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COLLEGE OF HEALTH SCIENCES, SCHOOL OF MEDICINE

DEPARTMENT OF MEDICAL BIOCHEMISTRY

Comparison of Immunohistochemistry with PCR Based Technology for Molecular Subtyping of Breast Cancer in Addis Ababa, Ethiopia

BY: Dessiet Oma

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Comparison of Immunohistochemistry with PCR Based Technology for Molecular Subtyping of Breast Cancer in Addis Ababa, Ethiopia

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This is to clarify that the thesis prepared by Dessiet Oma entitled, "***Comparison of Immunohistochemistry with PCR Based Technology for Molecular Subtyping of Breast Cancer in Addis Ababa, Ethiopia***" is submitted in partial fulfilment of the requirement for the degree of Master of Science in Medical Biochemistry, complies with the regulations of the University and meets the accepted standards concerning the originality and quality.

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List of Acronyms and Abbreviations

ASCO/CAP: American Society of Clinical Oncology/College of American Pathologists

BC: Breast Cancer

BCCL: Breast Cancer Cell Line

BRCA1: Breast Cancer Susceptibility Gene 1

CK5/6: Cytokeratin 5/6

DAB: 3, 3' Diaminobenzide

DCIS: Ductal carcinoma in situ

cDNA: Complementary Deoxyribonucleic acid

EGFR: Epidermal Growth Factor Receptor

ER/ESR1: Estrogen Receptor

FFPE: Formalin fixed, paraffin-embedded

FISH: Fluorescence in Situ Hybridization

HER2/ERBB2: Human epidermal growth factor receptor 2

HRP: Horseradish Peroxidase

IHC: Immunohistochemistry

IDC: Invasive or infiltrating ductal carcinoma

IRB: Institutional Review Board

Ki-67: Marker of cell proliferation

LCIS: Lobular carcinoma in situ

mRNA: Messenger Ribonucleic acid

NPA: Negative Percent Agreement

OPA: Overall Percent Agreement

PPA: Positive Percent Agreement

PR/PGR: Progesterone Receptor

RNA: Ribonucleic acid

RPL37-A: Ribosomal Protein L37A

RT-PCR: Reverse Transcription Polymerase chain reaction

SPMMC: St. Paul's Hospital Millennium Medical College

TASH: Tikur Anbessa Specialized Hospital

TNBC: Triple negative breast cancer

TNM: Tumor size, Number of positive lymph nodes, and Metastasis

WHO: World Health Organization

Abstract

Background: Breast cancer (BC) is a complex and heterogeneous diseases, characterized by several molecular subtypes with distinct morphologies and clinical implications that lead to differences in response to various treatment and clinical outcomes. Immunohistochemistry (IHC) is used for molecular subtyping of BC. However, IHC is subject to observer variability. Endpoint RT-PCR gene expression analysis may improve observer variability and diagnostic accuracy.

Objective: This study aimed to compare IHC with PCR based technology for molecular subtyping of breast cancer.

Method and Materials: In this comparative cross-sectional study a total of 54 BC tissue samples were collected from Tikur Anbessa Specialized Hospital (TASH), St. Paul's Hospital Millennium Medical College (SPHMMC), and Yekatit 12 Hospital Medical College. The sample was sent to a Clinic and Polyclinic for Gynecology, Martin-Luther University, Halle Saale, Germany for laboratory analysis. Out of 54 tissue samples, 41 were qualified for IHC analysis of ER, PR, HER2, and Ki-67 protein expression and endpoint RT-PCR analysis of ER, PR, and HER2 gene expression. Kappa statistics were used to assess the concordance between IHC and the endpoint RT-PCR. Furthermore, the socio-demographic and clinico-pathologic characteristics were evaluated.

Results: The overall percent agreement (OPA) between endpoint RT-PCR and IHC was 68.3% for ER (PPA 71.1%; NPA 33.3%), 39.0% for PR (PPA 14.3%; NPA 92.3%), and 82.9% for HER2 (PPA 62.5%; NPA 87.9%). Cohn's kappa values of 0.018 (<0.20), 0.045 (<0.200), and 0.481 (0.41-0.60) were generated for ER, PR, and HER2, respectively. Concordance for molecular subtypes was only 56.1% (23/41) and 0.20 kappa value. IHC and endpoint RT-PCR based molecular subtyping was shown to be discordant for 18/41 samples.

Conclusion: Molecular subtyping using endpoint RT-PCR was fairly concordant with IHC. Our result is promising that endpoint RT-PCR may provide a cost-effective, applicable BC molecular subtyping methods that can be better applied in low settings.

Keywords: Breast cancer, Molecular subtypes, IHC, RT-PCR

1. Introduction

1.1. Background

Breast cancer (BC) is the most prevalent form of cancer in women and the main cause of cancer mortality worldwide. Recent reports indicate that female BC has overtaken lung cancer as the most commonly diagnosed cancer, with an estimated 2.3 million new cases (11.7%), followed by lung cancer (11.4%). The same report confirmed that 684,996 (6.9%) new deaths occurred globally in the same year (Ghoncheh *et al*., 2016; GLOBO-CAN, 2020; Sung *et al*., 2021).

Cancer is one of the most common public health challenges in Africa, with an estimated 186,598 new BC cases and 85,787 deaths annually (GLOBOCAN, 2020). The incidence of BC in Africa is lower than developed countries; however, significantly high mortality is observed. This could be due to many reasons including racial, biological, socio-economic and socio-cultural factors (Bahnassy *et al*., 2020; Sung *et al*., 2021), in addition to limited awareness of the general population about BC and lack of advanced diagnostic and treatment means in Africa. In Sub-Saharan African countries including Ethiopia, BC is the most diagnosed cancer among women that followed by cervical cancer. Current incidence rate of BC in Ethiopia is 20.9 % (GLOBOCAN, 2020; Sung *et al*., 2021).

The majority of BC arises from the cells lining milk ducts (epithelium of milk ducts) and the minority develops from the lobules that provide milk to the ducts (American cancer society, 2021; WHO, 2021). Many different factors can affect the chance of getting BC; for example, being female, early menarche, older age, hormone replacement therapy, rad-iation, having children late or not at all, lack of physical activity, alcohol use, smoking, family history of breast cancer, and obesity (Kushi *et al*., 2012; WHO, 2021).

BC is not just a single disease; it is a heterogeneous disease made up of several different molecular subtypes. Each molecular subtype behaves differently, resulting in a varied reaction to different treatment options and clinical consequences. The method for mole-cular subtyping of BC is based on gene expression patterns. In 2000 Perou and his col-leagues classified BCs into molecular subtypes based on gene expression analysis using DNA microarrays from formalin fixed, paraffin-embedded (FFPE) tissue samples (Perou

et al., 2000). BCs are classified into five molecular subtypes based on estrogen receptor (ER), progesterone receptor (PR), and Human Epidermal Growth Factor (HER2) receptor expression levels: *luminal-A*, *luminal-B*, human epidermal growth factor receptor 2 (HER2)-*positive*, *basal-like* and *normal breast-like* (Perou *et al*., 2000; Sørlie *et al*., 2001; Zubair *et al*., 2021).

However, using DNA microarray methods to classify BC into molecular subtypes based on gene expression patterns is expensive. As a result, immunohistochemistry (IHC) is widely recognized as a suitable replacement for gene expression profiling techniques (Tang and Bourne, 2008). IHC, on the other hand, is susceptible to bias since it is highly subjective, vulnerable to technical and observer-based variability and the procedure is time taking. Thus the limitations of surrogate IHC molecular subtyping of breast cancer emphasize the need for more reliable, well standardized, and less subjective tests (Dai *et al*., 2014; Laible *et al*., 2016).

In Ethiopia, BC molecular subtyping is not done on regular basis because IHC is unavailable and expensive, resulting in a gap in the quality of BC treatment. In this study, we compared IHC and endpoint RT-PCR for molecular subtyping of BC based on the ER, PR, HER2, and Ki-67status using fresh frozen tissue samples and FFPE tissues from BC patients in terms of specificity, sensitivity and accuracy to increase the diagnostic coverage and to improve BC therapy in developing countries. We also assessed the socio-demographic and clinico-pathological characteristics of BC patients.

1.2. Literature reviews

BC is a condition in which cells proliferate and break out of control in the breast tissue. This type of cell proliferation results in a mass or lump known as a tumor, which can be benign or malignant. BC is a malignant tumor that begins in the breast cells. A malignant tumor is a group of cancer cells that can infiltrate surrounding tissues or spread to other parts of the body (metastasize). This disorder affects mostly women, although it can also affect men (American cancer society, 2021; WHO 2021).

Histologically, BC is defined by the cells that are affected in the breast. The majority of BC cases are carcinomas, particularly the invasive ductal carcinomas. These begin in the epithelial cells that coat the organs and tissues throughout the body. The other types of BC are *adenocarcinomas* that begin in the milk ducts or lobules (milk producing glands). There are also, other forms of BCs, such as *sarcomas* and *angiosarcomas* that arise from muscle, fat, or connective tissue cells, can also develop (American cancer society, 2021).

The most frequent BC is seen in cells lining milk ducts (85%) and lobules that provide milk to the ducts (15%). BCs that grow from the ducts are termed as ductal *carcinomas* and make up the majority of breast malignancies, while those that develop from lobules are known as lobular *carcinomas*. BC may be both invasive and noninvasive (in situ). BC that has spread to the surrounding tissues is known as invasive breast cancer. BC that is noninvasive (in situ) does not spread beyond the milk ducts or lobules of the breast (American cancer society, 2021; WHO 2021).

The benign breast carcinomas are classified into two types: ductal *carcinoma* in situ (DCIS) and lobular *carcinoma* in situ (LCIS). DCIS is only seen in the duct, whereas LCIS is only found in the lobules; it is a risk factor for developing invasive breast cancer. Invasive or infiltrating ductal carcinoma (IDC) is a kind of BC that has migrated outside of the duct or metastasized to other areas of the body via the lymphatic system and circulation. It is the most prevalent type of BC, accounting for 80 % of all invasive breast cancers. Invasive lobular *carcinoma* can spread to other parts of the body and comprises 15% of all invasive breast cancers (Allred *et al*., 2010; WHO 2021).

BC is usually identified via a screening process either before symptoms emerge or after a woman discovers a lump or lumps. Breast palpitation and loco-regional lymph node palpation are part of the clinical evaluation. The majority of masses are found on a mammography, as well as ultrasound of the breasts. Pathological analysis of breast tissue is required when cancer is suspected in order to make a diagnosis, identify the extent of the cancer stage, and describe the kind of disease. A needle biopsy or surgical incision can be used to collect tissue for pathological evaluation (Aebi *et al*., 2011).

1.2.1. Molecular biomarkers for breast cancer

Patients with a variety of cancer types, including BC, benefit from the use of molecular biomarkers in the detection and management of their disease (Duffy, 2013; Paoletti and Hayes, 2014). Biomarkers are particularly useful in BC for identifying individuals at higher risk of developing the disease within high-risk families, determining prognosis at the time of initial diagnosis, determining the most appropriate systemic therapy, post-operative surveillance, and monitoring therapy in advanced disease (Duffy *et al*., 2015).

ER, PR, HER2, and the Mib1/Ki-67 proliferation index are the most important molecular biomarkers associated with BC and are firmly established in the standard care of all primary, recurrent, and metastatic BC patients (Bertozzi *et al*., 2018). However, only three biomarkers (ER, PR, and HER2) are utilized in ordinary clinical practice worldwide, despite the fact that there are potentially vast pools of biomarkers on the horizon (Rakha *et al*., 2010).

Estrogen receptor (ER)

For BC molecular subtyping, ER is the most important and widely used biomarker. Ellwood Jensen of the University of Chicago discovered it in the late 1950s, and it has been applied in BC clinical therapy since the mid-1970s as a main indicator of endocrine response and a predictive factor for early recurrence (Jensen and Jordan, 2003; Rakha, 2010; Vuong *et al*., 2014). ER-positive BC accounts for about 80% of all cases of BC worldwide (Dai *et al*., 2016).

The ER is a cytoplasmic, ligand-regulated steroid nuclear receptor family that promotes cell proliferation, survival, and invasion in ER-positive BC. The DNA-binding domain of

ER regulates the transcription rates of target genes by binding with high affinity and specificity to estrogen response elements (ERE sequence) of DNA (Klinge, 2001). Once activated by estrogen, the ER is able to translocate into the nucleus, where it acts as a transcription factor, converting hormonal signals into a wide range of physiological responses in various target organs (Bertozzi *et al*., 2018).

