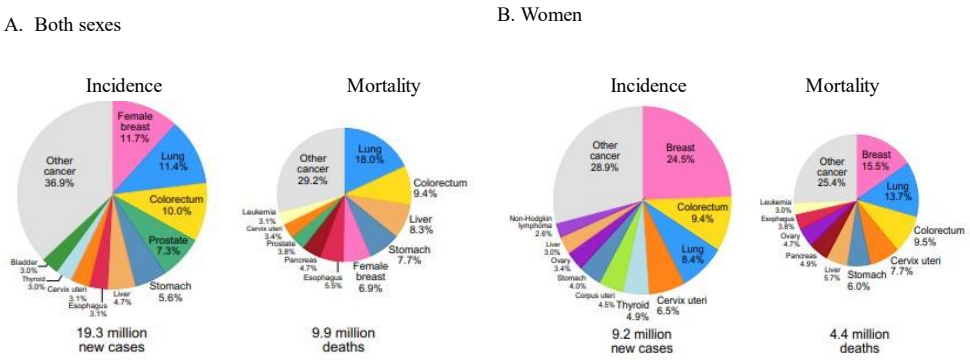


Figure 1. Distribution of cases and deaths for the top 10 most common cancers in 2020 for (A) both sexes and (B) women (Source: (Sung et al., 2021)).

2.2. The Hallmark of Cancer

The eight features of cancer include sustaining proliferative signals, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis (Hanahan and Weinberg, 2011), reprogramming cellular metabolism, and avoiding immune destruction. have been identified as "emerging hallmarks" in the most current revision of this concept (**Figure 2**) (Hanahan, 2022). There are three common molecular mechanisms for maintaining proliferative signals: modification of external growth signals, transcellular transducers of those signals, and

intracellular circuits that execute those signals (Hanahan and Weinberg, 2000). Tumor suppressors inhibit the cell cycle from moving the G1 to the S phase, thereby inhibiting cell cycle transitions. Tumor growth factor promotes proteins like p21 and p27 that prevent the action of cyclin-dependent kinases, which is necessary for the progression of the cell cycle, hence preventing the proliferation of cells. Cell attachment to an extracellular matrix can control the activation of many of these tumor suppressor pathways, overcoming this growth inhibition mechanism (Pickup et al., 2014). An increase in antiapoptotic molecules, a decrease in or a problem with the activity of pro-apoptotic proteins can all contribute to cancers evading apoptosis (Fulda, 2009). Tumor angiogenesis is the development of a blood vessel network that infiltrates malignant growths and transports nutrition, oxygen, and waste products. The angiogenic growth factors and cytokines that lead to neovascularization in malignancies include the gene families for vascular endothelial growth factor and angiopoietin (Nishida et al., 2006, Saaristo et al., 2000).

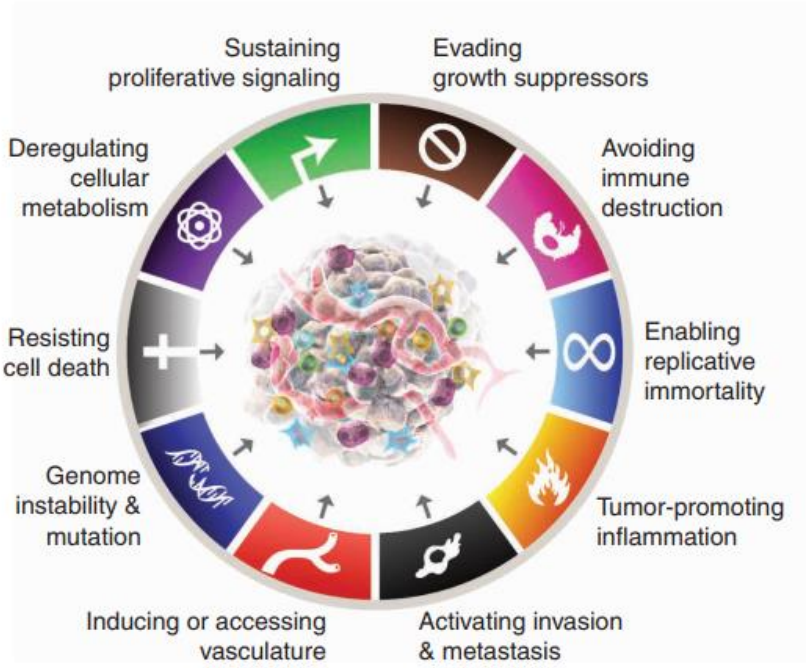


Figure 2. The eight hallmarks of cancer (source:(Hanahan, 2022))

2.3. Breast Cancer

Breast cancer is the leading cause of global cancer incidence in 2020, with an estimated 2.3 million cases and 685,000 deaths, accounting for 11.7% of cancer cases (Sung et al., 2021). The highest breast cancer incidence rates are in North America, Australia, New Zealand, and North and Western Europe (Torre et al., 2017, Ginsburg et al., 2017). The incidence of breast cancer is rising worldwide in the whole female population, but the growth rate is higher in young women in comparison to older ones (Palma et al., 2015). It is expected that by 2040, there will be 1 million deaths and more than 3 million new cases of breast cancer (Arnold et al., 2022) (**Figure 3**).

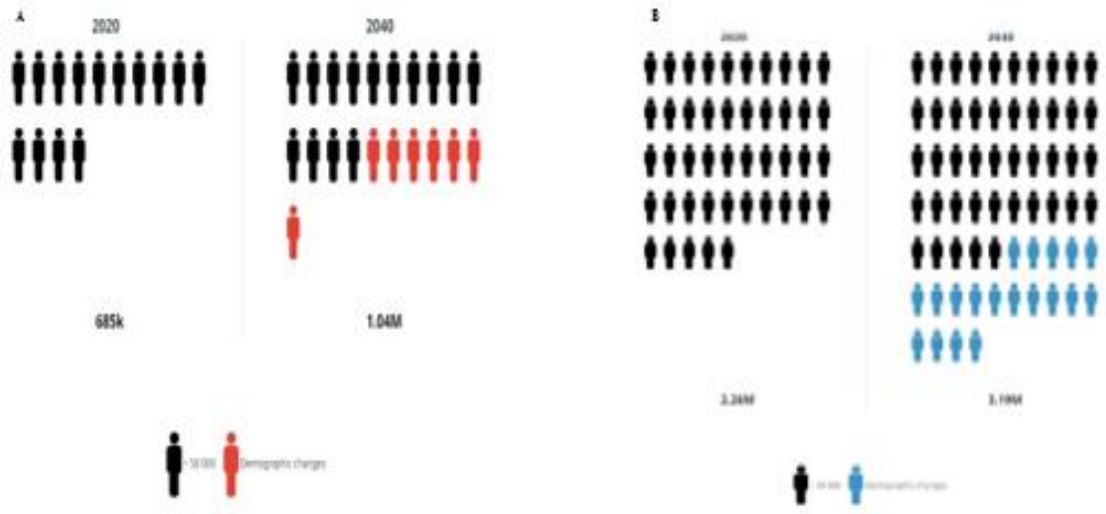


Figure 3. Estimated number of breast cancer deaths (A) and cases (B) from 2020 to 2040, females, age [0-85+] (Source:(Arnold et al., 2022)).

The highest age-standardized death rate is reported in Africa, including Ethiopia (**Figure 4**). In 2020, 85,787 deaths were reported in Africa (Arnold et al., 2022). The high number of deaths in Africa may be due to inadequate access to early detection, diagnosis, and treatment. It is also related to cultural differences in lifestyle behaviors, socioeconomic factors, and differences in the biological characteristics of breast cancer (Brewster et al., 2014).

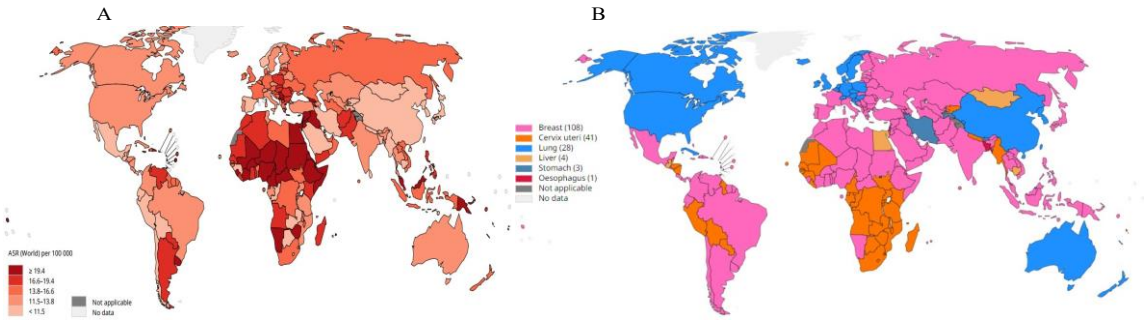


Figure 4. Estimated age-standardized mortality rates for breast, females, all ages (A) and top cancer per country of both sexes, all ages(B) (World) in 2020 (Source: (Lei et al., 2021, WHO, 2019)).

2.4. Breast Cancer in Ethiopia

In Ethiopia, the estimated number of cancer cases and deaths was 67, 573 and 47,954, respectively in 2018, and 1.42 million disability-adjusted life years of cancer in 2019. Breast cancer is the most common cancer constituting 33% of cancers in women, and 15,244 new cases and 8,159 deaths were reported in 2018 (Memirie et al., 2018, Awedew et al., 2022). Over 32,000 new cases of breast cancer and over 18,000 breast cancer deaths are predicted in Ethiopia by 2040 (**Figure 5**) (Arnold et al., 2022). The most common adult malignancies were those of the breast and cervix, colon, leukemia, non-Hodgkin lymphoma, prostate, thyroid, lung, stomach, and liver. Breast cancer accounted for 23% of all cancers found in the Addis Ababa cancer registry and 33% of cancers in women, making it by far the most common type of cancer (Memirie et al., 2018).

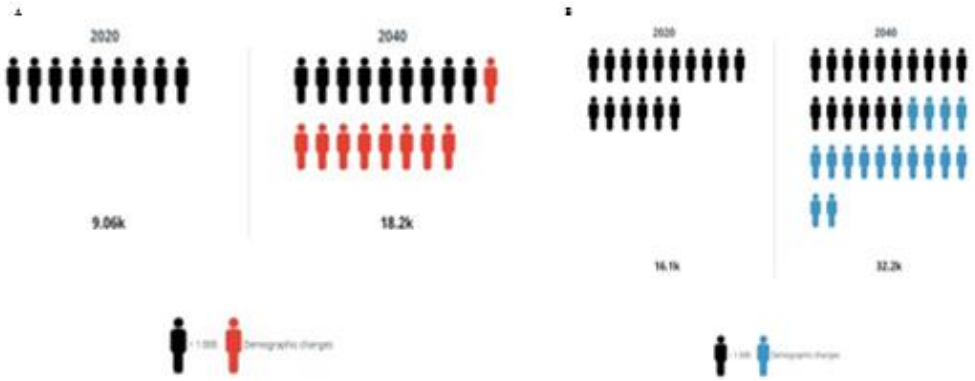


Figure 5. Estimated number of breast cancer deaths (A) and cases (B) in Ethiopia from 2020 to 2040, females, age [0-85+] (Source: (Arnold et al., 2022)).

Increasing trends of breast cancer cases are indicated in Ethiopia (Gebretsadik et al., 2021, Abate et al., 2018). Most of the patients were found to be advanced in stage at the time of diagnosis (Deressa et al., 2019, Gemta et al., 2019, Yoseph et al., 2021). An early diagnosis of breast cancer is necessary at all levels of the healthcare delivery system, as evidenced by the overall survival rates of 54.24% and 25.8% after two and five years, respectively (Tiruneh et al., 2021). According to another study, there were 9.8 deaths for every 100 people. After six years of observation, the estimated survival rate was 27%. Surgical margin involvement, poorly differentiated histological grade, advanced clinical stage, and positive lymph node status were all associated with a higher risk of death. On the other hand, chemotherapy, modified radical mastectomy, and adjuvant hormone therapy were protective (Areri et al., 2019). In south Ethiopia, the overall survival rate for patients is greater than two-thirds after two years. Living in a rural area, having advanced cancer, and not taking treatment as prescribed were all independent indicators of death. Therefore, enhancing breast cancer patient's ability to identify, diagnose, and receive treatment early on is crucial to prevent the issue with the right intervention strategies (Shita et al., 2020). The use of contraceptives, smoking, advanced stage, large tumor size, and high histological grade all significantly raise the chance of breast cancer recurrence (Biru et al., 2022, Bacha et al., 2020). Early menarche, living in a rural region, consuming packaged foods and beverages, having a family history of the disease, being overweight, and having one or

fewer children were additional risk factors for breast cancer (Tolessa et al., 2021, Tiruneh et al., 2021).

Breast cancer incidence can be reduced by practicing breastfeeding, increasing physical activity, and maintaining a balanced diet (Duche et al., 2021). According to a study, women with breast cancer have poor levels of mammography awareness, understanding, and practice. Participants' awareness of breast cancer risk factors was also inadequate (Duguma et al., 2022). Additional measures, such as raising awareness and launching advocacy campaigns regarding breast cancer self-examination, can be taken to improve early detection of the disease, which improves treatment outcomes (Dinegde, 2019).

Depression and anxiety were common among breast cancer patients, and it was discovered that inadequate patient-provider connections and a lack of financial support were important predictors (Belay et al., 2022). Treatment for breast cancer can cost up to USD 1,1157, which is a significant financial burden (Demeke et al., 2022).

2.5. Risk Factors for Breast Cancer

Risk factors for breast cancer can be broadly divided into two categories: extrinsic risk factors, which primarily include diets, lifestyle, and long-term medical intervention; whereas, inherent risk factors primarily include age, sex, race, and genetic composition. The extrinsic factors are essential for breast cancer prevention and treatment strategies that reduce breast cancer incidence (Kamińska et al., 2015). Early and more regular screening should be taken into consideration for those with a known genetic mutation or a family history of breast cancer. To choose the best screening plan for early diagnosis and prevention, people must talk to their healthcare providers about their risk factors (Lee et al., 2021).

2.5.1. Intrinsic factors

One of the main intrinsic factors of breast cancer risk in women is their age at diagnosis; older women are more susceptible (Kamińska et al., 2015). Most breast cancer diagnoses

occur in women 55 years of age and older (Feng et al., 2018). Breast cancer risk factors included older age at first parity and early menarche (Barańska et al., 2021). The other important intrinsic factor, inherited gene mutations, mostly involving BRCA1 and BRCA2, account for 5-10% of all cases of breast cancer (Majeed et al., 2014). The BRCA1 and BRCA2 mutations account for only around 20% of family breast cancer occurrences; the other 5-10% are caused by mutations in other uncommon susceptibility genes (Aloraifi et al., 2015). The following other gene mutations are also linked to an increased chance of developing breast cancer: ATM, BARD1, CDH1, CHEK2, MRE11A, NBN, TP53, PTEN, RAD50, RECQL, and RINT1 (Sheikh et al., 2015, Wisesty et al., 2020). Alterations to the extracellular matrix and the senescent fibroblast products are hypothesized to trigger the development of late-onset breast cancers (Benz, 2008). The breast cancer risk was greatly increased by family history, which also altered the patient's cancer characteristics (Brewer et al., 2017, Liu et al., 2021).

2.5.2. Extrinsic factors

Modern habits such as excessive drinking alcohol and eating too much fat may increase the likelihood of breast cancer. A daily alcohol intake of 35-44 grams is associated with a 32% increased risk of breast cancer. Smoking also increases the risk of breast cancer, especially in youngsters (Sun et al., 2017). Breast cancer risk was higher in women who used hormonal contraceptives either currently or previously, and the risk increased with longer use durations (Mørch et al., 2017, Hunter et al., 2010).

2.6. Molecular Pathogenesis of Breast Cancer

Normal human breast development is tightly regulated by complex signaling networks, many of which are dysregulated by cancer cells. These networks allow cells to interact with one another and their surroundings. Estrogen receptor (ER), HER-2, and canonical Wingless/Integrated (Wnt) signaling are the main signaling pathways that control normal mammary gland development and the activities of breast cancer stem cells (Feng et al., 2018).

2.6.1. Estrogen signaling

The two main mechanisms of ER-dependent gene transcription are estrogen/ligand-dependent and estrogen/ligand-independent (Lewis and Jordan, 2005, Sommer and Fuqua, 2001, Szostakowska et al., 2019).

2.6.1.1. Ligand-dependent

In ligand-dependent signaling mechanisms, the binding of estrogen with ER causes a conformational change, which allows various coregulators to stimulate the transcription of ER-target genes. The ligand/estrogen-dependent mechanism is further classified into direct genomic or classical, indirect genomic or non-classical, and non-genomic mechanisms of activation (VanHook, 2010, Bai and Gust, 2009, Castoria et al., 2010, Giraldi et al., 2010).

2.6.1.1.1. Direct genomic/classical

The direct genomic or classical pathway regulates the expression of ER target genes by the direct binding of estrogen-activated ERs to DNA binding at estrogen response elements (EREs). During estrogen binding with ER, the heat shock proteins (HSP70 and HSP90) dissociate ER from this binding in the cytosol, and change their conformation, then migrate as dimers into the nucleus to bind with EREs. This conformational change also allows helix 12 (H12) to accept coactivators and activate gene transcription (Giraldi et al., 2010, Puglisi et al., 2019, Menazza and Murphy, 2016, Bjornstrom and Sjoberg, 2005, Cui et al., 2013, Hayashi and Yamaguchi, 2008).

2.6.1.1.2. Indirect genomic/non-classical

In indirect genomic/non-classical pathways, estrogen receptors regulate the transcription of genes that do not contain EREs through indirect binding to DNA. The indirect ER binding is mediated by different co-factors (like SP-1, AP-1, and NF-κB) that stimulate gene transcription through interaction with DNA (Puglisi et al., 2019, Menazza and Murphy, 2016, Bjornstrom and Sjoberg, 2005, Cui et al., 2013, Hayashi and Yamaguchi, 2008).

Specificity protein 1 (Sp-1) is the main transcriptional factor that binds with ER and contributes to coactivator recruitment (Marino et al., 2006). Several genes like low-density lipoprotein receptor, progesterone receptor B, endothelial nitric oxide synthase, GATA binding protein 1, signal transducer and activator of transcription 5, activating transcription factor-2, c-jun, c Fos, ATF-1/cAMP response element-binding protein, nuclear transcription factor-Y, cyclin D1 and the retinoic acid receptor-1α are induced by estrogen *via* the Sp-1 mechanism (Fuentes and Silveyra, 2019, Marino et al., 2006). Activator protein 1 (AP-1) is also another main transcription co-factor that binds with ER and regulates target gene transcription. Genes like insulin-like growth factor-1 (IGF1), collagenase, IGF1-receptor, ovalbumin, and cyclin D1 are induced by the ER-AP-1 binding activation pathway(Fuentes and Silveyra, 2019), but ERβ inhibits the AP-1 dependent transcription of cyclin D1 (Cui et al., 2013).

2.6.1.1.3. Non-genomic/membrane-initiated

The non-genomic ER pathway can occur very quickly and is initially independent of genomic gene transcription. This rapid mechanism of action is mediated by the membrane-associated ER. Plasma membrane localization of ER is mediated by heat shock protein 27 (HSP27)(Zilli et al., 2009, Wilkenfeld et al., 2018), and associates with the membrane at caveolae lipid rafts through interactions with caveolin-1, Src, and striatin. The binding of membrane-localized ER and estrogen interact directly with RTK, the p85 regulatory subunit of PI3K, Src, and Shc to activate RAS/RAF/MEK1/2 and ERK1/2, PI3K/Akt/mTOR) signaling pathway. These kinase pathways not only induce cell survival and cell proliferation but also phosphorylate ER and its coregulators, which result in the activation of nuclear genomic transcription. Estrogen activates growth factor signaling *via* non-genomic actions of ER and the growth factor signaling, in turn, activates ER, hence forming a vicious cycle (Saxena and Sharma, 2010). Coregulatory proteins such as proline-glutamic acid, leucine-rich protein 1, and metastasis-associated proteins are important in activating non-genomic activity (Puglisi et al., 2019, Zilli et al., 2009, Arnal et al., 2017). G protein-coupled receptor 30 (GPR30) is also a membrane-localized receptor that has been observed to respond to estrogen to activate rapid signaling (Wilkenfeld et al.,

2018), such as PI3K and calcium signaling (Cui et al., 2013). ER-mediated, non-genomic signaling can also regulate nitric oxide, PKC, and calcium flux to promote autophagy, proliferation, apoptosis, survival, and differentiation. The calcium flux *via* membrane-localized ER leads to the activation of kinase pathways (Wilkenfeld et al., 2018). As a result, targeting this pathway could be one of the possible treatment strategies to reduce endocrine resistance.

2.6.1.2. Ligand-independent activation of ER

Growth factors interact with activated receptor tyrosine kinases (RTK) like human epidermal growth factor receptors, insulin-like growth factor-1 receptor (IGF-1R), and the fibroblast growth factor receptor (FGFR), which leads to activation of the phosphatidylinositol 3 kinases (PI3K) signaling pathway (Menazza and Murphy, 2016, Marino et al., 2006, Wilkenfeld et al., 2018). Phosphatidylinositol 3 kinase contains a catalytic domain (p110) and a regulatory domain (p85), and it phosphorylates phosphatidylinositol diphosphate (PIP2) to phosphatidylinositol triphosphate (PIP3), which in turn facilitates the phosphorylation of the Akt. Then, Akt activates mTOR *via* the inhibition of tuberous sclerosis 1/2 (TSC1/2). Tuberous sclerosis 1/2 is a tumor suppressor and heterodimer of tuberin and hamartin, which acts as a guanosine triphosphatase activating protein and negatively regulates Rheb-GTP by converting it into its inactive guanosine diphosphate-bound state (Saini et al., 2013, Liu et al., 2017, Paplomata and O'Regan, 2014). The tumor suppressor gene phosphatase and tensin homolog deleted on chromosome ten (PTEN) have an inhibitory effect on PI3K by dephosphorylating PIP3 to PIP2, and inositol polyphosphate 4-phosphatase type II (INPP4B) is also dephosphorylated PIP3 to PIP26 (Saini et al., 2013, Liu et al., 2017). Activation of the ER-target gene in the PI3K/Akt/mTOR pathway (**Figure 6**) is mediated by phosphorylation of ER on S167(Siersbæk et al., 2018). Taken together, activation of the PI3K/Akt/mTOR pathway plays a central role in breast cancer, and blocking of this pathway is an attractive treatment target, especially in endocrine-resistant ER-positive breast cancer.

Growth factors binding with the RTK receptors also lead to activation of the Ras/Raf/MEK/ERK signaling pathway (**Figure 6**) (Menazza and Murphy, 2016, Marino et al., 2006, Wilkenfeld et al., 2018). The binding of growth factor with RTK activates RAS. Activated RAS can then bind with RAF and activate the downstream signaling pathway (Degirmenci et al., 2020). When Raf is activated, its C-terminal catalytic domain can interact with MEK, and its catalytic VIII subregion is phosphorylated at the Ser218 and Ser222 activation loop, which activates MEK1/2. MEK1/2 is further activating ERK1/2 by phosphorylating the Tyr and Thr regulatory sites. Activated ERK1/2 are then translocated to the nucleus and promote phosphorylation of Ser 118 in the AF-1 domain of ER and activate its ER-target gene transcriptional activity (Guo et al., 2020). This pathway may be also a crucial target for the treatment of endocrine resistance ER-positive breast cancer

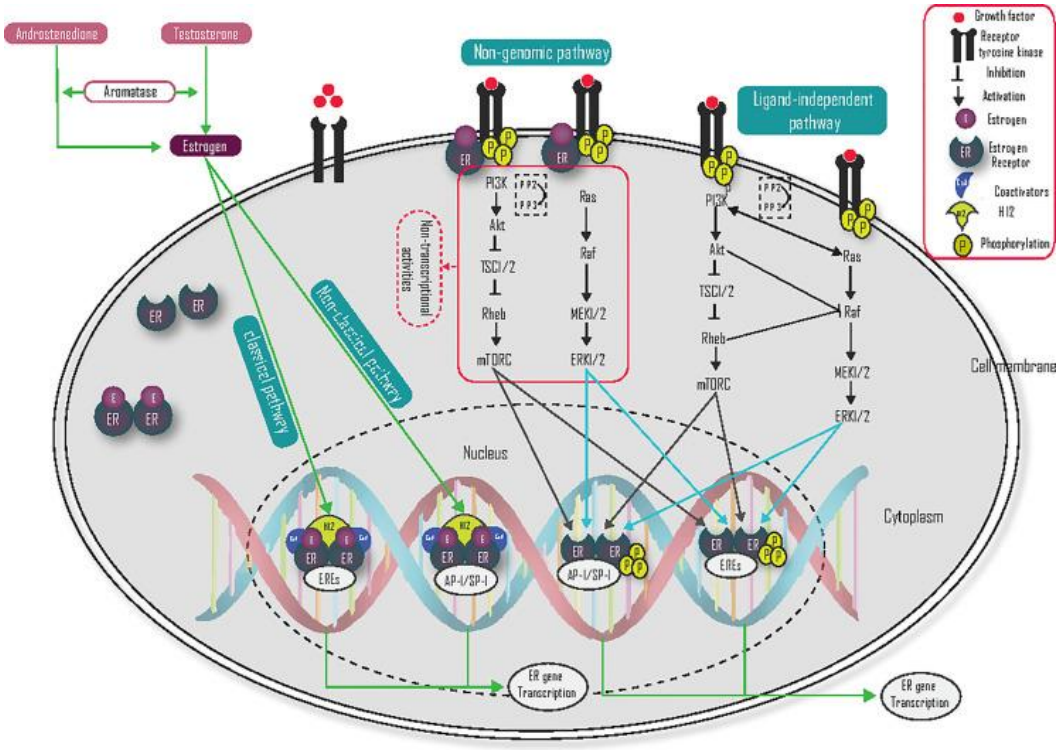


Figure 6. Signaling pathway of estrogen receptor (Source: (Belachew and Sewasew, 2021)).

2.6.2. HER-2 signaling

Membrane tyrosine kinase and the oncogene HER-2 are overexpressed and amplified in more than 20% of cases of breast cancer. In the HER-2 subtype of breast cancer, its overexpression is the main factor contributing to the growth and development of cancers (Gutierrez and Schiff, 2011). HER-2 intracellular domain dimerizes and becomes phosphorylated upon ligand attachment to its external domains. This then triggers several downstream signaling pathways, including RAS/MAPK and PI3K/Akt pathways (**Figure 7**). This pathway controls many genes related to invasion, metastasis, angiogenesis, cell division, proliferation, and survival (Feng et al., 2018, Gutierrez and Schiff, 2011, Hart et al., 2020). PTEN is a significant PI3K/Akt pathway inhibitor (Hart et al., 2020).

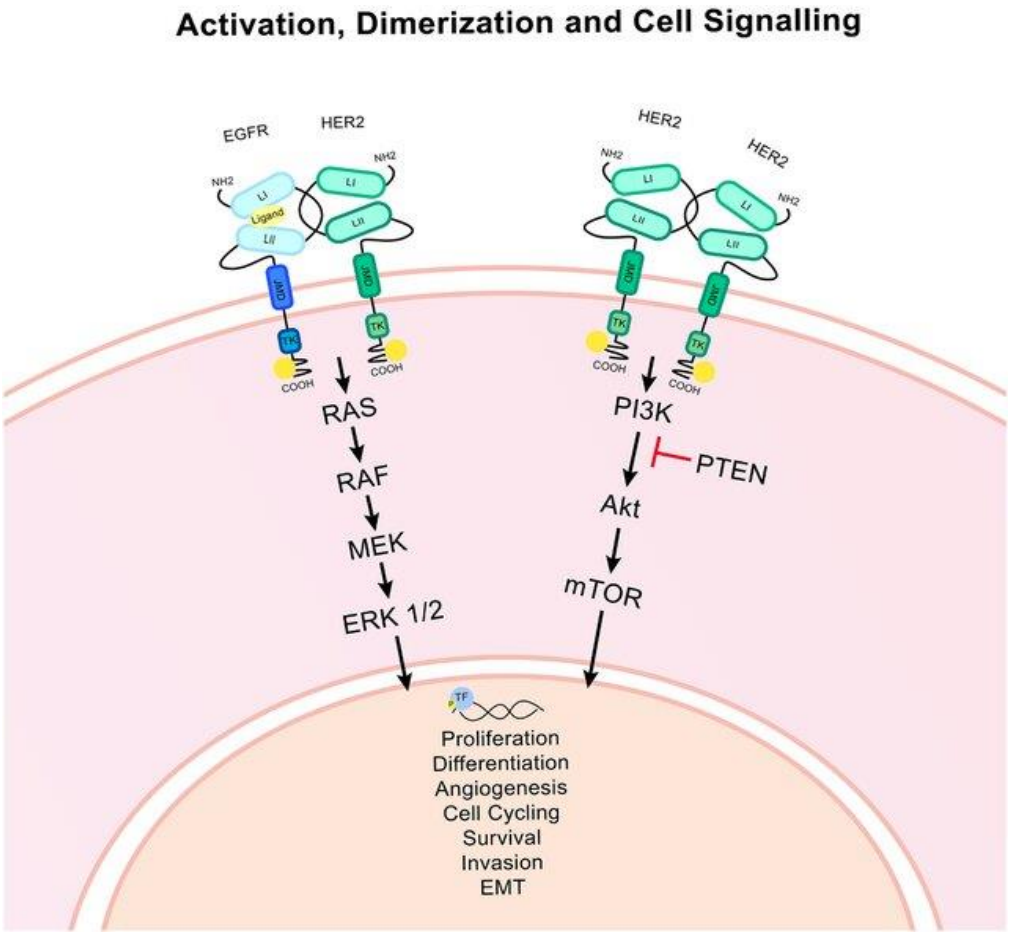


Figure 7. HER-2 signaling pathway (Source: (Feng et al., 2018)).

2.6.3. Wnt/β-catenin signaling

The mammary gland grows using Wnt signaling during embryogenesis, postnatal development, and pregnancy. The malignant progression of breast cancer, including metastasis, invasion, proliferation, and drug resistance, is mediated by wnt signaling. Triple-negative breast cancer is linked to Wnt/β-catenin signaling, which regulates key tumor-associated characteristics like migration, stemness, proliferation, and chemoresistance in TNBC cells (Pohl et al., 2017, Wen et al., 2020). In addition, Wnt signaling plays a critical role in immune microenvironment regulation, phenotypic development, treatment resistance, and stemness maintenance in breast cancer(Xu et al., 2020). The presence of Wnt ligands or antagonists, including DKK1, WIF1, or SFRPs, causes the dynamic degradation of β-catenin, which is controlled by the β-catenin destruction complex, keeping the Wnt signaling pathway in the off state. This multiprotein complex consists of the scaffolding proteins AXIN1/2 and APC, as well as the kinases CK1α and GSK3β. Proteases break down β-catenin after the two kinases phosphorylate it sequentially. On the other hand, Wnt ligands that bind with the FZD/LRP co-receptor complex lead to the disintegration of the β-catenin destruction complex. As co-repressors connected to TCF/LEF transcription factors are dislodged, β-catenin builds up in the cytoplasm and moves to the nucleus to start Wnt-target gene activation. R-spondins block the recycling of FZD receptors, which increases cellular sensitivity to Wnt ligands and amplifies the Wnt ligand response (Abreu de Oliveira et al., 2022) (**Figure 8**)

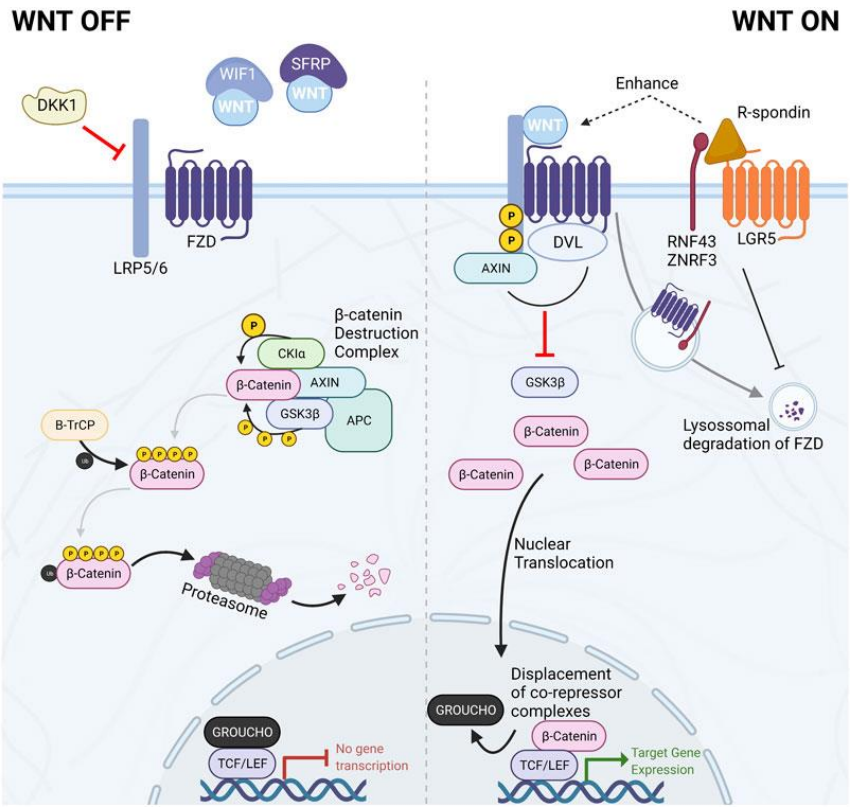


Figure 8. The classical wnt/β-catenin signaling pathway (Source: (Abreu de Oliveira et al., 2022)).

