ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

DEPARTMENT OF BIOCHEMISTRY

CIRCULATING BREAST CANCER-ASSOCIATED microRNA EXPRESSION IN SERUM AS BIOMARKER FOR BREAST CANCER DETECTION

A Thesis Submitted to Addis Ababa University, School of Graduate Studies, Department of Biochemistry in Partial Fulfillment of the Requirement for the Degree of Master of Sciences in Medical Biochemistry

By: Tariku Sime (BSc.)

January, 2019

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This is to clarify that thesis prepared by Tariku entitled, "Circulating Breast cancer-associated microRNA expression in serum as Biomarker for Breast cancer detection" is submitted in partial fulfillment of the requirement for the Degree of Master of Sciences in Medical Biochemistry, complies with the regulations of the university and meets the accepted standards with respect to the originality and quality.

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List of Abbreviations

AHRI	Armauer Hansen Research Institute
ALERT	All Africa Leprosy, Tuberculosis and Rehabilitation Training Centre
ANOVA	Analysis of Variance
AUC	Area under the Curve
BC	Breast Cancer
BRCA1	Breast cancer gene 1
BRCA2	Breast Cancer gene 2
CA 15-3	Carbohydrate Antigen 15-3
CEA	Carcinoembryonic Antigen
cDNA	complementary DNA
DGCR8	DiGeorge Syndrome chromosomal region 8
ER	Oestrogen Receptor
HER2	Human Epidermal growth factor Receptor 2
IARC	International Agency for Research on Cancer
IHC	Immunohistochemistry
MCF-7	Michigan Cancer Foundation-7
MDA-MB-468	Human Black Breast Adenocarcinoma
miRNAs	micro Ribonucleic Acid
PDCD4	programmed cell death 4
PR	Progesterone receptor (PR)
PTEN	phosphatase and tensin homolog
QRT-PCR	Quantitative Reverse transcriptase polymerase chain reaction
ROC	Receiver Operating Characteristic

TRBP	Transactivation response element RNA binding protein
TNM	Tumor, Node, Metastasis staging system
TASUH	Tikur Anbessa Specialized University Hospital

ABSTRACT

Background: Breast cancer (BC) is a type of cancer that develops/grows in breast tissues**.** It is one of the leading causes of death among women worldwide. Breast cancer is the most frequent cancer and the second leading cause of cancer death among women in industrialized countries. Although it is curable if diagnosed and treated appropriately at an early stage of the disease, diagnosis remains a challenge to the clinicians. The value of current histological prognostic indicators in predicting the course of the disease is weak and reliable markers are still lacking. Therefore, these gaps have led to significant interest in the quest for minimally invasive markers that are sensitive enough to detect early the presence of tumours in patients but not in healthy tumour-free individuals.

Objective: was to investigate the capability of circulating microRNAs in serum to discriminate breast cancer patients from healthy controls**.**

Methods: The study was conducted from June 2017 to August 2018 at Tikur Anbessa Specialized University Hospital.A case control study design was conducted involving 50 participants (25 cases, 25 controls). Five milliliter of blood samples were collected and serum was separated from each patient and healthy controls. miRNAs were extracted from serum samples and their relative expression levels were determined by quantitative real time *PCR*. Data were analyzed using SPSS software (version 25.0, Chicago, IL, USA) and Graph pad Prism version 7. After CT values were analyzed using Microsoft Excel 2010, independent t test and one way ANOVA was done to assess significance of the difference between patients and controls. Data were presented as mean ± SD. Values were considered statistically significant when P-value was less than 0.05.

Results: We reported that breast cancer specific circulating miRNAs level showed significant difference in breast cancer patients as compared to healthy controls. The expression level of miR-21 and miR-195 were significantly higher (P=0.003, P=0.042 respectively) in serum of breast cancer patients than healthy controls. While there was no significant difference in expression of miR-145 and miR-130b.Furthermore we showed that miR-21 and miR-195 expression level were higher in surgical operated breast cancer patients.

Conclusion: Circulating miR-21 and miR-195 are potential breast cancer biomarkers for breast cancer.

Keywords: Biomarkers, Breast cancer, Clinicopathological, Micro RNA

1 INTRODUCTION

1.1 Background

Breast cancer (BC) is a type of cancer that develops/grows in breast tissues (Truskett, 2014).Two different hypotheses about cell of origin of breast cancer have been proposed. One theory states that breast cancer originates from an epithelial stem cell and subsequent genetic changes determine the phenotype, while another hypothesis states that breast cancer could originate from different cells, both stem cells and progenitor cells(Olsson, 2013).Study conducted by Ince et al (2007) showed that cell of origin determines the metastatic potential of the tumor. The relation between cell of origin and hormonal status also presented by Olsson(2013) .

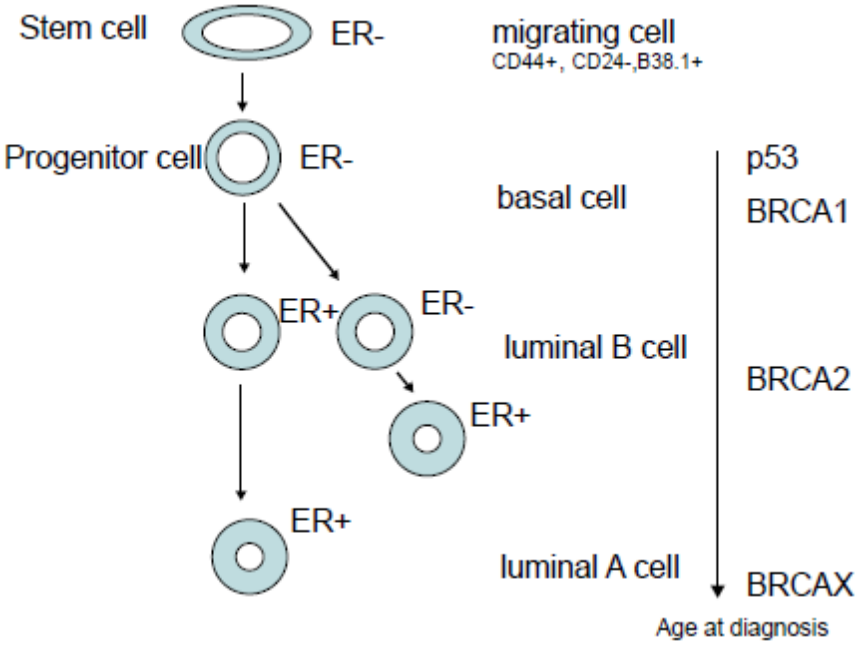


Figure 1:Suggested hierarchial order of breast cancer cell types (cell of origin) defined by expression analysis in relation to hormone receptor status (Olsson, 2013).

The above figure showed the postulation of the hypothesis which stated that breast cancer cell could originate from both stem cells and progenitor cells. Accordingly the figure showed that where tumours developing in germline mutations carriers of p53, BRCA1, BRCA2 and BRCAX preferentially have its cell of origin. On the other hand hypothesis also postulates that tumours originating in germline mutation carriers of BRCA1 mainly are ER-, in BRCA2 carriers mixed

ER+ and ER- and in BRCAX carriers mainly ER+. The origin of the BRCA1 tumors has been also associated with a luminal progenitor cell instead of a basal located stem cell. As the same time by gene expression studies BRCA1 associated tumors display a basal phenotype. The cell of origin is also related to age at diagnosis with receptor positive tumours initiated at a higher age in a more terminally differentiated tissue (Olsson, 2013).