Two types of ER (ERα and ERβ), are encoded by two distinct genes that are expressed differently in various tissues. Both ERα and ERβ bind estradiol in the normal mammary gland to govern cell proliferation and differentiation (Grober *et al*., 2011). ERα is also responsible for estrogen-induced mitogenic signaling in breast, uterine, and ovarian epithelial cells (Ali and Coombes, 2000) and is commonly expressed by BC cells (Renoir *et al*., 2013); whereas ERβ is usually associated with less aggressive tumors, as it inhibits both ER-mediated transcription and estradiol-induced proliferation in various types of cancer cells (Paruthiyil *et al*., 2004).

Progesterone receptor (PR)

The progesterone receptor (PR) is a ligand-dependent transcription factor found primarily in female reproductive tissues and the central nervous system. It belongs to the nuclear/steroid hormone receptor (SHR) family of transcription factors. PR controls the development, differentiation, and proliferation of target tissues as well as pathological processes in endocrine-based cancers by regulating gene networks in response to binding its cognate steroid hormone, progesterone (Obr and Edwards, 2012). PR-A and PR-B are two isoforms with different molecular weights. PR-B acts as a positive regulator of progesterone's actions, whereas PR-A works to counteract PR-B effects (Brisken, 2013).

The gene expression of PR is regulated by estrogen and it has been assumed that PR expression indicates an active estrogen-ER response system (Horwitz and McGuire, 1975; Yip and Rhodes, 2014).

PR positive BCs account for 65% to 75% of all cases (Dai *et al*., 2016). PR expressed by >50% of ER-positive tumors. Tumors expressing both ER and PR have better clinico-pathological, prognostic, and endocrine response characteristics (Patani *et al*., 2013). PR-positive tumors that lack the ER are rare, accounting for less than 1% of all occurrences

of BC. To rule out erroneous ER negativity, tumors having PR expression but no ER expression should be retested for ER status (Weigel and Dowsett, 2010). PR-negative BCs account for about 40% of ER-positive tumors. It is well established that tumors that are ER-positive/PR-negative are less responsive to tamoxifen than tumors that are positive for both receptors (Rakha, 2010; Vuong *et al*., 2014).

Human Epidermal Growth Factor Receptor 2 (HER2)

HER2, also known as ERBB2 and HER2/neu, is a transmembrane member of the tyrosine kinase epidermal growth factor receptors. These receptors are normally expressed at low levels in all epithelial cells in normal fetal and adult tissues, but they are also required for cancer proliferation and survival (Bertozzi *et al*., 2018). A healthy breast cell contains two copies of the HER2 gene. Certain types of breast cancer develop when a breast cell has more than two copies of HER2 gene, and those copies begin to overproduce the HER2 protein (Yardley *et al*., 2014).

EGFR/ErbB1, HER2/ErbB2, HER3/ErbB3, and HER4/ErbB4 are the four members of the HER family. These receptors have an extracellular ligand-binding domain, a transmembrane domain, and a cytoplasmic catalytic kinase domain that activates downstream signaling pathways like PI3K/Akt/mTOR and RAS/RAF/MEK/ERK (Bertozzi *et al*., 2018).

HER2 was initially discovered to be an indicator of patient's prognosis. HER2 gene amplification or protein overexpression has been linked to a poor prognosis and high recurrence rate and mortality in patients receiving systemic chemotherapy treatment (Rakha, 2010; Dai *et al*., 2016; Bertozzi *et al*., 2018).

Ki-67/Mib1

Mib1/Ki-67 is a proliferation indicator that can be utilized as a prognostic and predictive marker. It was discovered in the 1980s using a mouse monoclonal antibody against a nuclear antigen from a Hodgkin's lymphoma cell line. Ki-67 is a non histone nuclear protein that was called after the researcher's hometown; Ki denotes the University of Kiel in Germany, while 67 denotes the clone number on the 96-well plate (Kos and Dabbs, 2016). In breast cancer, Ki-67 has been used in conjunction with other markers to serve

as a prognostic and predictive tool and also it has been proposed as a clinical biomarker for distinguishing *luminal-A* and *luminal-B* subtypes (Kos and Dabbs, 2016; Maranta *et al*., 2020).

Ki-67 positivity is linked to a worse overall survival rate in breast cancer patients. The proliferative activity of the Ki-67 is essential in decision-making of adjuvant therapy in early breast cancer (Maranta *et al*., 2020). Ki-67 expression levels are calculated as the percentage of tumor cell nuclei positively stained. There is yet to be established an absolute standard technique or cutoff point and its reliability can be diminished by inter-observer variability. Originally, the Ki-67 antibody could only be used on fresh frozen tissue, but MIB-1, which targets the same epitope as the original and is the most widely used, led to the development of many additional antibodies that could be used on FFPE tissue (Mannell, 2016).

1.2.1. Molecular subtyping

Biologically, all BCs are not the same, despite the fact that they all arise from the same anatomic region and have a similar histological appearance. As a result, BCs comprise a varied and heterogeneous group of diseases. Because of this variability, diagnosing and treating it can be difficult. Clinical presentation, disease severity, and treatment response can all vary dramatically amongst BCs. BCs can also have diverse clinical features in different patients, leading to variable clinical outcomes (Perou *et al*., 2000; Sørlie *et al*., 2001; Zubair *et al*., 2021).

BC classification has traditionally been based on histopathological assessment, which includes histological type, grade, tumor size, lymph node involvement, and stage, and routinely used type of BC classification in clinical practice. The prognostic and predictive value of histopathological classification is limited. New methods are being developed to discover the molecular basis for BC heterogeneity. Hence, the gene expression profiling method and IHC have been validated for identifying BC subtypes and to make systemic therapy decisions (Rakha and Ellis, 2011; Vuong *et al*., 2014).

Molecular subtyping is the classification of BCs based on gene expression patterns. Because different molecular subtypes appear to have different prognoses and responses to

various treatment options, molecular subtyping can help improve outcomes (Pellicane, 2015).

Gene expression studies using DNA microarrays have revealed many distinct BC sub-types based on intrinsic genes that include 496 genes that differentiate BCs into separate groups using only the pattern of gene expression (Perou *et al*., 2000; Sørlie *et al*., 2001). The molecular subtypes of BC could be replaced by IHC because of its broad availability, while it is not possible to routinely employ gene expression profiling with DNA microar-rays (Tang and Bourne, 2008). IHC is a method that uses the idea of antibodies attaching selectively to antigens in biological tissues to examine frozen or FFPE BC tissues taken after biopsy or surgery. IHC is applied to demonstrate the expression of the proteins like ER, PR, HER2, and others on the surface of BC cells (Zaha, 2014).

BC was divided into five molecular subtypes with different clinical outcomes based on gene expression profiling. These are *luminal-A*, *luminal-B*, HER2-*positive*, *basal-like* and *normal breast-like* [Figure 1] (Perou *et al*., 2000; Sørlie *et al*., 2001; Malhotra *et al*., 2010; Sandhu *et al*., 210). The reasonable basis of such categorization is that the varia-tions among BC subtypes in the pattern of gene expression reflect the fundamental mole-cular diversity. These distinctions suggest that clinicians should examine BCs among the different subtypes in order to reach an appropriate treatment decision (Pellicane, 2015).

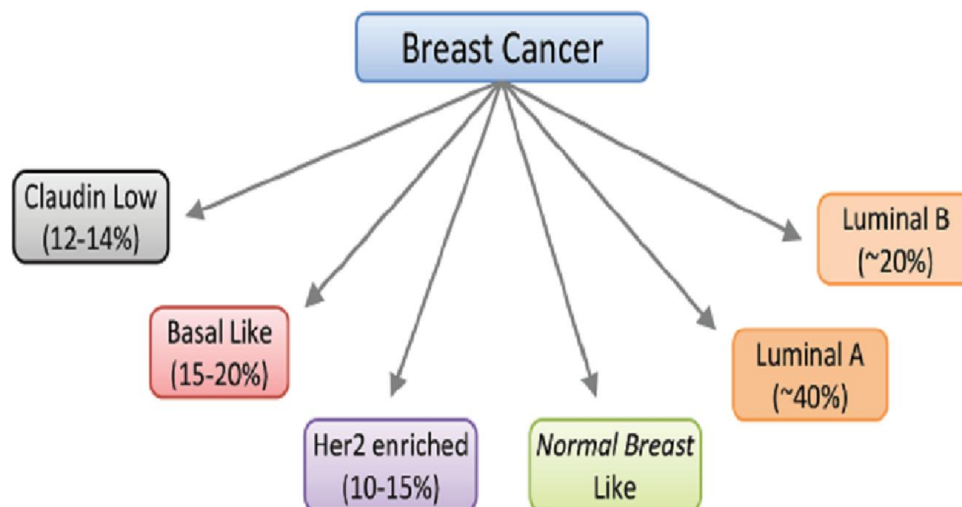

Figure 1: Molecular subtypes of breast cancer. Adopted from (Malhotra *et al*., 2010).

1.2.1.1. ***Luminal-A***

The luminal epithelial cells that line the mammary ducts and glands are over expressed in luminal BCs. The most prevalent subtype of BC is *luminal-A*, which accounts for 50–60% of all cases. It is described as ER-*positive*, PR-*positive,* and HER2-*negative* tumors with reduced levels of proliferation-related genes like Ki-67 (Peppercorn *et al*., 2008; Guiu *et al*., 2012; Yersal and Barutca, 2014).

Several studies have found that the *luminal-A* subtype is slow-growing and less aggressive than other subtypes, with a low histological grade, low degree of nuclear pleomorphism, and low mitotic activity. Patients with *luminal-A* BC have a better prognosis, especially in the near term, since they respond better to hormonal therapy (Kittaneh *et al*., 2013; Maisonneuve *et al*., 2014; Valla *et al*., 2016).

1.2.1.2. ***Luminal-B***

Luminal-B breast tumors are ER-positive and PR-positive, with variable HER2 expression and a significantly greater Ki-67 expression. *Luminal-B* tumors account for 15% to 20% of all BCs and have a more aggressive phenotype, higher histological grade, higher proliferative index, and a poor prognosis. When compared to the *luminal-A* subtype, this subtype has a greater recurrence rate and a lower survival rate following relapse (de Ronde *et al*., 2010; Kittaneh *et al*., 2013; Yersal and Barutca, 2014; Dai *et al*., 2015).

Gene expression profiling has identified heterogeneity within the *luminal-B* subtype, indicating two parts: *luminal-B* (HER2-*negative*) and *luminal-B* (HER2-*positive*). *Luminal-B* (HER2-*negative*) subtype includes all cases with ER and PR positivity, HER2 negativity and high Ki-67 but *luminal-B* (HER2-*positive*) subtype includes BC cases with positive ER and PR with positive HER2 and any Ki-67 level (Sørlie *et al*., 2001; Goldhirsch *et al*., 2013; Parise and Caggiano, 2019).

Untreated *luminal-B* BCs have a comparable overall survival rate to the *basal-like* and HER2-*positive* subtypes, which are commonly considered high risk tumors. Several studies have shown that *luminal-B* BC is less responsive to endocrine treatment than *luminal-A* BC and less sensitive to preoperative chemotherapy than HER2-*positive* and *basal-*

like BCs. *Luminal-B* BC, on the other hand, responds better to neoadjuvant chemotherapy than *luminal-A* BC (Maisonneuve *et al*., 2014; Abubakar *et al*., 2019).

Luminal-B BC patients are more likely to be younger, diagnosed at an advanced stage and grade, black, Hispanic, and women of lower socio-economic level (Parise and Caggiano, 2019). The study reveals that *Luminal-A* BC is the most common molecular subtype in Ethiopia. Furthermore, Ethiopian BC patients have highly proliferative *Luminal-B* tumors at young age (Hadgu *et al*., 2018).

1.2.1.3. HER2-*positive*

About 20% of the subtypes of BC are HER2-*positive* cancers. These tumors are distinguished by high expression of the HER2 gene and other HER2-related genes, as well as a lack of expression of the ER and PR genes (Peppercorn *et al*., 2008; Guiu *et al*., 2012; Ahn *et al*., 2020). Morphologically, these subtypes are highly proliferative, with a high histological and nuclear grade in 75% of cases. BCs that are HER2-positive develop and spread more aggressively than other subtypes and have a poor prognosis. They are more susceptible to neoadjuvant chemotherapy than *luminal* BCs (de Ronde *et al*., 2010; Kittaneh *et al*., 2013; Maisonneuve *et al*., 2014).