2.6.4. Other signaling pathways

Several other pathways are also crucial in controlling the development of breast cancer. Some of them are cyclin-dependent kinase (CKD), Notch, and others (Feng et al., 2018).

2.6.4.1. Cyclin-dependent kinases signaling

The class of serine/threonine kinases known as CDK is crucial for the regulation of cell cycle transition and has a significant involvement in the pathophysiology of cancer (Ghafouri-Fard et al., 2022). It has been shown that cyclins and CDKs play a critical role in the metastasis and proliferation of tumor cells (Sofi et al., 2022). Breast cancer is associated with overexpression of CDK 4/6 signaling. Such overexpression contributes to

chemoresistance indirectly through a PI3K/Akt/mTOR-dependent pathway and directly to cancer cell proliferation by inducing the G1-S transition (Nwabo Kamdje et al., 2014). Cyclin-dependent kinase (CDK) 4 and 6 inhibitors are promising cancer therapies for hormone receptor-positive and HER-2-negative metastatic breast cancer (Spring et al., 2020).

2.6.4.2. Notch signaling

Notch signaling controls the differentiation of breast epithelial cells through normal development. The overexpression or aberrant expression of Notch receptors and ligands is linked to the progression of breast cancer since it regulates angiogenesis (Kontomanolis et al., 2018). Elevated Notch signaling is present in all forms of breast cancer, however, it is most closely linked to triple-negative breast cancer (Edwards and Brennan, 2021). In the presence of ADAM/TACE and the γ-secretase enzymatic complex, Notch ligand-receptor interactions release the Notch intracellular domain. Following its translocation to the cell nucleus, the intracellular domain activates the CSL transcription factor. Signaling molecules implicated in angiogenesis, growth, survival, proliferation, energy metabolism, and chemoresistance of cancer cells are among the target genes (Nwabo Kamdje et al., 2014).

2.7. Classification of Breast Cancer

Historically, breast cancer was perceived as a single disease, but currently, gene expression profiling, genome, and exome sequencing have brought the concept that breast cancer is a complex, multifaceted disease having different risk factors, clinical presentation, histopathological and biological features, survival outcome, and responses to systemic therapy. Classification is significantly important for accurate diagnosis, treatment, and estimates of risk factors (Taherian-Fard et al., 2015). It is also important for the development of prognostic and predictive signatures that potentially allow individualized treatment (Vuong et al., 2014). Generally, a suitable classification of any disease must be

scientifically sound, clinically useful, easily applicable, and widely reproducible (Viale, 2012).

2.7.1. Histopathological classifications

Histopathologically, breast cancer is broadly classified into in situ carcinoma and invasive (infiltrating) carcinoma (**Figure 9**). In situ carcinoma is further sub-classified into ductal and lobular (Malhotra et al., 2010). Ductal in situ carcinoma is more common than lobular carcinoma (Burstein et al., 2004). Ductal in situ carcinoma has been further sub-classified into comedo, cribriform, micropapillary, papillary, and solid. Invasive carcinoma is also classified as invasive carcinoma of no special type, invasive lobular, ductal/lobular, mucinous (colloid), tubular, medullary, and papillary carcinomas. Of these, invasive carcinoma of no special type is the most common subtype and is further sub-classified as well-differentiated (grade I), moderately differentiated (grade II), or poorly differentiated (grade III) (Malhotra et al., 2010).

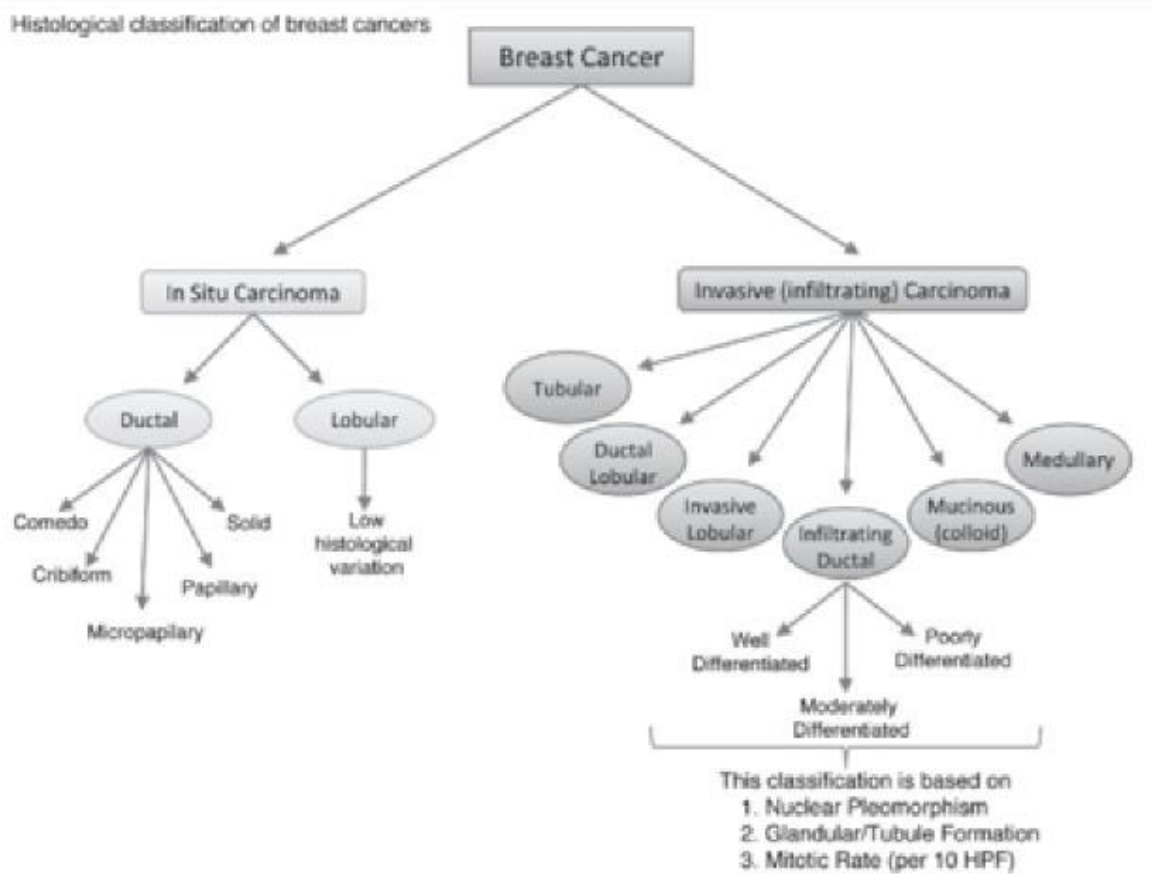


Figure 9. Histological classification of breast cancer subtypes (Source: (Malhotra et al., 2010).

2.7.2. Molecular classifications

Molecular profiling of breast cancer by gene expression studies has provided an important tool to classify breast cancer subtypes. Based on four main molecular biomarkers (PR, ER, HER-2, and Ki-67), breast cancer is classified as luminal A, luminal B, HER-2 -enriched, and triple-negative (**Figure 10**). These molecular subtypes are associated with distinct biological features, treatment response, and disease-specific outcomes (Hu et al., 2006, Sorlie et al., 2001), and show significant differences in the prediction of overall and disease-free survival (Malhotra et al., 2010). It should be noted that immunohistochemical and molecular classes do not overlap completely. For instance, some basal-like breast

cancers (according to the molecular classification) will not show the expected triple-negative immunophenotype and vice versa (Viale, 2012).

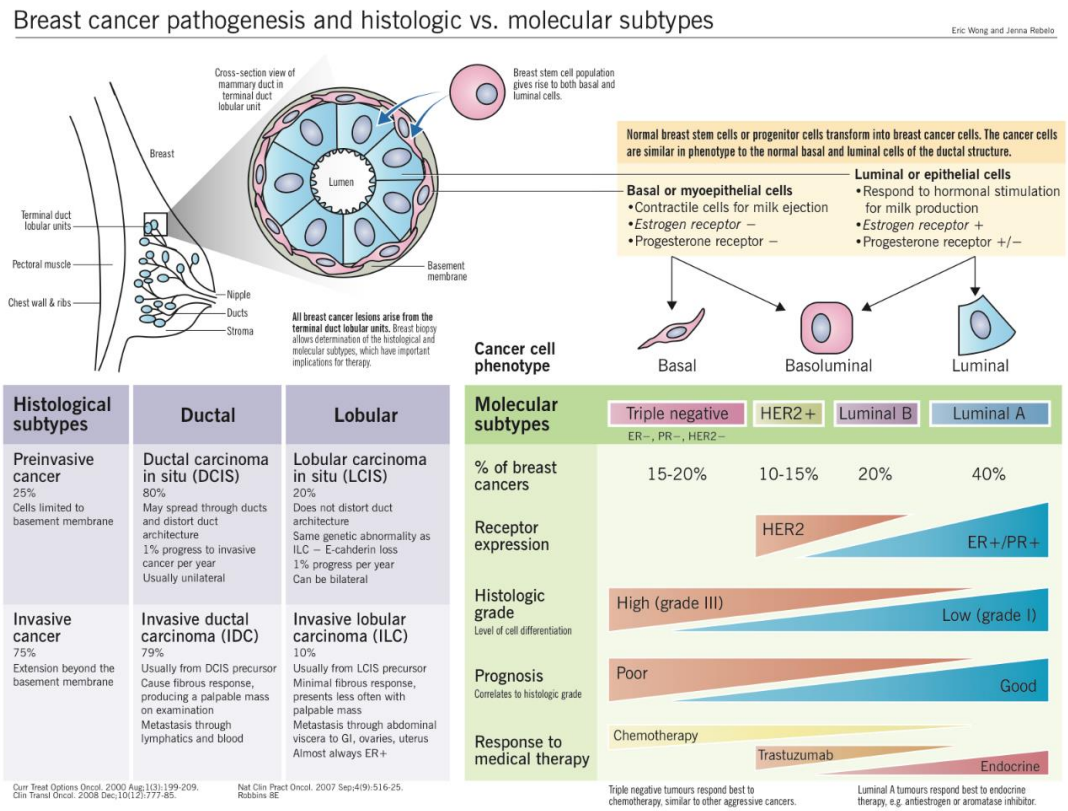


Figure 10. Breast cancer subtypes (Source: http://www.pathophys.org/breast-cancer/)

2.8. Biomarkers for Breast Cancer

2.8.1. Traditional prognostic biomarkers

The number of regional lymph nodes with metastasis, tumor size, tumor stage, and tumor grade are widely used and anticipated breast recurrence (Fung et al., 2017). Even though multiple molecular tests have been available in recent years, these features continue to be mandatory for determining prognosis and aiding therapy decisions (Nicolini et al., 2017).

2.8.1.1. Lymph node metastasis

The presence and number of axillary node metastasis have been the most important prognostic factors in breast cancer. Lymph node metastasis is an indicator of local recurrence, regional recurrence, and distant metastasis. Tumor survival status is decreased when lymph node involvement is increased (Carter et al., 1989, Nicolini et al., 2017). Nodal metastasis is also a marker of diagnosis and aggressive phenotype (Jatoi et al., 1999).

2.8.1.2. Tumor size

Measurement of tumor size is mandatory in assessing prognosis for breast cancer. Tumor size is a significant predictor of disease-free and overall survival. When tumor size is increased breast cancer patient survival is decreased (Carter et al., 1989, Crowe et al., 1992).

2.8.1.3. Tumor grade

The grading of breast cancer is based on the microscopic similarity of breast cancer cells to normal breast cells. Microscopic features such as nuclear pleomorphism, gland or tubule formation, and number of dividing cells are principally used for grading. Each of these factors assigned a score from 1 to 3 (with 1 being the closest to normal breast). These scores are then added together and, if the combined tumor score is between 3 and 5, it is well differentiated or grade I. If the combined score is 6 or 7, the tumor is designated as grade II or moderately differentiated, and if the combined score is 8 or 9, it is poorly differentiated or grade III (Parham et al., 1992, Giuliano et al., 2017). Tumor grades have prognostic significance and are primarily used to make decisions for lymph node-negative patients with borderline tumor sizes (Cianfrocca and Goldstein, 2004). However, the reproducibility of histologic grade assessment among pathologists is not uniformly reported in different series (Metzger Filho et al., 2011).

2.8.2. Molecular biomarkers

Molecular biomarkers listed below are the key drivers towards precise treatment, but they should be combined with conventional, histopathological, and clinical features such as

tumor size, tumor grade, lymph node status, and patient age (Duffy et al., 2016). In recent years, several molecular biomarkers that can provide prognostic information and predict response to therapy have been developed and validated (Nicolini et al., 2017).

2.8.2.1. Estrogen receptor

The ER is one of the successful tumor biomarkers in breast cancer and is present in about 70% of breast cancer cases (Gown, 2008). Estrogen receptor has a role in cellular growth, proliferation, and differentiation (Kabel, 2017). Clinical experience has shown that the expression of ER indicates strong biomarkers of response to tamoxifen treatment (Gown, 2008). Clinically, ER is useful for endocrine therapy of invasive breast cancer like the administration of selective modulators (tamoxifen), third-generation aromatase inhibitors (anastrozole, letrozole, or exemestane), luteinizing hormone-releasing hormone (LHRH) agonists (leuprolide, goserelin), pure ER down regulators (fulvestrant), oophorectomy and other endocrine therapies. Treatment of ER-negative patients with tamoxifen had no significant effect on breast cancer recurrence and mortality (Duffy et al., 2017). The European School of Oncology- European Society for Medical Oncology Consensus Conference for advanced breast cancer group also recommends administering endocrine therapy if any biopsy is ER-positive (Cardoso et al., 2014). Patients with ER-low breast cancer did have a statistically significantly lower overall survival when compared to patients with ER-positive breast cancer (Paakkola et al., 2021). The recent guidelines define a special subgroup called "ER low positive" for tumors with 1 to 10% ER expression (Allison et al., 2020). In trials involving adjuvant tamoxifen for five years, the proportional decreases in mortality and recurrence during a ten-year follow-up period were 28% and 50%, respectively (Abe et al., 1998).

2.8.2.2. Progesterone receptor

Protein products from PR target genes are involved in a variety of cellular activities, including transcription, steroid and lipid metabolism, cell growth, and apoptosis. Estrogen receptor-positive breast tumors that lacked PR expression were less responsive to endocrine therapy than those that expressed high levels of PR, but some authors

believe that PR is most useful as a prognostic indicator in ER+ cancer, not as a predictor of benefit from endocrine therapy (Kabel, 2017).

2.8.2.3. Human epidermal growth factor receptor-2

Human epidermal growth factor receptor-2 (HER-2) is a transmembrane glycoprotein in the epidermal growth factor receptor family with tyrosine kinase activity (Gown, 2008, Choi et al., 2009). Human epidermal growth factor receptor-2 (HER-2) is involved in cell proliferation, differentiation, and survival (Kabel, 2017). Human epidermal growth factor receptor-2 (HER-2) overexpression has been associated with more aggressive disease progression and a poorer prognosis (Eroglu et al., 2014). It is amplified and overexpressed in approximately 15% to 20% of primary breast cancers and 20% to 30% of all breast cancers (Wolff et al., 2013). Human epidermal growth factor receptor-2 (HER-2) -targeted therapy is recommended for patients with HER-2-positive advanced breast cancer. First-line treatments like trastuzumab, pertuzumab, and taxane, and second-line treatments like trastuzumab emtansine are recommended for HER-2-positive breast cancer. Adjuvant trastuzumab can reduce the risk of recurrence by one-half and mortality by one-third in early-stage breast cancer patients (Gown, 2008). A combination of HER-2 overexpression and the St Gallen classification is useful for predicting the risk of relapse in patients with lymph node-negative breast cancer (Choi et al., 2009). A high level of HER-2 receptor expression was associated with a significantly decreased survival rate in patients with breast cancer (Kabel, 2017).

2.8.2.4. Ki-67 proliferation

Assessment of the Ki-67 proliferation index by immunohistochemical staining has been widely used in the pathological evaluation of breast cancer clinical practice (Dowsett et al., 2011). This proliferation marker is important for distinguishing luminal A and luminal B breast cancer subtypes and advised adjuvant chemotherapy for luminal B but not for luminal A (Goldhirsch et al., Criscitiello et al., 2014). Although immunohistochemical

detection of the Ki-67 antigen has been used for many years to assess cancer proliferation (Penault-Llorca and Radosevic-Robin, 2017), the reproducibility and cutoff value of this biomarker are variable (Mikami et al., 2013, Polley et al., 2013). The Ki-67 remains one of the most promising yet controversial biomarkers in breast cancer (Kos and Dabbs, 2016).

2.9. Tumor Immune Response

Tumor clearance and progression are influenced by both innate and adaptive immune responses (Melvold and Sticca, 2007).

2.9.1. Innate immune response

The innate immune response is made up of immune cells from the myeloid or lymphoid lineages that undertake tumor-supportive or tumor-opposing actions. The phenotypes and activities of the innate immune response are elastic and vary when their immediate surroundings change (Maiorino et al., 2022).

2.9.1.1. Myeloid cells

Myeloid cells are a broad set of cells that regulate cancer progression from its inception to invasion and metastasis. Macrophages/monocytes, neutrophils, myeloid-derived suppressor cells, and dendritic cells are the most well-studied myeloid cells implicated in cancer immune response (Maiorino et al., 2022). Clinical trials are now being conducted to assess target therapeutics that harness the anti-tumor functions of myeloid cells (Yang et al., 2023). Infiltrating myeloid cell diversity determines oncological inhibition of tumor-associated macrophage recruitment, modulation of tumor-associated macrophage polarization, reduction of tumor-associated macrophage products, elimination of Myeloid-derived suppressor cells (MDSCs), and reduction of MDSC products (Cha and Koo, 2020).

2.9.1.1.1. Macrophage

Breast cancer cells secrete colony-stimulating factor 1 and chemokine (C-C motif) ligand 2 (CCL2) are contributors to recruiting monocytes from blood vessels. Under the influence

of the microenvironmental signals, the recruited monocytes polarize to different types of tumor-associated macrophages (Qiu et al., 2018). There are two types of macrophages: M1 macrophages, which are pro-inflammatory and tumoricidal, and M2 macrophages, which are specialized in controlling inflammation and repairing wounded tissues (Williams et al., 2016). Pro-inflammatory cytokines such as interferon-gamma (IFN-γ), tumor necrosis factor (TNF), and granulocyte-macrophage colony-stimulating factors, as well as pathogen-associated molecular patterns and endogenous danger signals, stimulate M1 macrophage polarization. The M1 macrophages exert tumor-killing functions by producing superoxide anions, nitrogen free radicals, and the immunogenic cytokines IFN-γ, TNF, interleukin (IL)-2, IL-6, and IL-12. It also aids in the presentation of tumor antigens to induce an adaptive T-cell response (Williams et al., 2016, Huang et al., 2022). Anti-inflammatory cytokines such as IL-4, IL-10, and IL-13 promote M2 macrophage polarization (Qiu et al., 2018). The M2 microphage is involved in tumor cell invasion and metastasis, angiogenesis, cancer stemness, and energy metabolism regulation. It also limits the invasion and function of anticancer CD8-positive T-cells and contributes to immunosuppression, and matrix remodeling, and hence favors tumor growth. High tumor-associated macrophage cell density in the primary tumor has been associated with a poor prognosis in patients (Huang et al., 2022, Qiu et al., 2018, Sousa et al., 2015). The macrophage polarization and its role in cancer are indicated in **Figure 11.**

CD68 tumor-associated macrophage was used as a human pan-macrophage marker, and its infiltration was linked to poor prognostic breast cancer characteristics like larger tumor size, higher tumor grade, lymph node metastasis, vascular invasion, hormone receptor negativity, HER-2 expression, and triple-negative breast cancer subtype. Furthermore, larger levels of infiltration were linked to poorer disease-free, breast cancer-specific, and overall survival (Qiu et al., 2018).

CD163 tumor-associated macrophage has been established as a marker for protumor M2-like macrophages, and significant infiltration is related to adverse clinicopathological features as well as an independent prognostic factor for worse disease-free and overall

survival in patients with both triple-negative and HER-2 positive breast cancers (Qiu et al., 2018).

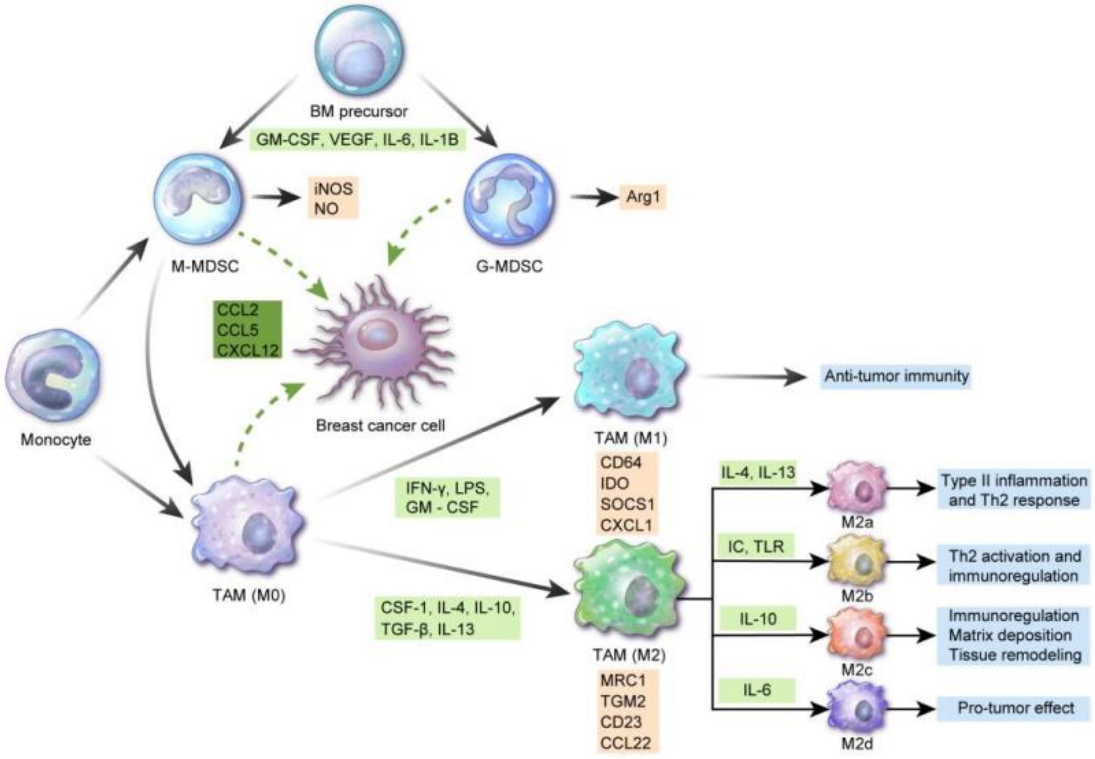


Figure 11. The macrophage polarization and its role in cancer (source:(Cha and Koo, 2020))

Tumor-associated macrophages produce proangiogenic factors such as vascular endothelial growth factor to create a network of vessels that nourish tumor cells and function as transport channels into the extracellular matrix. The tumor-associated macrophages also produce substances that suppress the local pro-inflammatory antitumor response, allowing tumor cells to evade detection (Obeid et al., 2013). Macrophage-targeted therapies are now being investigated in breast cancer, to increase macrophage tumor-killing activity or limit their recruitment and tumor-promoting function **(Figure 12)** (Qiu et al., 2018).

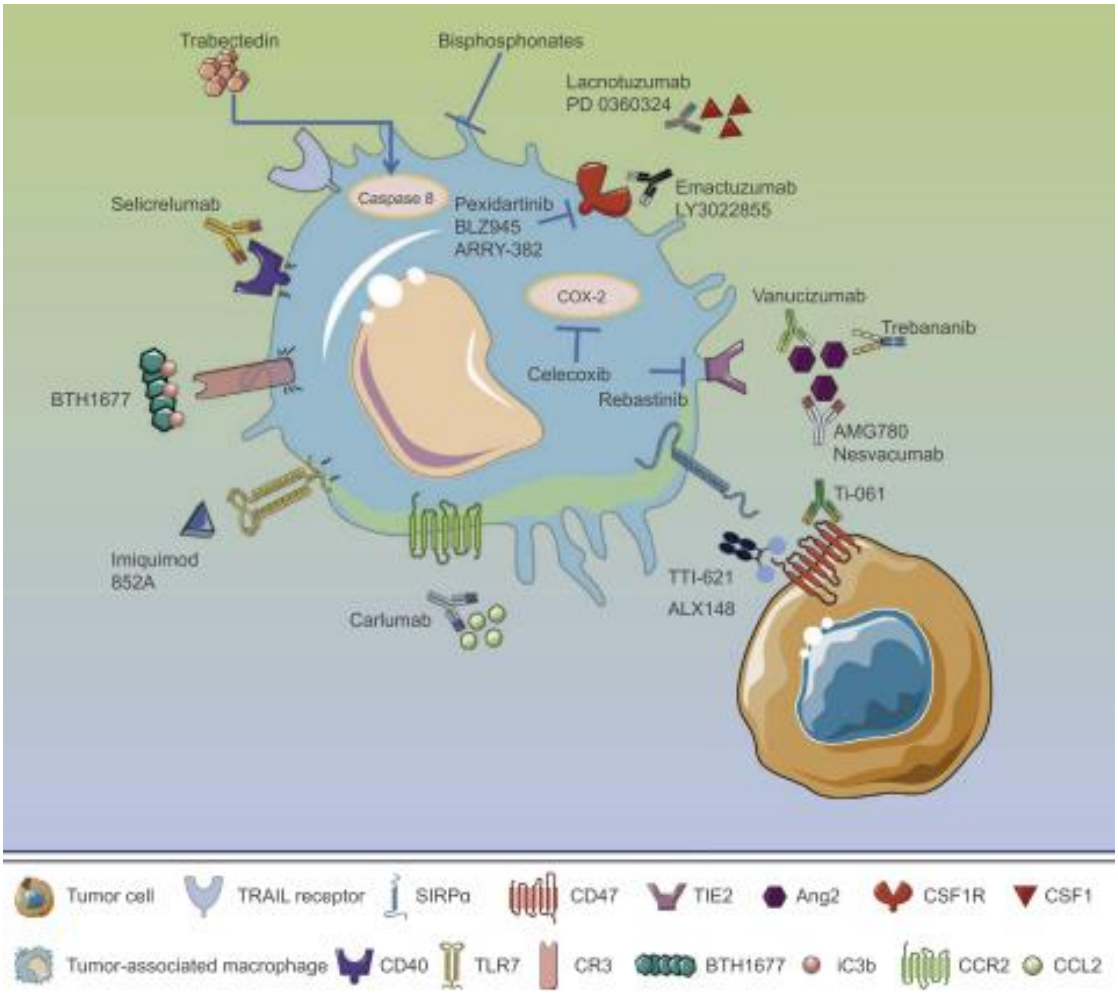


Figure 12. Mechanisms of macrophage-targeted therapies in breast cancer (Source: (Qiu et al., 2018))

2.9.1.1.2. Neutrophils

The first immune cells to be recruited to injured tissues are neutrophils. Tumor-associated neutrophils have been shown to promote metastasis by enhancing cancer cell proliferation, angiogenesis, and immunosuppression in the tumor microenvironment. They can, however, become antitumors by TGF-blockade using their plasticity (Maiorino et al., 2022). A high level of tumor-associated neutrophils is correlated with poor prognosis (Uribe-Querol and Rosales, 2015). Cancer cells stimulate neutrophils for a pro-NETotic phenotype by releasing pro-NETotic substances such as granulocyte colony-stimulating

factor and IL-8. The NETosis has been associated with increased disease progression and metastasis in breast cancer (Snoderly et al., 2019)

2.9.1.1.3. Myeloid-derived suppressor cells

Myeloid-derived suppressor cells express T-cell suppressor molecules such as l-arginase, inducible nitric oxide synthase, TGF-b, IL-10, cyclooxygenase-2, and indoleamine 2,3-dioxygenase (Maiorino et al., 2022). The monitoring of MDSC levels before and after primary tumor excision can improve the efficacy of immune-based therapies and the management of metastatic breast cancer (Bosiljcic et al., 2019).

2.9.1.1.4. Dendritic cells

Dendritic cells (DCs) play an important role in mediating the transition from innate to adaptive immunity. There are two types of DCs: conventional (or myeloid) DCs (cDCs) and plasmacytoid DCs(pDCs). The cDCs are antigen-presenting cells that are further subdivided into cDC1s and cDC2s, which stimulate CD8 and CD4 T cells, respectively. The cDC1s specialize in shaping anticancer immune responses by cross-presenting tumor-associated antigens to CD8 T cells, which identify them via MHC-I signaling, whereas cDC2s induce Th1, Th2, and Th17 polarization. Despite their limited antigen-presenting capacity, pDCs are the principal producers of type I interferons and are largely involved in antitumor immune responses (Maiorino et al., 2022, Del Prete et al., 2023).

2.9.1.2. Innate lymphoid cells

Innate lymphoid cells (ILCs) are the type of innate immune cells that lack antigen specificity and are classed as natural killer (NK) cells, ILC1s, ILC2s, ILC3s, and lymphoid tissue inducer (LTi) cells (Yuan et al., 2021). They are implicated in both protumor and antitumor responses (Maiorino et al., 2022). The ILC1s and NKs are also known as cytotoxic ILCs, whilst ILC2, ILC3, and LTi are known as helper ILCs (Sugimura and Wang, 2022)

2.9.1.2.1. NK cells

Even when not activated, NK cells can destroy target cells. Many cancers lose expression of MHC-I molecules after malignant transformation while retaining the synthesis of ligands that activate NK cells, making them extremely vulnerable to NK cell killing (Melvold and Sticca, 2007). The NK cells have the strongest cytotoxic activity because they produce granzymes, IFN-γ, and perforin. They have diverse cytotoxicity mechanisms and the ability to control the immune response through cytokine release, which plays an important role in antitumor immunity as well as in metastasis (Yuan et al., 2021, Laskowski et al., 2022, Roberti et al., 2012).

2.9.2. Adaptive immune response

2.9.2.1. T- lymphocytes

T-lymphocytes, which can be classified as helper, cytotoxic, and regulatory, are among the most significant cells in the tumor microenvironment (Zareinejad et al., 2023). The signal transducers and activators of the transcription pathway and cytokine stimulation lead naive CD4-T cells to differentiate into subtypes: T helper (Th)1, Th2, Th17, Regulatory T cell (Treg), follicular helper T (Tfh), Th19, Th9, Th22 and Th25. Both Th1 and Th2 cells release anti-tumor IFN-γ and IL-4. Th17 cells perform a dual role in immunosuppression, either by secreting chemokine ligand (CCL)-2 and CCL20 or by directly acting on the surface of tumor cells to promote tumor formation. The CD8-T cells mature into tissue-resident memory CD8-T cells that are capable of identifying E-cadherin on tumor cell surfaces and suppressing tumor development (Li and Cao, 2023). The Th1 cells are usually considered to be critical components of anti-tumor immune responses due to their ability to produce IFN-γ, activate macrophages, and increase killer CD8-T-cells; nevertheless, Th2 responses can promote cancer growth or metastasis (Zareinejad et al., 2023). The CD8-T-cells fight cancer by lysing and killing tumor cells. Higher CD8 T-cell levels are linked to a better prognosis and overall survival in triple-negative breast cancer (Amens et al., 2021)

The Th1 secretes inflammatory mediators such as IFN- γ and TNF, which delay the cell cycle in G1/G0 and promote apoptosis and senescence in breast tumor cells. It also

enhances CD8-T cell antigen presentation, proliferation, and cytolytic activity, activates M1 macrophages, and promotes dendritic cell maturation. The Th2 cells may direct carcinogenesis and epithelial-to-mesenchymal transition by secreting cytokines such as IL-3, IL-5, and granulocyte-macrophage colony-stimulating factor (GM-CSF). The Th17 also recruits and stimulates NK and dendritic cells, enhances MHC-I and II expression, suppresses MDSC proliferation, promotes apoptosis, and increases cytotoxic T lymphocyte activity. Other Th cells, such as Th22 and Th25, limit tumor growth and promote cell cycle arrest in tumor cells by decreasing extracellular signal-regulated kinase1/2 and protein kinase B phosphorylation and triggering apoptosis, respectively. The GM-CSF-producing T helper cells inhibit tumor progression and metastasis by interacting with monocytes and secreting soluble vascular endothelial growth factor receptors (VEGFRs)-1, which inactivates vascular endothelial growth factor (VEGF) and blocks angiogenesis. The T follicular helper is the principal source of chemokine (C-X-C motif) ligand (CXCL)-13 in breast malignancies, directing B-cells and enhancing lymphoid organization (Zareinejad et al., 2023). The overall anticancer activity of T-lymphocytes is shown in the figure below (**Figure 13**).