Breast cancer is a heterogeneous disease that can be classified according to histology, immunopathology, mRNA expression profiling, and miRNAs expression signature (Bertos and Park, 2011).

Among histological classification the commonest one includes:- ductal carcinoma in situ (DCIS), invasive ductal carcinoma (IDC) and invasive lobular carcinoma are the most frequent invasive carcinomas that breach the basement membrane (Al-Zawi et al., 2018).

Immunopathologically, BC are classified according to their receptor profile as Estrogen receptor (ER), Progesterone receptor (PR) and Human epidermal growth factor receptor 2 (HER2) (Stork et al., 2018;Nassar et al., 2017).

Based on mRNA expression profile, BC are also classified as luminal A subtype, normal-like subtype, luminal B, HER2-enriched subtype and basal subtype. Besides, miRNAs signature can sub-classify BC as Luminal A, Luminal B, Her2 and basal(Liu et al., 2014; Bhattacharyya et al., 2015).

Invasive breast cancer is a breast cancer that has spread from where it began in the breast ducts or lobules to surrounding normal tissue. Breast cancer occurs in both men and women, although male breast cancer is rare (Abbott et al., 2010).Breast cancer is the most common cancer among women. Since early detection reduces rate of mortality, women should visit health facilities as soon as they see the following warning sign on themselves: a lump in breast, pain in the armpit, pitting or redness of skin of breast, swelling (lump) in one of the armpits, an area of thickened tissue in a breast, discharge from one of the nipples, sunken or inverted nipple, peeling, scaling, or flacking of skin covering the breast or the nipples (Montazeri et al., 2008; Amjad et al., 2018).

There are many risk factors associated with breast cancer. Even-though, it is difficult to put exact causative agents, the major risk factors include: age and gender, family history of breast cancer, race and ethnicity, life style and diet, reproductive hormone levels, exposure to ionizing

radiation, smoking, weight gain, early age at menarche, late age at menopause, late age at first full-term pregnancy,environmental and genetic factors, physical inactivity, and the availability of early detection services(Norman, 2008; Eccles et al., 2013).

Breast cancer is diagnosed and surveyed by standard imaging modalities such as ultrasound and mammography. Moreover, axillary ultrasound is frequently used to assess axillary nodes at the time of initial diagnosis of breast cancer. The use of axillary ultrasound with percutaneous biopsy was initially used to determine clinical nodal stage in order to guide surgical staging and allow patients to proceed directly to axillary lymph node dissection and avoid sentinel lymph node surgery in cases with biopsy-proven nodal metastasis. However, standard imaging modalities may not detect early stage breast cancer in dense breast tissues, and may not be accessible for periodic check-ups and surveillance. In addition, biopsy samples taken through invasive procedures are required to confirm breast cancer diagnosis (Taplin et al., 2008).To this end, there is an intense interest in identifying serum (plasma) biomarkers that may delineate the presence, absence or extent of the disease when tumors cannot be visualized. Biomarkers are potentially useful for prognosis monitoring and they are conceivably valuable in surveillance. There is an attempt to identify biomarkers by different researchers and mounting evidence suggests that a variety of serum/plasma biomarker role for systematic clinical use to monitor breast cancer progression (Abbott et al., 2010; Boughey et al., 2016).

The study conducted by Cabeza et al(2017) elaborated biological role of non-coding RNAs in tumor genesis, disease prognosis and treatment outcome. MiRNAs, one class of non-coding RNAs, theoretically serve as a good candidate as biomarkers: they reflect recent expression status of the body as opposed to DNA and yet have comparable stability for laboratory handling; relatively lower cost of detection than protein biomarkers as there is no need to purify the target miRNAs to produce the assay kits (Andorfer et al., 2011).In addition, miRNAs have been associated with treatment outcome of breast cancer. For example, the effectiveness of doxorubicin, one of the drugs used for treatment of breast cancer disease, depends on miRNAs levels (Gao et al., 2016). Currently, the issue of treatment effectiveness depending on miRNAs forwarded that miRNAs are good candidates as minimally invasive breast cancer diagnosis and prognostic markers (Koduru et al., 2017).

1.2. Literature Review

1.2.1. Epidemiology and Burden of Breast cancer disease

Globally in 2008 approximately 1.3 million women develop breast cancer. As the same time breast cancer is the leading cause of cancer death among females accounting for 23% of the total cancer cases and 14% of the cancer deaths (Jemal et al., 2011). Youlden et al(2012) reported that female breast cancer resulted in a total of 5,884,000 loss of life globally in 2004.While the current global cancer statistics showed that BC remained the most frequently diagnosed cancer in women and the leading cause of cancer death worldwide, with an estimated 1.7 million new cases and 521,900 deaths in 2012. This accounts for 25% of total new cancer cases and 15% of all cancer deaths among females (Azubuike et al., 2018;Mar-Aguilar et al., 2013).It is the most frequent cancer and the second leading cause of cancer death among women in industrialized countries. American Cancer Society in 2014 reported that an estimated 231,840 (29%) new cases of invasive breast cancer were expected to be diagnosed among women in the US during 2015, compared with 105,590(13%) cases of lung cancer in the same population.

Yet the majority of breast cancer deaths occur each year in developing countries (Anderson et al., 2008).Despite the substantial improvement in breast cancer prognosis and survival, more than half of the breast cancer cases and 60% of the deaths are reported in economically low- and middle-income countries(Yip et al., 2008; Anderson et al., 2008).It is an urgent public health problem in high-resource regions and is becoming an increasingly urgent problem in low-resource regions, in which incidence rates have been increasing by up to 5% per year(Anderson et al., 2008).It is reported that beginning from 1990 global breast cancer incidence increased by 0.5% annually (Porter, 2008).

Over the past two decades, incidence and mortality trends had remained relatively stable and even decreasing in many developed countries. However, it had been on the rapid increase in many parts of Africa, Asia, central and South America(Shin et al., 2010; Jemal et al., 2011).

The burden of BC in sub-Saharan Africa in 2012 was approximately 100,000 occurrences per year and 49,000 deaths per year. In most countries of sub-Saharan Africa, following cervical cancer, breast cancer is the second most common cancer type among women(Trimble, 2017).

In many sub-Saharan African countries BC has now become the most commonly diagnosed cancer in women. In Ethiopia, breast cancer is typically a fatal disease with high mortality, unlike the experience of the western world where breast cancer is frequently treatable and with lower mortality (Dye et al., 2012).A cohort study conducted from 2005–2010 at Addis Ababa University Radiotherapy Center,on breast cancer survival showed that a 5-year distant metastasis-free survival was 72% for stages I and II and 33% for stage III (Kantelhardt et al., 2014). Breast cancer is the commonest cancer, constituting 33% of the cancers in women and 23% of all cancers identified from the Addis Ababa cancer registry (Memirie et al., 2018).

1.2.2. Breast cancer risk factors

To pin point why incidence is increasing is difficult; but there are some risk factors that contribute to the international variation in incidence rates and that increase one's chances of getting breast cancer: age and gender, family history of breast cancer, race and ethnicity, life style and diet, reproductive hormone levels, exposure to ionizing radiation, smoking, weight gain, early age at menarche, late age at menopause, late age at first full-term pregnancy,environmental and genetic factors, physical inactivity, and the availability of early detection services (Colditz et al., 2006; Eccles et al., 2013; Norman, 2008).Researchers also stated that breast cancer might be due to increased sensitivity to radiation in individuals who carry certain genetic defects, such as mutations in ATM (Ataxia telangiectasia mutated), BRCA1 (Breast cancer 1) or BRCA2gene (Colditz et al., 2006).