1.2.1.4. *Basal-like*

Basal-like BCs takes its name because they are caused by the over expression of genes associated with *basal* myoepithelial cells, which are found in a thin layer underneath the *luminal* cells (Stingl and Caldas, 2007; Cetin and Topcul, 2014). Because *basal-like* BC lacks expression of hormone receptors and HER2, it is referred to as *triple-negative* BC. These molecular subtypes, however, exhibit significant levels of expression of basal markers such as Cytokeratin (CK 5/6) and epidermal growth factor receptor (EGFR) (Dent *et al*., 2007; Rakha *et al*., 2007).

This molecular subtype is an aggressive, fast-growing tumor with a high risk of metastasis. Due to the *triple-negative* status of the receptor, *basal-like* BC is not responsive for endocrine treatment and trastuzumab (Herceptin) which are often prescribed for HER2-*positive* tumors. It is only susceptible to conventional chemotherapies; therefore due to the limited treatment choices it displays worse prognosis than other subtypes. As a result,

basal-like BC has a higher recurrence and cancer related mortality rate [Figure 2] (Pareja *et al*., 2016; Alexandrou *et al*., 2019).

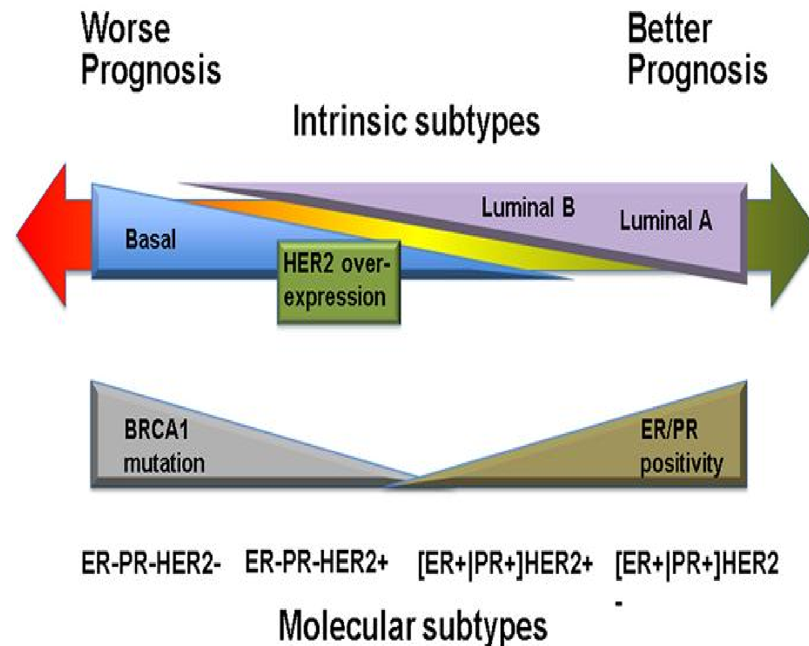

Figure 2: Patient outcome based on breast cancer molecular subtypes. Adopted from (Dai *et al*., 2015).

Basal-like BC accounts for up to 15% of BC cases and is most often diagnosed in pre-menopausal women under the age of 40, particularly those of African descent. Furthermore, *Basal-like* BCs have morphological and Immunohistochemical characteristics that are very similar to cancers that arise in BRCA1 mutation carriers. BRCA1 mutant malignancies are often characterized by a high histological grade, high proliferation markers, and a lack of ER, PR, and HER2 expression (Carey *et al*., 2006; Ihemelandu *et al*., 2007; Badve *et al*., 2011; Alexandrou *et al*., 2019).

1.2.1.5. *Normal breast-like*

Normal breast-like tumor is similar to *luminal-A* subtype which is ER+, PR+, HER2-, and low levels of Ki-67. *Normal breast-like* cancer has a good prognosis; however it is somewhat poorer than *luminal-A* subtype (Dai *et al*., 2015). Some researchers believe

that *normal breast-like* breast cancer is classified as TNBCs but they are not considered to be *basal-like* BCs as they are negative for CK5/6 and EGFR and others believe they are poorly characterized and have been categorized as molecular subtypes with normal breast samples, commonly known as unclassified BC (Weigelt *et al*., 2010).

Recently another molecular category discovered by gene expression profiling is claudin-low BC. It has been established that it predominantly displays a triple-negative phenotype and has limited expression of Ki-67, although only a small percentage of triple-negative breast tumors are claudin-low. Claudin-low tumors were linked to a young age, a higher tumor grade, a larger tumor size, widespread lymphocytic infiltrate, and a restricted tumor margin, as well as poor survival (Dias *et al*., 2017). According to several research, claudin-low is not merely a subtype similar to the intrinsic subtypes (*luminal-A*, *luminal-B*, *normal-like*, *basal-like*, and HER2-*positive*), but a complicated extra phenotype that may pervade BCs of diverse intrinsic subtypes (Fougner *et al*., 2020).

1.2.1.6. *Triple-negative* breast cancer

TNBCs are a form of aggressive BC that lacks expression of the ER, PR, and HER2. It is a highly metastasized, heterogeneous illness with a poor prognosis and a high relapse rate, accounting for 10–15 percent of overall BC cases. Because of the limited expression of three main receptors, targeted treatment approaches such as hormone therapy are unavailable. As a result, chemotherapy is the only therapeutic choice for TNBC (Qattan, 2020; Dass *et al*., 2021).

The majorities of *triple-negative* tumors is *basal-like* and have a clinical behavior similar to *basal-like*. But not all *basal-like* subtypes are TNBC.TNBCs is also significantly associated with BRCA1gene mutations. Pre-menopausal women, particularly young African-American women, are more likely to develop TNBC (Carey *et al*., 2006; Cetin and Topcul, 2014). Some studies showed that TNBCs are relatively low in Ethiopia and other Sub-Saharan African countries (Shenkutie *et al*., 2017; Hadgu *et al*., 2018).

1.3. Statement of the problem

Cancer is one of the leading causes of morbidity and mortality worldwide, with approximately 19.3 million new cases in 2020. The global cancer burden is expected to be 28.4 million cases in 2040, a 47% rise from 2020, due to demographic changes, although this may be further exacerbated by increasing risk factors associated with globalization and a growing economy (GLOBOCAN, 2020; Sung *et al*., 2021).

The burden of cancer is increasing in economically developing countries as a result of population aging and growth as well as increasingly, an adoption of western lifestyle including smoking, physical inactivity, and westernized diets (Siegel *et al*., 2014; WHO, 2021). Poverty, ignorance, inadequate diagnostic and treatment facilities has made cancer outcomes worse in sub-Saharan Africa countries in comparison with other world regions. The changes in population dynamics, lifestyles and diet across Africa have agreed with the increasing cancer incidence (Fregene and Newman, 2005; WHO, 2021).

One of the leading causes of cancer death around the globe is BC. It is the most common malignant tumor and the most frequently diagnosed cancer among women worldwide, with an estimated 2.3 million (11.7%) new cases in 2020 (GLOBOCAN, 2020; WHO, 2021).

Incidence rates of BC are 88% higher in economically developed countries than economically less developed like Africa (55.9 per 100,000 and 29.7 per 100,000). In contrast mortality rates in less developed countries have 17% higher when compared to developed countries (15.0 and 12.8 per 100,000 respectively) (Ferlay *et al*., 2015; Sung *et al*., 2021). Despite the lower incidence rate of BC in developing countries, the burden is alarmingly rising in these countries (Rahimzadeh *et al*., 2014). In addition, the mean age of BC patients in Africa are significantly lower (48 years), from which approximately two-thirds are premenopausal (Clarke *et al*., 2012; Sighoko *et al*., 2013).

Mortality rates of BC is higher in many low income countries than in high income countries, such as those in sub-Saharan Africa because of late stage at diagnosis and limited access to treatment (DeSantis *et al*., 2015). In sub-Saharan Africa, including Ethiopia BC is the most commonly diagnosed cancer in women followed by cervical cancer (Timote-

wos *et al*., 2018; Ba *et al*., 2020). Based on GLOBOCAN 2020 report the incidence rate of BC in East Africa is 3.57% (45,709 new cases). Notably, the cumulative risk of dying from cancer among women in 2020 was higher in Eastern Africa (11.0%) than in Northern America (8.2%), Western Europe (8.8%), and Australia/New Zealand (7.4%) (GLOBOCAN, 2020; Sung *et al*., 2021).

In Ethiopia, according to GLOBOCAN 2020 report BC incidence accounts 20.9% of all cancers in female, followed by cervical cancer 9.6% [Figure 3] (GLOBOCAN, 2020).

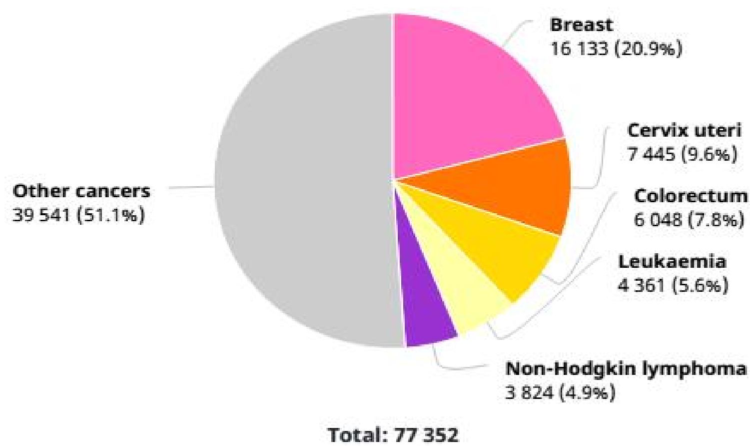

Figure 3: Ethiopian 2020 cancer report or incident. Adopted from (GLOBOCAN, 2020).

Unlike the Western world, where women present early and have a good chance of survival, women in Ethiopia usually present with very late-stage cancer and have low survival chance (Kantelhardt *et al*., 2014; Shenkutie *et al*., 2017). Furthermore, they are remarkably young (more than 50% of patients present with disease under the age of 40) compared to patients typical in the United States (Ersumo, 2006).

In general, the low survival rates of BC in developing countries like Ethiopia is partly attributed to the absence of screening programs and molecular subtyping means like IHC and other molecular techniques like PCR. This is critically hindering treatment strategies for BC patients (Kantelhardt *et al*., 2014; Shenkutie *et al*., 2017).

1.4. Significance of the study

This study is made with the aim to provide useful information to apply feasible, available, standardized and more accurate molecular subtyping technique in routine breast cancer diagnosis. The application of endpoint RT-PCR will be very important to improve the accuracy of the breast cancer diagnosis and to increase diagnostic coverage of breast cancer, which has vital role in management, as well as strategizing the treatment options for breast cancer patients with limited resources.

In addition to the stage of tumor, tumor grade, tumor size, lymph node involvement, and histological type; molecular subtyping based on ER, PR, HER2, and Ki-67 status is very important in decision making of breast cancer treatment. Although IHC is a gold standard for molecular subtyping of breast cancer, however routine diagnosis by IHC is a limitation for many laboratories, especially in developing countries. Endpoint RT-PCR has advantages over IHC, in terms of reducing the intra- and inter-observer based variation, it does not require experienced pathologists, and results are not affected by subjective interpretations. Furthermore, it is feasible, standardized and simple to implement. It Also enables the use of fresh frozen tissue samples which overcoming the challenges caused by fixation and paraffin embedding.

Finally, if endpoint RT-PCR applied in our country it will save breast cancer patients, health care centers, and the country money by reducing the cost of abroad laboratory analysis and most importantly it provide access to breast cancer diagnosis to all breast cancer patients, regardless of their economic status.

2. Objective

2.1. General Objective

The aim of this study was to compare immunohistochemistry with PCR based technology for molecular subtyping of breast cancer.

2.2. Specific Objectives

- To evaluate ER, PR, HER2, and Ki-67 using IHC for molecular subtyping of breast cancer.
- To evaluate ER, PR, and HER2 using endpoint RT-PCR for molecular sub-typing of breast cancer.
- To compare IHC and endpoint RT-PCR techniques for molecular subtyping of breast cancer.
- To assess socio-demographic and clinico-pathological characteristics of breast cancer patients in relation to the molecular subtyping.

3. Materials and methods

3.1. Study area

The study was carried out in three hospitals in Addis Ababa, Tikur Anbessa Specialized Hospital (TASH), St. Paul's Hospital Millennium Medical College (SPHMMC), and Yekatit 12 Hospital Medical College. TASH and SPHMMC are the country's largest national referral hospitals with cancer treatment centers. The rest is the regional referral hospital in Addis Ababa.