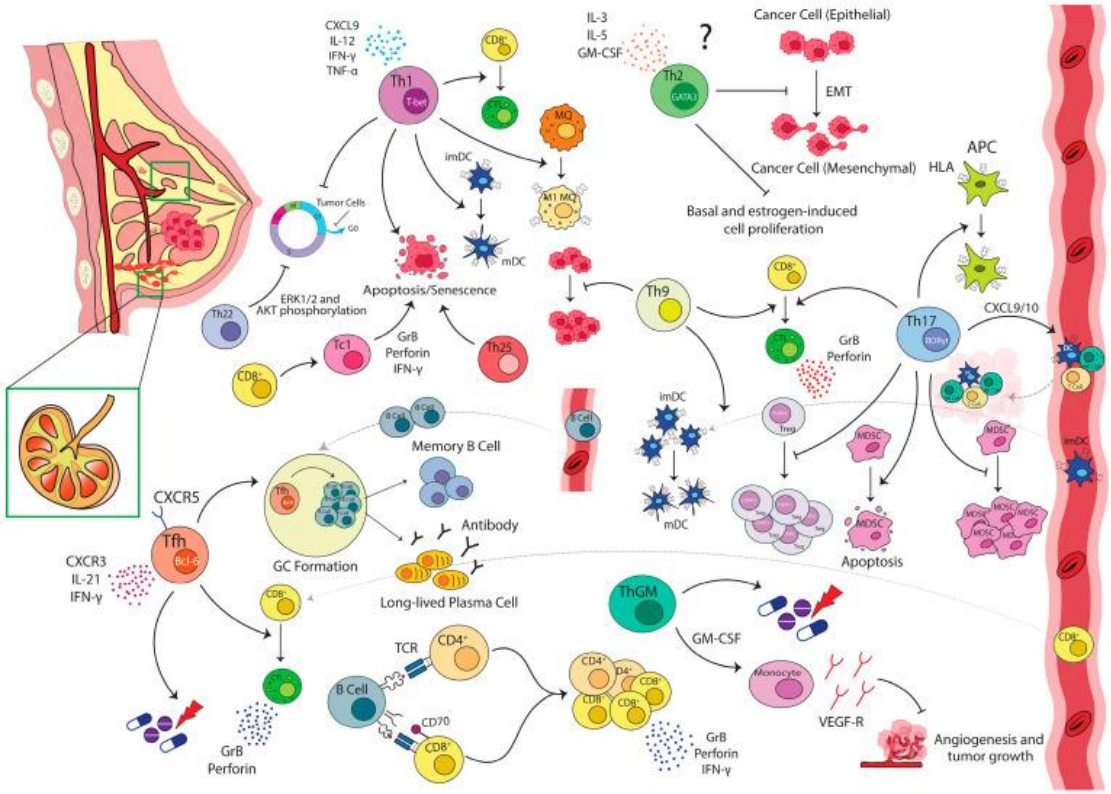


Figure 13. Anti-tumor functions of T-cell subsets in breast cancer (Source:(Zareinejad et al., 2023)).

The Th2 promotes tumor survival, growth, and metastasis by increasing the frequency of Tregs and triggering M2-macrophage differentiation. The Th17 controls angiogenesis generates pro-invasive factors, and promotes cancer cell proliferation, survival, and invasion. Th22 increases tumor cell motility by activating the STAT3/MAPK/AKT signaling pathway. Th25 suppresses the immune system by recruiting and activating type 2 immune cells. Th9 increases Treg immunosuppressive function while inhibiting immunological memory acquisition. Tfh plays a role in the regulation of epithelial-mesenchymal transition (EMT) during lymph node metastasis (Zareinejad et al., 2023)(**Figure 14**).

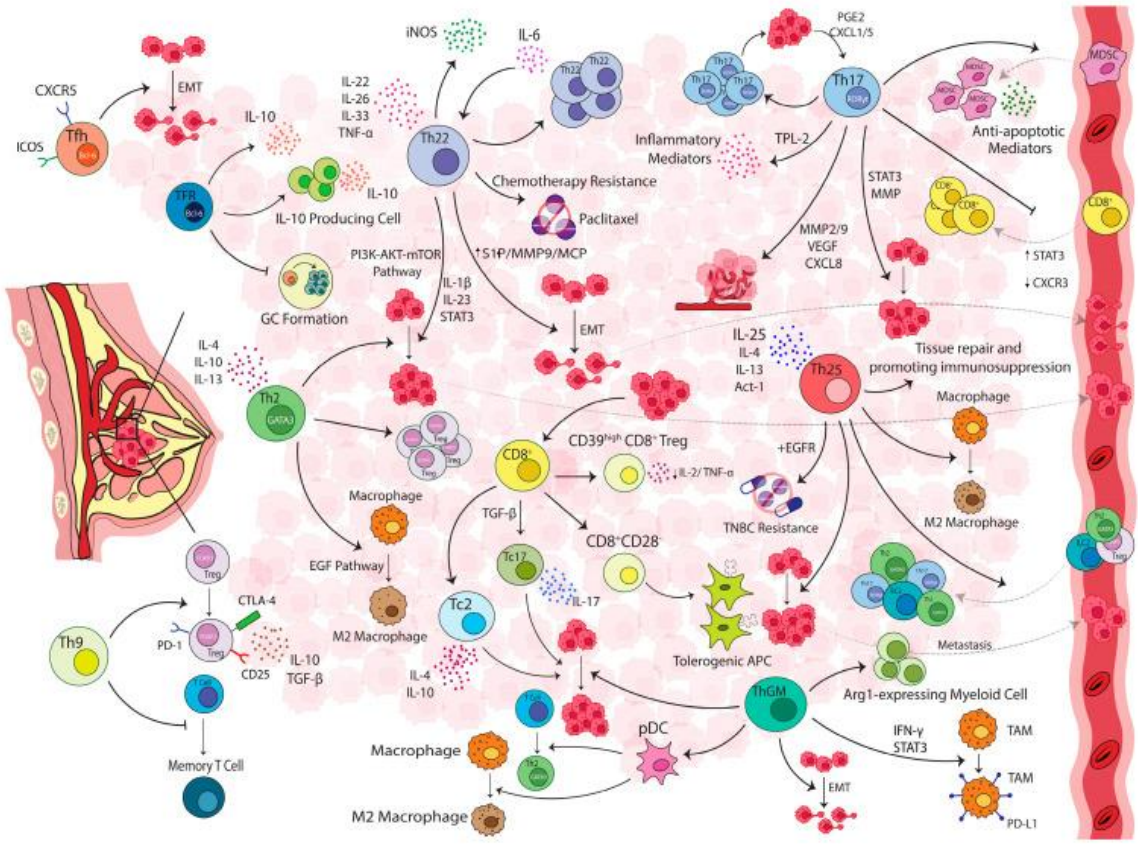


Figure 14. Pro-tumor functions of T-cell subsets in breast cancer (Source: (Zareinejad et al., 2023)).

Patients with high tumor-infiltrating lymphocytes and pathologic complete response (pCR) to neoadjuvant chemotherapy (NAC) have a great prognosis and may not require any more treatment beyond standard care. Immunotherapies such as checkpoint inhibitors or immune agonists may assist persons with high tumor-infiltrating lymphocytes at diagnosis. Patients with limited tumor-infiltrating lymphocyte penetration, either before or after NAC, require additional or other therapeutic options, such as adoptive cellular therapy or immunization regimens, to elicit an immunological response (**Figure 15**) (Dushyanthen et al., 2015).

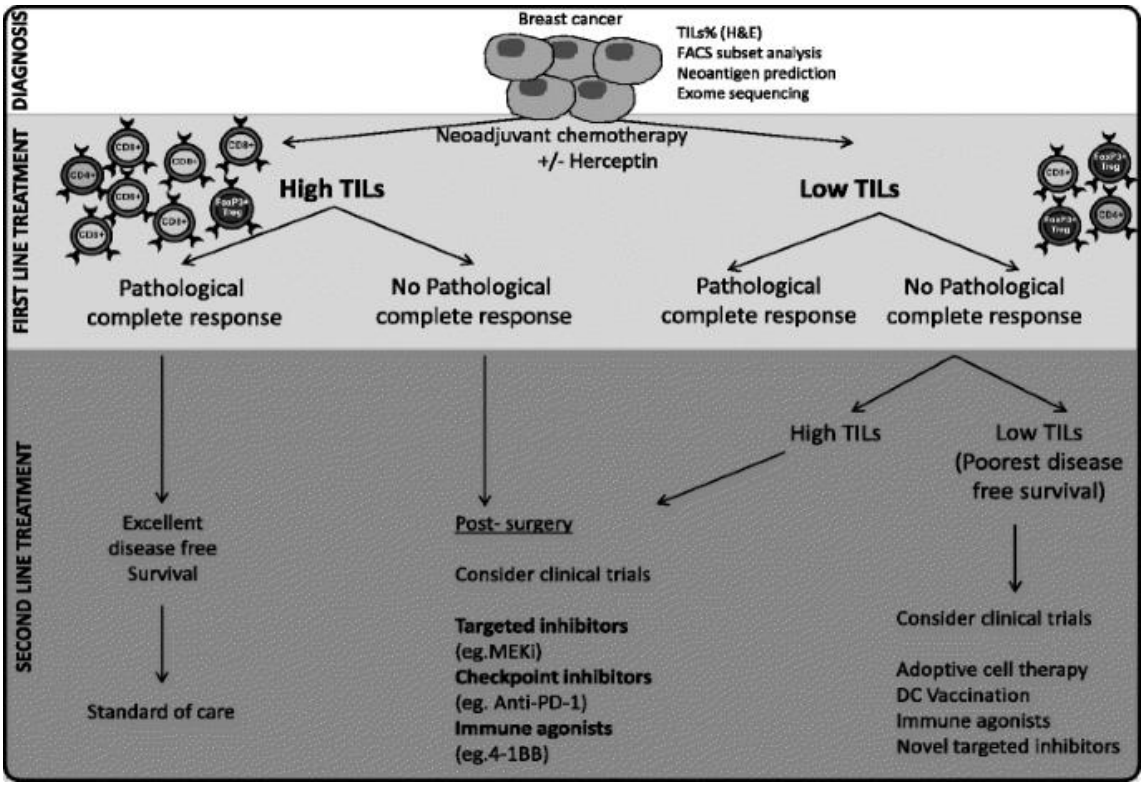


Figure 15. Prognostic and predictive role of TILS (Dushyanthen et al., 2015)).

2.9.2.1.1. Programmed cell death 1

Programmed cell death 1 (PD-1), is a transmembrane protein that is transcriptionally induced in activated T cells, B cells, and myeloid cells (Freeman et al., 2000). It plays an important role in down-regulating the immune system. One of the PD-1 ligands, programmed death-ligand 1 (PD-L1), can be expressed on tumor and immune cells. PD-L1/PD-1 binding physiologically promotes immune tolerance in peripheral tissues. Drugs interrupting immune checkpoints, such as anti-CTLA-4, anti-PD-1, anti-PD-L1, and others in early development, can mediate durable cancer regressions. The complex biology of immune checkpoint pathways still contains many mysteries, and the full activity spectrum of checkpoint-blocking drugs, used alone or in combination, is currently the subject of intense study (Topalian et al., 2015).

2.9.2.2. B-lymphocytes

B-cells are another type of adaptive immune cells that produce specific antibodies against tumor antigens, resulting in antibody-dependent cell-mediated and complement-mediated cytotoxicity (Melvold and Sticca, 2007). They may also aid in the growth and spread of breast cancer (Amens et al., 2021). A higher B-cell density suggests a better prognosis and response to treatment (Lam and Verrill, 2023).

2.10. MMP and Breast Cancer

Matrix metalloproteinases are a family of zinc-dependent endopeptidases that degrade extracellular matrix proteins. Matrix metalloproteinases play an important role in shaping the tumor microenvironment (Radisky and Radisky, 2015). Matrix metalloproteinases are produced as inactive zymogens and activated by proteinase cleavage (Egeblad and Werb, 2002). Interstitial collagenases, gelatinases, stromelysins, and membrane-type MMPs are the four categories of MMPs based on substrate specificity and domain organization. Matrix metalloproteinases play a role in breast cancer growth, invasion, and metastasis. High levels of MMPs have been linked to poor prognosis in breast cancer patients (Duffy et al., 2000). In breast cancer, MMPs can play both cancer-promoting and cancer-inhibiting roles. The MMP pathway, which is implicated in cancer promotion and inhibition is indicated in **Figure 16** (Kwon, 2022, Egeblad and Werb, 2002).

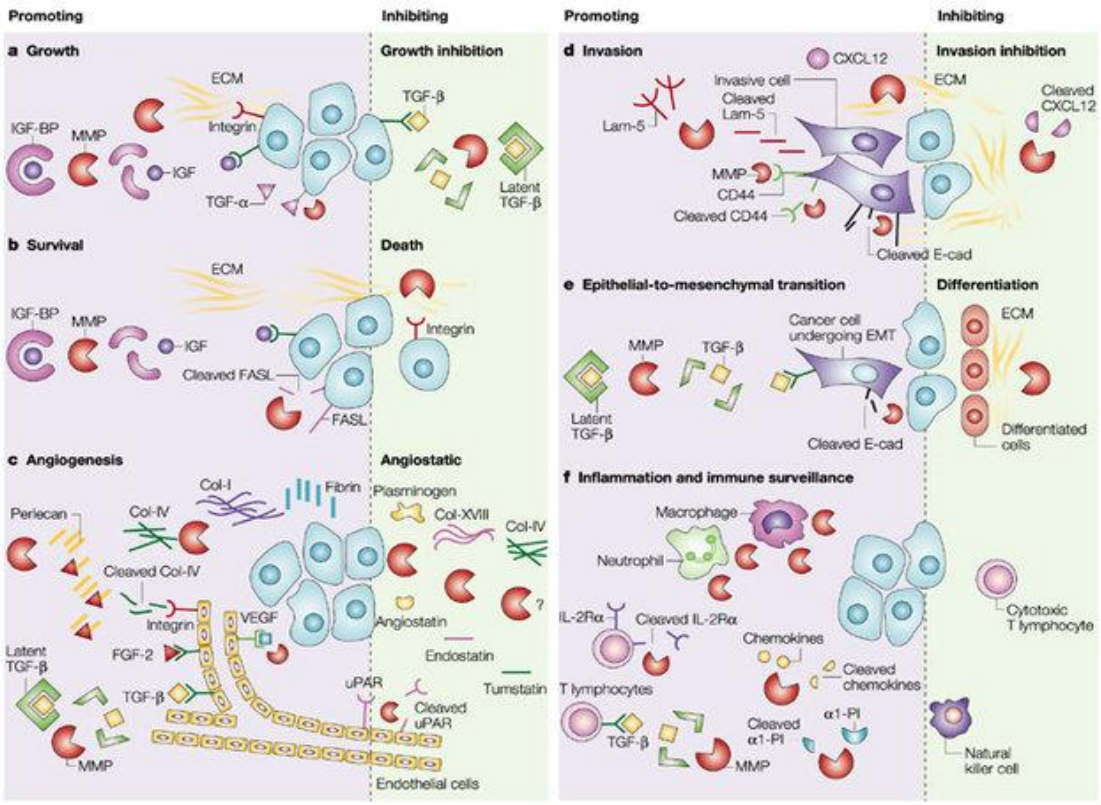


Figure 16. Cancer promoting and inhibiting pathway of MMPs (Source:(Egeblad and Werb, 2002)).

2.11. HPV and Breast Cancer

Human papillomavirus (HPV), a double-stranded circular DNA virus, is the cause of the most common sexually transmitted illness. Both men and women have a 50% chance of contracting the virus at some point in their lives. The involvement of HPV in cancer formation has received a great deal of attention, particularly in cervical cancer but also in other forms of neoplasms (Brianti et al., 2017). The oncoproteins E6, E7, and E5, which alter the growth regulation system and may interfere with apoptosis, are primarily responsible for the carcinogenicity of the HPV strains (Schiffman et al., 2016, Jiang and Yue, 2014). There is considerable debate and skepticism about the notion that HPV infection is the etiological factor contributing to the development of breast cancer (Kudela et al., 2019). The presence of HPV in malignant breast tumors has been reported, indicating

a potential role of HPV in the early stage of breast cancer and breast carcinogenesis (Maldonado-Rodríguez et al., 2022, Cavalcante et al., 2018, Khodabandehlou et al., 2019, Salman et al., 2017, Ngan et al., 2015). Two different ways work for HPV entry: direct contact between an infected epithelium and the mammary epithelium, and extracellular vesicles (EVs), which are released into the bloodstream by HPV-infected cells and can transport viral macromolecules to the breast. The exposure and sensitivity of mammary cells to estrogen, in conjunction with the presence of HPV genomes, may create an environment that is conducive to the activation of viral gene expression, resulting in abnormal overexpression of E6 and E7. On the other hand, HPV may influence ER signaling; the oncoproteins of HPV-18 E6 and E7 can directly interact with ER and increase the estrogen response element (ERE)-dependent transcriptional activity of ER. Consequently, the effective estrogen signaling that most breast cancer patients create from ER overexpression may have an impact on the expression of the HPV gene in those HPV-positive breast cells, promoting the initiation and progression of breast cancer (**Figure 17**) (Blanco et al., 2021).

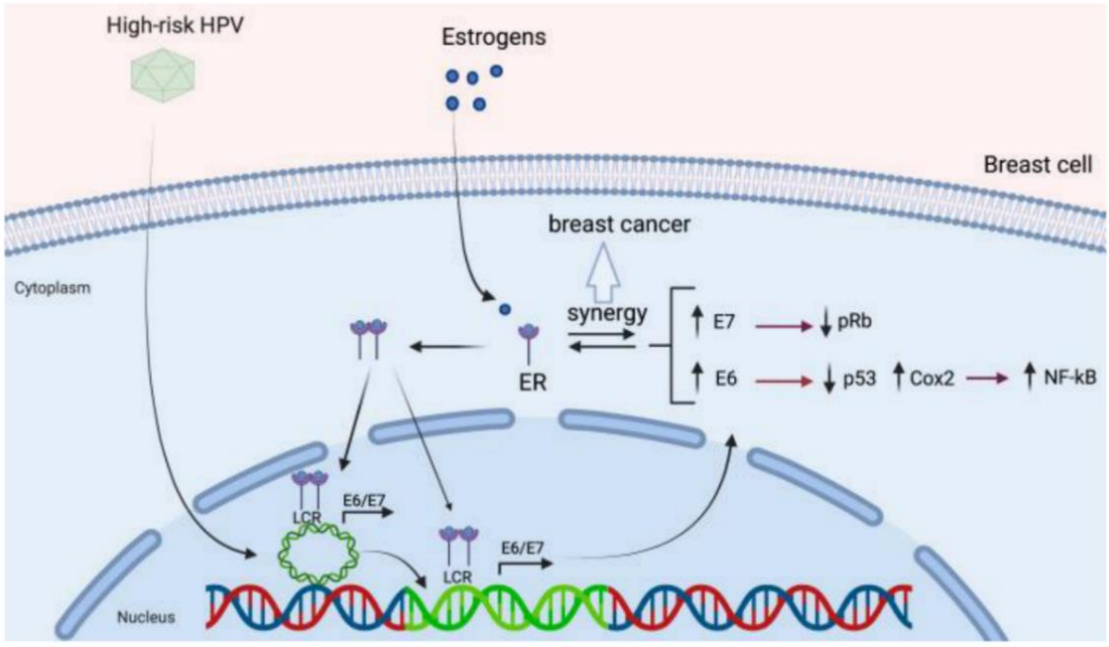


Figure 17. A suggested model of HPV-mediated breast carcinogenesis (Source: (Blanco et al., 2021)).

3. CHAPTER THREE: MATERIALS AND METHODS

3.1. Study Design, Study Area, and Samples

Retrospective (2015-2019) data were collected from 294 breast cancer patients using reports from the pathology departments of Jimma University Specialized Hospital and Hawassa University Specialized Referral Hospital for clinicopathological features assessment of invasive breast cancer. Only histopathological data of breast cancer from 2015 to 2019 were included.

A cross-sectional retrospective study involving 227 formalin-fixed paraffin-embedded (FFPE) tissue blocks was collected between 2015 and 2021 from four different regions: Hawassa Referral Hospital (Hawassa City, Southern Nations, Nationalities and Peoples (SNNP) region (N=46)), Jimma University Specialized Hospital (Jimma city, Oromia region (N=53)), Ayder Referral Hospital (Mekelle city, Tigray region (N=95)), and Hiwot Fana Specialized University Hospital (Harar city, Harar region (N=33)) to assess breast cancer subtyping and other clinicopathological characteristics (**Figure 18**).

For tumor-infiltrating lymphocytes, tumor-associated macrophages, and PD-L1 analysis, a total of 81 FFPE tissue blocks were used (32 from Ayder Referral Hospital (Mekelle City, Tigray region), 2 from Hiwot Fana Specialized University Hospital (Harer City, Hareri Region), 16 from Jimma University Specialized Hospital (Jimma city, Oromia region), 12 from Hawassa University Specialized Referral Hospital (Hawassa city, SNNP region) and 19 from ALERT Specialized Hospital (Addis Ababa).

For the MMPs expression study, a total of 58 FFPE tissue blocks were collected, 42 were from malignant breast cancer cases from referral hospitals (24 from Ayder Referral Hospital (Mekelle City, Tigray region), 8 from Hiwot Fana Specialized University Hospital (Harer city, Hareri Region), 4 from ALERT Specialized Hospital (Addis Ababa city), 3 from Jimma University Specialized Hospital (Jimma city, Oromia region), and 3 from Hawassa University Specialized Referral Hospital (Hawassa city, SNNP region). The 16 cases with benign tumors were from ALERT Specialized Hospital.

For HPV frequency assessment, a total of 120 FFPE tissue blocks were used; 66 from breast cancer cases and 54 from cases with non-malignant breast tumors, were collected. There were 66 breast cancer cases (23 from Ayder Referral Hospital (Mekelle City, Tigray region), 9 from Hiwot Fana Specialized University Hospital (Harer City, Hareri Region), 9 from ALERT Specialized Hospital (Addis Ababa city), 13 from Jimma University Specialized Hospital (Jimma city, Oromia region), and 12 from Hawassa University Specialized Referral Hospital (Hawassa city, SNNP region). The 54 cases of benign breast tumors were enrolled from ALERT Specialized Hospital.

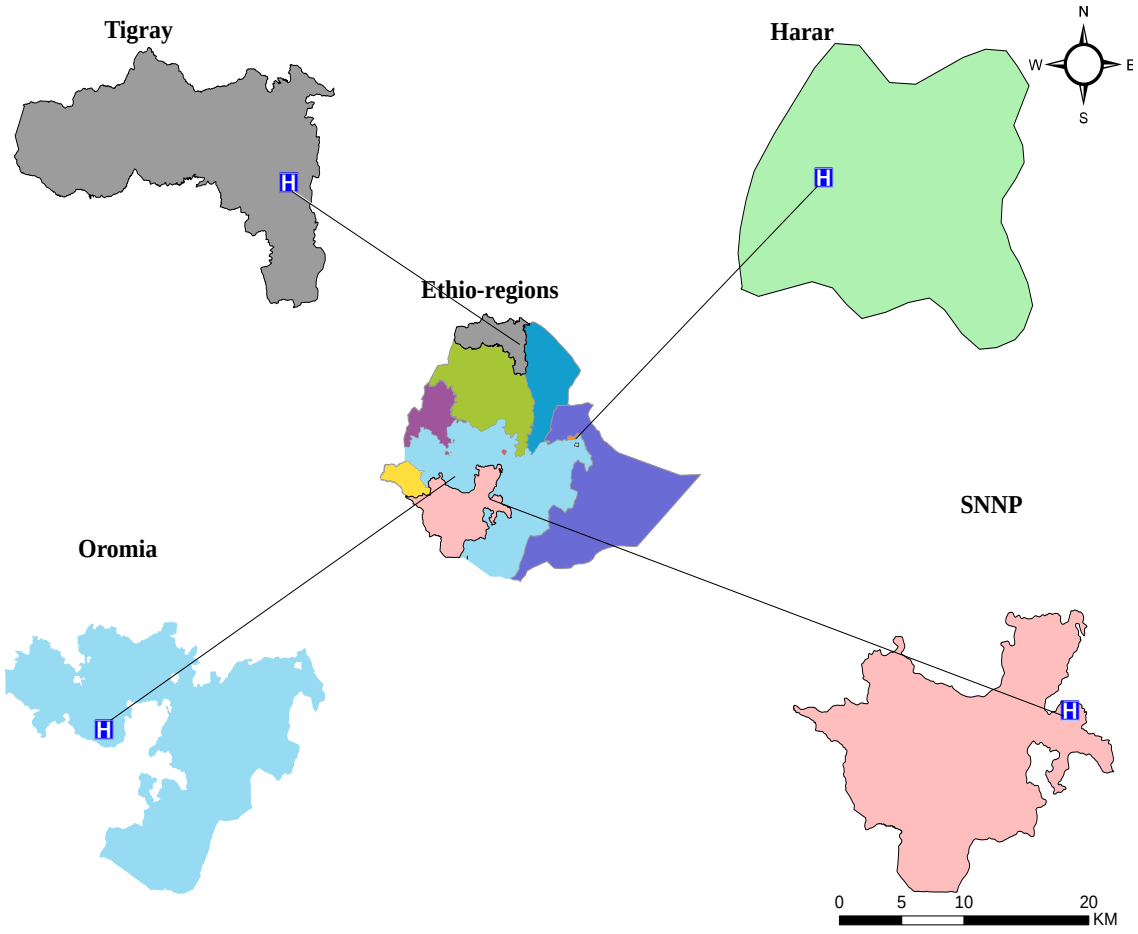


Figure 18. The study area and study centers (marked with an H to represent the hospitals) location map.

3.2. Sample Size

For breast cancer subtyping, the sample size was estimated using the single population proportion calculation, considering a 95% confidence level, a 5% margin of error, and a prevalence of 50% ER-positivity.

$N= (z_{\alpha/2})^2 p (1-p) /d^2$

$N= (1.96)^2 0.5(1-0.5) / (0.05)^2 =384$

Where n = sample size; Z α/2=Z score of 1.96 at 95% confidence interval (CI); p=prevalence ER positivity and d=margin of error. Subsequently, a 10% non-response rate was considered. In the end, 423 participants were planned for the study; however, we were only able to collect 227 tissue blocks because there were insufficient tissue block samples, mainly from Jimma University Specialized Hospital (Jimma city, Oromia region), Hiwot Fana Specialized University Hospital (Harer City, Hareri Region), and Hawassa University Specialized Referral Hospital (Hawassa city, SNNP region). Because we do not have enough resources, we can only analyze 81 immune cell cases and 58 metalloproteinase cases. For HPV detection, the sample size was calculated using Epi-info version 7.2.1.0, a statistical software. The characteristics that were taken into consideration were a 1:1 cases-to-control ratio, a 95% confidence level, 80% power, a 51.8% HPV prevalence among breast cancer cases, and an odds ratio of 3.0 (Delgado-García et al., 2017). Finally, a sample size of 130 was selected, of which 65 included cases and the remaining 65 controls. There were insufficient samples to collect the remaining 11 control cases, thus in the end, we used 66 cases, 54 controls, and 120 sample sizes together.

3.3. Data Collection

Demographic and clinicopathological data from biopsy reports of the pathology department in each hospital were collected using a data collection form (**Annex 1**). Demographic information, such as age, sex, and residence, and clinicopathological data, such as histologic type, stage, tumor size, grade, lymph node involvement, number of

involved lymph nodes, and nature of the specimen, were included. For clinicopathological features of invasive breast cancer assessment, 50 histopathology slides were inspected and reviewed at random; additionally, inclusion and exclusion criteria were set for clinicopathological features of invasive breast cancer assessment.

3.3.1. Inclusion criteria

Only invasive breast cancer was included in this study. A total of 294 patients with invasive breast cancer were included.

3.3.2. Exclusion criteria

Breast cancer patients with pathologies of non-invasive and/or in situ carcinoma were excluded.

3.4. Histopathological Grade and Stage

Histopathological grades were measured using Nottingham grading (Elston and Ellis, 1991). The evaluation of tubule formation, nuclear pleomorphism, and mitotic counts, each of which is scored 1-3, were used to determine the grade for each breast tumor. The scores for each category were added together to calculate the overall tumor grade, which has a range of 3-9. The following criteria were used to assess tumors: well-differentiated or grade I scores of 3-5; moderately differentiating or grade II scores of 6-7; and poorly differentiating or grade III scores of 8-9 (Elston and Ellis, 1991). The TNM staging method was used to perform histopathological stages. This method provides a “stage grouping” based on the size of the primary tumor (T), local lymph nodes (N), and distant metastases (M) (Edge et al., 2010) (**Table** 1). Histopathological grade and type were checked and confirmed at Armauer Hanssen Research Institute (AHRI) and Tikur Anbessa Specialized Hospital by three senior pathologists under principal investigator supervision. Histopathology assessment on FFPE sections stained with hematoxylin and eosin was performed at AHRI to confirm the diagnosis.

Table 1. The staging methods for breast cancer tumors.

Stages	Tumor Size (T)	Regional Lymph Nodes (N)	Distant Metastases (M)
0	Tis	N0	M0
IA	T1 *	N0	M0
IB	T0	N1mi	M0
	T1 *	N1mi	M0
IIA	T0	N1 **	M0
	T1*	N1 **	M0
	T2	N0	M0
IIB	T2	N1	M0
	T3	N0	M0
IIIA	T0	N2	M0
	T1 *	N2	M0
	T2	N2	M0
	T3	N1	M0
	T3	N2	M0
IIIB	T4	N0	M0
	T4	N1	M0
	T4	N2	M0
IIIC	Any T	N3	M0
IV	Any T	Any N	M1

T1* includes T1mi. **Only T0 and T1 tumors with nodal micrometastases are excluded from stage IIA and are instead classified as being stage IB (Source: (Edge et al., 2010)).

3.5. Immunohistochemistry Staining

The IHC staining for breast cancer subtyping was performed on 227 FFPE tissue blocks using an optimized IHC protocol. Sections of FFPE tissue were cut at a thickness of 4 µm and rehydrated in water. Heat-induced epitope retrieval was carried out using Dako FLEX, a low pH retrieval buffer for Ki-67, and a high pH retrieval buffer for ER, PR, and HER-2. The slides then underwent a 10-minute incubation with peroxidase-blocking solutions, followed by 30-minute incubations with specific primary antibodies and another 30 minutes with EnVision FLEX/HRP. DAB (3,3′-diaminobenzidine) chromogen was then applied for 5 minutes. The slides were counterstained for 30 seconds with hematoxylin and

mounted with DPX and coverslip (**Figure 19**). The principal investigator performed all sectioning and immunohistochemical staining. The detailed IHC procedure is indicated in **Annex 2**. Monoclonal rabbit anti-human ER (DAKO clone Ep1; Agilent Technologies, Denmark) and anti-human PR (DAKO clone PgR636, Agilent Technologies, Denmark) antibodies were used for the staining. If a tumor exhibited 1% or more of tumor cell nuclear staining, it was considered to be ER/PR positive (Allison et al., 2020). The HER-2/neu staining was performed using the HER-2/neu reagent (Polyclonal, Agilent Technologies, Denmark). The grading expression was based on recommendations from Wolff et al. (2018): specimens scored as 0 or 1+ were classified as HER2/neu negative, and specimens scored as 3+ were considered positive. Specimens with a score of 2+ were considered equivocal (Wolff et al., 2018). In multiple logistic regression analysis, the HER2/neu 2+ were excluded. According to the recommendation of the St. Gallen international panel of experts, a Ki-67 cut-off point of ≥ 20% was considered high (Goldhirsch et al., 2013). For ER and Ki-67 proliferation markers, we used DAKO mouse IgG1, Code X0931 negative control, and for PR and HER-2, we used DAKO rabbit immunoglobulin fraction (solid-phase absorbed), Code X0936. Both were diluted to the same IgG concentration as the primary antibody. Ductal epithelial cells from the breast were used as internal controls for ER and PR, and the mitotic index was used as an internal control for Ki-67 proliferation marker staining. Additionally, positive controls were performed using normal endometrial stroma for PR, cervix epithelial cells for ER, and tonsil for Ki-67 in each experiment. The HER-2 positive control was optimized using breast cancer samples used in this study.

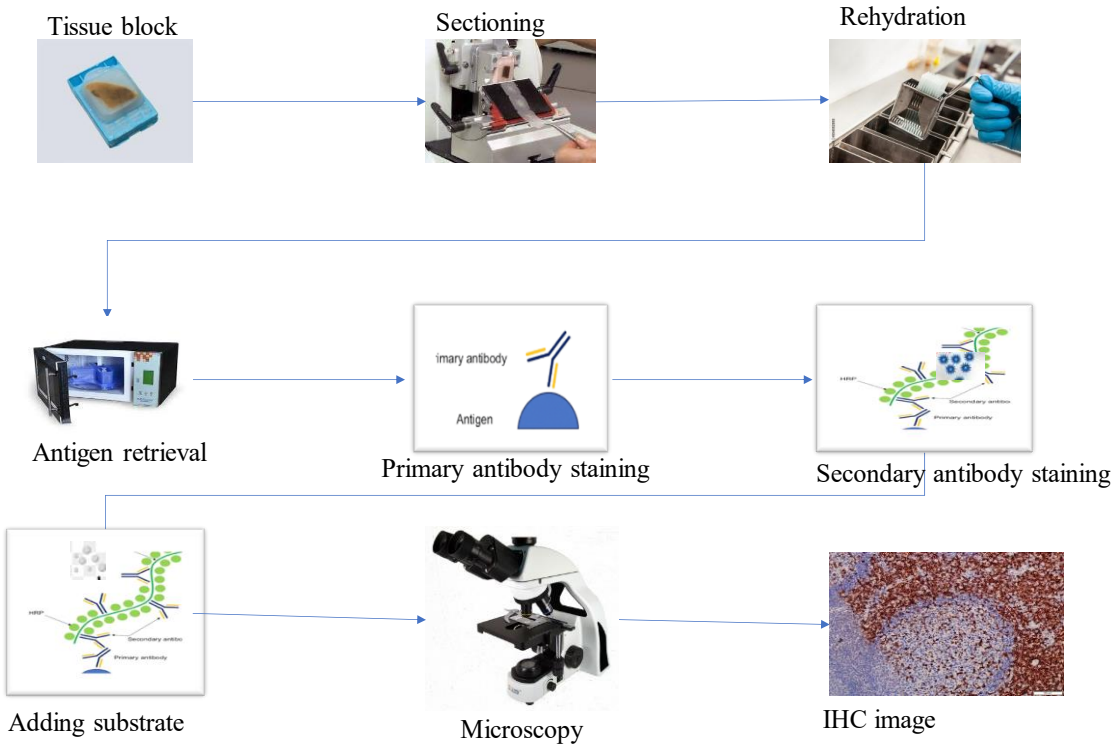


Figure 19. Outline of IHC protocol for FFPE tissue sections visualized by horse radish peroxidase (HRP) system.