Next to genetic mutations environmental factors have been reported as the major risk factors for the development of breast cancers. Mutations in genes like BRCA1, BRCA2, PIK3, RB, MDM2, TPK53 and HER2 that affect the body differently result in different types of breast cancer. Among this, genetic mutations in any of the two genes namely BRCA1 and BRCA2 account for 90% of breast cancer (Amjad et al., 2018).On the other hand, Kamińska et al(2015) reported that intrinsic factors such as age, sex, race, genetic makeup promoting familial occurrence of neoplastic disease or the occurrence of benign proliferative lesions of the mammary gland played role in as important risk factor. Extrinsic factors conditioned by lifestyle, diet or long-term medical intervention and their influence on the neoplastic process may be modified to a certain degree.

1.2.3. Types of Breast Cancer

Currently, it has been reported that different types of breast cancer are caused by different types of mutations in genes that affect the body differently (Amjad et al., 2018).Breast cancer is not a single disease but rather a heterogeneous one that could be classified according to histology, immunopathology, mRNA expression profiling, and miRNAs expression signature (Bertos and Park, 2011).

Histologically, ductal carcinoma in situ (DCIS) is the most common non-invasive breast cancer, while invasive ductal carcinoma (IDC) followed by invasive lobular carcinoma are the most frequent histotypes of invasive carcinomas that breach the basement membrane (Al-Zawi et al., 2018). On the other hand, less common histotypes includes: medullary, neuroendocrine, tubular, apocrine, metaplastic, mucinous, inflammatory, comedo, adenoid, cystic and micropapillary type (Nassar et al., 2017).

Immunopathologically, breast cancers are classified according to receptor profile that includes Estrogen receptor (ER), Progesterone receptor (PR) and Human epidermal growth factor receptor 2 (HER2) (Stork et al., 2018; Nassar et al., 2017; Bianchini et al., 2016). Receptor based classification will helps us in judgment of treatment outcome, level of prognosis, and optimal treatment. For estrogen receptor (ER)-positive patient, endocrine therapy (ET) should be considered complementary to surgery in the majority of patients.The effective treatment for HER2+ (ER-, HER2+) and triple positive (ER+, PR+, HER2+) tumours were HER2-targeted agents like trastuzumab monoclonal antibody. In addition to this, chemotherapy is the primary established systemic treatment for patients with triple-negative breast cancer (TNBC) in both the early and advanced-stages of the disease (Bianchini et al., 2016).

Based on mRNA expression profile breast cancer is classified as luminal A subtype, normal-like subtype, luminal B, HER2-enriched subtype and basal subtype. Besides, miRNAs signature can sub-classify breast cancer as basal-like and luminal tumors (luminal A and luminal B) (Liu et al., 2014, Dai et al., 2014).

1.2.4. Breast cancer Staging

By the first half of the 20th century, clinicians had become aware that they should stage breast cancer for effective treatment and better prognosis. The current methods of classifying breast cancer patients into prognostic groups, which influences therapeutic decisions, are based on clinicopathological parameters such as tumour size and grade, presence or absence of lymph node metastases, and distant metastases which are derived from careful histological analysis of primary breast cancer samples. The TNM (Tumor size, Nodes, and Metastasis) system, considered as gold standard method in routine use, integrates these into tumor stages that have major prognostic value (Singletary and Connolly, 2006; Weiss et al., 2018).

However the recent way of staging breast cancer according to the American Joint Committee on Cancer (AJCC) has published a staging system that incorporates contemporary biologic factors (biomarkers) into the traditional anatomic staging system. In addition to TNM, the receptor profile like ER, PR, HER2, grade, and multigene assays are widely used in practice to define prognosis and determine therapy (Giuliano et al., 2018).

1.2.5. Breast cancer diagnosis and treatment

The most available tool for the early detection and diagnosis of BC is mammography. However, it has limitations due to its use of ionizing radiation, and dense breast tissue decreases its detection sensitivity with optimal performance- only 20% among those <50 years of age. Moreover, the variable utilities of mammography to detect different types of tumors diminishes its effectiveness (Taplin et al., 2008; Board, 2018).

Tumor markers, which are proteins or enzymes produced by tumor cells or generated by host cells in response to tumorigenesis, are frequently used for screening and monitoring in oncology. The expression of tumor-specific antigens varies, and, tumor cells express several different unique antigens.At present, only two biomarkers, ER for predicting response to endocrine therapies and HER2 for predicting response to Trastuzumab, have been established and are assessed routinely in breast cancer marker (Pinto et al., 2013). So for effective cancer screening, combination of multiple markers are used. Furthermore, the most common technique to evaluate these hormone receptor statuses of breast tumours is immunohistochemistry (IHC) which relies

on recognition of the receptor protein by specific antibodies. But this method is subjective, time consuming and need professional personnel to perform unlike that of circulating tumor markers such as carcinoembryonic antigen (CEA) and carbohydrate antigen 15-3 (CA 15-3) which are clinically used as prognostic markers and to monitor breast cancer treatment success and follow-up. However, many studies do not recommend these markers for diagnosis due to low sensitivity and lack of detection ability for early stage disease (Uehara et al., 2008; Harris et al., 2007). Due to this, there is a need of new biomarkers that are easily accessible, and sensitive enough to detect early presence or absence of tumor. Extensive research has shown that microRNAs are dysregulated at all stages of breast cancer and microRNA detection is potentially one of minimal invasive method of detection of cancer (Zhu et al., 2009).

Breast cancer is treated in several ways. It depends on the kind of breast cancer and how far it has spread. People with breast cancer often get more than one kind of treatment. The treatment option for breast cancer patients includes: surgery, chemotherapy, hormonal therapy, biological therapy and radiation therapy (Pinto et al., 2013; Norman, 2008; Lumachi et al., 2015).

1.2.6. Breast cancer and microRNA

MicroRNAs (miRNAs) are a class of small, non-coding, endogenousRNA molecules (18–25 nucleotides in length) that regulate gene expression by interacting with target coding mRNAs either by degrading mRNA molecules on a post-transcriptional level or blocking their translation (Nassar et al., 2017; Jackson and Standart, 2007).By affecting gene regulation, miRNAs are likely to be involved in many human biological and cellular processes such as growth, apoptosis, development, hematopoietic cell differentiation, metabolism and tumorigenesis (Vidigal and Ventura, 2015; Kloosterman and Plasterk, 2006). Most of tumor-related miRNAs function as tumor suppressors or oncogenes and modulate many aspects of carcinogenesis, including cell proliferation, cell-cycle control, metastasis and angiogenesis as well as anticancer drug resistance (Landskroner-Eiger et al., 2013; Cho, 2010).

1.2.6.1 miRNAs Biogenesis

MiRNAs, generally transcribed by RNA polymerase II, are made as large RNA precursors, called pri-miRNAs. miRNA transcription genes are regulated through the modulation of various

transcription factors as that of protein-coding genes. Researchers have stated that there are 1048 human miRNAs which are encoded by 1000 miRNA genes (Fu et al., 2011).