TASH was established in 1972 and has been connected with Addis Ababa University's college of health sciences as a key teaching hospital since 1998. SPHMMC is tertiary referral hospital and the second largest hospital in Ethiopia. This hospital was first opened in 1968 and it is accountable to the Federal Ministry of Health. In 2007 it was re-established as SPHMMC to honor the Ethiopian Calendar's new Millennium era. Yekatit 12 Hospital Medical College is one of the hospitals under Addis Ababa City Administration Health Bureau that has been giving routine health services for Addis Ababa and other referral cases from different regional states of Ethiopia. It was established in 1915 and until 1970s the hospital was known as Haile Selassie I hospital, named after Emperor Haile Selassie I.

3.2. Study design and period

A comparative cross-sectional study was conducted to compare IHC with PCR based technology for molecular subtyping of breast cancer from April, 2019 to February, 2021.

3.3. Population

3. 3.1. Source population

The source population was all breast cancer patients who visited the referral hospitals (TASH, SPHMMC, and Yekatit 12 Hospital Medical College) in Addis Ababa, Ethiopia.

3.3.2. Study population

The study population was breast cancer patients who undergo surgery (lumpectomy or mastectomy).

3.4. Sampling technique and sample size determination

3.4.1. Sampling technique

A convenient non-probability sampling technique was applied in this study.

3.4.2. Sample size determination

The sample size was determined using G*power statistical software version 3.0.10 based on mean difference from constant (one sample case). It is founded on 95% confidence interval (two sided confidence level), 5% margin of error, 0.5 effect size, and 95% power (1-β). The minimum computed sample size was 54.

3.5. Variables

3.5.1. Dependent variables

- ✓ ER/ESR1
- ✓ PR/PGR
- ✓ HER2/ERBB2
- ✓ Ki-67/Mib1

3.5.2. Independent variables

- ✓ Sex, Age, Residential area, Education level, Histological grade, Tumor stage, Tumor site, Histological type, family history, Radiation exposure to chest, Smoking, Alcohol consumption, and Physical activity.

3.6. Inclusion and exclusion criteria

3.6.1. Inclusion criteria

All volunteer breast cancer patients who undergo surgery and age >18 were included.

3.6.2. Exclusion criteria

Breast cancer patients who have been treated with neoadjuvant therapy, breast cancer patients with pregnancy, and those with a history of any other malignancy were excluded.

3.7. Sample and data collection technique

3.7.1. Data collection

Data concerning socio-demographic variables were collected by trained nurses (data collectors) using structured questionnaire which was translated into local language that contains information about the socio-demographic characteristics of patient and clinico-pathological characteristics from patients' medical records.

3.7.2. Sample collection

3.7.2.1. Tissue sample collection and preparation

A total of 54 breast cancer tissue samples were collected from breast cancer patients that undergone surgery at the 3 hospitals. The collection of tumor tissue was done by attending surgeons. Portions of the solid tumor, free of fat, connective tissue, debris, and blood, was cut into pieces of approximately 3x 3 x 3 mm and it was transported using icebox then it was rapidly frozen at -80°c in deep freezer until laboratory analysis.

Formalin-fixed paraffin-embedded tissue sample preparation

Tissue samples were fixed with 10% formalin for 24-48 hours at room temperature following the standard protocol for sample preparation. Then trimming of fixed tissues into appropriate size and shape was performed. There after it was placed in embedding cassettes. Processing for paraffin embedding was taken (total 16 hours): 70% ethanol washes (two changes, 1 hour each), 80% ethanol wash (one change, 1 hour), 95% ethanol wash (one change, 1 hour), 100% ethanol washes (three changes, 1.5 hour each), xylene washes (three changes, 1.5 hour each), paraffin wax (58-60 ºC, two changes, 2 hours each).

Embedding tissues into paraffin blocks includes trimming of paraffin blocks as necessary and sliced into the required slices and place paraffin ribbon in water bath at about 40-45 ºC. Mount sections onto slides and allow sections to air dry for 30 minutes and then bake in 45-50 ºC oven overnight (IHC World, 2021).

3.8. Laboratory test principles

All laboratory analysis was performed at the Clinic and Polyclinic for Gynecology, Martin-Luther University, Halle Saale, Germany.

3.8.1. Hematoxylin & Eosin staining

Principle

Hematoxylin was used to stain FFPE tissue sections, a basic dye which stains basophilic components such as nucleic acids in the nucleus and results in a blue-purple contrast. The positively charged, acidophilic tissue components, such as amino groups in proteins, were then counterstained with an acidic dye, eosin. As a result, they become pink. Figure 4 shows representative H&E stained slide (Paramedics World, 2022).

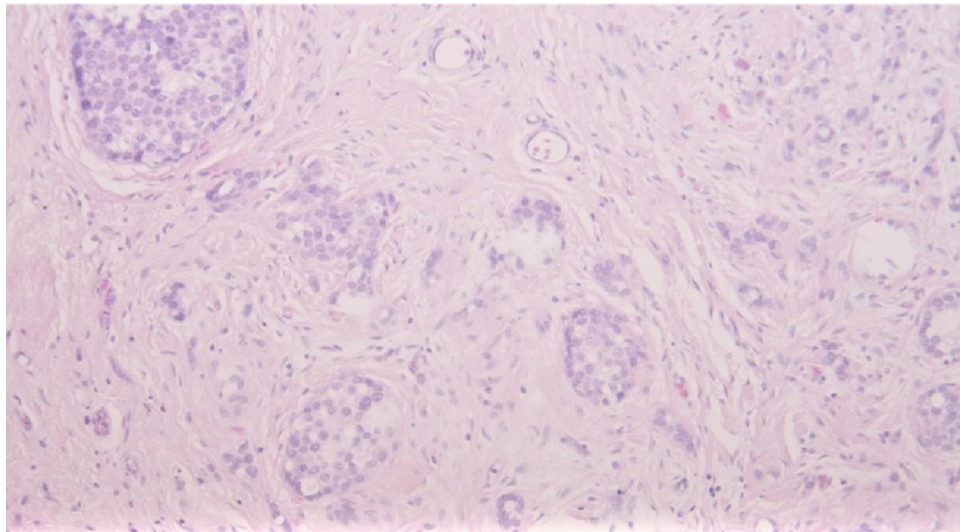

Figure 4: Representative image of H&E stained slide, 20X

3.8.2. Protein expression by IHC

Principle

FFPE tissue sections was first deparaffinised and rehydrated. Endogenous Peroxidase activity was blocked by incubation with 3% H_2O_2-solution (peroxide block). Epitopes of antigens were masked and no longer accessible for primary antibodies, because of the strong cross-links between proteins induced by formalin fixation. Epitope retrieval in buffer solutions of various compositions and pH values restores epitope structures, making them more accessible to specific antibodies. The next step was to incubate the sample with the appropriate primary antibody. The enhancing reagent "PostBlock" was applied and incubated after washing. Following a second washing, the Horseradish Peroxidase (HRP) polymer was applied. After incubation, any unbound HRP-polymer was completely washed away. The addition of the chromogenic substrate started the enzymatic reaction of the Peroxidase which leads to color precipitation where the primary antibody was bound. The color was observed with a light microscope. The chromogen used determines the color. The chromogen 3, 3'Diaminobenzidine (DAB) forms a dark brown precipitate.

Protocol

IHC was done in automated system using the Dako Autostainer Link (Agilent, USA) according to the manufacturer's procedure; FFPE sections were cut at 1-2µm thickness on a microtome and float in a 40°C water bath containing distilled water then transferred onto glass slides and allow the slides to dry in an oven at 60°C for one hour. Then the sections were deparaffinized with three xylene washes for 3 minutes each followed by 2x 96% ethanol 3 minutes each, 50% ethanol for 3 minutes, and distilled water for 3 minutes. Antigen retrieval was carried out with Sodium citrate buffer pH 6 at 89°C for 30 minutes for PR and Ki-67 and for 40 minutes for ER. After Endogenous Peroxidase activity was blocked with 3% H_2O_2 solution at room temperature for 7 min, the specific monoclonal antibodies (Ready to use) was applied for ER (clone Ab-11), PR (clone PgR 636), HER2 (clone NA) and Ki-67 (clone SP6) at room temperature for 20 minutes. The Dako FLEX + Rabbit EnVision System was used for detection. DAB chromogen was then applied for 10 minutes. Slides were counterstained with Mayer´s Hematoxylin for 30 seconds and then dehydrated and cover slipped [Figure 5].

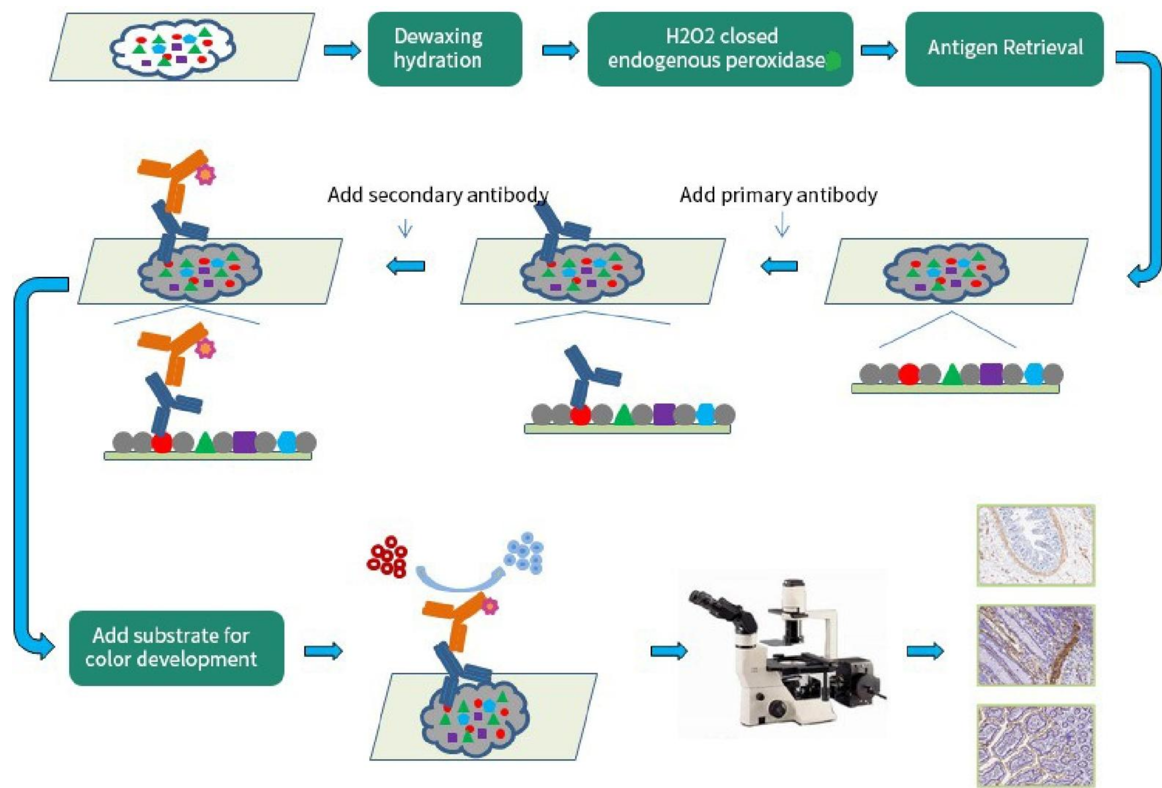

Figure 5: Immunohistochemistry principle. Adopted from (Sino Biological, 2022).

Immunohistochemical scoring

The breast cancer is considered positive for ER and PR if at least 10% of cancer cells showed positive nuclear staining of any intensity according to the American Society of Clinical Oncology/College of American Pathologists (ASCO/ CAP) guideline (Hammond *et al*., 2010).

HER2 was graded based on recommendations from Allred scoring system as described in the ASCO/ CAP as 0, 1+, 2+ or 3+. Scores of 0 and 1+ was considered negative and 3+ was considered positive. When a score of 2+ (weakly positive or equivocal) will be found, additional CISH or FISH testing was done to establish HER2 gene amplification status (Wolff *et al*., 2014).

Ki-67 grading was based on the proportion of positively stained cancer cell nuclei out of all cancer cell nuclei in the tissue section and the result was provided as a percentage

ranging from 0 to 100 %. To evaluate the expression of Ki-67, the positively stained nuclei of cancer cells was counted and expressed as the percentage designated the Ki-67 index. The Ki-67 index was considered low if the value is below 20% but high if it is equal or greater than 20% of cancer cells (Goldhirsch *et al*., 2013).