The IHC staining for surface marker expression of tumor-infiltrating lymphocytes, tumor-associated macrophages, and PD-L1 assessment was performed on 81 FFPE tissue blocks using an optimized IHC protocol. Heat-induced epitope retrieval was carried out using a high PH retrieval buffer for all markers (**Table 2)**. Six selected IHC images were used (two from low, two from moderate, and two from high staining percentage tissue) to calculate the percentage of positively stained cells for each marker using QuPath 0.4.4 software. Tonsil was used as positive controls for all markers except the CD-56 marker, for which we used appendices. The peroxidase-blocking, EnVision FLEX/HRP, DAB chromogen, and counterstained processes were performed according to the procedure stated in **Annex 2**.

Table 2. List and dilutions of tumor-infiltrating lymphocytes primary antibodies used for immune cell marker study.

Antibody clone	Item Code	Clone	Dilution	Manufacturer	Ag retrieval
CD3, Mouse anti-Human,	11324673	F7.2.38	1:40	Fisher Scientific (United Kingdom)	Microwave / EDTA buffer
CD4, Mouse anti-Human	11364603	4B12	1:40	Fisher Scientific (United Kingdom)	Microwave / EDTA buffer
CD8 alpha Mouse anti-Human	11364853	C8/144B	1:40	Fisher Scientific (United Kingdom)	Microwave / EDTA buffer
CD56 Mouse anti-Human	11520762	123C3	1:40	Fisher Scientific (United Kingdom)	Microwave / EDTA buffer
CD68 Mouse anti-Human	15217057	KP1	1:1000	Fisher Scientific (United Kingdom	Microwave / EDTA buffer
CD163 Mouse anti-Human	11334453	10D6	1:40	Fisher Scientific (United Kingdom	Microwave / EDTA buffer
PD-L1 (CD274), Rabbit anti-human	ACI3171C	CAL10	1:40	Zytomed Systems GmbH (United Kingdom)	Microwave / EDTA buffer
CD20cy Mouse Anti-Human	M075501-2	L26	1:200	Agilent Dako, Demark	Microwave / EDTA buffer

3.6. Breast Cancer Subtyping

Breast cancer subtyping in this study was performed based on the consensus of St. Gallen international experts that divided breast cancer into the following four subtypes (Goldhirsch et al., 2013): luminal A (ER and/or PR-positive, HER-2-negative and Ki-67 < 20%), luminal B (ER and/or PR-positive, HER-2-positive or ER and/or PR-positive, HER-2-negative and Ki-67 ≥ 20%), HER-2-enriched (ER and PR-negative, HER-2 positive) and triple-negative (ER-negative, PR-negative, and HER-2-negative).

3.7. RNA Extraction and Quantitative One-step RT-PCR

Total RNA was extracted from stored FFPE tissue specimens using the RNeasy® FFPE Kit (QIAGEN, Hilden, Germany) (Cat No 73504) according to the manufacturer's protocol for MMPs investigation (**Annex 3**). Ten tissue sections of 2µm thickness per sample were

used for RNA extraction. The quality of extracted RNA was checked using a Nanodrop 2000 spectrophotometer. All extracted RNA samples were then stored at -80°C until the RT-PCR test was performed.

Specific primers and fluorogenic probe sequences for MMP-2, MMP-9, and MMP-11 were taken from the literature (Decock et al., 2007) and their appropriateness was checked using the Primer-Blast tool in NCBI. The sequences for the MMP primers and probes are given in **Table 3**. PCR reactions were carried out on the CFX96 Deep well Real-time PCR instrument (Bio RAD, Singapore). All quantitative reverse-transcription PCRs were performed in duplicate using the SuperScript™ III Platinum™ One-Step qRT-PCR Kit (Invitrogen/Life Technologies Corporation, Carlsbad, CA 92008 USA) according to the manufacturer's instructions with an optimized volume of reaction. Each reaction was performed in a 25 µl reaction volume, containing 5 µL RNA, 12.5 µL 2x reaction mix, 0.5 µL SuperScript™ III RT/Platinum™ Taq Mix, 0.5 µL forward and reverse primers each (10 µM), 0.25 µL probe (10 µM) and 5.75 µL molecular grade water. Conditions for the PCR reaction were 15 minutes at 50°C, 2 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and 45 minutes at 58°C. The GAPDH gene was used as an endogenous control to normalize for differences in the amount of total RNA expression in each sample. To determine the relative RNA levels of expression within the samples, standard curves for the PCR reactions were performed.

Table 3. Taqman primers and probes sequence for the human MMPs and GAPDH.

Gene	**Gene bank accession no.**	**Primers and Probes (5'-3')**	**Sequence of primers and FAM-BHQ1 probes**	**Amplicon size (bp)**
MMP-2	NM004530	Forward primer	TGGCGATGGATACCCCTTT	83
		Reverse primer	TTCTCCCAAGGTCCATAGCTCAT	
		Probe	FAMCTCCTGGCTCATGCCTTCGCCCBHQ1	
MMP-9	NM004994	Forward primer	CCTGGGCAGATTCCAAACCT	54

		Reverse primer	GCAAGTCTTCCGAGTAGTTTTGGAT	
		Probe	FAMCTCAAGTGGCACCACCACAACATCACCBHQ1	
MMP-11	NM005940	Forward primer	CCGCCAGATGCCTGTGA	66
		Reverse primer	CGGAGGCGCCACACAA	
		Probe	FAMCCTCCTTTGACGCGGTCTCCACCBHQ1	
GAPDH	NM001357943	Forward primer	GAAGGTGAAGGTCGGAGTC	172
		Reverse primer	GAAGATGGTGATGGGATTTC	
		Probe	FAMCAAGCTTCCCGTTCTCAGCCBHQ1	

3.8. DNA Extraction and Multiplex Real-time PCR

DNA was extracted from stored (2015-2021) FFPE breast tissue specimens using GeneRead DNA FFPE Kit (Campay, QIAGEN GmbH, QIAGEN Str. 1, D-40724 Hilden) following the manufacturer's protocol (**Annex 4**). DNA extraction was performed on ten tissue sections of 2 µm thickness in each sample. The quality of extracted DNA was checked using a Nanodrop 2000 spectrophotometer. All extracted DNA samples were then stored at -20°C until the PCR test was performed.

The PCR reactions were carried out using the CFX96 Deep well Real-time PCR instrument (Bio RAD, Singapore). AnyplexTM II HPV28 Detection Kit (Seegene, Korea) was used to perform multiplex real-time PCR for the HPV genotyping as previously described (Seyoum et al., 2023). It allows for the simultaneous amplification, detection, and separation of target nucleic acids from 19 high-risk HPV types (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 69, 73, 82) and 9 low-risk HPV types (6, 11, 40, 42, 43, 44, 54, 61, 70) as well as an internal control (IC). Human housekeeping gene (human beta-globin) as endogenous IC was used to ensure extraction of DNA, verification of PCR reaction, and clarification of cell adequacy from each specimen.

3.9. Ethical Approval

Ethical approval for this study was obtained from the College of Natural Science Institutional Ethics Review Board (CNS-IRB) Addis Ababa University (No. IRB/032/2018), AHRI/ALERT Ethics Review Committee (AAERC) (No. PO/27/19) and Federal Democratic Republic of Ethiopia Ministry of Education (No.7/2.12/m259/35).

3.10. Statistical Analysis

The mean fold change expression for MMPs was calculated using a Microsoft Excel spreadsheet (Schmittgen and Livak, 2008). Statistical analysis for MMPs, tumor-infiltrating lymphocytes, tumor-associated macrophages, and PD-L1 was performed using GraphPad Prism version 8.0.0 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com). The assumption of normality was evaluated using the Shapiro normality test. Based on the skewed distribution of the dataset, a non-parametric T-test followed by a Mann-Whitney test was used for the comparison of different groups. Data collected from the pathology report, HPV PCR, and IHC results were entered and analyzed using SPSS Version-25 software. Univariate Chi-square tests were used to assess the hypothesis of the association between predictor and outcome variables of interest. Logistic regression was performed to determine associations between a given predictor and outcome variables after correcting for the effects of all other predictors. Statistical significance was defined as a p-value less than 0.05. For categorical measures, frequencies and percentages were examined. For continuous measures, mean, standard deviation, and range were examined.

4. CHAPTER FOUR: RESULTS

This chapter presented findings of five-year retrospective clinicopathological features, immunohistochemistry-defined subtypes, surface marker expression on tumor-infiltrating lymphocytes, tumor-associated macrophages and PD-L1, MMP expression, and HPV genotyping in Ethiopian breast cancer patients. In summary, this study found that breast cancer was more prevalent in young age groups (< 45 years) in Ethiopia, with invasive carcinoma of no special type as the most common histologic pattern. At the time of diagnosis, there was a high prevalence of lymph node involvement, advanced pathological stage, and grade. The proportions of ER-negative and PR-negative tumors were 48.3% and 53.2%, respectively. Triple-negative breast cancer was more common in southwest Ethiopia (Jimma) than in north Ethiopia (Mekele). The age groups, tumor size, histological grade, and Ki-67 proliferation index differed between the study sites. The non-luminal breast cancer subtype had a higher percentage of stromal CD20+, intratumoral CD3+ tumor-infiltrating lymphocytes, and CD68+ tumor-associated macrophages than the luminal subtype. The stromal and intratumoral PD-L1+, intratumoral CD3+ tumor-infiltrating lymphocytes, and CD163+ tumor-associated macrophages were also more commonly found in grade III breast cancer than in grade I and II breast cancer. The MMP-11 mRNA expression was associated with breast cancer. This study found no evidence that HPV contributes to the development of breast cancer; however, the level of HPV was significantly higher in ER-positive cases than in ER-negative cases of breast cancer.

4.1. Retrospective Clinicopathological Features

4.1.1. Demographic characteristics

A total of 589 patients presented with breast lumps at Hawassa University Comprehensive Specialized Referral Hospital and Jimma University Specialized Teaching Hospital from 2015 to 2019. Of these, 294 patients (49.9%) had a histopathological confirmed diagnosis of invasive carcinoma. From this, the stage of 32 patients, 39 tumor sizes, 161 grades, 8 lymph node involvements, 11 numbers of involved lymph nodes, 4 types of surgery, and 6 histological-type patient data were omitted because the information was not available in

their pathology reports. In addition to being absent on their medical records, tumor stage, tumor size, and lymph node involvement were not performed for every incisional biopsy. Moreover, in some cases of invasive breast cancer, lymph node dissection was not always performed. The age at diagnosis of breast cancer patients ranged from 17 to 100 years with a mean of 42.27(SD = ± 13.57) years. Most breast cancer patients (61.6%) were under 44 years of age (**Table 4**).

Table 4. Demographic characteristics of breast cancer patients at Hawassa University Comprehensive Specialized Referral Hospital and Jimma University Specialized Teaching Hospital from 2015 to 2019.

Variables		**Number (%)**
Age group	15-29	45(15.3%)
	30-44	136(46.3)
	45-59	77(26.2)
	60-74	28(9.5)
	>=75	8(2.7)
	Total	294(100.0)
Mean ± Sd (Minimum, Maximum) 42.27± 13.57(17,100)		
Sex	Female	284(96.6)
	Male	10(3.4)
	Total	294(100.0)
Patient residence	Hawassa	113(38.4)
	Jimma	181(61.6)
	Total	294(100.0)

4.1.2. Clinicopathological features

Most breast cancer patients (64.9%) were at pathological stage III, and 41.2% of the patients had a tumor size greater than 5 cm (pT3) at the time of diagnosis. It was observed that most patients (49.6 %) had a moderately differentiated tumor grade (grade II) followed by poorly differentiated (grade III) (28.6%) and well-differentiated (grade I) (21.8%).

Lymph node involvement was seen in 60.5 % of cases, and most of them (48.8%) had 4-10 involved lymph nodes. Mastectomy was the most common method of surgery, accounting for 84.5% of cases (**Table 5**).

Table 5. Distribution of tumor presentation at the time of diagnosis among breast cancer patients at Hawassa University Comprehensive Specialized Referral Hospital and Jimma University Specialized Teaching Hospital from 2015 to 2019.

Variables		**Frequency**
Stage	I	5(1.9)
	II	87(33.2)
	III	170(64.9)
	Total	262(100.0)
Tumor Size	pT1	8(3.1)
	pT2	71(27.8)
	pT3	105(41.2)
	pT4	71(27.8)
	Total	255(100.0)
Grade	I	29(21.8)
	II	66(49.6)
	III	38(28.6)
	Total	133(100.0)
Lymph node involvement	Positive	173(60.5)
	Negative	113(39.5)
	Total	286(100.0)
Positive lymph nodes at surgery	1-3	70(43.2)
	4-10	79(48.8)
	>10	13(8.0)
	Total	162(100.0)
Type of surgery	Mastectomy	245(84.5)

	Lumpectomy	2(0.7)
	Incisional biopsy	43(14.8)
	Total	290(100.0)

Invasive carcinoma of no special type was the most common histologic type of breast cancer (77.1%) followed by invasive lobular carcinoma (11.1%) and papillary carcinoma (2.8%) (**Figure 20).** The representative diagram of each histological type is indicated in **Figure 21.**

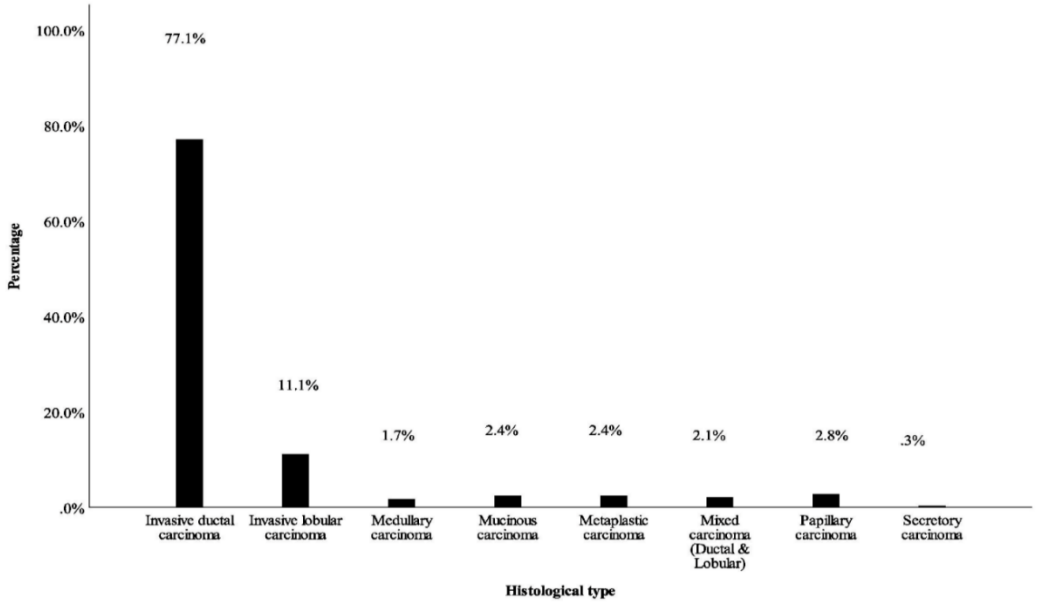


Figure 20. Distribution of histologic types of breast cancer: data pooled from Hawassa University Comprehensive Specialized Referral Hospital and Jimma University Specialized Teaching Hospital.

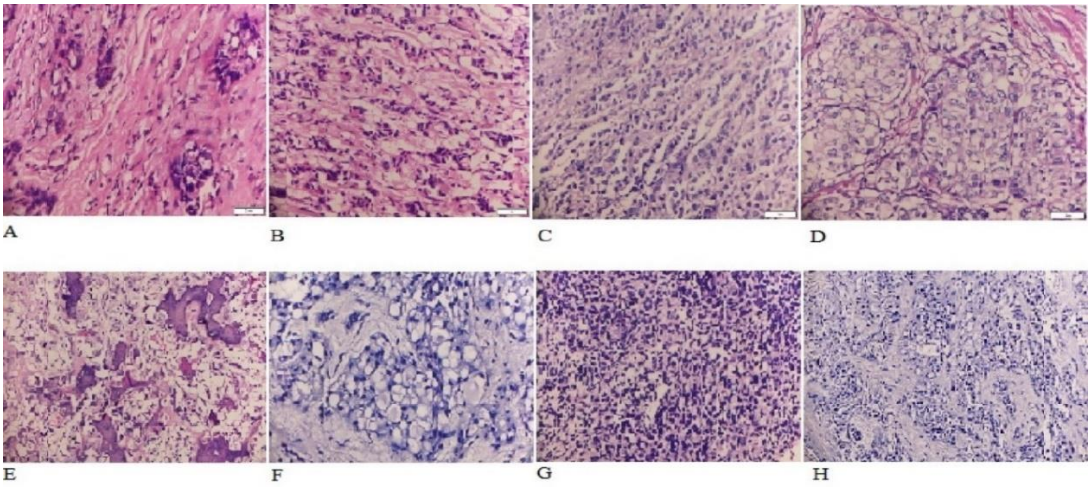


Figure 21. Histological types of breast cancer. (A) Invasive carcinoma of no special type, (B) Invasive lobular carcinoma, (C) Mixed (invasive ductal and lobular) carcinoma, (D) Medullary carcinoma, (E) Metaplastic carcinoma (F) Mucinous carcinoma (G) Papillary carcinoma (H) Secretory carcinoma.

4.1.3. Associated factors

Lymph node involvement was significantly associated with tumor size ($\chi 2 = 8.55$, $P = 0.033$), and type of surgery ($\chi 2 = 49.09$, $p < 0.001$), with higher lymph node involvement observed in patients undergoing mastectomy. Patients with lymph node involvement had a higher proportion of tumors with a size greater than 5 cm (46.6%), compared with those without lymph node involvement. Lymph node involvement was also higher among invasive carcinoma of no special type histologic types (**Table 6**).

Table 6. Lymph node involvement with stage, tumor size, and histologic type.

Variables	**N (%)**	**Lymph node involvement**		**χ2**	**P-value**
		Yes	No		
Tumor Size, n (%)					
≤ 2 cm(T1)	8(3.1)	3(1.9)	5(5.4)	8.55	0.033*
> 2 cm and ≤ 5 cm(T2)	70(27.6)	37(23.0)	33(35.5)		
> 5cm (T3)	105(41.3)	75(46.6)	30(32.2)		

Any size extension to the chest wall or skin(T4)	71(28.0)	46(28.6)	25(26.9)		
Total	254(100.0)	161(100.0)	93(100.0)		
Type of surgery, n (%)					
Mastectomy	242(85.5)	165(95.9)	77(69.4)	39.69	< 0.001*
Lumpectomy	2(0.7)	1(0.6)	1(0.9)		
Incisional biopsy	39(13.8)	6(3.5)	33(29.7)		
Total	283(100.0)	172(100.0)	111(100.0)		
Histologic type, n (%)					
Invasive carcinoma of no special type	218(77.3)	135(78.9)	83(74.8)		
Invasive lobular carcinoma	32(11.3)	19(11.1)	13(11.7)	7.4	0.37
Mixed carcinoma	6(2.1)	4(2.3)	2(1.8)		
Papillary carcinoma	7(2.5)	2(1.2)	5(4.5)		
Mucinous carcinoma	6 (2.1)	2(1.2)	4(3.6)		
Metaplastic carcinoma	7 (2.5)	5(2.9)	2(1.8)		
Medullary carcinoma	5 (1.8)	4(2.3)	1(0.9)		
Secretory carcinoma	1(0.4)	0(0.0)	1(0.9)		
Total	282(100.0)	171(100.0)	111(100.0)		

4.2. Breast Cancer Subtypes

4.2.1. Demographic and histopathological characteristics

In this study, 227 tumor specimens were collected. The mean age at diagnosis was 43.9 (SD = 13.9) years. A high rate of male breast cancer (4.8%) incidence was found in this study. The average age of breast cancer patients at the Hawassa study site was lower than those of other research sites (38.7 years) (**Table 7**).

Table 7. Basic demographic information of the study population at four study sites.

Variables		**Study sites**

		Frequency (%)	Hawassa	Jimma	Mekele	Harar
Age	< 50 years	151 (66.5)	40 (87.0)	31 (58.5)	59 (62.1)	21 (63.6)
	≥ 50 years	76 (33.5)	6 (13.0)	22 (41.5)	36 (37.9)	12 (36.4)
	Total	227 (100.0)	46 (100.0)	53 (100.0)	95 (100.0)	33 (100.0)
	Mean age ± SD	43.9 ± 13.9 years	38.7± 11.7 years	44.9± 13.9 years	45.2± 14.2 years	44.7± 13.6 years
Sex	Female	216 (95.2)	45 (97.8)	51 (96.2)	89 (93.7)	30 (93.9)
	Male	11 (4.8)	1 (2.2)	2 (3.8)	6 (6.3)	11 (4.8)
	Total	227 (100.0)	46 (100.0)	53 (100.0)	95 (100.0)	33 (100.0)

Tumor size greater than 5 cm (T3) at the time of diagnosis accounted for 28.9% of the cases, with a higher percentage (48.9%) in southwest Ethiopia (Jimma). The 19 and 35 cases of tumor size and lymph node involvement information were missed in their medical record and not included in this study, respectively. Any tumor size growing into the chest or skin (T4) was high in Harer (42.3%). Involvement of the lymph nodes was found in 63.7% of cases, with a higher percentage in northern Ethiopia (Mekele) (75.8%). Histological grades II and III accounted for 66% of the cases. Age, tumor size, and histologic grade were all substantially associated with study sites, with younger cases in southern Ethiopia (Hawassa), larger tumor size in southwestern Ethiopia (Jimma), and higher histological grade in northern Ethiopia (Mekele) (**Table 8**). Invasive carcinoma of no special type was the most common histomorphologic type of breast cancer (84.0%), followed by invasive lobular carcinoma.

Table 8. Distribution of histopathological, and immunohistochemical characteristics of breast cancer at the four study sites.

Variables*		**Frequency (%)**	**Study sites**				**p-value**
			Hawassa	**Jimma**	**Mekele**	**Harar**	
Tumor Size	T1	16 (8.0)	2 (4.9)	2 (4.3)	10 (11.5)	2 (7.7)	0.001
	T2	77 (38.3)	14 (34.1)	9 (19.1)	43 (49.4)	11 (42.3)	
	T3	58 (28.9)	13 (31.7)	23 (48.9)	20 (23.0)	2 (7.7)	

	T4	50 (24.9)	12 (29.3)	13 (27.7)	14 (16.1)	11 (42.3)	
	Total	201 (100.0)	41 (100.0)	47 (100.0)	87 (100.0)	26 (100.0)	
Grade	I	74 (33.9)	18 (39.1)	25 (47.2)	25 (26.3)	9 (27.3)	0.033
	II	70 (32.1)	16 (34.8)	16 (30.2)	25 (26.3)	12 (36.4)	
	III	74 (33.9)	12 (26.1)	12 (22.6)	45 (47.4)	12 (36.4)	
	Total	218 (95.6)	46 (100.0)	53 (100.0)	95 (100.0)	33 (100.0)	
Lymph node status	Positive	123 (63.7)	26 (56.5)	30 (56.6)	50 (75.8)	16 (59.3)	0.088
	Negative	70 (36.3)	20 (43.5)	23 (43.4)	16 (24.2)	11 (40.7)	
	Total	192 (100.0)	46 (100.0)	53 (100.0)	66 (100.0)	27 (100.0)	
ER	Positive	104 (51.7)	22 (48.9)	27 (51.9)	40 (52.6)	15 (53.6)	0.976
	Negative	97 (48.3)	23 (51.1)	25 (48.1)	36 (47.4)	13 (46.4)	
	Total	201 (100.0)	45 (100.0)	52 (100.0)	76 (100.0)	28 (100.0)	
PR	Positive	94 (46.8)	23 (51.1)	20 (38.5)	41 (53.9)	10 (35.7)	0.193
	Negative	107 (53.2)	22 (48.9)	32 (61.5)	35 (46.1)	18 (64.3)	
	Total	201 (100.0)	45 (100.0)	52 (100.0)	76 (100.0)	28 (100.0)	
HER-2	Positive	42 (22.0)	8 (18.2)	11 (21.6)	13 (19.1)	10 (35.7)	0.269
	Negative	128 (67.0)	30 (68.2)	38 (74.5)	45 (66.2)	15 (53.6)	
	Equivocal	21 (11.0)	6 (13.6)	2 (3.9)	10 (14.7)	3 (10.7)	
	Total	191 (100.0)	44 (100.0)	51 (100.0)	68 (100.0)	28 (100.0)	
Ki-67	Low	106 (57.0)	22 (56.4)	40 (81.6)	37 (52.9)	7 (25.0)	< 0.0001
	High	80 (43.0)	17 (43.6)	9 (18.4)	33 (47.1)	21 (75.0)	
	Total	186 (100.0)	39 (100.0)	49 (100.0)	70 (100.0)	28 (100.0)	
Subtype	Luminal A	41 (25.2)	12 (34.3)	17 (35.4)	9 (16.4)	3 (12.0)	0.114
	Luminal B	45 (27.6)	8 (22.9)	9 (18.8)	19 (18.8)	9 (36.0)	

	HER2 enriched	23 (14.1)	6 (17.1)	4 (8.3)	7 (12.7)	6 (24.0)	
	triple-negative breast cancer	54 (33.1)	9 (25.7)	18 (37.5)	20 (36.4)	7 (28.0)	
	Total	163 (100.0)	35 (100.0)	48 (100.0)	55 (100.0)	25 (100.0)	

* Variables are only shown for cases with known results. Differences of features among study sites assessed by X^2 test. There were differences in the sample sizes for each marker: of the 227 patients, 201 had ER and PR, 191 had HER-2, and 186 had KI-67 analysis. Missing data was caused by difficult-to-interpret results (internal control not working), falling tissue from IHC slides, and failure to obtain tissue samples for sectioning (for the few samples indicated to repeat, we can't get leftover tissue samples).

4.2.2. Tumor size

In univariate analysis, tumor size was determined in 201 cases (26 cases were missed), and Jimma was the region with the highest percentage of T3 and T4 tumors (76.6%) (**Table 8**). For multiple logistic regression analysis, 157 cases were included. Grade III tumors were 2.5 times more likely than grade I or II tumors to have a large (T3 or T4) tumor size (P = 0.025). The HER2-positive tumors were 4.1 times more likely than HER2-negative tumors to have a large (T3 or T4) tumor size (P = 0.007). Breast cancer cases from the south (Hawassa) and southwest (Jimma) of Ethiopia were 3.1 and 7.7 times, respectively, more likely to have T3 or T4 tumors than those from the north (Mekele) (**Table 9**).

Table 9. Multiple logistic regression analysis of demographic and histopathological parameters predicting tumor size.

Parameters*		All N (%) = 157	Large tumor size (T3 and T4) (n = 95) vs small tumor size (T1 and T2) (n = 62).	
			OR (95% CI)	p-value
Age group (years)	< 50 (ref) #	107 (68.2%)	1.40 (0.61–3.19)	0.430
	≥ 50	50 (31.8%)		
Grade	I or II (ref)	102 (65.0%)	2.57 (1.13–5.84)	0.025
	III	55 (35.0%)		
Lymph node involvement	Yes	102 (65.0%)	1.60 (0.75–3.45)	0.228
	No (ref)	46 (35.0%)		
HER-2	Negative (ref)	103 (65.6%)		
	Positive	34 (21.7%)	4.14 (1.47–11.67)	0.007
	Equivocal	20 (12.7%)	2.02 (0.70–5.81)	0.193
Study sites	Hawassa	40 (25.5%)	3.10 (1.13–8.47)	0.028
	Jimma	45 (28.7%)	7.7 (2.65–22.77)	< 0.0001
	Harar	22 (14.0%)	1.47 (048–4.57)	0.502
	Mekele (ref)	50 (31.8%)		

* Tumor size was the outcome variable. Binary categories of large (T3 or T4) and small (T1 or T2) were created with large tumor size as the reference value. The indicated dependent variables or parameters are listed in the left column. # The reference values for the predictor variables are indicated within parentheses.

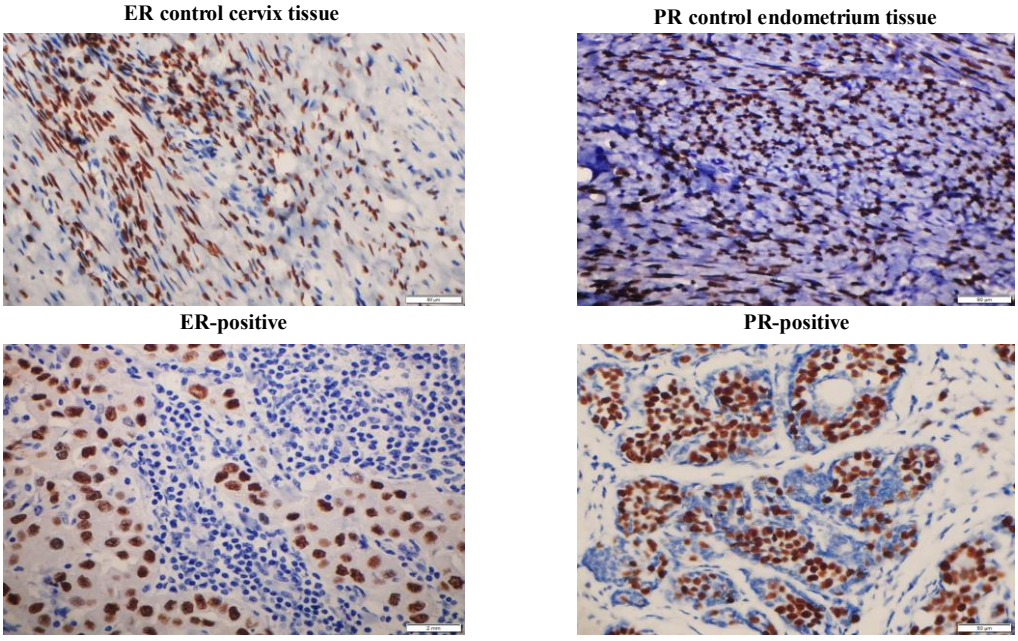
4.2.3. Estrogen receptor, HER-2, and Ki-67 proliferation

In univariate analysis, 201 cases were analyzed (26 cases were missed), and half of the specimens were ER and PR-negative (**Table 8**). In this study, 161 breast cancer cases were included in multiple logistic regression analysis, and the presence of an ER-positive tumor with a histological grade I or II was 2.9 times more common than that of a grade III tumor

(P = 0.005). The chance of having ER-positive breast cancer appears to be 2.1 times higher in older women (>50 years vs. <50 years) (P = 0.039) (**Table 10**).

Among a total of 191 specimens (36 cases were missed) included in univariate analysis, 22% of the cases were HER-2 positive, with the highest percentage (35.7%) reported from eastern Ethiopia (Harar) (**Table 8**). In the study of 137 breast cancer cases that were included for multiple logistic regression analysis, T3 or T4 tumors were 3.8 times higher than T1 or T2 tumors to be HER-2-positive (P = 0.01). Additionally, breast cancer cases in eastern Ethiopia (Harer) were 3.6 times more likely than cases in northern Ethiopia (Mekele) to be HER-2 positive (**Table 10**).

In univariate analysis, a total of 186 cases of breast cancer were analyzed (41 cases were missed), and high Ki-67 proliferation was observed in 43.0% of breast cancer cases (**Table 8**). In the multiple logistic regression analysis of 149 breast cancer patients, eastern Ethiopia (Harer) was 6.4 times more likely than northern Ethiopia (Mekele) to have high Ki-67 proliferation (**Table 10**). The representative IHC image for ER, PR, HER-2, and Ki-67 positive is shown in **Figure 22**.



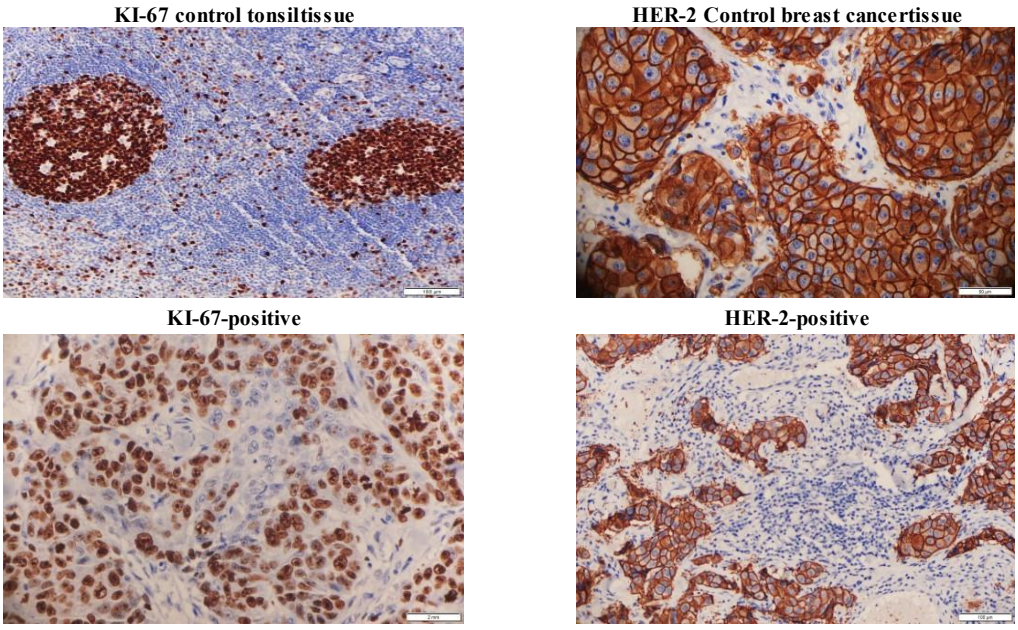


Figure 22. Representative IHC picture of ER, PR, HER-2, and Ki-67 positive breast cancer and control tissues**.**

Table 10. Multiple logistic regression analysis of positive ER and HER-2 status, and high Ki-67 proliferation index with other variables among 161 (ER), 137 (HER-2), and 149 (Ki-67) study participants.