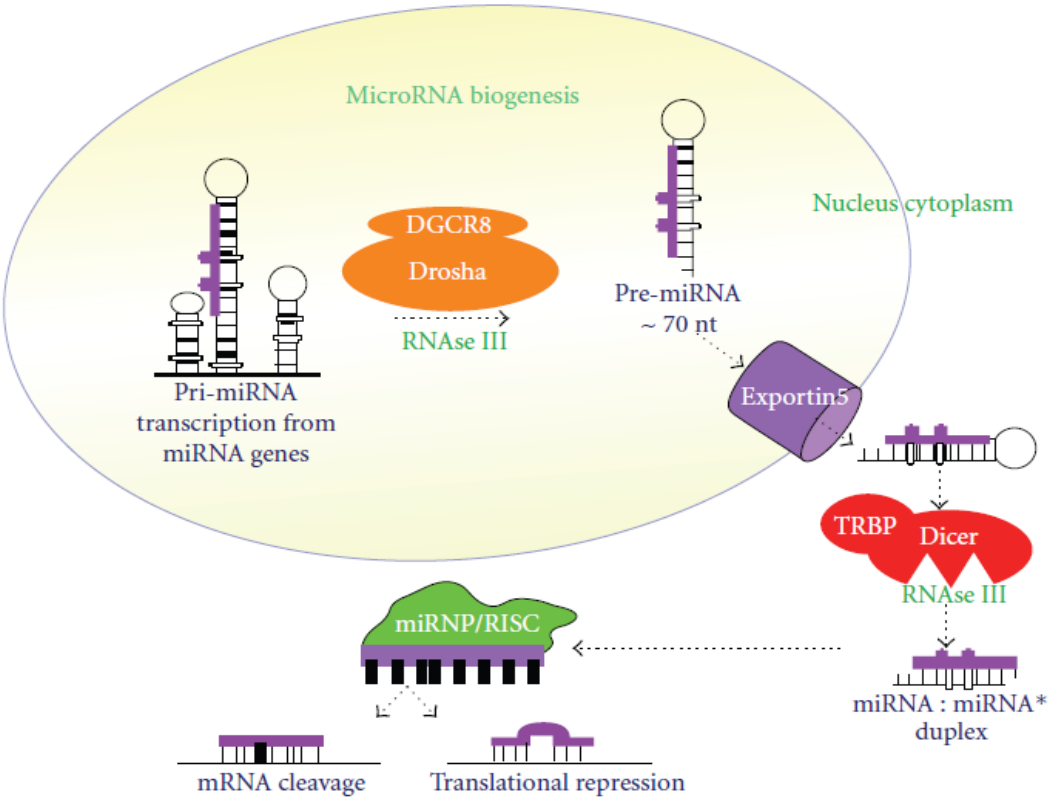


Figure 2:MiRNA biogenesis and processing in human cells *(*Heneghan *et al., 2010a)*.

The above figure elaborates a two-step process in human microRNA biogenesis. The nucleus process and the cytoplasm process. Accordingly, the multi-step process begins in the nucleus where the RNase III enzyme Drosha, coupled with its binding partner DGCR8, cleaves nascent miRNA transcripts (pri-miRNA) into ~70 nucleotide precursors (pre-miRNA).These pre-miRNAs consist of an imperfect stem-loop structure. Pre-miRNAs are then exported from the nucleus into the cytoplasm by Exportin 5.In the cytoplasm, the hairpin precursors are cleaved by Dicer and its binding partner the transactivator RNA-binding protein(TRBP)into a small, imperfect dsRNA duplex (miRNA :miRNA∗) that contains both the mature miRNA strand and its complementary strand. The miRNA strand is incorporated into the miRNP complex and

targets complementary mRNA sequences, exerting its functionality via mRNA cleavage or translational repression (Heneghan et al., 2010a;MacFarlane and Murphy, 2010).

1.2.6.2. miRNAs role in breast cancer

A single miRNA can regulate multiple mRNAs and a single mRNA can be targeted by a number of different miRNAs. Studies showed that half of the known mature human miRNAs are located in fragile chromosomal regions harboring DNA amplifications, deletions or translocations, and their expression is frequently deregulated during tumor development, which contributes to tumor progression(Fu et al., 2011; Calin et al., 2004). Researchers also forwarded that in breast cancer tissues, miRNAs have been shown to function as either oncogenes or tumor suppressors and circulating miRNAs may correlate with disease progression, therapeutic responses and patient survival(Han et al., 2017).

On the other hand, Iorio et al(2005) reported that 29 miRNAs were aberrantly expressed in human breast cancer patients' tissue compared to healthy controls; most significantly miR-10b, miR-125b, and miR-145 were down-regulated, whereas miR-21 and miR-155 were up-regulated. Asaga et al(2011) also reported in their study that the serum miR-21 had diagnostic and prognostic potential in breast cancer. Ever since 2008, when the presence of circulating miRNAs, was first demonstrated in cell-free bodily fluids such as plasma and serum (Lawrie et al., 2008), circulating miRNAs have been suggested to be able to distinguish breast cancer patients from healthy controls.

One study evaluated a panel of 7 miRNAs (miR-10b, miR-21, miR-145, miR-155, miR-195, let 7a, and miR-16). The evaluated miRNAs had different expression patterns in tumour tissues and blood samples of patients with primary BC compared to healthy controls. Of these elevated circulating miRNAs, miR-195 was found to be breast cancer specific and it could significantly differentiate breast cancer from other cancers and from healthy controls (Heneghan et al., 2010b).In addition, Mar-Aguilar and colleagues (2013) showed that circulating miRNAs have potential use as breast cancer biomarkers for early stage disease and they may also prove to be useful in clinical management during the peri-operative period. The same study found that miR-10b, miR-21, miR-125b, miR-145, miR-155, miR-191 and miR-382 had different expression

patterns in serum of breast cancer patients compared to healthy controls, with miR-145, miR-155 and miR-382 showing better elevation levels in BC serum than normal(Mar-Aguilar et al., 2013).

Even if the aforementioned studies and others suggested the presence of miRNAs in circulation and their potential use as novel molecular biomarker for breast cancer detection, further prospective validation of these blood-based miRNAs in breast cancer is needed to explore the exciting potential of circulating miRNAs to emerge as clinically useful novel biomarkers for breast cancer. Beyond these, miRNAs also play role in treatment of breast cancer. Scholars also stated that Multidrug resistance-associated protein 1 (MRP1) is an important efflux transporter and overexpression of MRP1 usually leads to chemo-resistance in breast cancer. However, the resulting chemo-resistance is resolved by miR-145.Overexpression of miR-145 sensitized breast cancer cells to doxorubicin. But they didn't associate with cardio toxicity effect of doxorubicin and the variation seen on miRNAs (Gao et al., 2016).

In addition, there have been some researches indicating that miRNAs could influence the milk fat metabolism. Additionally, overexpressing miR-130b impaired adipogenesis while inhibiting miR-130b expression enhanced adipogenesis in goat mammary epithelial cells(Chen et al., 2015). Wang et al(2018) reported the role of MiR-130b in bladder cancer, which promotes cell migration and invasion by targeting Phosphatase and Tensin Homolog Deleted from Chromosome 10 (PTEN). While in breast cancer,PTEN accelerates the malignant progress of triple-negative breast cancer by mediating the repression of the cyclin G2 gene 20.In ER-positive breast cancer, over expression of miR-130b can induce multidrug resistance, this result in treatment failure and poor prognosis.Beyond this miRNAs role was also mentioned in effectiveness and prognosis of the diseases by different scholars.