3.8.3. Gene expression by endpoint RT-PCR

RNA extraction from FFPE tissue samples

The RNA extraction was carried out by miRNeasy Mini Kit (QIAGEN®) which combines phenol/guanidine-based lysis of samples and silica membrane–based purification of total RNA. A piece of tissue was excised from FFPE tissue samples. Multiple sectioning of 8µm size was produced using microtome and was used for RNA extraction. Tissue samples were homogenized in QIAzol Lysis Reagent and after addition of chloroform, the homogenate was separated into aqueous and organic phases by centrifugation. RNA partitions to the upper, aqueous phase, while DNA partitions to the interphase and proteins to the lower, organic phase or the interphase. The upper, aqueous phase was extracted, and ethanol was added to provide appropriate binding conditions for all RNA molecules from 18 nucleotides upwards. The sample was then applied to the RNeasy Mini spin column, where the total RNA binds to the membrane and phenol and other contaminants were efficiently washed away. High-quality RNA was then eluted in RNase free water (Qiagen, 2021).

Protocol for gene expression analysis using endpoint RT-PCR

Breast cancer cell lines (BCCLS) including BT474 and BT-20 was used for optimization. For each run, a positive (BT474) and negative (BT-20) control cell lines with known ESR, PGR and ERBB2 expression was used. RPL37-A was used as a reference gene. Primer pair (forward and reverse) sequence for the corresponding gene of interest is listed in the table below (Table 1).

Table 1. Sequence of oligonucleotide primers (Denkert *et al*., 2011).

Gene name	**Primer**	**Sequences 5' -3'**
ESR1	ESR1 forward	5´-GCCAAATTGTGTTTGATGGATTAA-3´
	ESR1reverse	5´ GACAAAACCGAGTCACATCAGTAATAG-3´
PGR	PGR forward	5´-AGCTCATCAAGGCAATTGGTTT-3´
	PGR reverse	5´-ACAAGATCATGCAAGTTATCAAGAAGTT-3´
ERBB2	ERBB2 forward	5´-CCAGCCTTCGACAACCTCTATT-3´
	ERBB2 reverse	5´-TGCCGTAGGTGTCCCTTTG-3

Following the optimization on BCCLS, the end point RT-PCR experiment was done on the isolated RNA. The extracted RNA of each BCCLs and patient was used to synthesize cDNA using Biozym cDNA synthesis kit. To choose the optimum cDNA concentration amount required for PCR experiment, it was started with 100ng, 50ng and 25 ng cDNA. Moreover, variable PCR conditions including the number of cycles for each gene of interest was checked to set the optimum conditions for the establishment of the method. The PCR condition was set as follows: Denaturation: 95°C (1min); Annealing: 62°C (20sec); Extension: 72°C (20sec); PCR cycles: 30-40. Following the PCR experiment, gel documentation was done using Image Quant LAS 4000 luminescent analyzer.

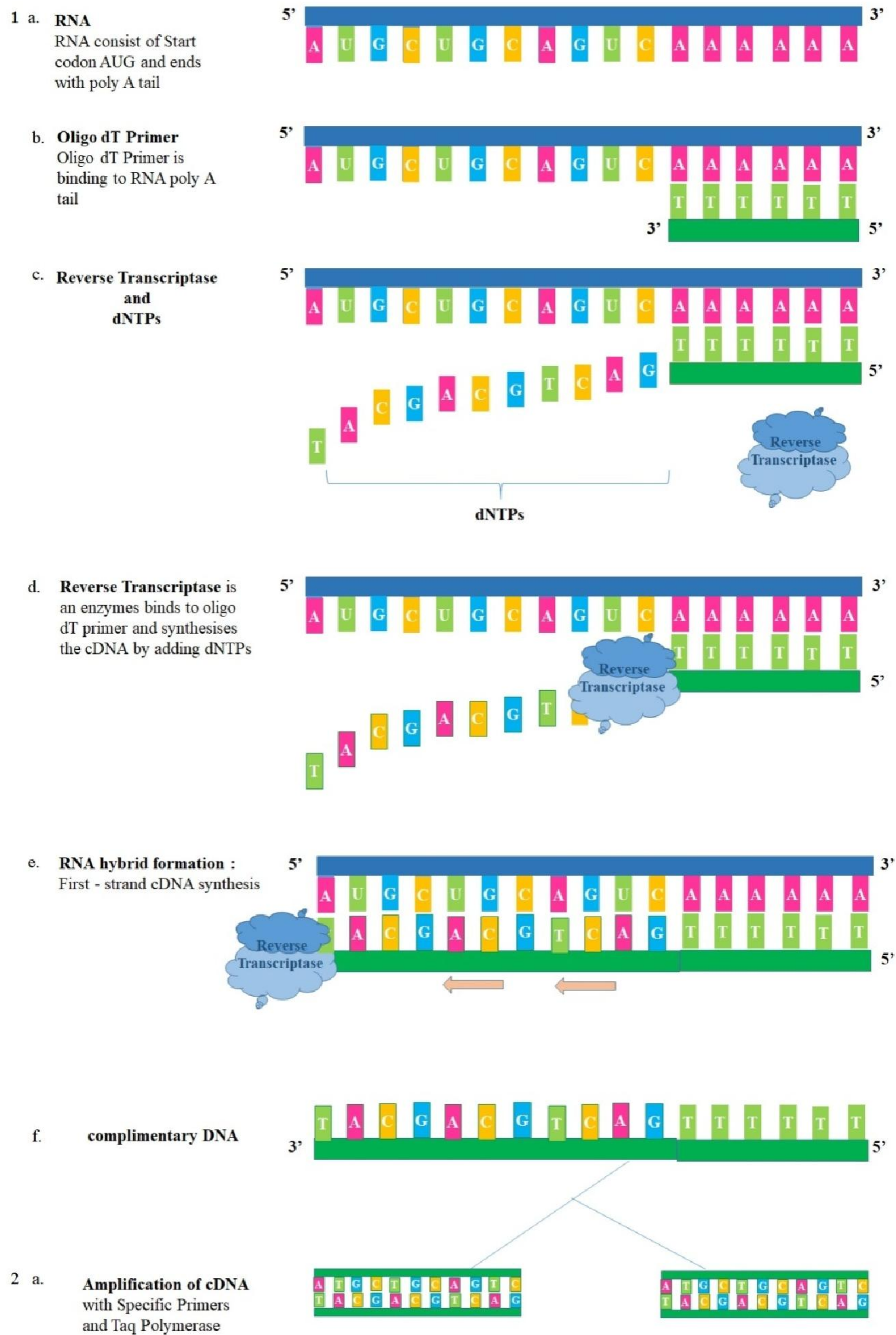

Figure 6: Endpoint RT-PCR principle. Adopted from (Thimmana, 2019).

3.9. Data quality assurance

The socio-demographic data and other preliminary data were taken by trained clinical nurses. The questionnaire was prepared in English first and translated into the local language. During data collection, regular supervision and follow-up was made. Samples were collected according to standard protocol. All cases under study were histopathologically classified based on the criteria of the WHO classification (Tavassoli and Devilee, 2003) by an experienced pathologist. The histological grade of breast tumor was determined using the Nottingham histological grading system (Rakha *et al*., 2008). Staging was performed using Tumor–Node–Metastasis (TNM) cancer staging system (Sobin *et al*., 2011). The standard operating procedure was followed in each and every procedure of laboratory assays.

3.10. Data analysis

Statistical analyses were performed using SPSS statistical software version 25.0. Overall percent agreement (OPA), positive percent agreement (PPA) or sensitivity and negative percent agreement (NPA) or specificity was calculated using 2×2 contingency tables. Kappa test was done to assess concordance between IHC and endpoint RT-PCR. Kappa statistic with 95% two-sided confidence intervals was calculated to estimate the overall agreement between the endpoint RT-PCR and IHC, with k-values categorized into poor (<0.2), fair (0.2–0.4), moderate (0.4–0.6), good (0.6–0.8) and very good (>0.8). Mean and median was used for the age of the study participants. Chi-square test was used to determine association of the socio-demographic characteristics, breast cancer risk factors, and clinico-pathological characteristics with the ER, PR, HER2, and Ki-67 status. Statistical significance was defined as *p*- value less than 0.05.

3.11. Ethical consideration

Ethical approval for all protocols used in this study was obtained from the Department of Medical Biochemistry ethics and research committee. The study also approved by Institutional Review Board (IRB) of Yekatit 12 Hospital Medical College, IRB of SPMMC and IRB of Addis Ababa City Administration Health Bureau. In addition, written informed consent was obtained from the study participants.

4. Results

4.1. Socio-demographic characteristics

Assessment of ER, PR, HER2, and Ki-67 using endpoint RT-PCR and IHC were performed on breast cancer tissue samples from 41 of 54 study participants. The remaining 13 samples were excluded because FFPE tissue was unqualified for the study and fresh frozen tissue sample was cancer free. There were 40 women and one male among the 41 study participants, with a median age of 38.5 years (range 28-71) and a mean age of 42.48 years (SD 9.89) or 42.48 ± 9.89. Only 2.4% of the study participants were under the age of 30, while 48.8% were 30-39 years old. The 53.7% of the study participants live in urban. The 34.1% of the study participants had only completed primary school, Illiterate 22.0%, College and above 17.1%, and High school 9.8%. The socio-demographic characteristics of the study participants are summarized in Table 1.

Table 2. Socio-demographic characteristics of the study participants (n=41)

Variables		Frequency (%)
Sex	Female	40 (97.6)
	Male	1 (2.4)
Age group Mean=42.48 (SD 9.89) Median=38.5 (range 28-71)	<30	1 (2.4)
	30-39	20 (48.8)
	40-49	11 (26.8)
	50-59	5 (12.2)
	≥ 60	3 (7.3)
	Missing	1 (2.4)
Residential area	Urban	22 (53.7)
	Rural	12 (29.3)
	Missing	7 (17.1)
Education level	Illiterate	9 (22.0)
	Primary school	14 (34.1)
	High school	4 (9.8)
	College and above	7 (17.1)
	Missing	7 (17.1)

4.2. Breast cancer risk factors

Premenopausal women made up 24 (58.5 %) of the study participants. Five (12.2%) of the patients had a family history of breast cancer, and 6 (14.6%) had radiation exposure to the chest. Physical activity, cigarette smoking, and alcohol consumption were 4 (9.6%), 0 (0.0%), and 1 (2.4%), respectively (Table 2).

Table 3. Breast cancer risk factors of the study participants

Variables		Frequency (%)
Menopausal status	Pre-menopausal	24 (58.5)
	Post-menopausal	13 (31.7)
	Missing	4 (9.8)
Family history of breast Cancer	Yes	5 (12.2)
	No	29 (70.7)
	Missing	7 (17.1)
Radiation exposure to chest	Yes	6 (14.6)
	No	26 (63.4)
	Missing	9 (22.0)
Physical exercise	No	31 (75.6)
	Occasionally	1 (2.4)
	3 hours a week	1 (2.4)
	5 hours a week	1 (2.4)
	Other	1 (2.4)
	Missing	6 (14.6)
Smoking	Yes	0 (0.0)
	No	35 (85.4)
	Missing	6 (14.6)
Alcohol consumption	Yes	1 (2.4)
	No	34 (82.9)
	Missing	6 (14.6)

4.3. Clinico-pathological Characteristics of the study participants

The frequency of clinico-pathological characteristics of the study participants is summarized in the table below (Table 3).

Table 4. Clinico-pathologic characteristics of the study participants

Variables		Frequency (%)
Stage at time of diagnosis	0	1 (2.4)
	I	0 (0.0)
	II	14 (34.1)
	III	21 (51.2)
	IV	0 (0.0)
	Missing	5(12.2)
Histological type	IDC	37 (90.2)
	ILC	1 (2.4)
	Mixed	1 (2.4)
	DCIS	1 (2.4)
	Micropapilary	1 (2.4)
Histological grade	I	1 (2.4)
	II	16 (39.0)
	III	23 (56.1)
	Missing	1 (2.4)
Sites of breast cancer	Right	15 (36.6)
	Left	24 (58.3)
	Missing	2 (4.8)

4.4. Evaluation of ER, PR, HER2, and Ki-67 using IHC

There were 38 (92.7%) ER-*positive*, 27 (65.9%) PR-*positive*, and 8 (19.5%) HER2-*positive* breast cancers. There was High Ki-67 expression 25 (61.0%) (Table 4). Figure 7 shows representative images of positive IHC for ER, PR, HER2, and Ki-67.