Parameters*		**ER-positive (n = 79) ER-negative (n = 82)**		**HER-2-positive (n = 34) HER-2-negative (n = 103)**		**High Ki-67 proliferation (n = 59) Low Ki-67 proliferation (n = 90)**	
		OR (95% CI)	p-value	OR (95% CI)	p-value	OR (95% CI)	p-value
Age group (years)	< 50 (ref)#	2.18 (1.04–4.58)	0.039	0.61 (0.2–1.61)	0.317	1.65 (0.70–3.90)	0.253
	≥ 50						
Grade	I or II		0.005		0.727		

	III (ref)	2.96 (1.40–6.26)		1.18 (0.47–2.96)		0.20 (0.08–0.46)	<0.0001
Tumor Size	T1 or T2 (ref)	0.98 (0.49–1.98)	0.956	3.85 (1.39–10.68)	0.010	0.82 (0.79–4.15)	0.158
	T3 or T4						
Lymph node involvement	Yes	1.60 (0.80–3.20)	0.185	0.99 (0.41–2.41)	0.981	0.67 (0.230–1.51)	0.337
	No (ref)						
Study sites	Hawassa	1.18 (0.48-2.93)	0.719	0.82 (0.23-2.92)	0.760	1.69 (0.58-4.91)	0.333
	Jimma	0.71 (0.29-1.76)	0.461	1.04 (0.32-3.43)	0.947	0.42 (0.14-1.25)	0.118
	Harar	1.51 (0.51–4.47)	0.456	3.61 (1.01–12.87)	0.048	6.39 (1.85–22.09)	0.003
	Mekele (ref)						

***** Binary logistic regression was performed with ER, HER-2 positivity, and high Ki-67 proliferation marker as the outcome variables (with marker negativity and low Ki-67 proliferation as the reference value), and predictor variables listed in the parameter column at left.

The reference values for the predictor variables are indicated within parentheses.

4.2.4. IHC subtypes distribution

In univariate analysis, 163 samples with all IHC available the IHC subtypes showed the following distribution: 33.1% triple-negative breast cancer, 27.6% luminal B, 25.2%

luminal A, and 14.1% HER-2 enriched (**Table 8**). Among the 131 breast cancer patients included in multiple logistic regression analysis, luminal A subtypes were 10.4 times more likely to have histological grade I or II than grade III (P = 0.002). Luminal A subtypes of breast cancer in southern Ethiopia (Hawassa) were 3 times more likely than in northern Ethiopia (Mekelle) (P = 0.109). We observed cases with tumor size T3 or T4 were 4.8 times higher to have HER2 enriched subtypes than tumor size T1 or T2 (**Table 11**). In univariate analysis, triple-negative breast cancer was found in the highest number of cases from southwestern Ethiopia (Jimma) (37.5%), followed by cases from northern Ethiopia (Mekele) (36.4%) (**Table 8**). Using a multiple logistic regression model, after controlling for other variables, triple-negative breast cancer in southwestern Ethiopia (Jimma) was 2.1 times more likely than in northern Ethiopia (Mekelle), though this did not show statistical significance (P = 0.18) (**Table 11**).

Table 11. Multiple logistic regression analysis of demographic and histopathological parameters, taken as predictive variables for individual IHC subtypes compared to others (N = 131).

Parameters		**All N (%) = 131**	**Luminal A (n = 30)**		**Luminal B (n = 37)**		**HER2-enriched (n = 19)**		**Triple-negative breast cancer (n = 45)**	
			OR (95% CI)	p-value	OR (95% CI)	p-value	OR (95% CI)	p-value	OR (95% CI)	p-value
Age group (years)	< 50	90 (68.7%)	0.42 (0.15–1.19)	0.105	0.95 (0.39–2.328)	0.906	1.48 (0.44–5.03)	0.527	1.48 (0.62–3.57)	0.382
	≥ 50 (ref)	41 (31.3%)								
Tumor size	T1 or T2 (ref)	51 (38.9%)	0.52 (0.20–1.38)	0.188	1.16 (0.49–2.73)	0.742	4.84 (1.23–19.03)	0.024	0.61 (0.25–1.47)	0.267
	T3 or T4	80 (61.1%)								

Grade	I or II	85 (64.9%)	10.43 (2.36–55.39)	0.002	1.64 (0.67–4.03)	0.280	0.72 (0.24–2.14)	0.554	0.22 (0.09–0.54)	0.001
	III (ref)	46 (35.1%)								
Lymph nodes involved	Yes	86 (65.6%)	1.04 (0.39–2.72)	0.944	1.34 (0.57–3.14)	0.507	1.33 (0.42–4.18)	0.628	0.66 (0.29–1.65)	0.336
	No (ref)	45 (34.4%)								
Study sites	Hawassa	31 (23.7%)	2.94 (0.79–10.93)	0.109	0.76 (0.25–2.38)	0642	1.03 (0.24–4.46)	0.968	0.53 (0.17–1.71)	0.290
	Jimma	42 (32.1%)	1.829 (0.35–4.80)	0.702	0.55 (0.17–1.72)	0.302	0.49 (0.11–2.28)	0.363	2.13 (0.71–6.41)	0.180
	Harar	20 (15.3%)	0.37 (0.06–2.27)	0.284	1.87 (0.59–5.99)	0.290	2.18 (0.49–9.67)	0.304	0.41 (0.10–1.63)	0.205
	Mekele (ref)	38 (29.0%)								

4.3. Surface Marker Expression Tumor-infiltrating Lymphocytes, Tumor-associated Macrophages, and PD-L1

4.3.1. Demographic, clinicopathological, and IHC characteristics

Breast cancer was detected at a mean age of 44.7 years. Breast cancer patients had a high frequency of grade I & II, tumor size (T3 and T4), lymph node involvement, invasive carcinoma of no special type histological type, ER negative, PR negative, HER-2 negative, low Ki-67 proliferation index, and luminal subtype (**Table 12**).

Table 12. Demographic, clinicopathological, and immunohistochemical characteristics of breast cancer patients.

Characters		**N (%)**
Age	< 50 years	50(61.7)

	≥ 50 years	31(38.3)
	Mean ± Sd (Minimum, Maximum) = 44.7 ± 13.6(22,75)	
Sex	Female	78(96.3)
	Male	3(3.7)
Tumor size	T1 and T2	29(40.8)
	T3 andT4	42(59.2)
Grade	I and II	51(63.0)
	III	30(37.0)
Lymph node involvement	Yes	52(75.4)
	No	17(24.6)
Histological type	Invasive carcinoma of no special type	74(91.4)
	Invasive lobular carcinoma	1(1.2)
	Mucinous carcinoma	2(2.5)
	Mixed carcinoma (Ductal & Lobular)	1(1.2)
	Metaplastic & SCC keratinizing	1(1.2)
	Papillary carcinoma	2(2.5)
ER	Positive	36(44.4)
	Negative	42(51.9)
PR	Positive	39(48.1)
	Negative	42(51.9)
HER-2	Positive	16(19.8)
	Negative	59(72.8)
	Equivocal	6(7.4)
Ki-67 proliferation index	High	31(38.3)
	Low	50(61.7)
Subtype	Luminal	45(60.0)
	Non-luminal	30(40.0)

4.3.2. Intratumoral tumor-infiltrating lymphocytes, tumor-associated macrophages, PD-L1, and breast cancer subtypes

A significant association between intratumoral CD3+ tumor-infiltrating lymphocytes (P = 0.0069) and CD68+ tumor-associated macrophages (P = 0.0223) and breast cancer subtype was observed, with a higher percentage in the non-luminal breast cancer subtype. A high percentage of intratumoral CD4+ tumor-infiltrating lymphocytes (P = 0.1862) and CD163+ tumor-associated macrophages (P = 0.0938) were found in the non-luminal breast cancer subtype; however, the difference was not statistically significant (**Figure 23**). Representative microscopic image of IHC staining of CD3+, CD4+, CD8+, CD20+, CD56+, PD-L1+, CD68+, and CD163+ breast cancer and control tissues is indicated in **Figure 31.**

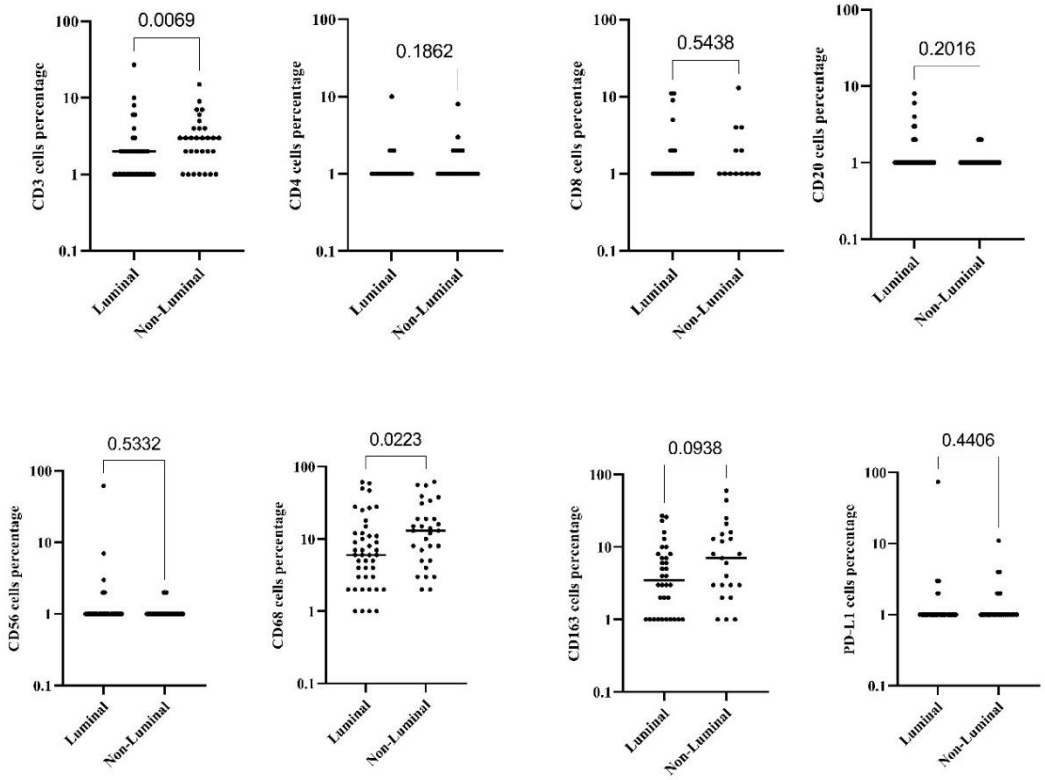


Figure 23. Intratumoral tumor-infiltrating lymphocytes, tumor-associated macrophages, PD-L1, and subtypes in breast cancer patients. The intratumoral percentage of CD3, CD4,

CD8, CD20, CD 56, CD68, CD163, and PD-L1 expression among breast cancer subtypes was log-transformed with median values indicated for each group by the horizontal lines.

4.3.3. Stromal tumor-infiltrating lymphocytes, tumor-associated macrophages, PD-L1, and breast cancer subtypes

A significant association was found between stromal CD20+ tumor-infiltrating lymphocytes (P = 0.0224) and the breast cancer subtype, with a greater number in the non-luminal breast cancer subtype. The non-luminal breast subtypes had a higher percentage of stromal CD3+ (P = 0.0893), CD8+ (P = 0.0570), CD56+ (P = 0.1009) tumor-infiltrating lymphocytes, and CD163+ tumor-associated macrophages (P = 0.1084), but the difference was not statistically significant (**Figure 24**).

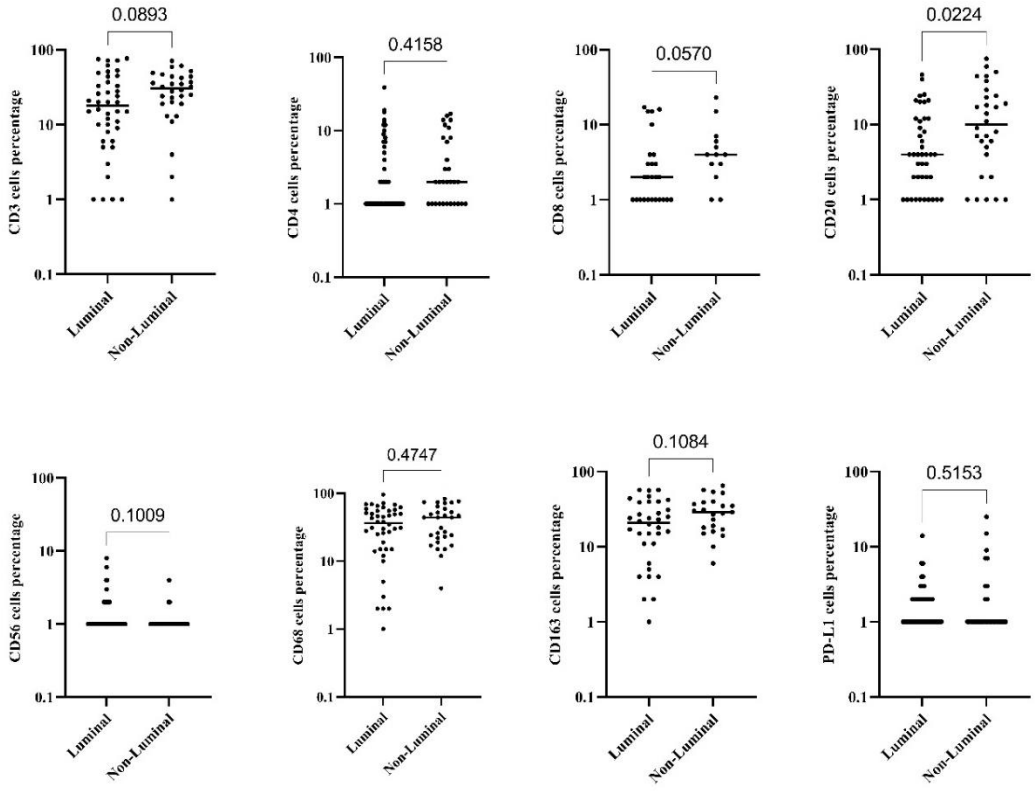


Figure 24. Stromal tumor-infiltrating lymphocytes, tumor-associated macrophages, PD-L1, and subtypes in breast cancer patients. The stromal percentage of CD3, CD4, CD8,

CD20, CD 56, CD68, CD163, and PD-L1 expression among breast cancer subtypes was log-transformed with median values indicated for each group by the horizontal lines.

4.3.4. Intratumoral tumor-infiltrating lymphocytes, tumor-associated macrophages, PD-L1, and estrogen receptor

This study found a significant association between intratumoral CD3+ tumor-infiltrating lymphocytes (P = 0.0065) and CD68+ tumor-associated macrophages (P = 0.0184) and ER, with a higher percentage of ER-negative breast cancer. A high percentage of intratumoral CD4+ tumor-infiltrating lymphocytes (P = 0.0919), CD163+ tumor-associated macrophages (P = 0.1086), and PD-L1+ (0.0855) were found among ER-negative breast cancer, although it was not statistically significant (**Figure 25**).

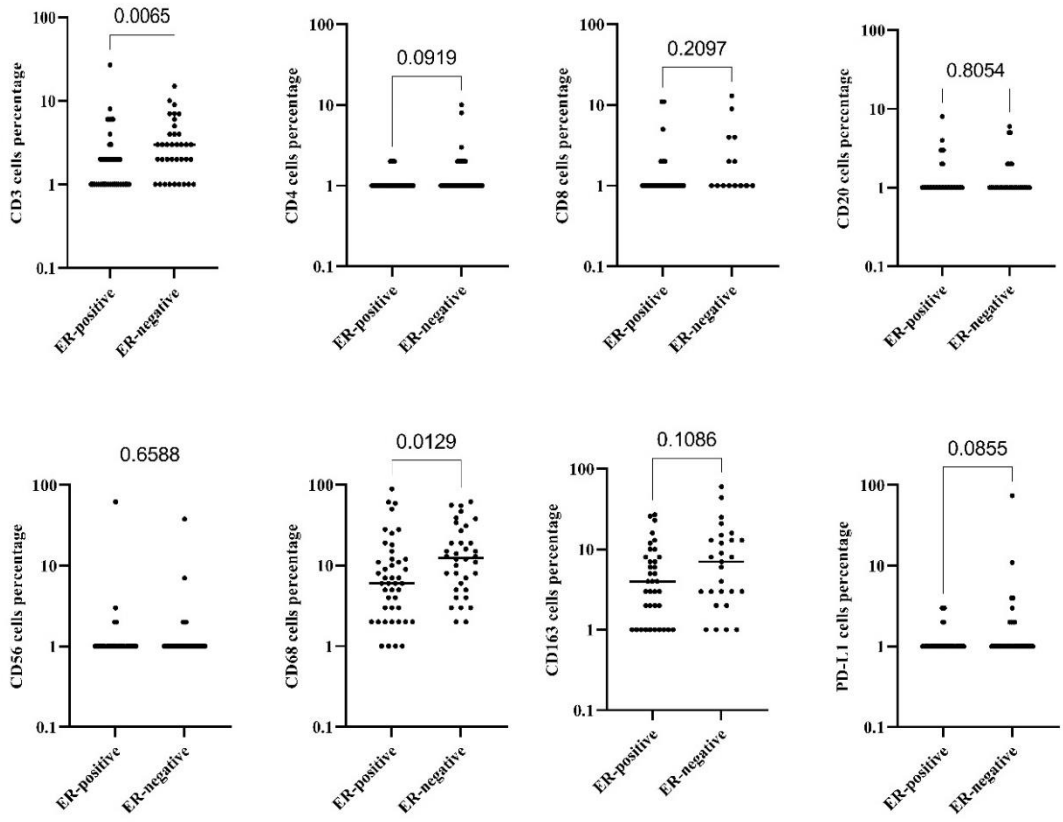


Figure 25. Intratumoral tumor-infiltrating lymphocytes, tumor-associated macrophages, PD-L1, and ER in breast cancer patients. The intratumoral percentage of CD3, CD4, CD8, CD20, CD 56, CD68, CD163, and PD-L1 expression among ER-positive and ER-negative breast cancer was log-transformed with median values indicated for each group by the horizontal lines.

4.3.5. Stromal tumor-infiltrating lymphocytes, tumor-associated macrophages, PD-L1, and estrogen receptor

There was a significant association between stromal CD3+ tumor-infiltrating lymphocytes (P = 0.0179), CD8+ tumor-infiltrating lymphocytes (P = 0.0260), CD20+ tumor-infiltrating lymphocytes (P = 0.0144), and CD163+ tumor-associated macrophages (P = 0.0292) and ER, with a higher percentage of ER-negative breast cancer. Higher percentages of stromal CD4+ tumor-infiltrating lymphocytes (P = 0.0795) and other immune cells were discovered in ER-negative breast cancer; however, this was not statistically significant (**Figure 26**).

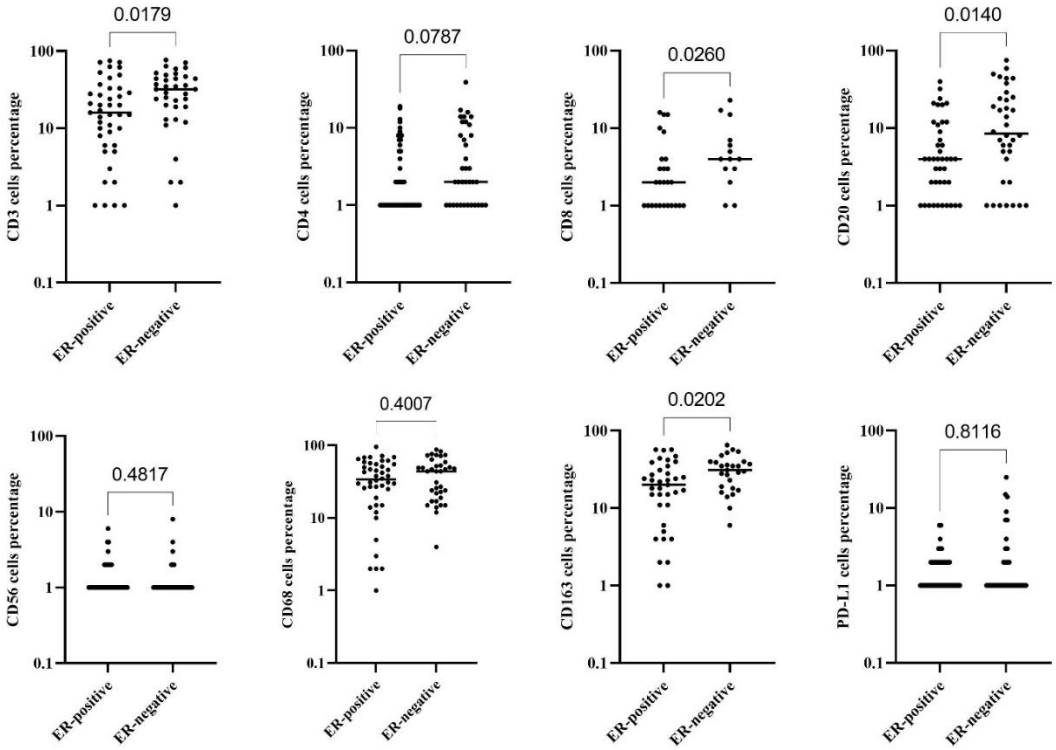


Figure 26. Stromal tumor-infiltrating lymphocytes, tumor-associated macrophages, PD-L1, and ER in breast cancer patients. The stromal percentage of CD3, CD4, CD8, CD20, CD 56, CD68, CD163, and PD-L1 expression among ER-positive and ER-negative breast cancer was log-transformed with median values indicated for each group by the horizontal lines.

4.3.6. Intratumoral tumor-infiltrating lymphocytes, tumor-associated macrophages, PD-L1, and grade

The was a significant association between intratumoral CD3+ tumor-infiltrating lymphocytes (P = 0.0222), CD163+ tumor-associated macrophages (P = 0.0138), PD-L1+ (P = 0.0280), and TNM tumor grade, with a high percentage among grade III breast cancer. A high percentage of intratumoral CD8+ tumor-infiltrating lymphocytes (P = 0.1375) and CD 20+ tumor-infiltrating lymphocytes (P = 0.1060) were also found among grade III breast cancer, but not statistically significant (**Figure 27**).

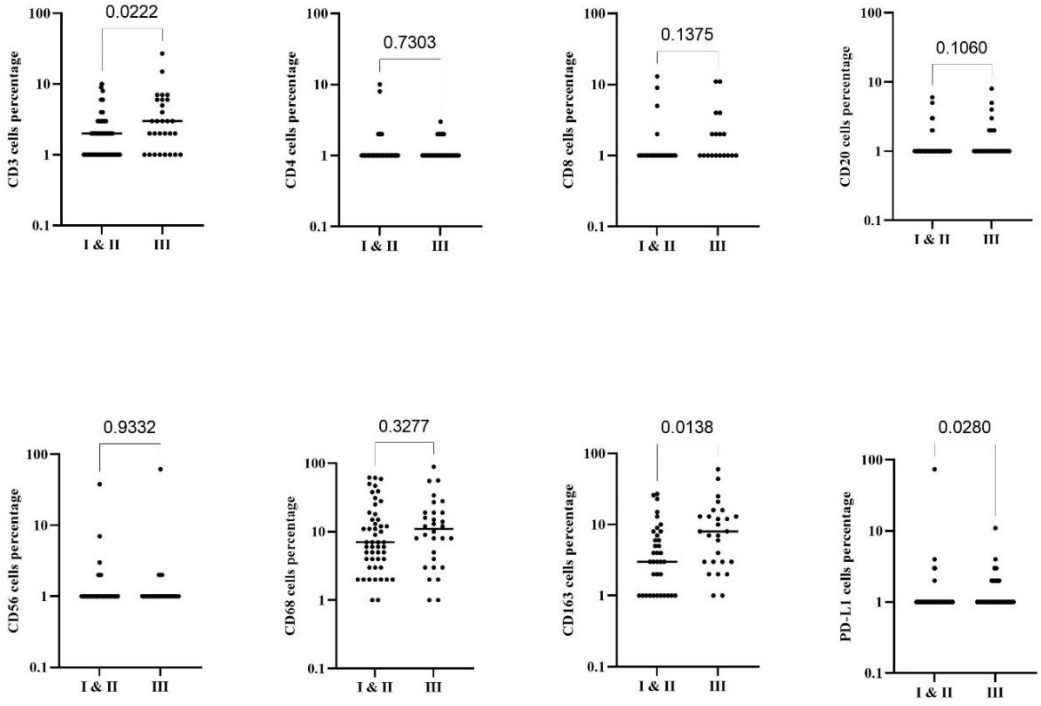


Figure 27. Intratumoral tumor-infiltrating lymphocytes, tumor-associated macrophages, PD-L1, and grade in breast cancer patients. The intratumoral percentage of CD3, CD4, CD8, CD20, CD 56, CD68, CD163, and PD-L1 expression among different tumor grades was log-transformed with median values indicated for each group by the horizontal lines.

4.3.7. Stromal tumor-infiltrating lymphocytes, tumor-associated macrophages, PD-L1, and grade

There was a significant association between stromal PD-L1+ (P = 0.0232) and TNM tumor grade, with a higher percentage among grade III breast cancer. The percentage of CD8+ tumor-infiltrating lymphocytes (P = 0.0891) and CD163+ tumor-associated macrophages (P = 0.1563) were higher among grade III breast cancer than grade I & II, but not statistically significant (**Figure 28**).

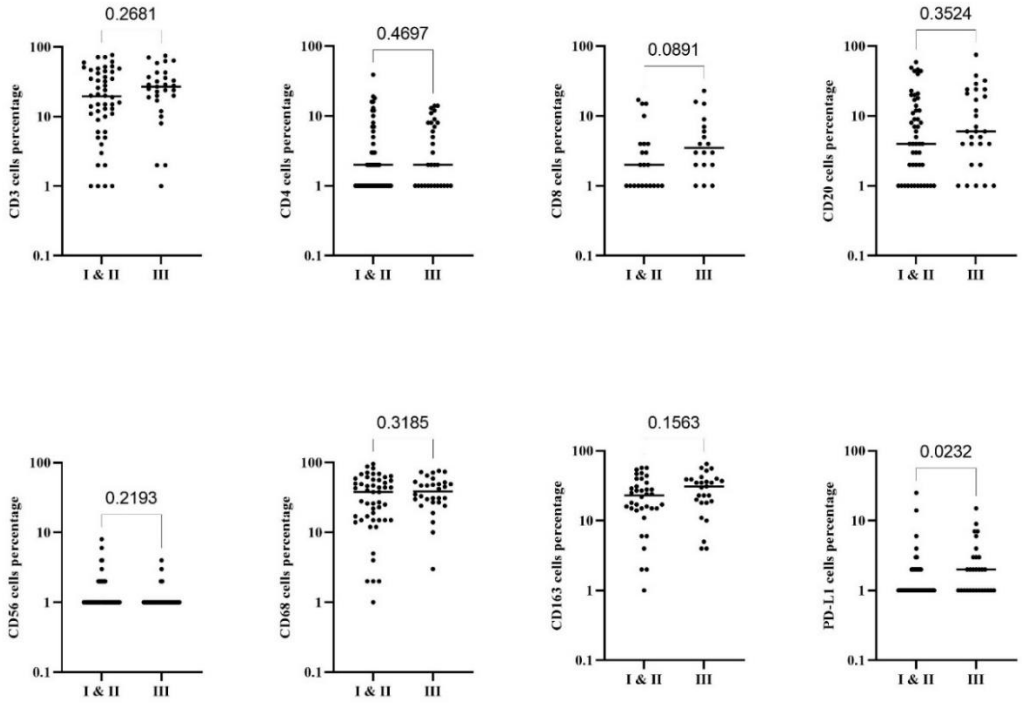


Figure 28. Stromal tumor-infiltrating lymphocytes, tumor-associated macrophages, PD-L1, and grade in breast cancer patients. The stromal percentage of CD3, CD4, CD8, CD20, CD 56, CD68, CD163, and PD-L1 expression among different tumor grades was log-transformed with median values indicated for each group by the horizontal lines.

4.3.8. Intratumoral tumor-infiltrating lymphocytes, tumor-associated macrophages, PD-L1, and Ki-67

A high percentage of intratumoral CD3+ (P = 0.1773), CD8+ (P = 0.0993), CD20+ (P = 0.0778), and CD56+ (P = 0.956) tumor-infiltrating lymphocytes were also found among high Ki-67 proliferation index of breast cancer, but not statistically significant (**Figure 29**).

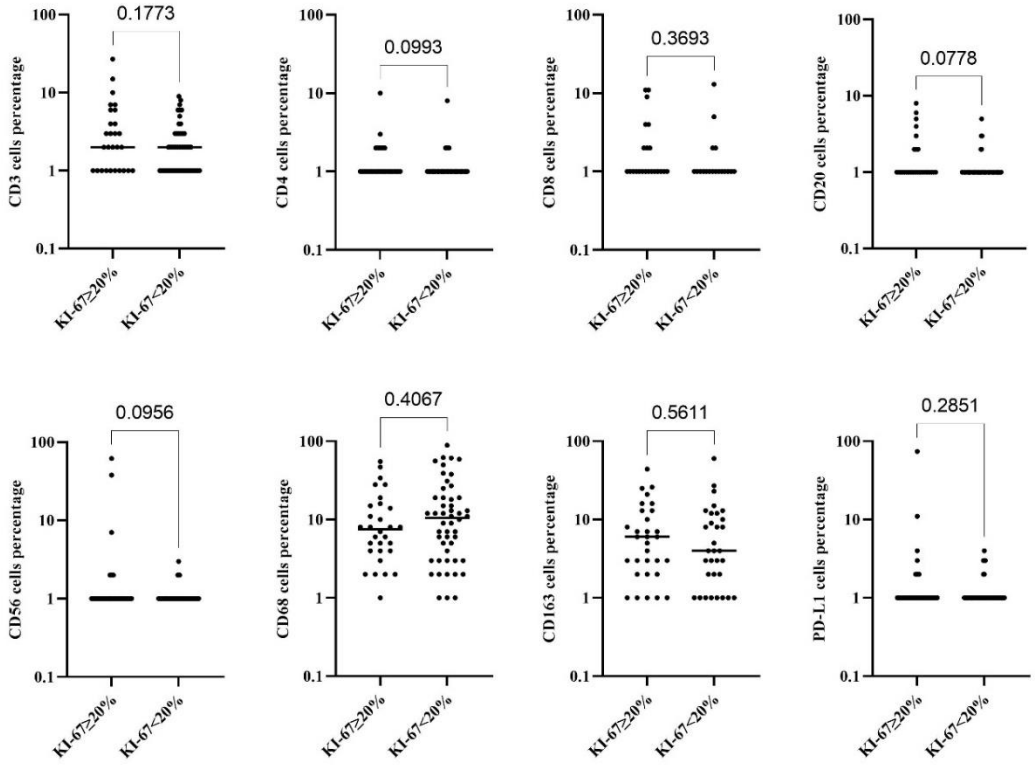


Figure 29. Intratumoral tumor-infiltrating lymphocytes, tumor-associated macrophages, PD-L1, and Ki-67 proliferation index in breast cancer patients. The intratumoral percentage of CD3, CD4, CD8, CD20, CD 56, CD68, CD163, and PD-L1 expression among high and low Ki-67 proliferation index was log-transformed with median values indicated for each group by the horizontal lines.

4.3.9. Stromal tumor-infiltrating lymphocytes and Ki-67

There was a significant association between stromal PD-L1+ (P = 0.0331) and the Ki-67 proliferation index, with a higher percentage among the high Ki-67 proliferation index of breast cancer. The percentage of CD8+ (P = 0.1033) and CD20+ (P = 0.1912) tumor-infiltrating lymphocytes were higher among high and low Ki-67 proliferation index of breast cancer, respectively, but not statistically significant (**Figure 30**).