In the present study, qRT-PCR assay was performed to detect the expression of miR-21, miR-130b, miR-145, miR-195. Hs-RNU6 was used as endogenous control in BC and corresponding healthy control serum sample.

1.3. Statement of the problem

Globally, breast cancer is the most common cancer among women, comprising 23% of the total cancer cases.It is also the primary cause of cancer-related deaths in low-income countries. One in eight women born today will be diagnosed with breast cancer at some time in her life (Birhane et al., 2015). Researchers also showed that BC is the most common diagnosed cancer next to skin cancers among women in the United States and it accounts for nearly 1 in 3 cancers. It is also the second leading cause of cancer death among women after lung cancer (DeSantis et al., 2014). Breast cancer is also one of the public health problems in developing countries like Ethiopia(Dye et al., 2012). Breast cancer has now become the most commonly diagnosed cancer in women in several sub-Saharan African countries. The burden of BC is likely to increase, reflecting the increase in the prevalence of known risk factors associated with urbanization. In Ethiopia, estimated age-standardized death rates of 11.8 per 100,000 females is reported (Kantelhardt et al., 2014). Breast cancer is the commonest cancer, constituting 33% of the cancers in women and 23% of all cancers identified from the Addis Ababa cancer registry (Memirie et al., 2018).

There are challenges in the management of breast cancer because of it is a phenotypically complex and diverse genetic disease, involving a variety of changes in gene expression and structure (Fu et al., 2016). The value of current histological prognostic indicators in predicting the course of the disease is weak and many of the molecular mechanisms underlying breast cancer progression remain poorly understood (Heneghan et al., 2010b). These dilemmas prevail in the management of breast cancer patients including unpredictable response and development of resistance to adjuvant therapies. Reliable markers are still lacking, and the most promising serum markers, CEA and CA15-3, are not sensitive or specific enough for the detection of breast cancer at an early stage (Uehara et al., 2008).Although early detection of breast cancer is vital to reduce the mortality of this disease, even with the most acceptable methods such as mammography for breast cancer detection, concerns remain for rates of misdiagnosis, missed diagnosis and over-diagnosis. In short, there is still problem in early diagnosis of breast cancer (Taplin et al., 2008).

These deficits have led to significant interest in the quest for sensitive, minimally invasive markers that can be exploited to detect early neoplastic changes thus facilitating the detection of

breast cancer at an early stage, as well as for monitoring the progress of patients with breast cancer and their response to treatments. Furthermore, this leads to an increased interest in identifying and validating serum-based biomarkers for the diagnosis of breast cancer.

1.4. Significance of the study

The primary involvement of miRNAs in the aetiology and progression of breast cancer diseases heralds these molecules as significant novel markers with potential use as diagnostic, prognostic and therapeutic tools. The identification of miRNAs might be of potential use in the development of a multimarker blood-based test to complement and improve early detection of breast cancer. Such a multimarker blood based test might for instance provide a pre-screening tool, especially for younger women, to facilitate decisions about which individuals to recommend for further diagnostic tests.

The capacity of miRNAs expression profiles to classify breast tumours according to clinicopathological variables currently used to predict disease progression highlights their potential as reliable prognostic indicators which may contribute to improved selection of patients for adjuvant therapy. Their most important advantages include the possibility of their repeated measurement in a non-invasive manner (without distress to patients) as well as their remarkable stability in plasma/serum make them attractive for breast cancer detection.

The investigation of existing miRNAs molecules coupled with the identification of novel miRNAs, and elucidation of their downstream targets, will provide a better understanding of their functional effects and thus provide greater insight into the complex and poorly understood mechanisms underlying diseases such as breast cancer. Furthermore this will provide clinicians and scientists with a new repertoire of molecular targets which could be exploited for potential therapeutic intervention or this might have potential applicability in the clinical setting as a point-of-care blood test.

1.5. Hypothesis

Selected serum circulating miRNAs level will discriminate between breast cancer patients from healthy controls, which could be useful for breast cancer detection.

2. OBJECTIVE

2.1. General Objective

The main objective of this study is to investigate the capability of circulating miRNAs in serum to discriminate breast cancer patients from healthy controls.

2.2. Specific Objective

- To determine the type and level of circulating miRNAs in breast cancer patients compared to age matched healthy controls.
- To examine the relationship between selected circulating miRNAs levels and existing clinicopathological features of breast cancer.
- To examine association between miRNAs markers in breast cancer patient with history of mastectomy.

3. MATERIALS AND METHODS

3.1. Study Area

The study was conducted in Tikur Anbessa Specialized University Hospital (TASUH) from June 2017 to August 2018. TASUH is found in Lideta sub city, Addis Ababa. This hospital gives service to the community through different departments that are actively engaged in treating patients with different types of cancer and other diseases. This University Hospital is the only oncology center currently available in Ethiopia. All cancer patients are referred and evaluated from all over the country at TASUH.Consequently, this hospital was selected for conscripting study participants to be incorporated in this study, which is expected to represent patients who are coming from all over the country.

3.2. Study Design

A case control study was designed to investigate circulating miRNAs in serum as minimal invasive biomarker for breast cancer detection.Two separate groups of study participants were included in this study: Study participants with breast cancer who came to take chemotherapy treatment and the healthy age-matched female volunteers who served as controls with no history of malignant diseases, no blood donations received and no current inflammatory condition on the basis of self-report. On the other hand, the case group was selected by the Oncologist on the basis of their clinical diagnosis. Blood sample was collected from each patients and healthy control for serum preparation. The serum sample was processed for miRNAs extraction and analyses of expression level of candidate biomarker serum miRNAs in cases against control group. Details of all patients' relevant demographic information were obtained from data collected by a PhD student at TASUH. All patients and healthy controls were enrolled into this study after obtaining their written informed consent.

3.3. Study population and participants

The study participants were grouped into patient group and control group. Patient group were females from different regions of Ethiopia diagnosed with breast cancer at TASUH. The control group were healthy women and healthy control within the study period.

I) Inclusion Criteria

Patient

- Females with breast cancer who were above 18 years old.
- Patients with breast cancer and who were willing to give informed consent.

Healthy control

- Age matched health control who was selected by clinician without any family history of cancer disease.
- Healthy controls who were willing to give informed consent.

II) Exclusion criteria

Patient

- Females with breast cancer who were less than 18 years old.
- Females with breast cancer who were with severe infection, active clinical comorbidities
- History of any other malignancy.
- Not willing to give informed consent.

Healthy control

- Age less than 18 years old.
- Not willing to give informed consent.

3.4. Study Collaborating Institutes and their Role

This study was carried out at TASUH 'with the ongoing Breast cancer study in collaboration with Armauer Hansen Research Institute (AHRI). The thematic breast cancer study project in Department of Biochemistry supported this study by coordinating health care workers (physicians, nurses, lab technicians for clinical data collection) to adhere to the specific guidelines for this study. Prior to the initiation of participants recruitment; the process of clinical data collection, identification of the participants and laboratory practice training was given to the clinical worker and laboratory technicians who were selected to participate in this study at

TASUH by the Breast cancer study project coordinators. Sample collection was performed by these trained personnel. In this study, patients' data and blood samples were shared with the ongoing Breast cancer study explicitly with the one PhD student, who is under the breast cancer study project who was working on different objective with this study. The healthy Control samples were collected from Tekle Haimanot health center around Tikur Anbessa specialized university hospital from Adult out-patient department. After blood sample collection in serum separator tube, the protocol up to serum isolation was done at sample collection site and the serum was taken to AHRI for miRNA extraction, reverse transcription and quantitative PCR analysis.