Table 5. Evaluation of ER, PR, HER2, and Ki-67 using IHC (n=41)

Variables		Frequency (%)
ER	Positive	38 (92.7)
	Negative	3 (7.3)
PR	Positive	27 (65.9)
	Negative	14 (34.1)
HER2	Positive	8 (19.5)
	Negative	33 (80.5)
Ki-67	High	25 (61.0)
	Low	16 (39)

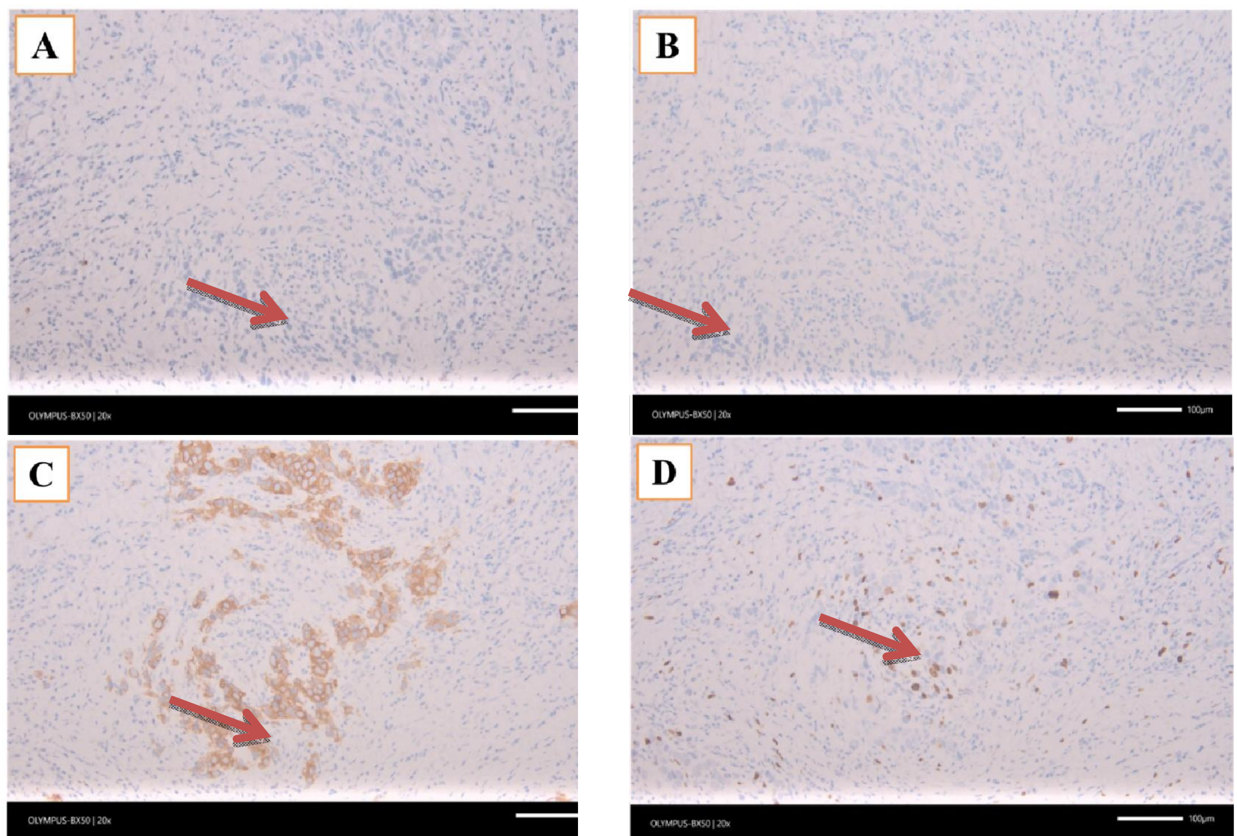

Figure 7: Representative images of IHC of ER, PR, HER2 and Ki-67 in human breast cancer tissue (20X). Arrows showing abundantly stained areas for different receptors in

breast cancer tissues. Breast cancer tissue was stained with antibodies specific for ER, PR, HER2 and Ki-67. Images depict ER positive (**A**), PR positive (**B**), HER2 positive (**C**) and Ki-67 positive (**D**).

4.5. Evaluation of ER, PR, and HER2 using endpoint RT-PCR

There were 29 (70.7%) ER-*positive* breast cancers, 5 (12.2%) PR-*positive* breast cancers, and 9 (22.0%) HER2-*positive* breast cancers (Table 5). Figure 8 shows representative images of positive endpoint RT-PCR for ER, PR, and HER2.

Table 6. Evaluation of ER, PR, and HER2 using endpoint RT-PCR (n=41)

Variable		Frequency (%)
ER	Positive	29 (70.7)
	Negative	12 (29.3)
PR	Positive	5 (12.2)
	Negative	36 (87.8)
HER2	Positive	9 (22.0)
	Negative	32 (78.0)

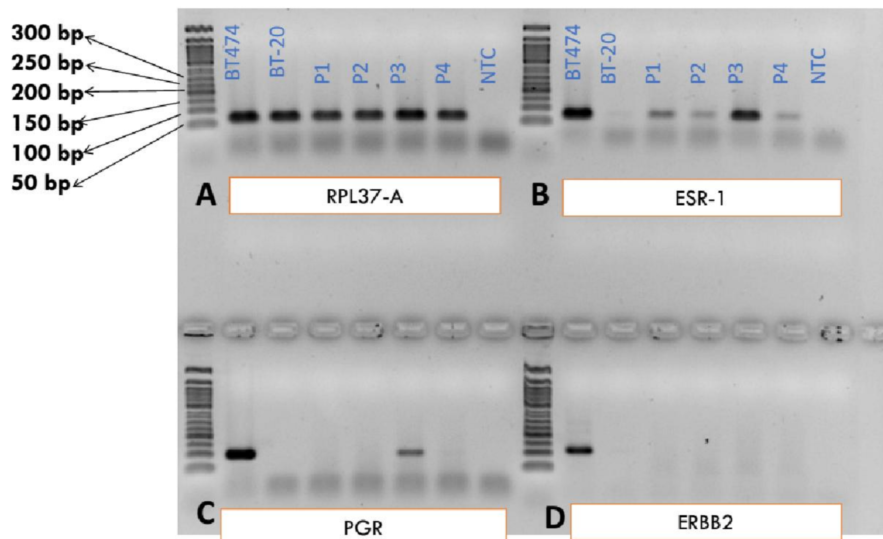

Figure 8: Gel document of endpoint RT-PCR. **A)** RPL37-A (reference gene); BT474: positive control, BT-20: negative control, P1-P4 patient samples, and NTC (No Template

Control). **B)** ESR-1gene; BT474: positive control, BT-20: negative control, P1-P4 positive sample. **C)** PGR gene; BT474: positive control, BT-20: negative control, P3 positive sample, P1, P2 and P4 negative sample. **D)** ERBB2 gene; BT474: positive control, BT-20: negative control, P1-P4 negative sample.

4.6. Association of socio-demographic characteristics, Breast cancer risk factors, and clinico-pathological characteristics with ER, PR, HER2, and Ki-67 status

There was no statistically significant association in the distribution of the socio-demographic characteristics, breast cancer risk factors, and clinico-pathological characteristics with the ER, PR, HER2, and Ki-67 status and it is summarized in Table 6,7, and 8, respectively.

Table 7. Association between socio-demographic characteristics and ER, PR, HER2, and Ki-67 status

Socio-demographic characteristics	**ER**		**PR**		**HER2**		**Ki-67**	
	ER-	**ER+**	**PR-**	**PR+**	**HER2-**	**HER2+**	**High**	**Low**
Age groups								
<30	0	1	1	0	1	0	0	1
30-39	3	17	7	13	16	14	11	9
40-49	0	11	2	9	10	1	8	3
50-59	0	5	3	2	3	2	4	1
>=60	0	3	0	3	2	1	1	2
n=40								
p-value	0.518		0.176		0.618		0.383	
Residential area								
Urban	3	19	6	16	19	3	11	11
Rural	0	12	5	7	8	4	8	4
n=34								
p-value	0.180		0.391		0.175		0.350	

Education level								
Illiterate	1	8	6	3	5	4	8	1
Primary school	1	13	4	10	11	3	6	8
High school	1	3	1	3	4	0	1	3
College and above	0	7	0	7	7	0	4	3
n=34								
p-value	0.555		0.390		0.112		0.090	

***p*-value <0.05 is statistically significant**

Table 8. Association between breast cancer risk factors and ER, PR, HER2, and Ki-67 status

Breast cancer risk factors	**ER**		**PR**		**HER2**		**Ki-67**	
	ER-	**ER+**	**PR-**	**PR+**	**HER2-**	**HER2+**	**High**	**Low**
Physical exercise								
Yes	0	4	0	4	4	0	2	2
No	3	28	11	20	24	7	18	13
n=35								
p-value	0.515		0.150		0.288		0.759	
Alcohol consumption								
Yes	0	1	0	1	1	0	1	0
No	3	31	11	23	27	7	19	15
n=35								
p-value	0.756		0.492		0.612		0.380	
Smoking								
Yes	0	0	0	0	0	0	0	0
No	3	32	11	24	28	7	20	15
n=35								
p-value								

Menstrual status								
Premenopausal	3	21	9	15	19	5	14	10
postmenopausal	0	13	4	9	10	3	8	5
n=37								
p-value	0.184		0.682		0.874		0.850	
Family history of breast cancer								
Yes	1	4	3	2	4	1	1	4
No	2	27	8	21	23	6	18	11
n=34								
p-value	0.340		0.152		0.972		0.080	
Radiation exposure to chest								
Yes	0	6	1	5	5	1	2	4
No	3	23	9	17	20	6	15	11
n=32								
p-value	0.382		0.393		0.732		0.281	

***p*-value <0.05 statistically significant**

Table 9. Association between clinico-pathologic characteristics and ER, PR, HER2, and Ki-67 status

Clinico-pathologic characteristics	**ER**		**PR**		**HER2**		**Ki-67**	
	ER-	**ER+**	**PR-**	**PR+**	**HER2-**	**HER2+**	**High**	**Low**
Stage at time of diagnosis								
0	0	1	1	0	1	0	1	0
II	1	13	4	10	12	2	10	4
III	2	19	7	14	15	6	13	8
Missing	0	5	1	4	5	0	1	4
n=41								
p-value	0.864		0.831		0.617		0.479	

Histological type								
IDC	3	34	10	27	29	8	23	14
ILC	0	1	0	1	1	0	0	1
Mixed	0	1	1	0	1	0	1	0
DCIS	0	1	1	0	1	0	1	0
Micropapilary	0	1	0	1	1	0	0	1
n=41								
p-value	0.981		0.121		0.898		0.351	
Histological grade								
I	0	2	1	1	2	0	1	1
II	0	16	4	12	13	3	8	8
III	2	20	7	15	17	5	15	7
Missing	0	1	0	0	0	0	0	0
n=41								
p-value	0.423		0.739		0.734		0.506	
Sites of breast cancer								
Right	1	14	7	8	11	4	8	7
Left	2	22	6	18	2	4	14	5
Missing	0	2	0	2	2	0	3	4
n=41								
p-value	0.654		0.091		0.330		0.269	

***p*-value <0.05 statistically significant**

4.7. Molecular subtyping based on IHC and endpoint RT-PCR

Based on the simplest classification (expression of ER, PR, and HER2), out of 41breast cancer tissue samples *luminal*-A (ER+ and/or PR+, HER2-) was more prevalent which was 75.6% and HER2-*positive* (HER2+, ER-, and PR-) was less prevalent (2.4%). However, according to 2013 St. Gallen international expert consensus, which includes Ki-67, the *luminal-B* subtype was the most prevalent (58.5%), followed by *luminal-A* (34.1%), TNBC (4.9%), and HER2-*positive* (2.4%).

On the other hand, since Ki-67 was not evaluated by endpoint RT-PCR, the molecular subtyping using endpoint RT-PCR was based on the expression of hormone receptors and HER2, we found that *luminal-A* was more prevalent which 51.2% and HER2-*positive* was less prevalent 2.4% (Table 9).