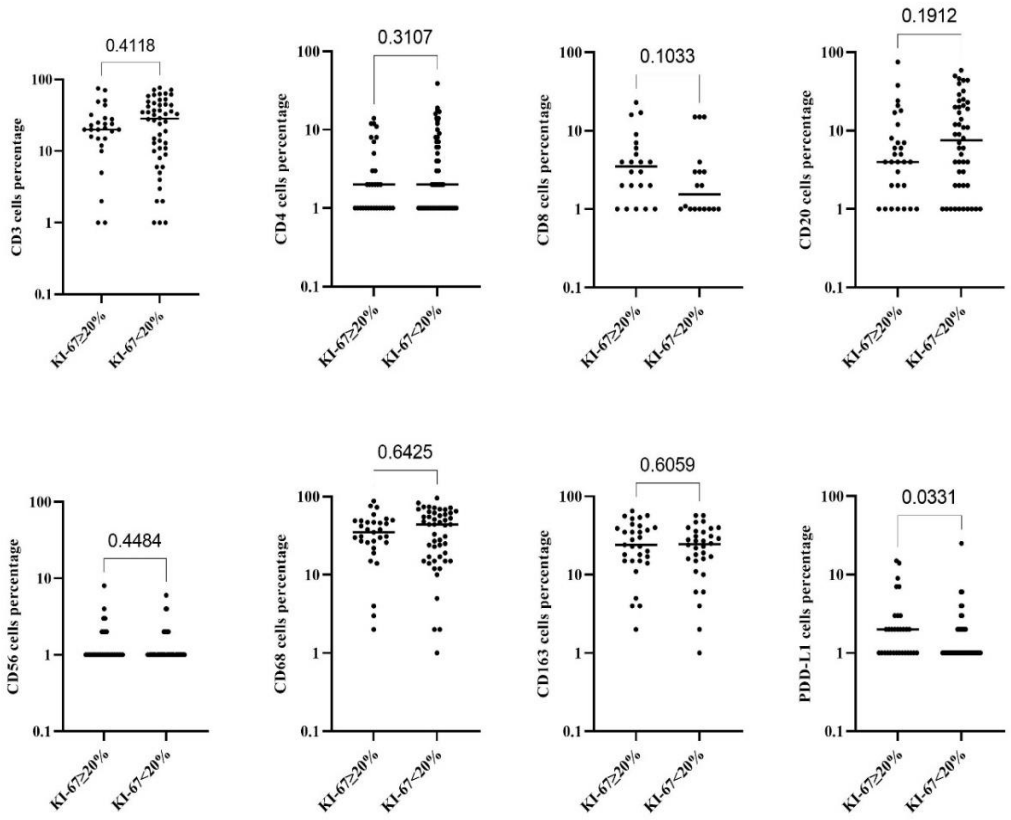
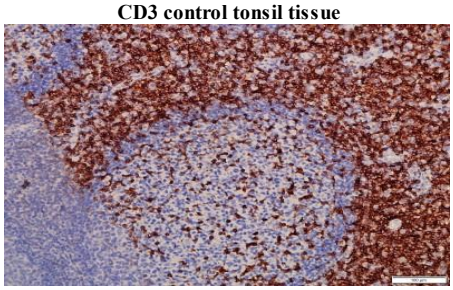
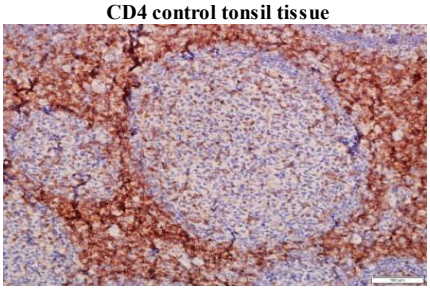


Figure 30. Stromal tumor-infiltrating lymphocytes, tumor-associated macrophages, PD-L1, and Ki-67 proliferation index in breast cancer patients. The stromal percentage of CD3, CD4, CD8, CD20, CD 56, CD68, CD163, and PD-L1 expression among high and low Ki-67 proliferation index was log-transformed with median values indicated for each group by the horizontal lines.

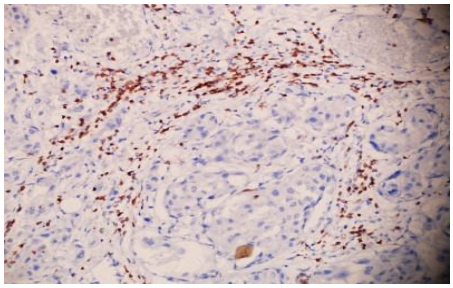
CD3 control tonsil tissue



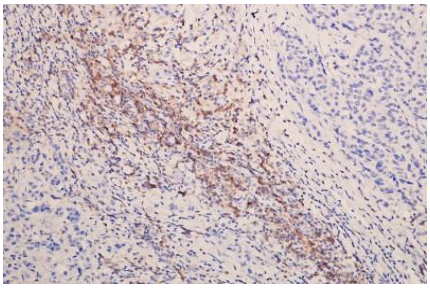
CD4 control tonsil tissue



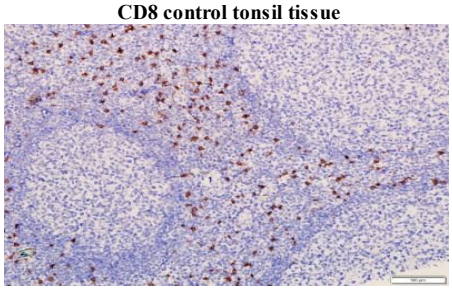
CD3-positive



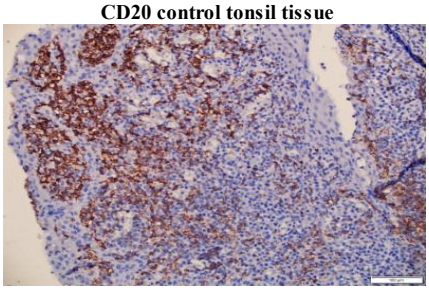
CD4-positive



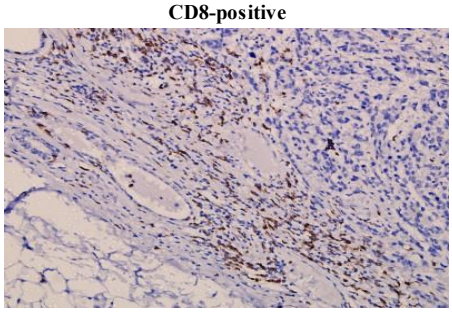
CD8 control tonsil tissue



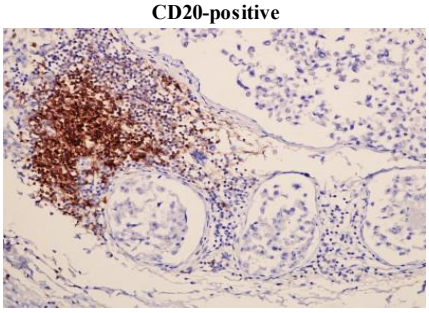
CD20 control tonsil tissue



CD8-positive



CD20-positive



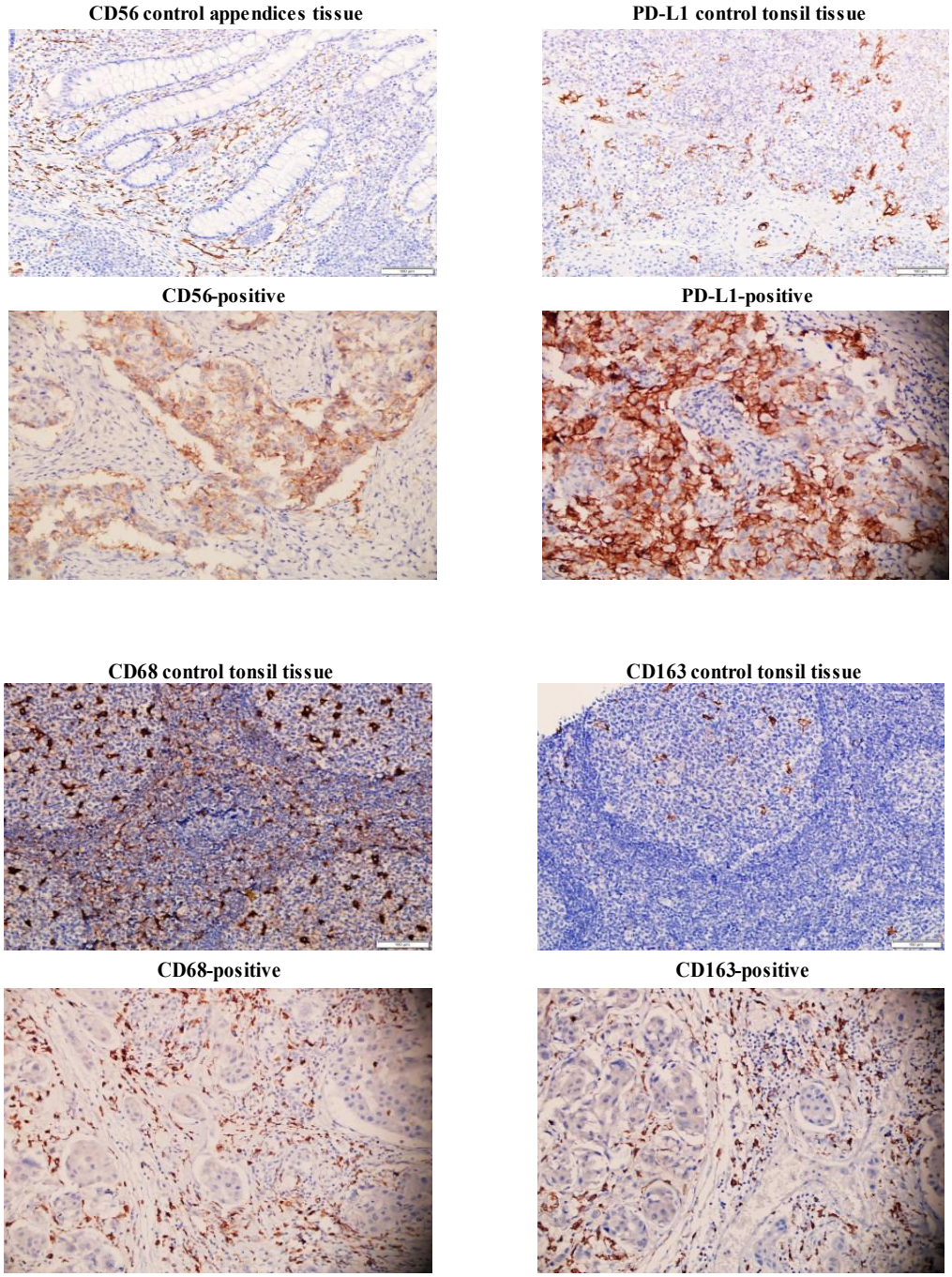


Figure 31. Representative IHC picture of CD3, CD4, CD8, CD20, CD56, PD-L1, CD68 and CD163 positive breast cancer and control tissues**.**

4.4. MMPs Expression

4.4.1. Demographic and clinical characteristics

A total of 58 study participants were involved in this study, of which 42 (72.4%) and 16 (27.6%) had breast cancer and benign breast tumors, respectively. The mean age at diagnosis was 36.6 (SD ± 13.5) years (**Table 13**). Grade III breast cancer accounted for 42.9% and the size of T3-T4 accounted for 45.2%. Lymph node positivity was seen in 66.6% of breast cancer cases. The most common histomorphological type was invasive carcinoma of no special type (85.7%). The ER and PR positivity was 59.5% and 50.0%, respectively. Human epidermal growth factor receptor-2 positivity was 19.0%. The most common immunohistochemistry-defined subtype was the luminal subtype (luminal A and B) which accounted for 47.6% (**Table 14**).

Table 13. Demographic characteristics of study participants with benign breast tumor and breast cancer.

Variables		**Frequency**	**Percent (%)**
Age group	15-29	16	28.6%
	30-44	26	46.4%
	45-59	9	16.1%
	≥60	5	8.9%
	Total	56	100.0%
	Missing	2	
	Mean ± Sd (Minimum, Maximum) = 36.6 ± 13.5(15,70)		

Table 14. Clinical characteristics of study participants with breast cancer. Differences of features among cases assessed by the Mann-Whitney test.

Variables		**Frequency (%)**	**MMP-2**	**MMP-9**	**MMP-11**
			P-value		
Grade	I-II	24(57.1)	0.8112	0.4423	0.4689
	III	18(42.9)			

	Total	42(100.0)			
Tumor Size	T1-T2	14(33.3)	0.4828	0.5773	0.5708
	T3_T4	19(45.2)			
	Not assessed	9(21.5)			
	Total	42(100.0)			
Lymph node	Positive	28(66.6)	0.5421	0.5656	0.1096
	Negative	9(21.5)			
	Not assessed	5(11.9)			
	Total	42(100.0)			
Histomorphological type	Ductal carcinoma	36(85.7)	0.6611	0.1112	0.0221
	Others	6(14.3)			
	Total	42(100.0)			
ER	Positive	25(59.5)	0.4164	0.1528	0.0514
	Negative	17(40.5)			
	Total	42(100.0)			
PR	Positive	21(50.0)	0.8813	0.7088	0.1123
	Negative	21(50.0)			
	Total	42(100.0)			
HER2	Positive	8(19.0)	0.5913	0.7935	0.6910
	Negative	26(62.0)			
	Equivocal	8(19.0)			
	Total	42(100.0)			
HER2 Score	IHC0	17(40.5)	0.2523	0.3413	0.2499
	IHC 1+ negative	9(21.5)			
	IHC 2+ equivocal	8(19.0)			
	IHC 3+ positive	8(19.0)			
	Total	42(100.0)			
Ki-67	Low	20(47.6)	0.1162	0.9505	0.6494
	High	22(52.4)			
	Total	42(100.0)			
IHC defined	Luminal A	9(21.5)	0.0706	0.7768	0.6292
	Luminal B	11(26.1)			

breast cancer subtypes	HER2	5(11.9)			
	Triple-negative breast cancer	9(21.5)			
	Not determine	8(19.0)			
	Total	42(100.0)			

4.4.2. Relative mRNA expressions of MMPs in breast cancer and benign breast tumor cases

The mRNA expression of MMP-11 was 5.1 times higher in breast cancer than in benign breast tumors cases and the difference was statistically significant (P = 0.012). Higher mRNA expression of MMP-9 was also seen in breast cancer (P = 0.105 (**Figure 32**). To confirm the presence of the desired PCR product, a conventional PCR was performed (**Figure 33**).

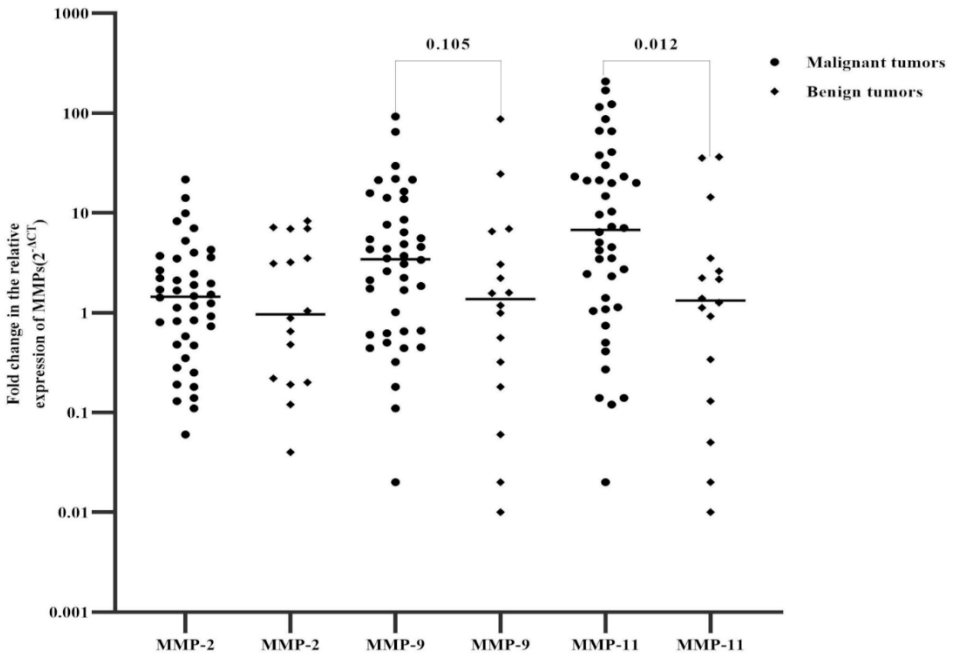


Figure 32. Expression of MMP-2, MMP-9, and MMP-11 in BREAST CANCER and benign breast tumor cases. Fold change in the relative levels of MMP-11 was log-transformed with median values indicated for each group by the horizontal lines.

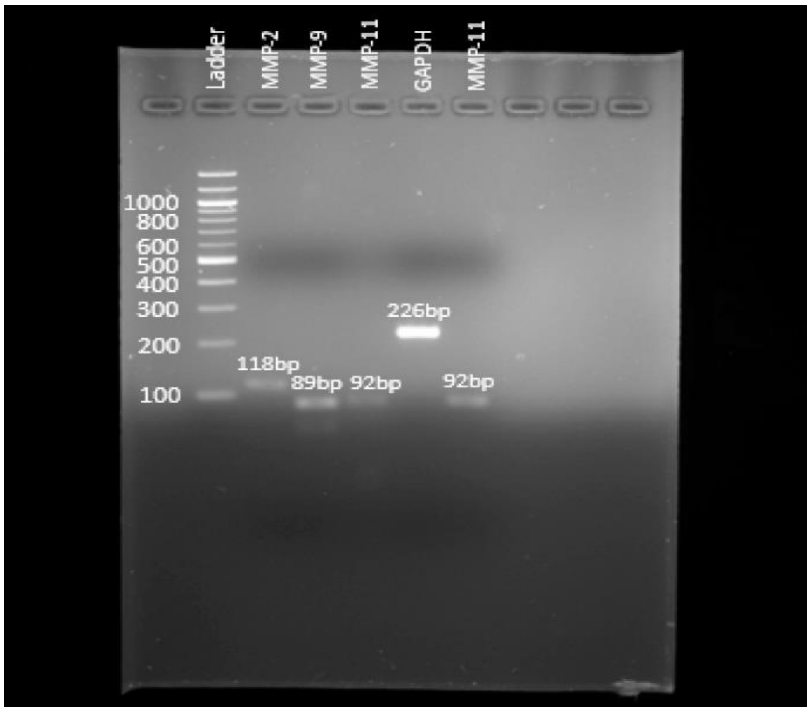


Figure 33. Representative PCR amplification result for MMP-2, MMP-9, MMP-11, and GAPDH.

4.4.3. Relative expression of MMP-11 mRNA on breast cancer grouped by Ki-67 expression, grade, and lymph node status

The expression of MMP-11 was 2.4 times higher in breast cancer cases with lymph node positivity than in cases with negative lymph nodes (P = 0.1096). The MMP-11 expression showed no statistically significant difference compared with grade I or II breast cancer cases (**Figure 34**).

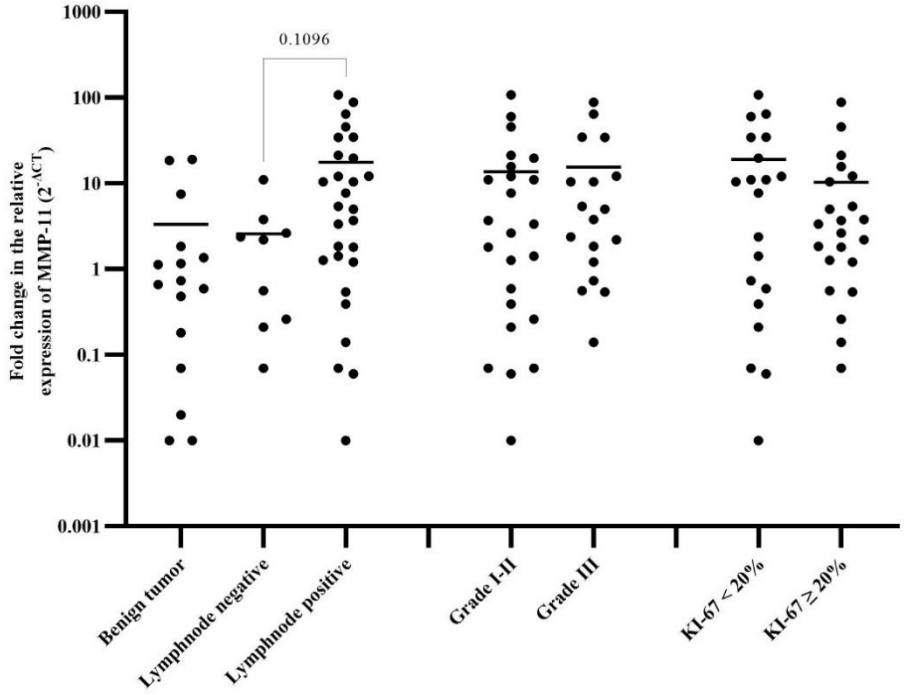


Figure 34. Expression of MMP-11 in cases of breast cancer categorized by Ki-67+ cell percentage, grade, and lymph node status. Log transformed values with median are denoted by horizontal lines.

4.4.4. MMP-11 relative mRNA expressions in groups of ER, PR, HER-2 status, and subtypes in breast cancer

The expression of MMP-11 was 5.7 times higher in ER-positive than ER-negative breast cancer cases (P = 0.0514). The MMP-11 expression was 2.4 times higher in HER-2-negative breast cancer cases than in HER-2-positive cases. Luminal A breast cancer subtypes had higher MMP-11 expression than benign breast tumors and other subtypes of breast cancer (**Figure 35**).

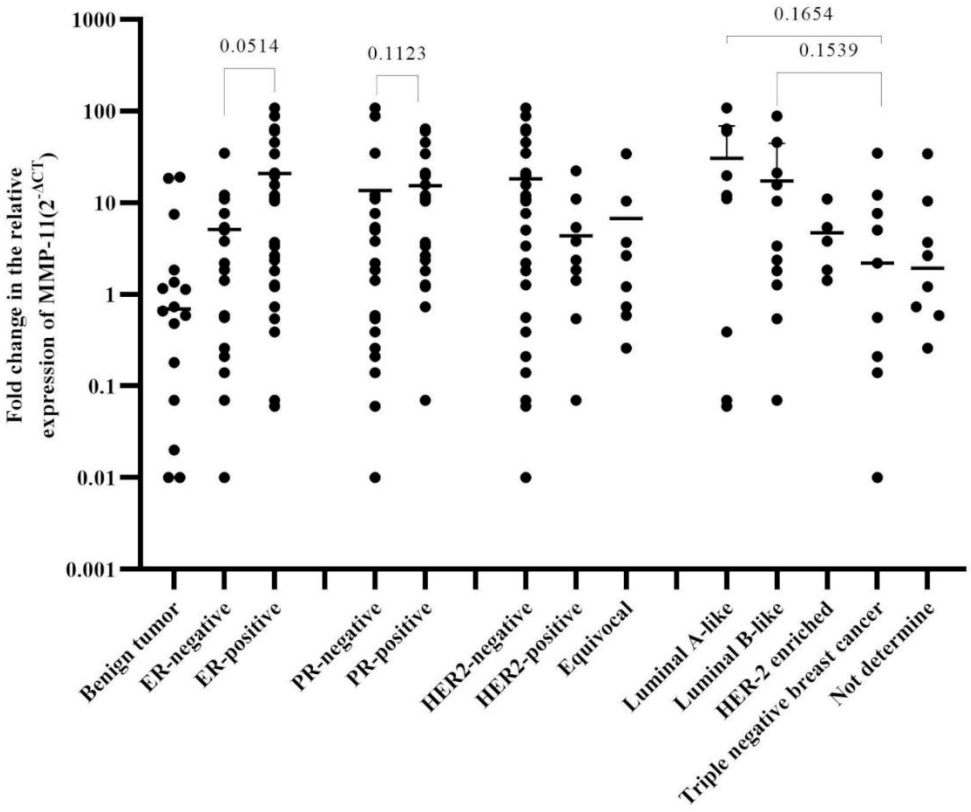


Figure 35. Expression of MMP-11 in cases of breast cancer categorized by ER, PR, HER-2 status, and IHC-defined breast cancer subtypes. Log transformed values with median are denoted by horizontal lines.

4.4.5. MMP-2 relative mRNA expressions of breast cancer grouped with Ki-67, grade, lymph node, ER, PR, HER-2 status, and subtypes

The breast cancer cases with lymph node-positive had MMP-2 expression levels that were 1.6 times higher than those with lymph node-negative breast cancer. The MMP-2 expression was 2 times higher in low Ki-67 proliferation cases than in high Ki-67 proliferation (**Figure 36**).

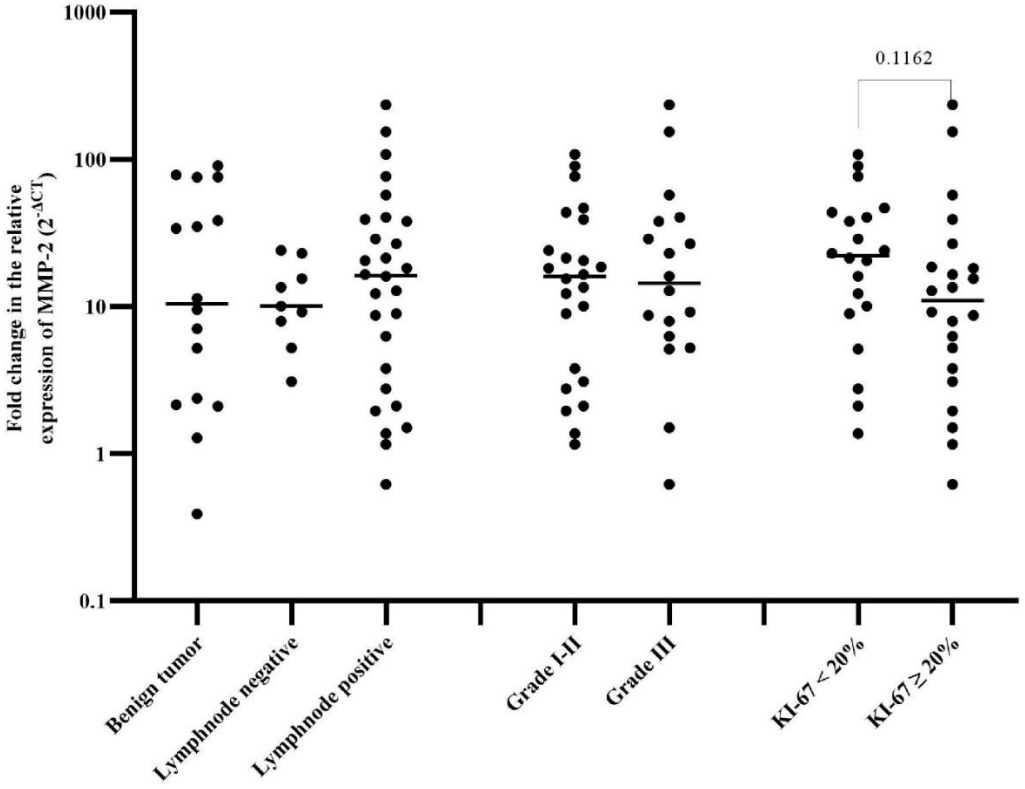


Figure 36. Expression of MMP-2 in cases of benign and malignant breast cancer categorized by Ki-67+ cell percentage, grade, and lymph node status. Log transformed values with median are denoted by horizontal lines

The MMP-2 expression was 1.3 times higher in HER-2-negative breast cancer patients compared to HER-2-positive breast cancer cases, but the difference was not statistically significant (**Figure 37**)

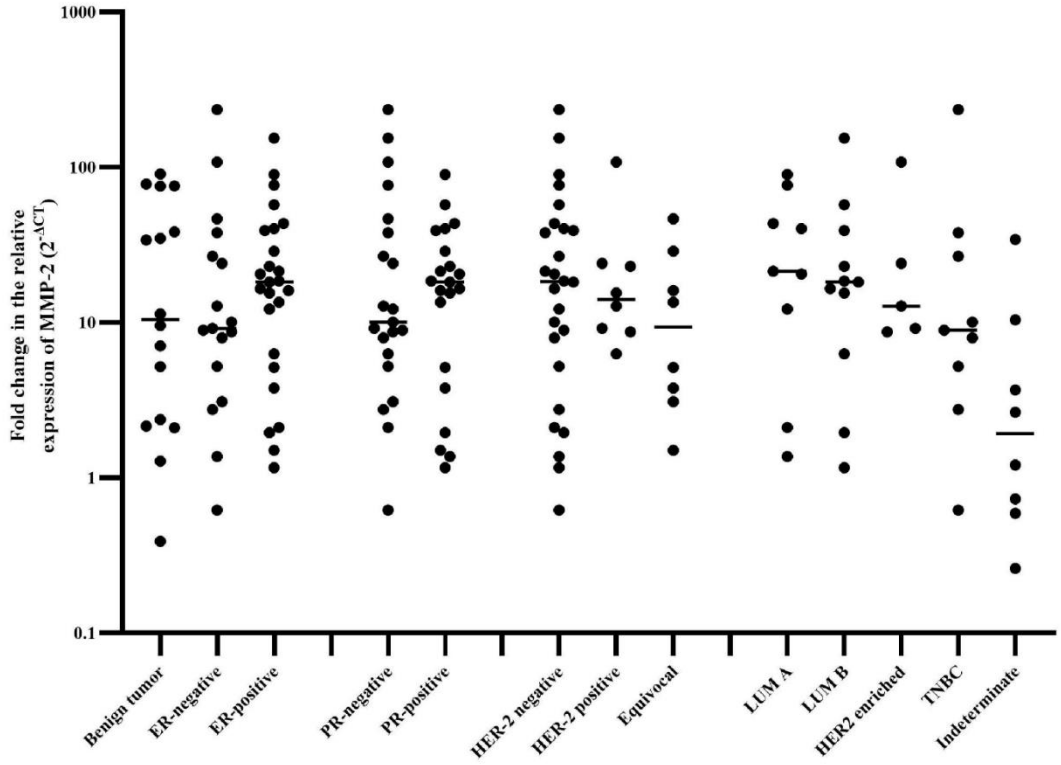


Figure 37. Expression of MMP-2 in cases of benign and malignant breast cancer categorized by ER, PR, HER-2 status, and IHC-defined breast cancer subtypes. Log transformed values with median are denoted by horizontal lines.

4.4.6. MMP'-9 relative mRNA expressions of breast cancer grouped with Ki-67, grade, lymph node, ER, PR, HER-2 status, and subtypes

The MMP-9 expression was higher in grade III breast cancer cases than in Grade I-II breast cancer cases, with a 1.9 times higher difference (**Figure 38**).

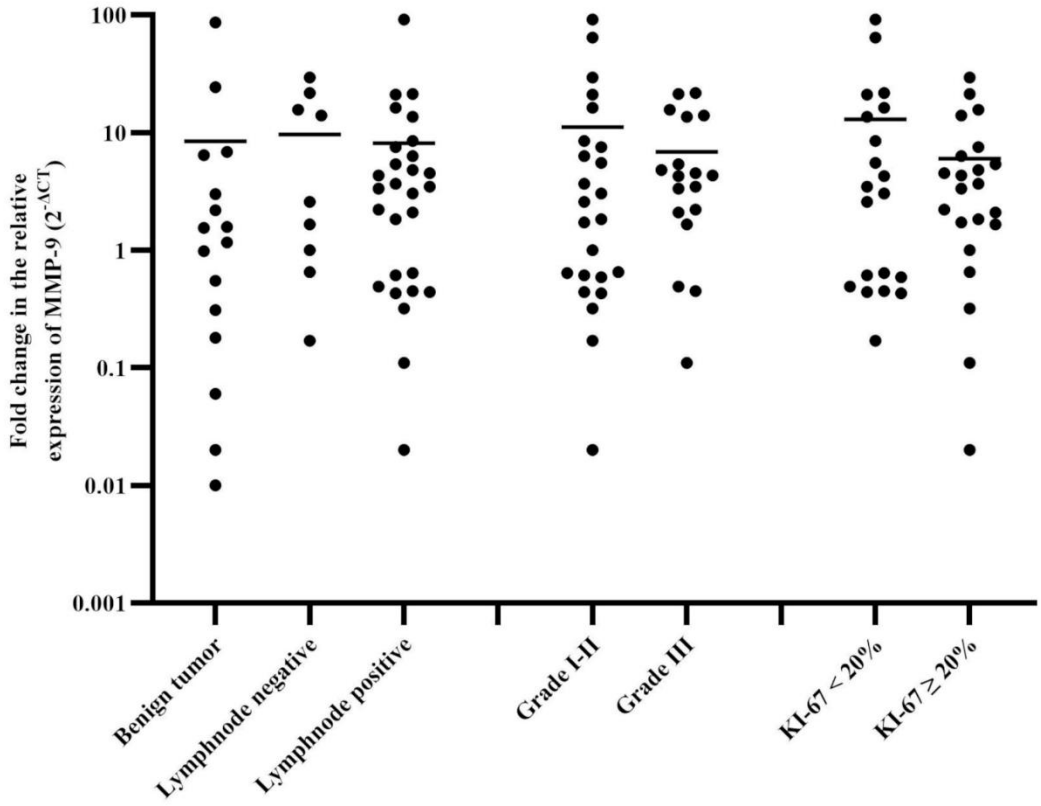


Figure 38. Expression of MMP-9 in cases of malignant breast cancer categorized by Ki-67+ cell percentage, grade, and lymph node status. Log transformed values with median are denoted by horizontal lines.

The ER-positive breast cancer cases had MMP-9 expression that was 2 times higher than ER-negative breast cancer cases. MMP-9 expression was higher in luminal A breast cancer subtypes compared to benign breast tumors and other subtypes (**Figure 39**).