3.5. Candidate microRNA selection

A panel of five breast cancer associated miRNAs were selected for investigation in this study, on the basis of their reported relevance to breast cancer. These miRNAs were also previously documented to be dysregulated in breast tumour tissues and circulation compared to healthy control. Moreover, these miRNAs target complementary mRNA sequences of specific genes, exerting their functionality via mRNA cleavage or translational repression. The present study includes miR-21, miR130b, miR-145,miR-195 and Hs-RNU6 was selected as an endogenous control(housekeeping gene)for normalization of expression level detected by real time PCR(Li et al., 2017).

Table 1: Selected miRNAs

miRNAs of interest	References
miRNA-21	(Frankel et al., 2008, Asaga et al., 2011, Motawi et al., 2016)
miRNA-145	(Sachdeva et al., 2009)
miRNA-195	(Yang et al., 2013)
miRNA130b	(Chen et al., 2015)
Hs-RNU6	(Asaga et al., 2011, Yang et al., 2013, Li et al., 2017)

3.6. Sample size Determination

Sample size was determined by the following formula:

$$n = \frac{2 * \sigma^2 (Z_{\alpha/2} + Z\beta)^2}{(\mu 1 - \mu 2)^2}$$

Where; $\sigma^2 = \frac{S_1^2 - S_2^2}{2}$ for Pooled Sample

n - Sample size required in each group

Z_α- the tabulated value of standard normal (95%)

Zβ -the tabulated value of power of test (80%)

μ1= mean for case (5.05)

μ2= mean for control (0.45)

S_1=Variance for case (12.17)

S_2 = variance for control (0.9)

σ= standard deviation

$$\sigma^2 = \frac{12.17^2 - 0.9^2}{2} = 73.65$$

$$n = \frac{(1.96+0.84)^2 \, 2 * 73.65}{(4.6)^2}$$

$= 54.576 \approx 55$

In this study 25 Breast cancer patients and 25 Healthy controls were included. Total sample size was 50. This was reduced from 55 in each group to 25 in each group due to insufficient availability of reagents.

The standard deviation and mean of difference were determined based on the previous case control study on breast cancer conducted in Ireland (Heneghan et al., 2010).

3.7. Sampling procedure and clinical data collection

Five millilitre blood samples were collected prospectively from a group of 25 female with breast cancer study participants and 25 age-matched healthy controls, respectively in Vacutainer Serum Separator Tubes II (Becton Dickinson).Blood sampling technique was minimally invasive and performed by trained laboratory personnel under aseptic conditions from all the cases with BC at the time-point when they come for chemotherapy and healthy age-matched female volunteers. The relevant demographic and clinical data were recorded at the same time of specimen being received for each patient and healthy control.All control individuals were also interviewed by a clinician prior to being enrolled in this study to ensure they were eligible for inclusion criteria in this study.

3.8. Specimen Processing

After clotting samples at room temperature for 30 minutes, specimens were centrifuged at 1,200g for 15 minutes. The supernatant was transferred into new micro -centrifuge tubes and Sera were stored at -20°C until use.

3.8.1. miRNAs extraction from serum

The miRNeasy serum/plasma kit (Catalog number (Cat.no.) 217184, Qiagen) protocol was used to extract miRNAs from serum; In brief the procedure was as follows: first, we thawed the serum before transferring200 μL of serum to new collection tube. One millilitre of Qiazol lysis reagent was added to the serum and mixed by vortexing. After incubating, at room temperature (RT) for 5 minutes, 3.5 μL of spike-In control (micro RNA mimic) was added. Two hundred microliter of chloroform was then added, and the mixture was shaken vigorously before incubating at RT for 3 minutes. Subsequently, the mix was centrifuged for 15 minutes at 12,000g at 4℃ to separate it into three phases. Only the upper aqueous phase was transferred in to new collection tube and 1.5 volumes of 100% ethanol was added and mixed by pipetting. Seven hundred microliter of the resulting mix was transferred to RNeasy MinElute spin column in a 2 ml collection tube and centrifuged at 12000g for 15 seconds. The flow-through was discarded and 700 μL washing buffer RWT was added and centrifuged at 12000g for 15 seconds. After discarding the flow–through, 500 μ L of the second wash buffer RPE was added, the tubes centrifuged at 12000g for

15 seconds, and the flow–through discarded. To wash the spin column membranes, 500 µL of 80% ethanol was added and centrifuged at 12000g for 2 minutes. Then RNeasy MinElute spin column were placed in a new 2 ml collection tubes and centrifuged at 18000g for 5 minutes with the lids of the spin column open to dry the membrane. Then we transferred the RNeasy MinElute spin column into a 1.5 ml collection tube and eluted by adding 14 µL of RNase free water with centrifugation at 18000g for 1 minute. Finally, the RNeasyMin Elute spin column was discarded and the eluted RNA stored at -20 ℃ for 30 minute until used for cDNA conversion. The cDNA conversion was done in the same day of extraction.

3.8.2. Reverse transcription: miRNA to complementary DNA (cDNA)

Reverse transcription of small RNA was performed by using miScript II RT Kit(Cat.no.218161 ,Qiagene).To prepare cDNA we followed the protocol provided by the maker of the kit. In short, freez-stored RNA,10x miScript Nucleics Mix, RNasefree-water and 5x miScript HiFlex Buffer were thawed at RT. The reverse transcription master mix was prepared on ice. A total volume of 20 µL of reaction mix, consisting of 4 µL of 5x miScript HiFlex Buffer, 2 µL of 10x miScript Nucleics Mix, 7 µL of RNase-free water, 2 µL of RTmix and 5 µL of template RNA, was mixed thoroughly by pipetting.After this, the mix was incubated for 60 mins at 37ºC followed by 5 min of inactivation at 95ºC. The resulting cDNA was stored at –20ºC freezer until use.

3.8.3. Real-Time PCR for Detection of Mature miRNAs

The quantitative real-time PCR (qRT-PCR) assay was carried out using miScript SYBR® Green PCR Kit (Cat.no.218073, Qiagen).Each reaction was performed in a final volume of 20 µL by following kit protocol. First, all reagents (2x QuantiTect SYBR Green PCR Master Mix, 10x miScript Universal Primer, 10x miScript Primer Assay, template cDNA, and RNase-free water) were thawed at RT and transferred on to ice before mixing. Then, master mix was prepared in a total volume of 20 µL containing 10 µLof 2x SYBR Green Mix,2 µL Universal primer, 2 µL miRNA specific primer and 1 µL RNase free water. After mixing the reaction mix thoroughly but gently, 15 µL volumes were dispensed into the reaction tubes. Then 5 µL of template cDNA was added to the reaction tubes and loaded into Rotor-Gene^{TM}3000 programmable thermal cycler (Corbett Life Science) real time PCR machine. We programed the real-time cycler as follows: Initial taq activation at 95 ºC for 15 min, followed by 40 cycles of 94 ºC for15 sec,55 ºC for 30

sec and 70 ºC for 30sec.Finally we placed Rotor-Disc in the real-time cycler and started the cycling program. At the end of cycle, we extracted CT value for each reaction. The runs were done in duplicates, and runs were repeated when the duplicate values differed by 0.5 or more. The level of miRNAs gene expression was analysed using the CT values generated form the qPCR run and automatically generated when we set the threshold at 0.01 and normalized by using Hs-RNU6-2 as an endogenous control (housekeeping gene).