Table 10. Molecular subtyping based on IHC and endpoint RT-PCR (n=41)

Molecular subtypes	**Based on IHC**		**Based on endpoint RT-PCR**
	Using the expression of hormone receptors and HER2	**Using St. Gallen 2013 classification**	**Using the expression of hormone receptors and HER2**
	Frequency (%)	Frequency (%)	Frequency (%)
Luminal-A	31 (75.6)	14 (34.1)	21 (51.2)
Luminal-B	7 (17.1)	24 (58.5)	8 (19.5)
TNBC	2 (4.9)	2 (4.9)	11 (26.8)
HER2+	1 (2.4)	1 (2.4)	1 (2.4)
Total	41 (100)	41 (100)	41 (100)

4.8. Comparison of IHC with endpoint RT-PCR for molecular subtyping of breast cancer

Comparison of IHC and endpoint RT-PCR for molecular subtyping of breast cancer was based only on ER, PR, and HER2. Ki-67 is excluded because it was only performed by IHC.

4.8.1. Concordance between IHC and endpoint RT-PCR based on ER, PR, and HER2 status

ER protein and ESR1 mRNA expression showed a slight overall agreement of 63.3% (PPA 71.1%; NPA 33.3%) while 13 cases were discordant, with 11 cases each positive by IHC and negative by endpoint RT-PCR; while 2 cases were negative by IHC and positive by endpoint RT-PCR. Overall agreement for PR protein and PGR mRNA expression was 39.0% (PPA 14.3%; NPA 92.3%). For PGR, 25 cases were discordant, with 24 cases

each positive by IHC and negative by endpoint RT-PCR, while 1case was negative by IHC and positive by endpoint RT-PCR. HER2 protein and ERBB2 mRNA expression showed moderate overall agreement with OPA 82.9%; PPA 62.5% and NPA 87.9%. Table 10 shows the summarized concordance between IHC and endpoint RT-PCR based on ER, PR, and HER2 status.

Table 11. Concordance between IHC and endpoint RT-PCR based on ER, PR, and HER2 status

Variables	Concordance				
	OPA	**PPA**	**NPA**	**Kappa value**	***p*-value**
ER	68.3% (28/41)	71.1% (27/38)	33.3% (1/3)	0.018(<0.20)	>0.05
PR	39.0% (16/41)	14.3% (4/24)	92.3% (12/13)	0.045(<0.20)	>0.05
HER2	82.9% (34/41)	62.5% (5/8)	87.9% (29/33)	0.481(0.41-0.60)	<0.05

OPA=Overall Percent Agreement, **PPA**=Positive Percent Agreement, **NPA**=Negative Percent Agreement

4.8.2. Concordance of molecular subtyping with IHC and endpoint RT-PCR

IHC and endpoint RT-PCR molecular subtypes showed 56.1% overall concordance and kappa of 0.20 (fair agreement). Out of 31 cancers that were classified as *luminal-A* by IHC, only 19 (61.3%) were similarly classified, the 12 discordant cases being classified as either *luminal-B* (n=4, 12.9 %) and TNBC (n=8, 25.8 %). Out of the 7 cases that were classified as *luminal-B* by IHC, 3 (42.9 %) were similarly classified, the 4 discordant cases being classified as *luminal-A* (n=2, 28.6 %), HER2+ (n= 1, 14.3%) and TNBC (n= 1, 14.3%). HER2+ molecular subtype were not similarly classified. Out of 2 samples which were classified as TNBC by IHC, 1(50.0%) was similar, 1 was discordant case classified as *luminal-A* (50.0%). Table 11 shows the summarized concordance of molecular subtyping using IHC and endpoint RT-PCR.

Table 12. Concordance of molecular subtyping with IHC and endpoint RT-PCR

IHC based	**Endpoint RT-PCR based**					**Kappa value**	**OPA**
	Luminal-A	*Luminal-B*	HER2+	TNBC	Total		
Luminal-A	19 (61.3%)	4(12.9%)	0(0.0%)	8(25.8%)	31(100%)	0.200*	56.1% (23/41)
Luminal-B	2(28.6%)	3(42.9%)	1(14.3%)	1(14.3%)	7(100%)		
HER2+	0(0.0%)	1(14.3%)	0(0.0%)	0(0.0%)	1(100%)		
TNBC	1 (50.0%)	0(0.0%)	0(0.0%)	1(50.0%)	2(100%)		
Total	22(53.7%)	8(19.5%)	1(2.4%)	10(24.4%)	41(100%)		

=** ***p**-value <0.05 is statistically significant**

5. Discussion

The management and outcome of breast cancer is partly influenced by the appropriate, accurate and sensitive diagnostic means. The application of specific diagnostic modalities for breast cancer varies from laboratories to laboratories based on the available resources in a given settings. Revisiting the existing diagnostic techniques of breast cancer is essential to increase the accuracy and sensitivity in addition to minimizing the cost incurred on the patient and health system. To this end, we evaluated the potential use of the endpoint RT-PCR technique in substituting the conventional IHC technique for breast cancer detection.

It is possible to measure ER, PR, and HER2 gene expression on an automated, practical diagnostic method that is also simple to use, feasible, accessible, standardized, and give rapid result. These characteristics suggest that such an approach could be useful in the treatment of breast cancer patients in low- and middle-income countries, where access to more standardized diagnostic procedures like IHC and FISH is limited (Wu *et al*., 2018). Therefore, we evaluated ER, PR, and HER2 gene expression by endpoint RT-PCR and compared with the IHC. Furthermore, we estimated molecular subtypes using endpoint RT-PCR and compared the result with the subtypes classified based on IHC.

We showed that age, residential area and education level had no significant association with ER, PR, and HER2 status and with each molecular subtypes of breast cancer. The age of the participants in this study ranged from 28 to 71 years old, and the mean age of the study participants was 42.48 years, and 58.5% of the female study participant was pre-menopausal. This result considerably similar with the other studies done in Ethiopia (Kantelhardt *et al*., 2014; Hadgu *et al*., 2018) also the relatively younger age of women with breast cancer was already reported in several studies in Africa (Miguel *et al*., 2017; Traoré *et al*., 2019, Uyisenga *et al*., 2020; Sayed *et al*., 2021) unlike developed countries where the older age is more prevalent (Abdulrahman and Rahman, 2012). The causes of breast cancer in Ethiopian and other African patients at younger age could be due to environmental or genetic factors, such as rapid urbanization, adoption of unhealthy lifestyles, as well as the fact that African countries have a higher number of younger populations (Brinton *et al*., 2014; Uyisenga *et al*., 2020). In addition, the study

done among black South African women showed the use of oral and injectable hormonal contraceptives might be one of the causes of breast cancer at young age (Urban *et al*., 2012).

Similar to other African and Western countries, our study showed IDC was the predominant histological type (90.2%) (Miguel *et al*., 2017; Traoré *et al*., 2019; Adani-Ifè *et al*., 2020). Stage III (51.2%) was the most frequent and high tumor grade (Grade III) reported in 56.1 % of our study participants, which is similar to previous Ethiopian studies (Kantelhardt *et al*., 2014; Shenkutie *et al*., 2017). This is also consistent with findings from Rwanda and Angola, where stage III tumors was more prevalent (62 % and 68.6%, respectively) (Miguel *et al*., 2017; Uyisenga *et al*., 2020). This is mostly due to the fact that the majority of cancer cases in developing countries present at advanced stages of the disease due to a low educational level, lack of awareness, undermining symptoms, cultural beliefs, lack of medical facilities and trained personnel, (Shenkutie *et al*., 2017; Uyisenga *et al*., 2020).

Breast cancer on the left breast was more common in our study (58.3%). It was also seen in patients from other African countries, as well as Caucasian and African American patients. However, there is no obvious explanation; it could be because the left breast is 5 to10% more likely to develop cancer than the right breast, and family history of breast cancer, particularly among first-degree relatives, and age at diagnosis have been explored as possible explanations (Amer, 2014; Cheng *et al*., 2018).

Using the standard IHC classification recommended by the 2013 St. Gallen panel of experts (Goldhirsch *et al*., 2013), we found that *luminal-B* (58.5%) was the most prevalent molecular subtype of breast cancer in our study, which is supported by studies that showed high prevalence of *luminal-B* molecular subtypes in European (57.1%) and Chinese (54.3%) women (Maisonneuve *et al*., 2014; Wei *et al*., 2017). Similarly recent studies in Africa like, Mozambique (49%), South Africa (42.2%), and Kenya (35.8%) showed relatively predominant *luminal-B* molecular subtype (Brandão *et al*., 2020; Kakudji *et al*., 2021; Sayed *et al*., 2021).

However, when we classified tumors by the expression of ER, PR, and HER2, *luminal-A* became the most frequent subtype (75.6%) followed by *luminal-B* (17.1%), TNBC (4.9%) and HER2-*positive* (2.4%), which is similar with the previous studies done in Ethiopia that have shown the most prevalent subtype was the *luminal-A* (40%-54%) followed by *luminal-B* (Shenkutie *et al*., 2017; Hadgu *et al*., 2018) and also considerably in agreement with studies done in Algeria (50.6%) and Guinea (34.5%) (Cherbal *et al*., Traoré *et al*., 2019). These might be due to differences in the classification methods between studies and there could be inter-laboratory variation in assessment of the expression of ER, PR, HER2, and Ki-67 (Maisonneuve *et al*., 2014).

Our study showed that the concordance between endpoint RT-PCR and IHC was poor for ER with kappa value < 0.20 and OPA 68.3% and for PR 39.0% OPA and <0.20 kappa value, while moderate concordance for HER2 with 82.9% OPA and 0.481 kappa value. Our finding was relatively less when compared to previous studies which showed good to high concordance with highest OPAs for ER (91.8-99%), PR (82.5-94%), and HER2 (86-97%) (Bastien *et al*., 2012; Wirtz *et al*., 2016; Sinn *et al*., 2017; Wu *et al*., 2018; Janeva *et al*., 2021) while PR and HER2 showed high specificity 92.3% and 87.9% respectively and it was relatively similar with previous studies, 87.7% for PR and 93.5% for HER2 (Bastien *et al*., 2012; Wirtz *et al*., 2016; Wu *et al*., 2018).

The molecular subtyping with IHC and endpoint RT-PCR showed fair agreement (OPA 56.1%, 0.20 kappa value). Relatively less when compared to other studies 91.6% concordance with 0.885 kappa value. In our study, *Luminal-A* molecular subtype similarly classified with 61.3% (19/31) by both IHC and endpoint RT-PCR. These finding was relatively approximated with other study that showed 65.4% similar classification (Wirtz *et al*., 2016). In addition, our result showed similar molecular subtyping with 42.9% *Luminal-B* and 50% TNBC, which shows relative discrepancies when compared to previous studies 61.2%, and 85.7% of *luminal-B* and 87.1% and 62.5% of TNBC (Wirtz *et al*., 2016; Janeva *et al*., 2021). In This study HER2-*positive* subtype didn't show similar classification (however, we can't say that there was no agreement regarding the HER2-*positive* subtype because from the entire cases there was only one HER2-*positive* sub-

type) in contrast to previous studies (79.6% and100%) (Wirtz *et al*., 2016; Janeva *et al*., 2021).

The main explanation for the less OPA compared to previous studies may be because this study used endpoint RT-PCR to analyze gene expression, whereas other studies use real time RT-qPCR. Variability in the management of pre-analytical samples and the scoring technique could also be a factor (Wu *et al*., 2018). Another reason for discrepancies in ER, PR, and HER2 assessment using both methods, as well as misclassification of breast cancer molecular subtyping using both methods, is that IHC may be biased in favor of selected or representative tumor areas, whereas PCR gene expression assays frequently analyze gene levels in the entire tumor mass (which is a reflection of the average gene expression in the entire tissue slice) (Bastien *et al*., 2012; Sinn *et al*., 2017; Janeva *et al*., 2021).

Furthermore, discrepancies between mRNA expression as measured by endpoint RT-PCR and protein expression as measured by IHC, such as high mRNA expression but low corresponding protein, or vice versa. This may be due to the imbalance between post-transcriptional and post-transnational modifications. And mRNAs are less stable than proteins; this might be a contributing factor to low mRNA expression despite high protein expression. As well as protein degradation may be the factor (Greenbaum *et al*., 2003; Sinn *et al*., 2017).

6. Conclusion

A total of 41 eligible cases of breast cancer were enrolled in this study. The goal of this study was to compare IHC to PCR based technology for breast cancer molecular subtyping. Breast cancers in our study were high grade tumors diagnosed at an advanced stage and at a young age. However, there was no statistically significant association between socio-demographic characteristics, clinico-pathologic characteristics, and breast cancer risk factors and IHC markers and molecular subtypes. Hormone receptor positive patients were more common, and IHC revealed a high expression of Ki-67. *Luminal*-B breast cancer was more common than *luminal*-A, according to IHC expression of ER, PR, HER2, and Ki-67 classification of molecular subtypes. The ER, PR, and HER2 status, as well as molecular subtypes approximated based on the abovementioned three biomarkers, were used to compare the endpoint RT-PCR and IHC in our study. Our findings indicated that endpoint RT-PCR and IHC had an overall fair level of agreement. This study suggests that endpoint RT-PCR may have a promising future role in bringing a potential solution to the problem of limited access to breast cancer diagnostics in low-resource countries, like Ethiopia.