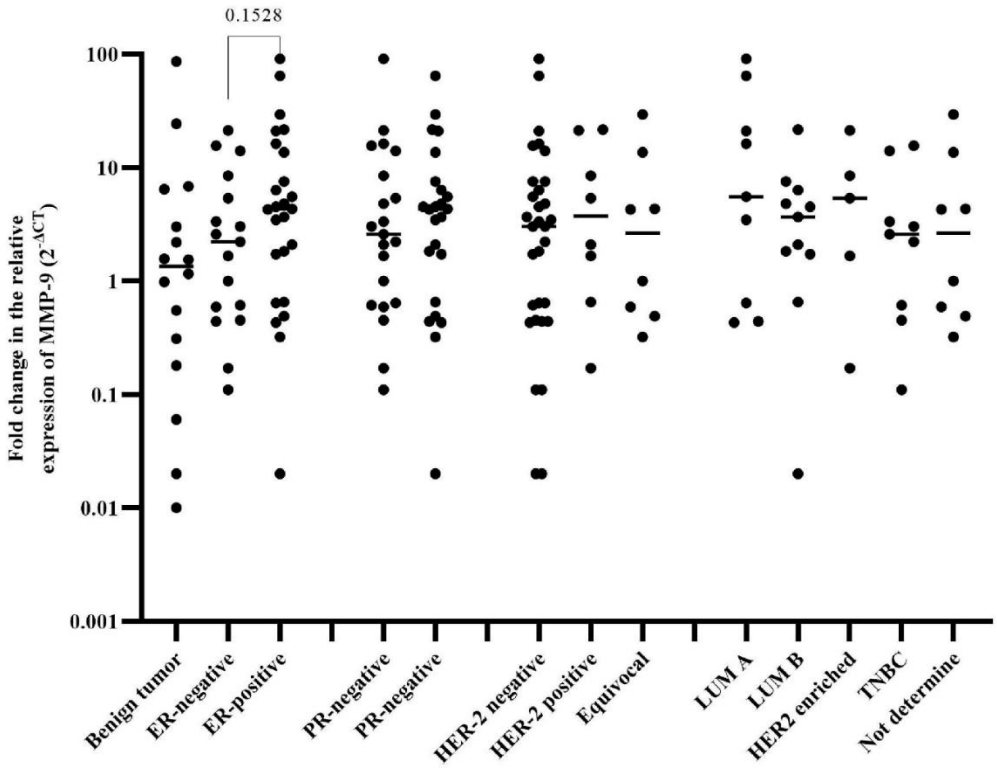


Figure 39. Expression of MMP-9 in cases of malignant breast cancer categorized by ER, PR, HER-2 status, and IHC-defined breast cancer subtypes. Log transformed values with median are denoted by horizontal lines.

4.5. HPV Detection and Genotyping

4.5.1. Magnitude of HPV infection and genotypes

A total of 120 study participants were enrolled in this study, of which 30 (25.0%) were positive for HPV infection. The magnitude of HPV among breast cancer cases and non-malignant breast lesions were 20.6% and 29.6%, respectively. The high-risk HPV 16 genotype was dominant with 85.6% and 68.7% accounting for breast cancer cases and benign tumors, respectively (**Table 15**).

Table 15. The magnitude of HPV infection and genotypes among breast cancer and non-malignant breast cases.

Characteristics		**Case**	**Control**	**Total**
HPV infection				
	HPV positive	14(20.6%)	16(29.6%)	30(25.0%)
	HPV negative	52(76.4%)	38(70.4%)	90(75.0%)
	Total	66(100%)	54(100%)	120(100%)
HPV genotype	HPV 16	12(85.6%)	11(68.7%)	23(76.6%)
	HPV 44	1(7.2%)	0(0%)	1(3.3%)
	HPV 59	1(7.2%	0(0%)	1(3.3%)
	HPV 6	0(0%)	1(6.3%)	1(3.3%)
	HPV 16 and 42	0(0%)	1(6.3%)	1(3.3%)
	HPV 31	0(0%)	1(6.3%)	1(3.3%)
	HPV 31 and 45	0(0%)	1(6.3%)	1(3.3%)
	HPV 42	0(0%)	1(6.3%)	1(3.3%)
	Total	14(100%)	16(100%)	30(100%)

4.5.2. Associated factors for HPV infection

Estrogen receptor-positive breast cancer had a significantly higher proportion of HPV infection than estrogen receptor-negative breast cancer (P = 0.022. The luminal (luminal A and Luminal B) IHC subtype has also a significantly higher proportion of HPV than other subtypes (P = 0.018). The percentage of HPV was also higher among HER-2 grades 0 than in other grades; however, this was not statistically significant. The low Ki-67 proliferation index has a higher HPV percentage than the high Ki-67 proliferation index, but this only reached borderline significance (P = 0.056). There was no significant association between age, tumor size, lymph node involvement, and HPV infection (**Table 16**).

Table 16. Associated factors for HPV infection among breast cancer and non-malignant breast cases.

		HPV infection		**Total**	**P-value**
		Positive	Negative		
Age	< 50 years	27(90.0%)	75(83.3%)	102(85.0%)	0.376
	≥ 50 years	3(10.0%)	15(16.7%)	18(15.0%)	
	Total	30(100%)	90(100%)	120(100%)	
Study group	Case	14(46.7%)	52(57.8%)	66(55.0%)	0.289
	Control	16(53.3%)	38(42.2%)	54(45.0%)	
	Total	30(100%)	90(100%)	120(100%)	
Laterality	Right	14(46.7%)	46(51.1%)	60(50.0%)	0.673
	Left	16(53.3%)	44(48.9%)	60(50.0%)	
	Total	30(100%)	90(100.0%)	120(100%)	
Tumor Size	T1 & T2	3(30.0%)	19(44.2%)	22(41.5%)	0.412
	T3 & T4	5(70.0%)	24(55.8%)	31(58.5%)	
	Total	10(100%)	43(100%)	53(100%)	
Lymph node involvement	Yes	7(50.0%)	26(55.3%)	33(54.1%)	0.726
	No	7(50.0%)	21(44.7%)	28(45.9%)	
	Total	14(100%)	47(45.9%)	61(100%)	
Grade	I	5(35.7%)	22(42.3%)	27(40.9%)	0.824
	II	4(28.6%)	11(21.2%)	15(22.7%)	
	III	5(35.7%)	19(36.5%)	24(36.4%)	
	Total	14(100%)	52(100%)	66(100%)	
ER	Positive	12(85.7%)	27(51.9%)	39(59.1%)	0.022
	Negative	2(14.3)	25(48.2%)	27(40.9%)	
	Total	14(100%)	52(100%)	66(100%)	
PR	Positive	7(50.0%)	24(46.2%)	31(47.0%)	0.798
	Negative	7(50.0%)	28(53.8%)	35(53.0%)	
	Total	14(100%)	52(100%)	66(100%)	
HER-2	Positive	2(16.7%)	14(30.4%)	16(27.6%)	0.342

	Negative	10(83.3%)	32(69.6%)	42(72.4%)	
	Total	12(100%)	46(100%)	58(100%)	
HER-2 Grade	IHC 0	9(64.3%)	23(44.2%)	32(48.5%)	0.400
	IHC1	2(14.3%)	9(17.3%)	11(16.7%)	
	IHC 2	2(14.3%)	6(11.5%)	8(12.1%)	
	IHC3	1(7.1%)	14(26.9%)	15(22.7%)	
	Total	14(100%)	52(100%)	66(100%)	
Ki-67	Low proliferation	11(78.6%)	26(50.0%)	37(56.1%)	0.056
	High proliferation	3(21.4%)	26(50.0%)	29(43.9%)	
	Total	14(100%)	52(100%)	66(100%)	
Subtype	Luminal	11(91.7%)	25(55.6%)	36(62.1%)	0.018
	Non-luminal	1(8.3%)	21(44.4%)	22(37.9%)	
	Total	12(21.2)	45(100%)	58(100%)	

Differences of features among HPV infection assessed by X^2 test.

In multivariate analysis, a total of 58 breast cancer cases were analyzed, and the ER-positive breast cancer cases were almost 10 times more likely to have HPV than ER-negative breast cancer cases, and this retained statistical significance (P = 0.043). Cases with high Ki-67 proliferation index were not statistically significantly different in the adjusted multivariate model (**Table 17**).

Table 17. Multivariate logistic regression of ER, Ki-67 proliferation index, and HER-2 status, taken as predictive variables for HPV positive than HPV negative.

Parameters		**HPV positive**	**HPV negative**	**Total**	**COR (95% CI)**	**p-value**	**AOR (95% CI)**	**p-value**
ER	Positive	12(85.7%)	27(51.9%)	39(59.1%)	5.56(1.13-27.32)	0.035	9.61(1.07–86.32)	0.043
	Negative Ref.	2(14.3)	25(48.2%)	27(40.9%)				
HER-2	Positive Ref.	2(16.7%)	14(30.4%)	16(27.6%)	2.19(0.42-11.31)	0.350	1.01(0.16–6.46)	0.989

	Negative	10(83.3%)	32(69.6%)	42(72.4%)				
Ki-67	Low proliferation	11(78.6%)	26(50.0%)	37(56.1%)	3.67(0.92-14.69)	0.066	2.29(0.49–10.69)	0.291
	High proliferation Ref.	3(21.4%)	26(50.0%)	29(43.9%)				

5. CHAPTER FIVE: DISCUSSION

5.1. Retrospective Clinicopathological Features Study

This study found that breast cancer is more common in younger age groups, with invasive carcinoma of no special type, a high incidence of lymph node involvement, advanced pathological stage, and grade. The mean age for breast cancer (42,3 years) in this study was comparable with previous studies conducted in Ethiopia, which indicated a mean age range of 43-45 years (Kantelhardt et al., 2014a, Ersumo, 2006, Gemta et al., 2019). Reports from other African countries indicated a low mean age range of 47 to 52 years in West Africa (Menkiti et al., 2021, Ezike et al., 2021, Adani-Ifè et al., 2020), 41 to 53 years in East Africa (Gnanamuttupulle et al., 2021, Ntirenganya et al., 2021), 50 to 56 years in South Africa (Fourati et al., 2014, Kakudji et al., 2021), and 46-51 years in North Africa (Gusbi et al., 2020, Salhia et al., 2011). In contrast to these studies, the mean age of breast cancer patients at diagnosis was reported as 57 years in Germany (Arndt et al., 2002) and 62 years in the United Kingdom (Alwan et al., 2018), indicating a higher age range than the current report. Hereditary predisposition and low life expectancy will probably enhance the occurrence of breast cancer at an early age in this study.

Most of the breast cancer patients (64.9%) were diagnosed with TNM stage III breast cancer in this study. This finding is similar to other studies conducted in Ethiopia, Pakistan, and Haiti that reported 56.7%, 57.4%, and 55.5% stage III breast cancer, respectively (Hadi and Jamal, 2016, DeGennaro Jr et al., 2018, Gemta et al., 2019). In contrast, early-stage presentation is more commonly reported in Europe and India (Abdulrahman and Rahman, 2012, Takalkar et al., 2016). Previous studies from Ethiopia also revealed that only a small proportion of the women were found to perform breast self-examination and clinical examination (Legesse and Gedif, 2014, Black and Richmond, 2019), which could be the possible reason for having advanced-stage breast cancer in this study. In addition, lack of awareness and screening could be also a plausible reason based on observation during the current study.

Most (49.6%) patients had moderately differentiated (grade II) breast cancer. This finding was in line with earlier studies carried out in African countries, where grade II tumors were reported to account for 52.6% of cases in Nigeria (Adeniji et al., 2020), 51.3% in the Republic of the Congo (Malanda et al., 2021)), and 53.8% in South Africa (Toma et al., 2021). Conversely, poorly differentiated (grade III) breast cancer was common in another study conducted in Ghana (49%) (Titloye et al., 2016). Grade III is the most common grade in both African and African-American populations, but European descent has lower grades, which may be related to racial disparities, lower socioeconomic status, environmental or lifestyle factors, and late diagnosis in the African population (Amend et al., 2006).

This study found that most breast cancer patients (60.5%) had lymph node involvement at the time of diagnosis. In contrast to this study, 41.2% of lymph node involvement was seen in Mexico (Maffuz-Aziz et al., 2017), probably due to early diagnosis in this country than in Ethiopia. In this study, lymph node involvement showed the highest frequency among invasive carcinoma of no special type (78.9%). Involvement of lymph nodes was also significantly associated with tumor size ($\chi 2 = 8.55$, $P = 0.033$), and the highest frequency of lymph node involvement was seen in tumors with size greater than 5 cm (46.6%). The exact estimation of the size of the tumor is necessary before surgery to make the best decision for the management of patients (Orang et al., 2013). Lymph node involvement was also significantly associated with the type of surgery ($\chi 2 = 39.69$, $p < 0.001$), with the highest frequency among mastectomy types of surgery (95.9%).

Globally, more than 75 % of breast carcinoma is histologically invasive carcinoma of no special type (Society, 2019). Similarly, invasive carcinoma of no special type was the most common (77.1%) histologic type of breast cancer in this study.

Tumor size greater than 5cm (pT3) was the most common (41.2%) at the time of diagnosis in this study. The five-year mortality rate among breast cancer patients with a tumor size greater than 5 cm is a 48% increase compared to those having a tumor size less than 5 cm in a study in Ghana (Mensah et al., 2016), indicating the predictive importance of tumor size.

Mastectomy was the most performed surgery, accounting for 84.5% of breast cancer patients in this study. In contrast, lumpectomy has been the most common (69%) in Europe. This could be due to the early detection/presentation of patients with smaller tumor sizes, the practice of breast-conserving surgeries, and/or better diagnostic techniques. The mastectomy rate in Africa is more than 85%, compared to 30% in Europe (Abdulrahman and Rahman, 2012).

5.2. Breast Cancer Subtypes

Immunohistochemical markers are frequently used to guide treatment choices, classify breast cancer into biologically distinct subtypes, and serve as prognostic and predictive markers (Zaha, 2014). The IHC staining procedures to determine therapeutic biomarkers status have recently been introduced into clinical practice in Ethiopia but are still not available in all regions of the country. We chose these study sites because genetic research, despite its lack of specificity, has demonstrated that Ethiopian genetic diversity reflects linguistic stratification and diverse influences on the Ethiopian gene pool (Pagani et al., 2012). Our research was conducted in regional areas of Ethiopia with limited oncology care. This study found a high proportion of breast cancers with advanced clinical and pathologic characteristics, such as a high prevalence of lymph node involvement, large tumor size, and high histological grade. The percentage of ER- and PR-negative results reported in this study was higher than in earlier Ethiopian studies (Hadgu et al., 2018, Kantelhardt et al., 2014b, Reibold et al., 2021). The triple-negative breast cancer was seen to be more frequent in southwest Ethiopia (Jimma) and north Ethiopia (Mekele). Study sites showed the different composition of age groups, tumor size, histological grade, and Ki-67 proliferation.

In this study, the mean age for breast cancer patients at diagnosis was 43.9 years. Most patients were premenopausal (younger than 50 years old), with the highest frequency (87.0%) in Hawassa. Common young age is comparable to other earlier studies carried out in Ethiopia, which reported patients with mean ages of 43 to 47 years (Shenkutie et al., 2017, Reibold et al., 2021, Hadgu et al., 2018) and other African studies reported mean

ages less than 50 years of age (Malanda et al., 2021, Rweyemamu et al., 2021, Reibold et al., 2021). According to a comparative study, Sudanese patients were 10 years younger than those from Germany and Italy (Sengal et al., 2018, Awadelkarim et al., 2008), and patients in Nigeria were 21 years younger than those in the UK (Gukas et al., 2006). This could be due to the young population structure in Ethiopia and Africa, with a predominance of people below the age of 60 years.

Although male breast cancer accounts for fewer than 1% of all cases of breast cancer (Copur et al., 2021), this study revealed 4.8% of male cases. Over the previous three decades, there has been some improvement in male breast cancer survival rates. According to research assessing men diagnosed with breast cancer between 1988 and 2017, overall survival rose from 64.61% in 1988–1997 to 69.05% in 2008–2017 at five years (Leone et al., 2023). Male breast cancer patients have a lower overall survival rate than female patients. Finding new therapeutic targets requires a better understanding of the transcriptional and epigenetic landscape to address the heterogeneity of tumors (Wiśniewska et al., 2023).

Histological grades II and III were found in most patients in the current study, with the highest proportion in northern Ethiopia (Mekele). A considerable percentage of cases (53.8%) had tumor sizes that were T3 or T4, with southwestern Ethiopia (Jimma) reporting the largest number of cases (75.6%). In this study, lymph nodes were involved in 63.7% of breast cancer patients, with northern Ethiopia (Mekele) having the highest frequency (75.8%). The histological grade is now taken into account when selecting the therapy strategy (Weigelt et al., 2010). Compared with European cohorts, grade I tumors were most common (Spitale et al., 2009, Luyeye Mvila et al., 2015). Lack of knowledge and awareness of early detection, a poor perception of breast cancer, lack of financial and social support, absence of adequate population screening, poor support system, and sociocultural factors including tradition, belief, and fear all contribute to the severity of breast cancer in Africa (Akuoko et al., 2017, Getachew et al., 2020). According to a study done in Ethiopia, women hide tumors from their families because a mastectomy is related to a perception of premature death, infertility, and divorce (Getachew et al., 2020). In the present study, a

high proportion of patients under the age of 50 years, a high degree of lymph node involvement, and a high degree of Ki-67 proliferation all suggest that appropriate chemotherapy should be initiated in these settings with limited resources. These tumor features may increase cancer mortality, demanding a comprehensive approach that includes raising cancer awareness, upgrading cancer infrastructure, and providing prompt treatment.

Breast cancer histomorphological characteristics have been well-documented as a significant prognostic factor. By far, the most common is invasive carcinoma of no special type. The other forms of breast cancer have slightly better outcomes (Makki, 2015, do Nascimento and Otoni, 2020, Nagao et al., 2012). The most prevalent histomorphologic type of breast cancer in the current study is invasive carcinoma of no special type, accounting for 84% of the cases. A similar finding has been reported in other countries (Alsughayer et al., 2022, Atta Manu et al., 2020, Elfagieh, 2020).

Molecular subgroups were also significant predictors of breast cancer mortality (Haque et al., 2012). Poorer outcomes have been linked to the triple-negative and HER-2 subtypes (Su et al., 2011). Triple-negative breast cancer has a poor prognosis, high levels of invasiveness, and metastatic potential. Additionally, they are resistant to endocrine- and HER-2-targeted therapies (Yin et al., 2020). A higher percentage of triple-negative breast cancer subtypes (33.1%) was reported in this study, which is higher than 23% (Hadgu et al., 2018, Desalegn et al., 2022) and 24.8% (Eber-Schulz et al., 2018) reported in earlier Ethiopian studies from the capital city. Triple-negative breast cancer subtypes were found on average in 26.4% of patients from African countries, with 22.8% in East Africa, 14.9% in Middle Africa, 22.6% in North Africa, and 16.6% in South Africa. However, west Africa had a substantially higher rate, accounting for 45.7% (Hercules et al., 2022). Comparative research showed Sudan had a triple-negative breast cancer rate of 34.5%, while Germany had a rate of 14.2% (Sengal et al., 2018). Compared to populations of European heritage, populations of African descent had the greatest reported prevalence of triple-negative breast cancer (Jiagge et al., 2018). One important factor is the higher prevalence of triple-negative breast cancers in younger age groups. Additionally, this could be explained by hereditary factors, such as the founder's BRCA gene mutation (Siddharth and Sharma,

2018, Hayat et al., 2021), not been reported from Ethiopia yet. Another study has also revealed the connection between African ancestry and the immunologic profile of triple-negative breast cancer (Martini et al., 2022).

Luminal A subtypes have the best prognosis, and the most effective therapy for this subtype is tamoxifen or aromatase inhibitors (Orrantia-Borunda et al., 2022). Luminal B subtypes are more severe and have a worse prognosis than Luminal A subtypes (Orrantia-Borunda et al., 2022). In the present study, the percentage of luminal B breast cancer was 27.6%, which is comparable with a prior study in Ethiopia, where it was 26% (Hadgu et al., 2018). In this study, the percentage of luminal A subtype was 25.2%. An earlier study conducted in Ethiopia found a higher proportion of luminal A at 40% (Hadgu et al., 2018). The comparative study conducted between Africa and Europe also showed a higher luminal A subtype in Leuven than in Kinshasa with 64.5% and 40.2%, respectively (Luyeye Mvila et al., 2015). Another study also reported a higher percentage of luminal A in Germany than in Sudan with 68.4% and 36.9%, respectively (Sengal et al., 2018). This is probably due to the lack of the older age group who have a high proportion of luminal A subtypes in the African setting.

The HER-2-enriched breast cancer subtype is more aggressive and has a worse prognosis than luminal subtypes (Orrantia-Borunda et al., 2022), especially before the availability of modern HER-2 neu-directed therapies. The development of anti-HER-2-targeted drugs has significantly increased patient survival rates for this subtype (Dieci et al., 2020). The current study found 14.1% of breast cancers to be HER-2-enriched subtypes, which is greater than the 10% (Hadgu et al., 2018) and 9.5% (Eber-Schulz et al., 2018) found in an earlier study in Ethiopia. A comparative study revealed that the HER-2-enriched subtype is higher in Sudan (15.7%) than in Germany (6.8%) (Sengal et al., 2018). This study found 11% of cases that were HER-2 2+ or equivocal, a substantial proportion. We did not perform fluorescent in-situ hybridization (FISH) for equivocal cases; however, we recommend that FISH should be performed in a future investigation to determine the precise number of HER2-enriched breast cancer subtypes. This study provides important data that can be used to advocate for the appropriate allocation of resources to support

developing pathology capacity. This is particularly timely, as the patents of the technology backbone for HER2-directed therapies have expired and global access to HER2 neu-directed therapies is expected to increase.

Endocrine therapy is a significant part of treatment for breast cancers that are ER-positive (Nicholson and Johnston, 2005). Tamoxifen and an aromatase inhibitor should be a regular component of endocrine therapy for the majority of premenopausal and postmenopausal women with receptor-positive breast cancer, respectively (Colleoni and Giobbie-Hurder, 2010). The 15-year mortality rates of breast cancer were reduced by around 30% and 40% by tamoxifen and aromatase inhibitors in adjuvant settings, respectively (Krauss and Stickeler, 2020). ER-positivity is found in 51.7% of the patients in the current study. A higher percentage of ER-positivity, with values of 65.5% (Shenkutie et al., 2017), 73% (Reibold et al., 2021), 65% (Hadgu et al., 2018), and 65.3% (Kantelhardt et al., 2014b), were observed in prior Ethiopian studies. In a systematic review from sub-Saharan Africa, 42% of breast cancer cases were ER-positive, with 35.0% in West Africa (Eng et al., 2014). Higher rates of ER-positive breast cancer were reported in other nations 77.8% - 87.9% (Acs et al., 2021, Johansson et al., 2021, Khabaz, 2014). There was a significant correlation between histological grade and ER status, with a higher histological grade more likely to be ER-negative, in this study and confirmed by other studies (Sofi et al., 2012). Based on our findings, receptor testing availability should be a priority to offer the best treatment for breast cancer patients.

5.3. Tumor-infiltrating Lymphocytes, Tumor-associated Macrophages, and PD-L1 Expression

The presence of a high percentage of tumor-infiltrating lymphocytes in breast cancer predicts a better prognosis, particularly in the HER-2-enriched and triple-negative breast cancer subtypes (He et al., 2020). The high percentage of tumor-infiltrating lymphocytes reduced the chance of recurrence of breast cancer (Rathore et al., 2014, Kuroda et al., 2021). Tumor-associated macrophages will be a potential target for breast cancer therapy and are involved in angiogenesis, migration, metastasis, and immune evasion in the

development of breast cancer (Huang et al., 2022, Williams et al., 2016, Qiu et al., 2018). A high level of tumor-associated macrophages is associated with poor prognosis (Allison et al., 2023). PD-L1 is an effective immunotherapeutic target for patients with metastatic triple-negative breast cancer (Alkaabi et al., 2023, Zhang et al., 2023). Currently, Pembrolizumab and atezolizumab, PD-L1 inhibitors, have been used in combination with chemotherapy to treat PD-L1-positive metastatic triple-negative breast cancer (Emens and Loi, 2023). In this study, the non-luminal breast cancer subtype had a higher percentage of stromal CD20+ tumor-infiltrating lymphocytes, intratumoral CD3+ tumor-infiltrating lymphocytes, and CD68+ tumor-associated macrophages than the luminal subtype. The stromal PD-L1+, intratumoral CD3+ tumor-infiltrating lymphocytes, intratumoral CD163+ tumor-associated macrophages, and intratumoral PD-L1 + were also more commonly found in grade III breast cancer than in grade I and II breast cancer.

The current study found a significant association between intratumoral CD3+ tumor-infiltrating lymphocytes and CD68+ tumor-associated macrophages and the breast cancer subtype, with a higher percentage in the non-luminal breast cancer subtype. Other research findings supported the predominance of tumor-infiltrating lymphocytes in HER-2-positive and triple-negative breast cancer patients (Stanton and Disis, 2016). The intratumoral tumor-infiltrating lymphocyte frequencies were also observed to be low in the luminal A subtype (Dimitrova et al., 2021). Other studies have found a high frequency of CD3+ tumor-infiltrating lymphocytes in HER-2 and triple-negative breast cancer patients (Koletsa et al., 2020). Increased CD68+ tumor-associated macrophage expression in the tumor has been associated with a shorter overall and relapse-free survival (Morita et al., 2017, Eiró et al., 2012). High numbers of stromal and intratumoral CD68+ tumor-associated macrophages have been inversely associated with the luminal A breast cancer subtype (Medrek et al., 2012, Wang et al., 2022, Zhao et al., 2017). The number of stromal and intratumoral CD68+ tumor-associated macrophage cells was higher in the triple-negative and HER-2-positive groups (Chen et al., 2023). In triple-negative and HER-2 enriched subtypes, a higher level of positive CD3 and CD68 may suggest a better response to targeted immunotherapy. Additional information about the mechanisms behind this subtype could guide the development of novel immune-targeted treatments.

A significant association was found between stromal CD20+ tumor-infiltrating lymphocytes and the breast cancer subtype in this study, with a greater number in the non-luminal breast cancer subtype. Other research findings suggested a positive correlation between a higher percentage of intratumoral and stromal CD20+ tumor-infiltrating lymphocytes from triple-negative breast cancer (Mahmoud et al., 2011, Mahmoud et al., 2012). In HER2-positive and triple-negative breast cancer, the presence of CD20+ tumor-infiltrating lymphocytes improves disease-free and overall survival (Garaud et al., 2019). The triple-negative breast cancer was found to have a large percentage of CD20+ tumor-infiltrating lymphocytes (Rathore et al., 2014). Even though the tumor microenvironment has a high density of tumor-infiltrating lymphocytes, triple-negative breast cancer does not react well to immunotherapy. Therefore, decreasing PD-L1 expression in patients may enhance their anti-tumor immunity, which in turn may benefit triple-negative breast cancer treatment (Li et al., 2017a).

The presence of high percentages of tumor-infiltrating lymphocytes has been associated with ER-negative breast cancer (Mohammed et al., 2012). The current study found a higher percentage of intratumoral CD3+ tumor-infiltrating lymphocytes and CD68+ tumor-associated macrophages in ER-negative breast cancer than in ER-positive. Other research also examined the high frequency of CD68+ tumor-associated macrophages in ER and PR-negative breast cancer (Ni et al., 2019, Zhao et al., 2017). A meta-analysis study found a high density of both CD68+ and CD163+ tumor-associated macrophages in breast cancer with low ER or PR levels, which could be prognostic indicators in non-metastatic breast cancer (Ni et al., 2019). Estrogen plays an essential role in inhibiting NF-κB signaling and reorienting helper T cells to their more repair-oriented Th2 signature (Harding and Heaton, 2022), which could be responsible for the high level of CD3 and C68 positive in ER-negative breast cancer.

In this study, there was a significant association between higher percentages of stromal CD3+ tumor-infiltrating lymphocytes, CD8+ tumor-infiltrating lymphocytes, CD20+ tumor-infiltrating lymphocytes, and CD163+ tumor-associated macrophages and, ER-negative (Mutka et al., 2023). A higher number of total CD20+ tumor-infiltrating

lymphocytes was associated with better breast cancer-specific survival, a longer disease-free interval in ER, and PR-negative breast cancer (Rathore et al., 2014, Mahmoud et al., 2011). A high level of stromal CD163+ tumor-associated macrophages was reportedly associated with ER-negativity (Medrek et al., 2012), and it was found to be an independent prognostic factor for relapse-free and overall survival (Jamiyan et al., 2020).

In this study, there was a significant association between intratumoral CD3+ tumor-infiltrating lymphocytes and CD163+ tumor-associated macrophages and TNM tumor grade, with a high percentage in grade III breast cancer. In another study, a higher percentage of CD3+ tumor-infiltrating lymphocytes were found in advanced histological-grade breast cancer (Mutka et al., 2023). Inflammatory cell infiltrates have been linked to high-grade breast cancer (Mohammed et al., 2012), and a meta-analysis and other study findings have also revealed significantly higher intratumoral and stromal CD163+ tumor-infiltrating lymphocytes in advanced histological grade III breast cancer (Ni et al., 2019, Medrek et al., 2012, Mutka et al., 2023).

There was an association between intratumoral and stromal PD-L1+ and TNM tumor grade in the current study, with a high percentage of PD-L1+ in grade III breast cancer. PDL-1+ is an important inhibitory checkpoint involved in cancer immune modulation (Angelico et al., 2023). A high level of tumoral PD-L1 positivity has been associated with longer disease-free and disease-specific survival (Oner et al., 2021). In a phase three trial of Japanese breast cancer patients who received the anti-PDL1 drug atezolizumab, there was a 6-month difference in progression-free survival, among the PD-L1+ subset with metastatic triple-negative breast cancer (Iwata et al., 2019). Another study also found a 3.5-month progression-free survival (Schmid et al., 2018). PD-L1 expression was considerably higher in patients with high tumor-grade breast cancer (Li et al., 2019, Lou et al., 2017, Punhani and Ahluwalia, 2023). Another study found no statistically significant association between tumoral or microenvironmental PD-L1+ expression and breast cancer grade (Doğukan et al., 2019). According to this result, PD-L1 may play a role in tumor grade-based stratification of patients who benefit from therapy that targets the PD-1 pathway.

5.4. MMPs Expression

The MMPs have proteolytic activity and break down the extracellular matrix, promoting angiogenesis, and controlling the growth and metastasis of tumor cells (Gialeli et al., 2011, Nöel et al., 1996). They are also associated with the initiation, invasion, and metastasis of breast cancer (Duffy et al., 2000). In the present study, the MMP-11 expression was shown to be significantly higher in breast cancer cases compared to benign breast tumors. Several studies have observed MMP-11 expression at higher levels in breast cancer than in nearby normal breast tissues (Benson et al., 2013, Peruzzi et al., 2009). MMP11 hindered SMAD family member 2 from being degraded in the tumor growth factor signaling pathway, which facilitated the growth of breast cancer (Zhuang et al., 2021). Low levels of CD8+ T cells, CD4+ T cells, and B cells are also correlated with high MMP-11 expression (Kim et al., 2021). The MMPs also increase the availability of growth factors and cytokines (Gialeli et al., 2011) that could play a role in cancer initiation and progression.

In this study, there was a higher mRNA expression of MMP-2 and MMP-9 in breast cancer patients compared to benign breast tumors, though the difference was not significant. Other studies observed higher levels of MMP-2 expression in breast cancer than in nearby non-cancerous tissues (Köhrmann et al., 2009, Zhang et al., 2013, Benson et al., 2013, Mohammadian et al., 2020). The significant link between increased angiostatin and the upregulation of MMP-2 and MMP-9 (Chung et al., 2006) suggests possible involvement in cancer initiation, progression, and invasion.

The current study found that the expression of MMP-11 in breast cancer was about 2.4 times higher in lymph node-positive than in lymph node-negative. The MMP-11 increased cell motility of oral cancer cells through the focal adhesion kinase/SRC kinase pathway (Hsin et al., 2017), and it is plausible that this pathway could be involved in breast cancer metastasis. The expression of MMP-2 was about 1.6 times higher in breast cancer patients with lymph nodes positive than in lymph nodes negative in this study. Increased cell migration and invasion are promoted by interactions between the tumor cell surface

epidermal growth factor (EGF) receptors and its ligand EGF via upregulating MMP-2 expression (Majumder et al., 2019).

The MMP-11 and MMP-9 mRNA expressions were higher in grade III tumors than in grade I-II in the current investigation. Similar to this study, grade III breast cancer has been associated with increased MMP-11 mRNA expression (Cheng et al., 2010). The MMPs may promote tumor spread, invasion, and growth in breast cancer by destroying cytokines and cell adhesion molecules and increasing angiogenesis and growth factors (Ren et al., 2015), which may lead to a worse prognosis.

The expression level of mRNA of MMP-11 was 5.7 times higher in ER-positive breast cancer than in negative ones. This finding is supported by other studies (Zhuang et al., 2021, Cheng et al., 2010). Cell survival mediated by MMP-11 depends on the p42/p44 MAPK and AKT pathway (Fromigué et al., 2003). According to Marino et al. (2006), the primary transcriptional factor that interacts with ER and promotes the recruitment of coactivators is specificity protein 1 (Marino et al., 2006), specificity protein 1 is also implicated in the basal production of MMP-11 (Barrasa et al., 2012).

According to this study, HER2-negative breast cancer had higher levels of MMP-2 and MMP-11 mRNA expression than HER2-positive breast cancer. In contrast, other studies reported HER2-positive breast cancer with increased mRNA expression of MMP-11 (Zhuang et al., 2021, Sathyanarayanan et al., 2020). The role of MMP-11 in HER2-positive breast cancer through interaction with cancer cells, monocytes, and endothelial cells is also indicated (Kang et al., 2022).

The expression of MMP-9 and MMP-11 was higher in luminal A than in other breast cancer subtypes. The higher immunohistochemical protein expression of MMP-9 among luminal A breast cancer was also reported in another study (Kalavska et al., 2021). In contrast, high levels of MMP-9 protein expression were found in triple-negative (Joseph et al., 2020) and HER 2 enriched breast cancer (Yang et al., 2018).