CT values were calculated as follows: By Livak and Schmittgen (2001) method.

By using average Ct value of each gene for each sample, we calculated fold change as follows

ΔC_T= (C_T target gene - C_T housekeeping gene)

$\Delta\Delta C_{T\ patient}$= ($\Delta C_T$ of patient target gene - Average ΔC_T of control target gene)

$\Delta\Delta C_{T\ control}$= ($\Delta C_T$ of control target gene - Average ΔC_T of control target gene)

Fold change for patients $=_2^{-\Delta\Delta CTpatient}$

Fold change for control $=_2^{-\Delta\Delta CTcontrol}$

Where: C_T target gene stands for C_T of mir21, mir145, mir130b, or mir195

C_T housekeeping gene stands for C_T of Hs-RNU6-2

3.9. Study variable

3.9.1. Dependent Variable

- Level of miRNA21
- Level ofmiRNA130b
- Level of miRNA145
- Level of miRNA195

3.9.2. Independent variable

- Age
- Weight
- Height
- BMI

3.10. Operational Definition

Body Mass Index (BMI): A measurement used to assess nutritional status and measured as weight divided by height square. For adults over 20 years old, BMI falls into one of the following categories.

Table 2*:* Body mass index categories

BMI	Nutritional status
Below 18.5	Underweight
18.5–24.9	Normal weight
25.0–29.9	Pre-obesity
30.0–34.9	Obesity class I
35.0–39.9	Obesity class II
Above 40	Obesity class III

(Kivimäki et al., 2017).

Mastectomy: Mastectomy is the removal of the whole breast.

3.11. Data Entry and Statistical Analysis

Data were analyzed using SPSS software (version 25.0, Chicago, IL, USA) and GraphpadPrism version 7.After CT values were extracted, the values were analyzed using Microsoft Excel 2010.Indepent t test and One way ANOVA test were used to check statistical significance of change in fold of miRNAs between patients and healthy control. Data were presented as mean ± SD .Values were considered statistically significant when P-value was less than 0.05.

3.12. Data quality assurance

The socio-demographic characteristics and related data were collected by professional nurses and all the laboratory procedures were handled appropriately. Tools used to measure and analyze the variables were standardized and automated. Thedata were entered in to SPSS version 25 and cleared and checked for correctness and completeness before analysis.

3.13. Ethical Clearness

The study protocol was reviewed and approved by the research and ethics committee of Medical Biochemistry department and institutional review board of Health Science of Addis Ababa University. The Ethical clearance was also obtained from AHRI/ALERT Ethics Review Committee. Permission for data collection was obtained from oncology department of Tikur Anbessa Specialized Hospital. Written informed consent was obtained from all participants.

3.14. Dissemination and Utilization of Results

Hard and soft copies will be submitted to the department of Medical Biochemistry. The findings from the study will be presented to the department of Medical Biochemistry, College of Health Sciences, Addis Ababa University and other concerned and scientific bodies. Moreover, the study findings will be shared with concerned bodies at the zonal, regional and national level and submitted to a peer-reviewed journal for publication.

4. RESULTS

4.1. Socio-Demographic Characteristics of the Respondents

A total of 50 females, 25 breast cancer patients and 25 healthy controls participated in this study. The mean age of the participants was 37.78 ± 9.74 with a minimum age of 20years and a maximum age of 65years.Regarding the age category of the study participants, 21/50 (42%) of our study participants were in the age range of 35-41 years (Table3).

Table 3: Frequency distribution of age category of study participant

Age	Frequency	Percent (%)
20-27	8	16
28-34	8	16
35-41	21	42
42-48	6	12
49-55	4	8
56-62	2	4
>63	1	2
Total	50	100

When we consider Body Mass Index (BMI) of the study participants, most of the study participants (52%) had normal body weight (Figure3).

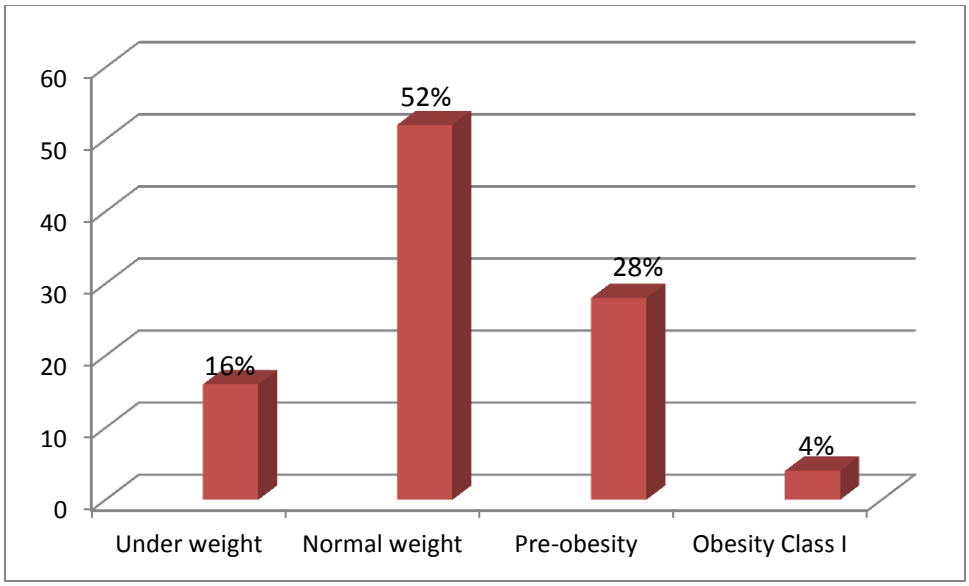


Figure 3*:* Frequency distribution of BMI of participants.

Table 4: Mean and Standard deviation of participant age, weight, height and BMI profile

	Age	Weight	Height	BMI
Mean	37.78	58.32	1.60	22.95
Std.Dev	9.74	11.06	0.06	4.19
N	50	50	50	50

4.2. Clinical and Laboratory Result

Participants were asked the time they visited health professionals after experiencing the signs and symptom of the disease. Among 25 breast cancer study participants, 8/25 (32%), were diagnosed in 6-12 month after development of symptoms (Table5).

Table 5: Frequency Distribution of Patient stay with disease before diagnosis.

Duration of symptoms before diagnosis	Frequency	Percent (%)
<Month	6	24
1-5 Months	4	16
6-12Months	8	32
>12Months	7	28
Total	25	100

Considering history of surgery, 17/25(68%), patients underwent mastectomy (Table6).

Table 6: Frequency Distribution of Patients' history of Mastectomy

Surgery	Frequency	Percent (%)
YES	17	68
NO	8	32
TOTAL	25	100

Staging of breast cancer status was done by a professional Pathologist at the department of pathology for the participants. Fifty-two percent of breast cancer study participants had stage III disease (Table7).