7. Strength and limitation of the study

7.1. Strength of the study

Our study is unique in Ethiopia since it is the first to compare endpoint RT-PCR with IHC for the molecular classification of breast cancer. Normal adjacent tissue was used as a healthy control to show molecular changes associated with cancer. Another strength of this study is that Ki-67 expression level was measured using IHC. In addition, there was no borderline positivity or equivocal for HER2 as determined by IHC.

7.2. Limitation of the study

Due to the lack of literatures on molecular subtyping of breast cancer using endpoint RT-PCR, we have discussed our result with that of real time RT-qPCR. Some fresh frozen tissue samples were fat (didn't contain cancer cells) which result in decreased sample size. FFPE tissues were used for endpoint RT-PCR which could have had the impact on the result. And I didn't take part in the laboratory analysis, which was crucial for gaining experience and exposure to practical laboratory work.

8. Recommendation

Our findings demonstrate the molecular status of breast cancer and imply that hormone therapy may benefit the majority of breast cancer patients in Ethiopia. Based on this, we believe there is a need to incorporate hormone receptor, HER2, and Ki-67 testing into our routine clinical practice in order to deliver the right breast cancer treatment. Finally, more concordance research is needed in the future, with a focus on the impact of sample handling and fixation methods on the outcomes.

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ANNEXES

Annex 1. Questionnaire

Please answer every question in the questionnaire by marking “X” in the space or filling the necessary information.

I. Personal information

1. Patient code__________________
2. Age (in yr) ______________
3. Gender

Male ☐ Female ☐

4. Residential area

□ Urban □ Rural

5. If any underlying disease (specify)__________________________

II. Body Mass Index

1. Weight (in Kg) ________________
2. Height (m)_____________________
3. Body Mass Index__________________________

III. Physical Activity

1. Do you perform physical exercise?
2. □ Yes □ No
3. If Yes, how often per week?
4. □ Occasionally □ 4days □ 3days □ 2days □ Other_____

IV. Alcohol consumption

1. Do you consume alcohol?

□ Yes □ No

2. If yes, how often?

□ Occasionally □1 Bottle alcohol daily □ 2-5 Bottle alcohol daily □ other___

V. Smoking

1. Do you smoke?

□ Yes □ No

1. If yes, how often?

□ Occasionally □1 cigar daily □ 1 pack per day □ Other_________

VI. Personal and family history of breast cancer

1. Have you ever had certain breast cancer conditions before?

□ Yes □ No

2. Have any of your close blood relatives ever had breast cancer?

□ Yes □ No

3. If yes, whom

□ One of your first degree relatives (mother, sister or daughter)

□ One of your distantly related relatives (specify) __________________________

4. Have you ever had radiation therapy to the chest area as treatment before?

□Yes □ No

VII. Pathological information (tumor characteristics)

1. Tumor stage________________________
2. Tumor size___________________
3. Histological type___________________
4. Histological grade___________________
5. Lymph node involvement___________________
6. Metastasis___________________
7. Sites of breast cancer______________________
8. Menopausal status premenopausal ☐ postmenopausal ☐

Annex 2. Study information sheet (English Version)

Research title: Comparison of Immunohistochemistry with PCR based Technology for Molecular Subtyping of Breast Cancer in Addis Ababa, Ethiopia.

Investigator: Dessiet Oma

Address: Phone: 0922346930

E-mail: dessiet.oma@aau.edu.et

Introduction

You are invited to take part in a research study. Before you decide, it is important for you to understand why the research is will be done and what it will involve. Please take a time to read or listen the following information carefully and discuss it with others if you wish.

Purpose of the study

The aim of this study is to compare IHC and PCR based technology for molecular sub-typing of breast cancer. This will be important to implement available, less expensive and more accurate breast cancer molecular subtyping technique, which will be important for better treatment strategies and improve prognosis of breast cancer patients in the future.

Procedure

If you agree to participate in this study, we will require the following:

- We will collect tissue sample from breast tumor after surgery and we will use it for different tests.

- We will ask you various questions related to the study.

Potential risks and Discomforts

There will be no discomfort during tissue sample collection. Because tissue sample will be collected from removed tumor mass after surgery completed.

Confidentiality

We respect your privacy and confidentiality. Any information that identifies you will not be shared with anyone else outside the study team. The information we will collect from you as part of the study will be kept in a locked file cabinet, or be protected by a password on the computer only accessible to personnel involved in the study. There is no sensitive issue that you will be asked related with your social desirability but any information that is obtained in connection with this study and that can be identified with you will remain confidential.

Sharing the result

At the end of the study, the study findings will be published and they will be disseminated to other scientists through publication, and also to your doctor, if needed, so that further medical care can be pursued.

The right to refuse

The participation is completely voluntary and you have the right not to participate in this study. You may withdraw at any time and place without consequences of any kind. You may also refuse to give any sample. You can ask any questions regarding to this study and you have a right to get a laboratory diagnosis result for free.

Annex 3. Consent Form (English Version)

Patient code number ________________

By name below, I confirm that I have read and understood this informed consent. I understand that this is a research study and that my participation is voluntary. I understand that I may change my mind about participating at any time, without my medical care or legal rights being affected. I have had the opportunity to ask questions and my questions have been answered. I have been given adequate explanation and understand the purpose, procedures, risks and benefits of the research study. By signing this form, I give my permission for the researchers to have access to my tissue sample from breast tumor removed after surgery for the study.

____________________	________________	________________
Name of participant	date	signature
____________________	________________	________________
Name of investigator	date	signature

Annex 4. Study information sheet (Amharic version)

አዲስ አበባ ዩኒቨርሲቲ

የጤና ሳይንስ ኮሌጅ

የሕክምና ፋካልቲ

ባዮኬሚስተሪ ት/ክፍል

የጥናቱ ተሳታፊዎች የመረጃ ቅፅ

የጥናቱ ርዕስ፡- የጡት ካንሰር አይነቶችን የሚለይ የምርመራ አይነትን (Immuno-chemistry and PCR based technology) **ለማወዳደር ፡፡**

የጥናቱ ባለቤት፡- ደሴት ኦማ

አድራሻ፡- ሞባይል 0922346930

ኢ-ሜይል፡- dessiet.oma@aau.edu.et

መግቢያ፡-እባክዎ በዚህ ጥናት ላይ ለመሳተፍ ከመወሰኖ በፊት ጥናቱ ለምን እደሚካሄድና ምን ምን ዓይነት ነገሮች እንደሚያስፈልጉት ለማወቅ ጥቂት ግዜ ይወስዱና የሚከተለውን ስለጥናቱ በተመለከ ተመረጃ ይመልከቱ፡ አስፈላጊ ከሆነም ከሌሎች ሰዎች ጋር ይወያዩ፡፡ ማንኛውም ግልፅ ያልሆነ ነገር ካለ ወይም ተጨማሪ መረጃ ከፈለጉ የጥናቱ ባለቤትን መጠየቅ ይችላሉ፡፡

የጥናቱ አላማ፡-የዚህ ጥናት ዋና አላማ የጡት ካንሰር አይነቶችን የሚለይ የምርመራ አይነት በማወዳደር በዋጋ፣ በጥራት እና በብቃት የተሻለውን የምርመራ አይነት እዚህ አገራችን ላይ ተግባራዊ ለማድረግ፤ ይህም ለጡት ካንሰር ህመምተኞች የተሻለ የምርመራ ዘዴን በመፍጠር ከትክክለኛ መድሓኒት ጋር እንዲገናኙ ያደርጋል፡፡

የጥናቱ ሂደት፡-በዚህ ጥናት ላይ ለመሳተፍ ፍቃደኛ ከሆኑ ከእርሶ የሚጠበቁት የሚከተሉት ናቸው፡፡

1. ምርምሩን ለመስራት የሚያስፈልገው የtissue ናሙና ከቀዶጥገና በሃላ ጥናቱን ለመስራት ብቻ እሚውል፡፡

2. የጥናቱ ባለቤት ጥናቱን በተመለከተ አንዳንድ ጥያቄዎችን ሊጠይቅ ይችላል፡፡

ጉዳት፡- ከዚህ ጥናት ጋር በተያያዘ በጤናም ሆነ በሚያገኙት ተገቢ ህክምና ምንም አይነት ጉዳት ስለማያስከትል አይስጉ፡፡

ሚስጥራዊነት፡- ማንኛውም ከዚህ ጥናት ጋር የተያያዘ የግል መረጃ ሚስጥራዊነት የተጠበቀ ነው፡፡ ስለዚህ የጥናቱ መረጃ ይፋ የሚሆነው ለእርሶ ብቻ ነው፡፡ስለሚወሰደው ማንኛውም መረጃዎች ሆነ የጥናት ውጤት ለማሰራጨት በስም ሳይሆን በሚስጥር (ኮድ) የሚመዘገብ ይሆናል፡፡

የተሳትፎ መብት፡-በዚህ ጥናት መሳተፍ ሙሉ በሙሉ በርሶ ፍቃድ የተመሰረተ መሆኑን ልናሳስብ እንወዳለን፡፡ በመሆኑም በማንኛውም ግዜ ምንም ዓይነት ምክንያት ሳይሰጡ ከጥናቱ ራስን የማግለል መብት የተጠበቀ ነው፡፡የሰጡት tissue ናሙና ለዚህ ጥናት እንደሚውል ማድረግ በእርሶ ሙሉ ፈቃድ ብቻ ሲሆን በጥናቱ ላይ ለመሳተፍ መወሰን ወይም አለመወሰን መድሐኒት ወይም ሌላ የጤና አገልግሎት የማግኘት መብት አሁንም ሆነ ለወደፊቱ ምንም አይነት ተፅእኖ አያሳድርብዎትም፡፡

Annex 5. Consent Form (Amharic version)

የተሳታፊዎች ስምምነት ማረጋገጫ

የሚስጥር ቁጥር ___________________

የተሳታፊዉ ስም ___________________

እኔ ስሜ ከላይ የተጠቀሰው ተሳታፊ "የጡት ካንሰር አይነቶችን የሚለይ የምርመራ አይነትን (Immunochemistry and PCR based technology) **ማወዳደር ፡፡" ጥናት ላይ በቂ ገለጻ ተደርጎልኛል፡፡ ለጥናቱም የ**tissue **ናሙና እንደሚያስፈልግ ተገልጾልኛል፡፡ የጥናቱንም አላማዎችም ተረድቻለሁ፡፡**

በመጠይቁ ላይ የገለጽኳቸው መረጃዎች በሙሉ በሚስጥር የተጠበቁ እንደሚሆኑ ተነግሮኛል፡፡ በጥናቱ ላይ ያለመሳተፍና ማንኛውንም መረጃ ለመስጠት እንዲሁም በማንኛውም ጊዜ ከጥናቱ ራሴን የማግለል መብቴ የተጠበቀ እንደሆነ ተገልጾልኛል፡፡ ስለዚህ ለዚህጥናት መረጃና የስምምነት ቃሌን የሰጠሁት በአጠቃላይ ሁኔታውን በመረዳትና በፍጹም ፍቃደኝነት ነው፡ በተጨማሪም ጥያቄ ለመጠየቅ ተፈቅዶልኝ ለማወቅ የፈለኩትን ያህል ማብራሪያ አግኝቻለሁ፡፡ የዚህጥናት ተሳታፊ በመሆኔ የማገኘው ጥቅም የሁሉንም ምርመራ ውጤት በነጻ ማግኘት እንደሆነ ተረድቻለሁ፡፡በ አጠቃላይ እኔ ከላይ በመተማመኛ ቅፅ የተጠቀሱትን ሁሉ በሚገባና በተረጋጋ መንፈስ አንብቤዋለሁ፡፡ ስለዚህ በዚህ ጥናት ለመሳተ ፍፈቃደኛ መሆኔን በፊርማዬ አረጋግጣለሁ፡፡

ፊርማ ___________________ **ቀን** ___________________

(**የስምምነት ቅጹን ማንበብ ለማይችሉ ተሳታፊዎች**)

የአማካሪ ነርስ ስም __________________________ **ፊርማ** _________________

ቀን ___________________