In general, our result showed that MMP-11, which is a member of the stromelysin subgroup, has a stronger association with breast cancer progression than MMP-2 and MMP-9. MMP-11 is secreted in its active form (Cui et al., 2017), suggesting that MMP-11 may play a unique role in early tissue remodeling processes in breast cancer progression. MMP-11 has also a significant role in tumor cell survival rather than in proteolytic action (Rio et al., 1996, Nöel et al., 1996), which may be another reason for the high expression of MMP-11 in breast cancer progression. The breast cancer stromal cells, particularly peritumoral fibroblasts, express significant levels of MMP-11 and may be associated with the early stages of aggressiveness of breast cancer (Wolf et al., 1993, Basset et al., 1990).

5.5. HPV Detection and genotyping

Persistent HPV infection causes the host genome to integrate with the viral genome, increasing genomic instability and the inactivation of cell cycle checkpoints, which ultimately leads to the development of cancer (McBride and Warburton, 2017). The controversial role of HPV in the development of breast cancer is indicated (Kudela et al., 2022). This study provides no proof that HPV contributes to the development of breast cancer in that both non-malignant HPV presences; however, among breast cancer lesions the magnitude of HPV was significantly higher in ER-positive than ER-negative cases

The overall percentage of HPV infection was 25.0% in this study, and non-malignant breast lesions had a greater rate of HPV infection (29.6%) than breast cancer (20.6%), which implies that HPV infection and breast cancer are not linked. The existence of a causal link between HPV infection and breast cancer is still subject to debate; while some research revealed a higher risk and suggested that HPV may have played a role in the development of breast cancer (Lin et al., 2023, Sher et al., 2020, Ren et al., 2019), other studies found no such connection (Usman et al., 2022, Bakhtiyrizadeh et al., 2017). In a different analysis, it was discovered that 2.7% of Ethiopian breast cancer cases had HPV DNA (Gebregzabher et al., 2021) which is a far lower level than in the current study. The percentage of HPV positivity was higher in the current study than in Sudan (8.6%) and

Congo (15%) (Elagali et al., 2021). The difference could be explained by the sensitivity of the PCR employed or other HPV-causing risk factors. The common genotype in this study was HPV-16, also reported in many other studies (Manzouri et al., 2014, Elagali et al., 2021). The specific role played by this virus in the pathogenesis of breast cancer needs careful consideration and determined by larger epidemiologic studies, as this study does not provide evidence to support HPV's involvement in the development and progression of breast cancer.

According to the current study, ER-positive breast cancer has an HPV positivity rate that is 9.6 times greater than ER-negative breast cancer. A connection between the HPV virus and ER-positive breast cancer was also reported by other investigations (Balci et al., 2019, Ilahi et al., 2016). Few studies have shown that ER expression may encourage cervical neoplasia by altering the expression of the HPV genes (Blanco et al., 2021, Fernandes et al., 2015). This process may involve overexpression and/or mutations in APOBEC3B which are associated with breast cancer (Ohba et al., 2017). Estrogen exposure causes DNA double-strand breaks that ultimately result in genomic instability and carcinogenesis via G protein-coupled receptor 30 for cervical cancer and cervical dysplasia; however, the exact mechanism is unknown yet (Riley et al., 2003, Son et al., 2014, Spurgeon et al., 2017, Ogawa et al., 2023). These arguments might also apply to breast cancer, but further molecular studies are needed to comprehend if and how HPV infections are associated with ER-positive breast cancer.

Previous studies have explored possible mechanisms in which HPV could be associated with HER-2 and Ki67 levels. In vitro studies with long-term breast cancer, cell lines indicated that transfected HPV E6 and E7 could enhance HER-2 expression and confer an EGF-independent in vitro proliferation, suggesting a possible mechanism for how HPV infection might contribute to breast cancer progression (Ignatoski et al., 2005, Yasmeen et al., 2007). Ki67 expression is related to the proliferation index of breast cancer cases. A study observed that breast cancer with low proliferation indices had high HPV prevalence (Fernandes et al., 2015). Similarly, a low histopathological grade of vaginal carcinoma is associated with high HPV prevalence (Hellman et al., 2014). In our study, we observed

differences between HER2 and Ki-67 proliferation index among HPV-positive and negative breast cancer cases; however, these differences were not statistically significant, particularly after adjustment in a multivariate model.

5.6. Limitation

The small sample size, retrospective nature, and absence of analysis of HER-2 equivocal data using fluorescent in-situ hybridization are the major limitations of this work. Larger studies in the future studies to solidify our study findings are warranted. We were not able to perform FISH on 11% of our samples. The pre-analytical factors, in particular tissue fixation, may not have been completed in less than one hour of cold ischemic time, and it was not possible to guarantee that these variables were optimized or standardized across these sites. Furthermore, novel markers that could be unique to the Ethiopian population could have been identified using gene expression studies such as microarray or transcriptomics. We see strength in performing centralized IHC for all samples of regions that have not been studied before.

5.7. Conclusion

The difference in breast cancer subtypes found in different Ethiopian regions suggests the need for further study. There was a higher percentage of infiltrating immune cells in the non-luminal subtype. MMPs were elevated in breast cancer compared with benign tissue, and high in grade III and luminal A breast cancer subtypes. There is no proof of linking HPV infection to breast cancer, but more investigation is required to examine this possibility.

5.8. Recommendation

- The considerably high rates of triple-negative breast cancer and hormone receptor-negative tumors (still showing half the patients with endocrine-sensitive disease) in our study need special attention and suggest that indiscriminate use of hormone receptor therapy should be discouraged without measuring hormone receptor levels.

- Half the patients were ER-positive in this study, indicating that receptor status testing and availability of endocrine treatment need to be prioritized in cancer control programs.
- A different pattern of age, tumor size, histological grade, and Ki-67 proliferation index was found between the study sites, showing the need for each tertiary center to monitor the composition of features among their respective patients
- The higher rate of male breast cancer in this study needs further prospective study to see the actual incidence.
- Future research should be conducted prospectively on tumor-infiltrating lymphocytes, tumor-associated macrophages, and PD-L1 along with their role in survival and immunotherapy.
- The role of MMPs in breast cancer pathophysiology should be investigated further.
- The relatively high fraction of patients with advanced disease reinforces general recommendations that a level of awareness of breast cancer self-examination and screening are recommended, and improvements in health care and expertise are needed.
- Further research is necessary to examine any potential link between HPV and breast cancer, particularly the high frequency of HPV in ER-positive breast cancer warrants further attention.
- The general result of the study indicates an advanced disease stage of breast cancer and a high number of triple-negative cancers. This information suggests that policymakers in Ethiopia should revisit their cancer policy to address the growing prevalence of advanced breast cancer and triple-negative breast cancer.

5.9. Future directions

Further studies focusing on mutation analysis in young populations are necessary to understand the development of breast cancer. Advanced techniques like microarray, transcriptomics, and metabolomics can be used by researchers to identify novel markers and treatment targets for breast cancer.

5.10. Funding

This study was funded by Armauer Hansen Research Institute, Addis Ababa University, and Mizan Tepi University. Additionally, this study received some funding from Else-Kroener-Foundation through Martin-Luther-University, Halle-Wittenberg, Germany, grant No. 2018_HA31SP and a Grant from Hospital Partnerships Funding Program through Deutsche Gesellschaft für Internationale Zusammenarbeit (GIZ) commissioned by the German Federal Ministry for Economic Cooperation and Development (BMZ) No. 81281915.

5.11. Acknowledgments

The authors acknowledged all staff of the pathology department and driver officers (Mr. Shambel and Mr. Solomon Gebre) and drivers from Armauer Hansen Research Institute for their contributions.

5.12. Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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APPENDIXES

APPENDIX 1

Data collection form for breast cancer subtype study

Title of the study: Immunohistochemistry-derived subtypes of breast cancer distribution in four regions of Ethiopia

Part 1: Socio-demographic characteristics

1.1. Code no_____ biopsy number: _____

1.2. Phone number_____

1.3. Age_____ Sex_____

1.4. Region _____ Residence area: Rural_____Urban_____

1.5. Education level: Illiterate __High school or less____College or above_____

1.6. Socioeconomic status: High____ Middle_____ Low_____

1.7. Marital status: Single_____ Married_____Widowed_____

1.8. Height_____ Weight_____ BMI_____

Part 2: Clinico-pathological characteristics recording sheet

2.1. Duration of symptom in month _____

2.2. Duration of illness _____

2.3. Nature of specimen: Lumpectomy___Mastectomy____Others (Specified)_____

2.4. Family history of breast cancer: Yes____ No ____ if yes,
Mother_____Grandmother_____Sister_____Aunt_____Others_____

2.5. Family history of other type of cancer: Yes___ No ____ if yes, please specify: __

2.6. How was the lump found?

- ✓ Self accidentally_____
- ✓ Self as part of regular physical examination

- ✓ Routine physical examination_____
- ✓ Mammographic screening_____
- ✓ Unknown_____

2.7. Physical findings at the presentation

- ✓ Nipple retraction_____Bloody discharge_____
- ✓ Palpable axillary lump_____Fixation to overlying skin_____
- ✓ Fixation to the underlying muscle_____ Erythema of skin_____
- ✓ Peaud' orange_____ Satellite nodules_____

2.8.History of benign breast disease _____ Prior mammographic screening _____

2.9.Alcohol consumption_____ Smoking Status _____

2.10. Age at menarche_____ Age at menopause_____

2.11. Menstrual status: Pre_____Post_____

2.12. Childbirth: Never _____ Have _____ (Number_____)

2.13. Age at first full-term pregnancy_____

2.14. Ever been breastfeeding any child: Yes_____ No_____

2.15. Oral contraceptive used: Yes_____ No_____

2.16. Procedure_____

2.17. Focality_____

2.18. Size of largest tumor_____

2.19. Histology of breast cancer:

- ✓ Infiltrating ductal_____
- ✓ Intraductal_____
- ✓ Lobular_____
- ✓ Others_____

2.20. Degree of differentiation:

- ✓ Well differentiated_____
- ✓ Moderately differentiated_____

- ✓ Poorly differentiated_____
- ✓ Lymph node involvement-Yes___ No___ if Yes:-Number of lymph nodes positive__

2.21. Presence of in-situ lesion_____

2.22. Lymphovascular invasion_____

2.23. Site

- ✓ Upper Outer quadrant_____
- ✓ Upper inner quadrant_____
- ✓ Lower outer quadrant_____
- ✓ Lower inner quadrant_____
- ✓ Central_____

2.24. Surgical Margin Status

2.25. Pathological stage of disease at diagnosis: I___II_____III_____IV_____

2.26. Laterality÷ Right _____ Left_____

APPENDIX 2

The SOP for immunohistochemistry staining on breast cancer Biomarkers

1. Section preparations

   - ✓ Cut the sections at 4 microns & mount them on poly-L-lysine coated slides.
   - ✓ Dry the slides overnight at 37°C.
   - ✓ Store slides at room temperature until the next use. **Note**: Do not store more than 6 weeks for HER-2 and 2 months for another staining is recommended.

2. Staining procedure
   1. Heat the slides at 60°C for 30 minutes
   2. Deparaffinize slides in xylene for 2 changes, 10 min each
   3. Transfer slides to 100% alcohol, for 2 changes, 5 min each
   4. Transfer once through 95%, 80%, and 70% alcohols respectively for 5 min each
   5. Transfer the slides to distilled water for 5 minutes
   6. Place the slides in a microwave slide dish and cover them with EnVision™ FLEX Target Retrieval Solution, High pH or Low PH as described.
   7. Microwave on high power (800W) until boiling, 10 minutes for ER, PR, and Ki67, and 20 minutes for HER-2.
   8. Reduce the effect to 200 W and run for 12 minutes for ER, PR, and Ki67 and 24 minutes for HER-2.
   9. Cool for a minimum of 20 minutes. **The cooling step is critical!**
   10. Remove the slides and rinse in tap water for 5 minutes
   11. Place in wash buffer for 5 minutes
   12. Drain excess wash buffer from slides. Take proper care not to dry the tissue!
   13. Block the sections with 100ul endogenous peroxidase blocking buffer for 10 minutes

14. Rinse with wash buffer for 2 changes.
15. Drain excess wash buffer from slides. Take proper care not to dry the tissue!
16. Apply 100 µl appropriately diluted primary antibody and negative control solution to the sections on the slides and incubate in a humidified chamber at room temperature for 30 minutes
    Dilution: ER, PR and Ki-67(1:200) and HER2(1:600)
17. Wash the slides with wash buffer for 2 changes
18. Drain excess wash buffer from slides. Take proper care not to dry the tissue!
19. Apply 100 µl EnVision™ FLEX /HRP to the sections on the slides and incubate in a humidified chamber at room temperature for 30 min (keep protected from light).
20. Wash slides with wash buffer for 2 changes
21. Drain excess wash buffer from slides. Take proper care not to dry the tissue!
22. Apply 100 µl EnVision™ FLEX DAB+ Chromogen- Substrate Buffer solution for 10 minutes at room temperature

Caution: DAB is a suspect carcinogen. Handle with care!

23. Wash slides with DW 2 changes.
24. Drain excess wash buffer from slides. Take proper care not to dry the tissue!
25. Counterstain slides with 300ul hematoxylin for 30 second
26. Rinse the slides in tap water 3 changes
27. Dehydrate the tissue slides with alcohol (95%, 95%, 100%, and 100%), for 5 min each.
28. Clear the tissue slides in 2 changes of xylene for 5 minutes each
29. Coverslip using a mounting solution

APPENDIX 3

SOP for RNA extraction

1. Using a scalpel, trim excess paraffin off the sample block.
2. Cut sections 5-20 µm thick, discard the first 2–3 sections.
3. Immediately place the sections in a 2 ml microcentrifuge tube, & close the lid.
4. Add 1 ml xylene. Vortex vigorously for 10 s and centrifuge at full speed for 2 min.
5. Carefully remove the supernatant by pipetting without disturbing the pellet.
6. Add 1 ml ethanol (100%) to the pellet, mix by vertexing, & centrifuge at full speed for 2 min.
7. Carefully remove the supernatant by pipetting without disturbing the pellet.
8. Keep the lid open, and incubate at room temperature **(15-25°C) or at up to 37°C.** Incubate for 10 min or until all residual ethanol has evaporated.
9. Add 240 µl Buffer PKD, and mix by vertexing.
10. Add 10 µl Proteinase K. Mix gently by pipetting up and down.
11. Incubate at 56°C for 15 min, then at 80°C for 15 min. If using only one heating block, leave the sample at room temperature after the 56°C incubation until the heating block has reached 80°C.

Note: Complete digestion of tissue by Proteinase K is not required for maximum RNA yield; however, the 80°C incubation step is crucial.

Important: Ensure that the heating block has reached 80°C before starting the 15 min incubation. The 15-minute incubation at 80°C is critical for the reversal of crosslinks and optimal RNA performance in downstream applications, such as real-time RT-PCR. The incubation at 80°C in Buffer PKD partially reverses the formaldehyde modification of nucleic acids. Longer incubation times or higher incubation temperatures may result in more fragmented RNA but may also result in slightly lower CT values in downstream applications, such as real-time RT-PCR.

12. Incubate on ice for 3 min. Then, centrifuge for 15 min at 20,000 x g (13,500 rpm).

13. Transfer the supernatant to a new microcentrifuge tube, taking care not to disturb the pellet. The pellet contains insoluble tissue debris, including crosslinked DNA. If processing more than 2 sections, a larger tube may be necessary.
14. Add DNase Booster Buffer equivalent to a tenth of the total sample volume (25 µl) and 10 µl DNase I stock solution. Mix by inverting the tube. Centrifuge briefly to collect residual liquid from the sides of the tube.

    Note: DNase I is supplied lyophilized and should be reconstituted as described in

    Note: DNase I is especially sensitive to physical denaturation. Mixing should only be

    carried out by gently inverting the tube. Do not vortex.
15. Incubate at room temperature for 15 min.
16. Add 500 µl Buffer RBC to adjust binding conditions, and mix the lysate thoroughly.
17. Add 1200 µl ethanol (100%) to the sample, and mix well by pipetting. Do not centrifuge. Proceed immediately to step 18. Precipitates may be visible after the addition of ethanol. This does not affect the procedure.
18. Transfer 700 µl of the sample, including any precipitate that may have formed, to an RNeasy MinElute spin column placed in a 2 ml collection tube. Close the lid gently, & centrifuge for 15 s at ≥8000 x g (≥10,000 rpm). Discard flow-through. * Reuse the collection tube in step 19.
19. Repeat step 18 until the entire sample has passed through the RNeasy MinElute spin column. Reuse the collection tube in step 20.
20. Add 500 µl Buffer RPE to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm). Discard the flow-through.

    Ensure that Buffer RPE is at room temperature (15–25°C).

    Note: Buffer RPE is supplied as a concentrate. Reuse the collection tube in step 21.
21. Add 500 µl Buffer RPE to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 2 min at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. Discard the collection tube with the flow-through.

Note: After centrifugation, carefully remove the RNeasy MinElute spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol occurs.

22. Place the RNeasy MinElute spin column in a new 2 ml collection tube. Open the lid of the spin column, and centrifuge at full speed for 5 min. Discard the collection tube with the flow-through.

To avoid damage to their lids, place the spin columns into the centrifuge with at least one empty position between columns. Orient the lids so that they point in a direction opposite to the rotation of the rotor (e.g., if the rotor rotates clockwise, orient the lids counterclockwise).

It is important to dry the spin column membrane because residual ethanol may interfere with downstream reactions. Centrifugation with the lids open ensures that no ethanol is carried over during RNA elution.

23. Place the RNeasy MinElute spin column in a new 1.5 ml collection tube. Add 14–30 µl RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at full speed to elute the RNA. Elution with smaller volumes of RNase-free water leads to higher total RNA concentrations, but lower RNA yields. The dead volume of the RNeasy MinElute spin column is 2 µl: elution with 14 µl RNase-free water results in a 12 µl eluate.

APPENDIX 4

SOP for DNA extraction

1. Using a scalpel, trim excess paraffin off the sample block and cut up one section, up to 10 µm thick. If the sample surface has been exposed to air, discard the first 2–3 sections. Immediately place the section in a 1.5 ml or 2 ml microcentrifuge tube (not supplied).
2. Add 160 µl Deparaffinization Solution, vortex vigorously for 10 s, and centrifuge briefly to bring the sample to the bottom of the tube. 2. Incubate at 56°C for 3 min, then allow to cool to room temperature. If too little Deparaffinization Solution is used or if too much paraffin is carried over with the sample, the deparaffinization Solution may become waxy or solid after cooling. If this occurs, add additional Deparaffinization Solution, and repeat the 56°C incubation.
3. Add 55 µl RNase-free water, 25 µl Buffer FTB, and 20 µl proteinase K (a master mix comprising these components may be prepared in advance). Vortex and briefly centrifuge the sample. Deparaffinization Solution will form a layer above Buffer FTB with the addition of proteinase K.
4. Incubate at 56°C for 1 hour.
5. Incubate at 90°C for 1 hour. If using only one thermomixer, leave the sample at room temperature (15–25°C) after the 56°C incubation in step 4, until the heating block has reached 90°C for step 5.
6. Briefly centrifuge the tube to remove drops from inside the lid and transfer the lower, clear phase into a new microcentrifuge tube (not provided).
7. Add 115 µl RNase-free water and mix.
8. Add 35 µl UNG to the sample, vortex, and incubate at 50°C for 1 hour in a thermomixer. If using only one thermomixer, leave the sample at room temperature (15–25°C) after the 90°C incubation in step 5, until the thermomixer has reached 50°C for step 8.
9. Briefly centrifuge the tube to remove drops from inside the lid.
10. Add 2 µl RNase A (100 mg/ml), mix, and incubate for 2 min at room temperature. Continue with step 11 in part 2 of the protocol.

11. Add 250 µl Buffer AL to the sample and mix thoroughly by vortexing. Then add 250 µl ethanol (96–100%) to each sample and mix thoroughly by vortexing. Centrifuge briefly to remove drops from inside the lid.
12. Transfer 700 µl lysate to the QIAamp MinElute column (in a 2 ml collection tube), close the lid, and centrifuge. Discard the flow-through and reuse the collection tube
13. Repeat step 12 until the complete lysate is used up.
14. Add 500 µl Buffer AW1 to each spin column and centrifuge. Discard the flow-through and reuse the collection tube.
15. Add 500 µl Buffer AW2 to each spin column and centrifuge. Discard the flow-through and reuse the collection tube.
16. Add 250 µl ethanol (96–100%) to the spin column and centrifuge.
17. Discard the flow-through and collection tube.
18. Place the spin column into a new 2 ml collection tube (supplied) and centrifuge to remove any residual liquid.
19. Place the QIAamp MinElute column into a clean 1.5 ml microcentrifuge tube (not provided) and discard the collection tube containing the flow-through.
20. Open the lid of the QIAamp MinElute column and apply 20–40 µl Buffer ATE to the center of the membrane. IMPORTANT: Ensure that Buffer ATE is equilibrated to room temperature. Dispense Buffer ATE onto the center of the membrane to ensure complete elution of bound DNA. The volume of eluate will be up to 5 µl less than the volume of Buffer ATE that was applied to the column.
21. Close the lid and incubate at room temperature (15–25°C) for 1 min, then centrifuge. Incubating the QIAamp MinElute column loaded with Buffer ATE for 5 min at room temperature before centrifugation generally increases DNA yield.

APPENDIX 5

The first page of articles that have been published.

medicines

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Brief Report

Clinicopathological Features of Invasive Breast Cancer: A Five-Year Retrospective Study in Southern and South-Western Ethiopia

Esmael Besufikad Belachew [1,2,3,*], Adey Feleke Desta [2], Dinksira Bekele Deneke [3], Bizunesh Dires Fenta [4], Alemwosen Teklehaymanot Alem [4], Abdo Kedir Abafogi [5], Fekade Yerakly Lukas [4], Mesele Bezabih [5], Dareskedar Tsehay Sewasew [3], Eva J. Kantelhardt [6], Tesfaye Sisay Tessema [7] and Rawleigh Howe [3]

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Abstract: Abstract**Background:** Breast cancer (BC) is the most common type of cancer in Ethiopia. The incidence of BC is also rising, but the exact figure is still poorly known. Therefore, this study was conducted to address the gap in epidemiological data on BC in southern and southwestern Ethiopia. **Materials and Methods:** This is a five-year (2015–2019) retrospective study. The demographic and clinicopathological data were collected from biopsy reports of different kinds of breast carcinomas in the pathology department of Jimma University Specialized Hospital and Hawassa University Specialized Referral Hospital. Histopathological grades and stages were conducted using Nottingham grading and TNM staging system, respectively. Collected data were entered and analyzed using SPSS Version-20 software. **Results:** The mean age of patients at diagnosis was 42.27 (SD = 13.57) years. The pathological stage of most BC patients was stage III, and most of them had tumor sizes greater than 5 cm. Most patients had moderately differentiated tumor grade, and mastectomy was the most common type of surgery at the time of diagnosis. Invasive ductal carcinoma was the most common histological type of BC, followed by invasive lobular carcinoma. Lymph node involvement was seen in 60.5% of cases. Lymph node involvement was associated with tumor size ($\chi^2 = 8.55$, $p = 0.033$) and type of surgery ($\chi^2 = 39.69$, $p < 0.001$). Conclusions: This study showed that BC patients in southern and southwestern Ethiopia displayed advanced pathological stages, relatively young age at diagnosis, and predominant invasive ductal carcinoma histological patterns.

Keywords: breast cancer; histological type; invasive ductal carcinoma; lymph node involvement; stage and grade

Citation: Belachew, E.B.; Desta, A.F.; Deneke, D.B.; Fenta, B.D.; Alem, A.T.; Abafogi, A.K.; Lukas, F.Y.; Bezabih, M.; Sewasew, D.T.; Kantelhardt, E.J.; et al. Clinicopathological Features of Invasive Breast Cancer: A Five-Year Retrospective Study in Southern and South-Western Ethiopia. *Medicines* **2023**, *10*, 30. https://doi.org/10.3390/medicines10050030

Academic Editor: William Cho

Received: 4 March 2023
Revised: 10 April 2023
Accepted: 24 April 2023
Published: 4 May 2023

1. Background

Cancer is characterized by an uncontrolled and invasive growth of cells that spreads to other parts of the body through blood circulation and lymphatic vessels [1]. The sustaining proliferative signals, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis are the main features of cancer [2].

There were an estimated 19.3 million new cases and 9.9 million deaths from cancer in 2020. A high percentage of breast cancer (BC) cases (11.7%), followed by lung (11.4%),

Belachew *et al. BMC Research Notes* (2023) 16:253
https://doi.org/10.1186/s13104-023-06518-5

BMC Research Notes

RESEARCH NOTE Open Access

The expression of matrix metalloproteinase 2, 9 and 11 in Ethiopian breast cancer patients

Esmael Besufikad Belachew[1,2,3*], Adey Feleke Desta[2], Dinikisira Bekele Deneke[3,4], Tewodros Yalew Gebremariam[4], Dessalegn Abeje Tefera[3], Fikadu Alemu Atire[3], Dawit Hailu Alemayehu[3], Tamirayehu Seyoum[3], Marcus Bauer[5,6], Selfu Girma[3], Dareskedar Tsehay Sewasew[3], Eva J. Kantelhardt[7,8], Tesfaye Sisay Tessema[9] and Rawleigh Howe[3]

Abstract

Introduction Matrix metalloproteinases (MMPs) play a pathophysiological role in cancer initiation and progression. Numerous studies have examined an association between MMP-2, MMP-9, and MMP-11 expression and clinicopathological characteristics of breast cancer (BC); however, no research has been done on the MMP expression levels in BC cases from Ethiopia.

Materials and methods A total of 58 formalin-fixed paraffin-embedded breast tissue samples encompassing 16 benign breast tumors and 42 BC were collected. The RNA was extracted and quantitative reverse-transcription PCR was performed. GraphPad Prism version 8.0.0 was used for statistical analysis.

Results The MMP-11 expression levels were significantly higher in breast cancer cases than in benign breast tumors (P = 0.012). Additionally, BC cases with positive lymph nodes and ER-positive receptors had higher MMP-11, MMP-9, and MMP-2 expression than cases with negative lymph nodes and ER-negative, respectively. The MMP-11 and MMP-9 expressions were higher in grade III and luminal A-like tumors than in grade I-II and other subtypes, respectively.

Conclusion The MMP-11 expression was higher in BC than in benign breast tumors. Additionally, MMP-11, MMP-9, and MMP-2 were higher in BC with positive lymph nodes and estrogen receptors. Our findings suggest an important impact of MMPs in BC pathophysiology, particularly MMP-11.

Keywords BC, Benign breast tumor, Ethiopia, Matrix metalloproteinases, mRNA expression

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frontiers | Frontiers in Endocrinology

TYPE Original Research
PUBLISHED 09 November 2023
DOI 10.3389/fendo.2023.1250189

OPEN ACCESS

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RECEIVED 29 June 2023
ACCEPTED 20 October 2023
PUBLISHED 09 November 2023

CITATION
Belachew EB, Desta AF, Gebremariam TY, Deneke DB, Ashenafi S, Yeshi MM, Fenta BD, Alem AT/H, Alemu A, Abafogi AK, Desta T, Chanyalew M, Beshah D, Taylor L, Bauer M, Tsehay D, Girma S, Melka DS, Tessema TS, Kantelhardt EJ and Howe R (2023) Immunohistochemistry-derived subtypes of breast cancer distribution in four regions of Ethiopia.
Front. Endocrinol. 14:1250189.
doi: 10.3389/fendo.2023.1250189

Immunohistochemistry-derived subtypes of breast cancer distribution in four regions of Ethiopia

Esmael Besufikad Belachew[1,2,3*], Adey Feleke Desta[2†], Tewodros Yalew Gebremariam[4], Dinikisira Bekele Deneke[4], Senait Ashenafi[4], Melisachew Mulatu Yeshi[5], Bizunesh Dires Fenta[6], Alemwosen T/Hayimanot Alem[6], Addisu Alemu[7], Abdo Kedir Abafogi[8], Tigist Desta[3], Menberework Chanyalew[3], Daniel Beshah[9], Lesley Taylor[10], Marcus Bauer[11,12], Dareskedar Tsehay[3], Selfu Girma[3], Daniel Seifu Melka[13], Tesfaye Sisay Tessema[14], Eva J. Kantelhardt[15,16†] and Rawleigh Howe[3†]

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Purpose: Different biological characteristics, therapeutic responses, and disease-specific outcomes are associated with different molecular subtypes of breast cancer (BC). Although there have been different studies on BC in the Ethiopian capital city of Addis Ababa, there have been few studies in other parts of the nation, and none have evaluated biological characteristics in other locations in the context of the extensive ethnic and genetic diversity found in Ethiopia. This study was carried out to evaluate the distribution of immunohistochemistry (IHC) subtypes of BCs throughout four Ethiopian regions.

Methods: A total of 227 formalin-fixed paraffin-embedded (FFPE) tissue blocks were collected from tertiary hospitals in four Ethiopian regions between 2015 and 2021. The IHC staining was performed for subtyping, ER, PR, HER2, and Ki-67 proliferation markers.

Results: The mean age at diagnosis was 43.9 years. The percentage of ER and PR-negative tumors were 48.3% and 53.2%, respectively. The IHC subtypes showed the following distribution: 33.1% triple-negative breast cancer (TNBC), 27.6% luminal B, 25.2% luminal A, and 14.1% HER2 enriched. In multiple logistic regression analysis, grade III and HER2 positivity were associated with larger tumor size, and also originating from Jimma compared to Mekele.

Conclusion: Patients with ER-negative, PR-negative, and TNBC were found in 48.3%, 53.2%, and 33.1% of cases, respectively, showing that half the patients could potentially benefit from endocrine treatment. A considerably high prevalence of TNBC was reported in our study, demanding additional research that includes genetic predisposition factors. Additionally, aggressive tumors were found in a high percentage of younger age groups, which must be considered when planning personalized treatment strategies.

KEYWORDS

breast cancer, estrogen receptor, immunohistochemistry, subtype, Ethiopia, Africa

frontiers in Endocrinology

REVIEW
published: 25 March 2021
doi: 10.3389/fendo.2021.599586

Molecular Mechanisms of Endocrine Resistance in Estrogen-Receptor-Positive Breast Cancer

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The estrogen receptor is a vital receptor for therapeutic targets in estrogen receptor-positive breast cancer. The main strategy for the treatment of estrogen receptor-positive breast cancers is blocking the estrogen action on estrogen receptors by endocrine therapy but this can be restricted *via* endocrine resistance. Endocrine resistance occurs due to both *de novo* and acquired resistance. This review focuses on the mechanisms of the ligand-dependent and ligand-independent pathways and other coregulators, which are responsible for endocrine resistance. It concludes that combinatorial drugs that target different signaling pathways and coregulatory proteins together with endocrine therapy could be a novel therapeutic modality to stop endocrine resistance.

Keywords: acquired resistance, breast cancer, endocrine resistance, endocrine therapy, estrogen receptor, *de novo* resistance

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Cancer Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *28 September 2020*
Accepted: *15 February 2021*
Published: *25 March 2021*

Citation:
Belachew EB and Sewasew DT (2021) Molecular Mechanisms of Endocrine Resistance in Estrogen-Receptor-Positive Breast Cancer. Front. Endocrinol. 12:599586. doi: 10.3389/fendo.2021.599586

INTRODUCTION

The estrogen hormone is important in maintaining the function of the reproductive system, bone metabolism, cardiovascular maintenance, central nervous systems, and lubrication of the vaginal lining. Overexpression of the estrogen hormone is associated with an increased risk for breast cancer (1, 2).

Abbreviations: AD, Activation domains; AF1, Activation Function 1; AF-2, Activation Function 2; AIB1, Amplified in Breast Cancer 1; AP-1, Activator Protein; BLACAT1, Bladder Cancer-associated Transcript 1; CARM-1, Coactivator-associated arginine methyltransferase-1; CBP, CREB Binding Protein ; CCL2, Chemokine (C-C motif) Ligand 2; CDK, Cyclin-dependent kinase; CYP2D6, Cytochrome P450/2D6; DBD, DNA Binding Domain; ER, Estrogen receptor; EREs, Estrogen Response Elements; ERα, Estrogen Receptor α; ERβ, Estrogen Receptor β; FGFR, Fibroblast Growth Factor Receptor; GCN5, General control non-derepressible 5; GPR30, G protein-coupled receptor 30; H12, Helix 12; HAT, Histone acetyltransferase; HDAC, Histone deacetylase; HSP27, Heat shock protein 27; IGF, Insulin-like growth factor; IGF-1R, Insulin-like growth factor-1 receptor; IL-1B, Interleukin-1B; LBD, Ligand-binding Domain; MAPK, Mitogen-activated Protein Kinase; CoA, Coactivator; CoR, Corepressor; NCOR, Nuclear corepressors; NR2F2, Nuclear receptor subfamily 2, group F, member 2; PI3K, Phosphatidylinositol 3 kinases; PKA, Protein Kinase A; PRMT, Protein arginine methyltransferase; PTEN, Phosphatase and tensin homolog deleted on chromosome ten; RIDs, Receptor interaction domain; RTK, Receptor tyrosine kinase; SERDs, Selective ER Down-regulators; SERMs, Selective ER Modulators; SMRT, Silencing Mediator of Retinoic Acid and Thyroid Hormone Receptor; Sp1, Specificity Protein 1; TAM, tumor-associated macrophages; TGFβ, Transforming Growth Factor-beta; TME, Tumor microenvironment; TNFα, Tumor Necrosis Factor α; TSC, Tuberous sclerosis.