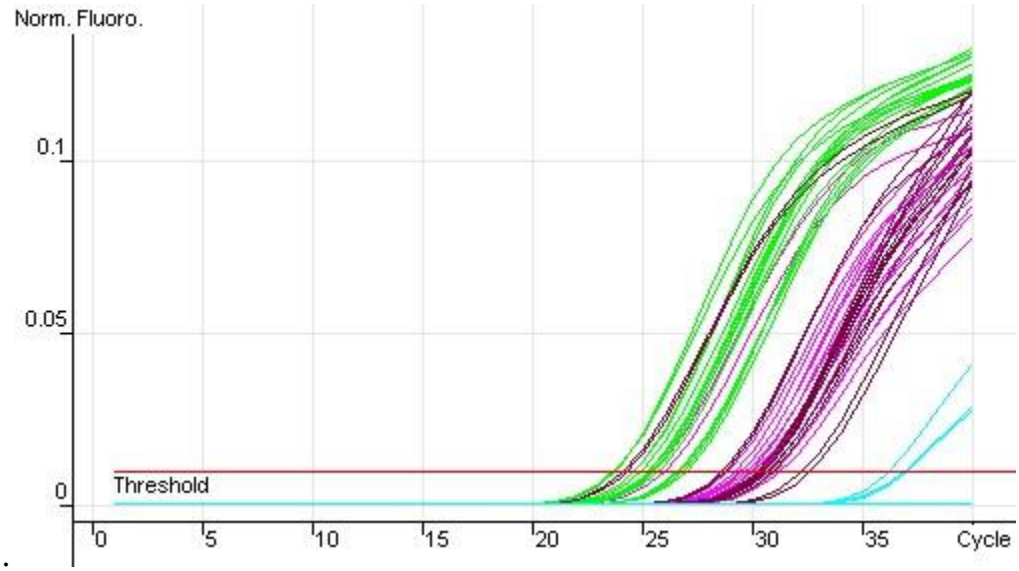
Table 7: Frequency Distribution of breast cancer patient stages

Stage	Frequency	Percent
II	5	20
III	13	52
IV	7	28
Total	25	100

4.3. Detection of miRNAs in the Circulation of Breast Cancer Patients

Expressions of 5 miRNAs, (miR21, miR145, miR195, miR130b and Hs-RNU6-2) chosen for their established relevance to breast cancer, were detectable in all serum samples from breast cancer study participants (n=25) as well as healthy controls (n =25). Hs-RNU6-2 expression was stable and reproducible across all 50 participants and was used to normalize RT-qPCR data.RT-

qPCR was optimized before real sample was run. The Ct value was extracted for analysis of fold change (Figures 4, 5 and 6).



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Figure 4: Amplification of target gene of BC sample in linear scale during optimization.

We construct the melt curve to rollout primer dimer .As figure below

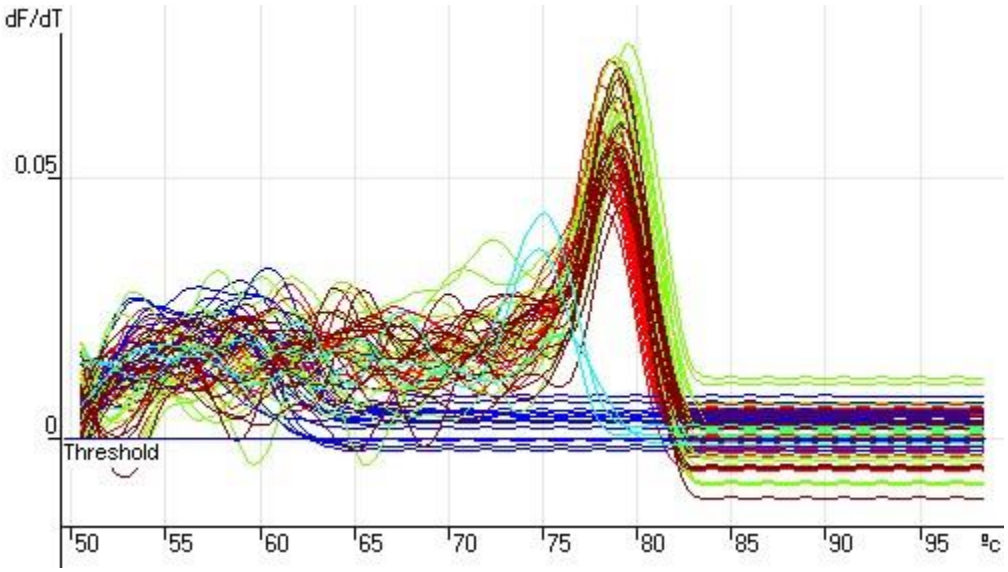


Figure 5: Melt curve of target genes of BC sample. We have only one pick so it is not primer dimer rather it is real amplification

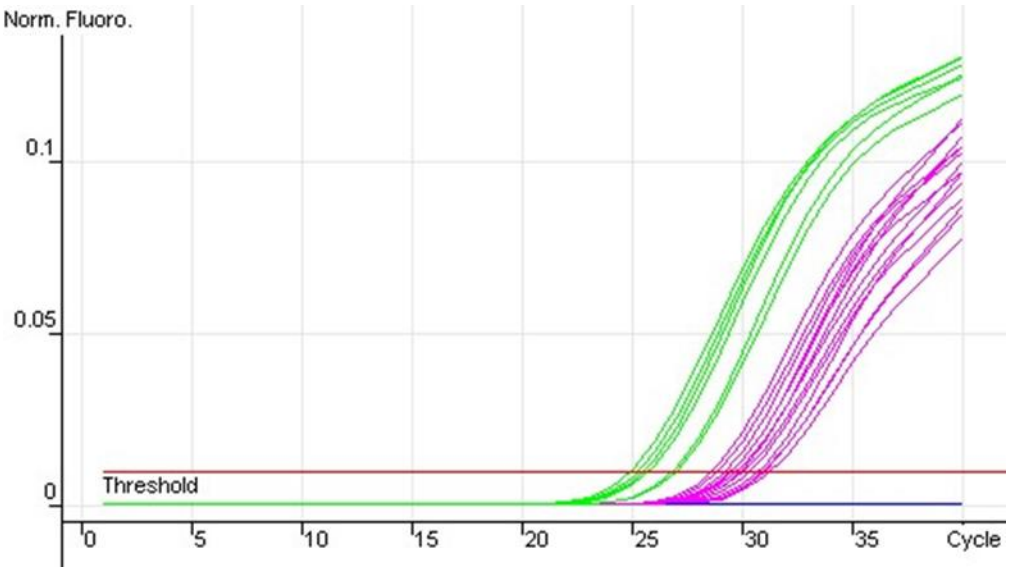


Figure 6: Amplification of Target gene after optimization.

All reactions were run in duplicates and Ct values of each duplicate reaction were averaged after ensuring there was no variation in Ct values of duplicate runs.

We investigated the levels of 4 circulating target miRNAs in serum of 25 breast cancer study participants and compared with those of 25 healthy controls to explore relative expression level of circulating miRNAs.

As shown in Table 8, the levels of two miRNAs (miR-21and miR-195) were significantly higher in breast cancer study participants than in healthy controls (p=0.003, =0.042, respectively).

Table 8: Mean relative quantification expression levels with SD of target miRNAs in serum from breast cancer patients compared with serum from healthy controls.

Target miRNAs	Patients (n =25)	Controls(n =25)	Mean Fold Change in miRNAs	P value
miR21	4.237(0.2094)	1.375(0.8898)	4.237	0.003
miR145	1.65(0.2274)	1.858(0.3765)	1.65	0.6384
miR195	6.364(2.316)	1.508(0.265)	6.364	0.042
miR130b	1.143(0.2955)	1.468(0.2421)	1.143	0.3988

The level of expression of miRNAs in patients versus controls with their mean ±SD is also presented graphically as follows (Figure7).

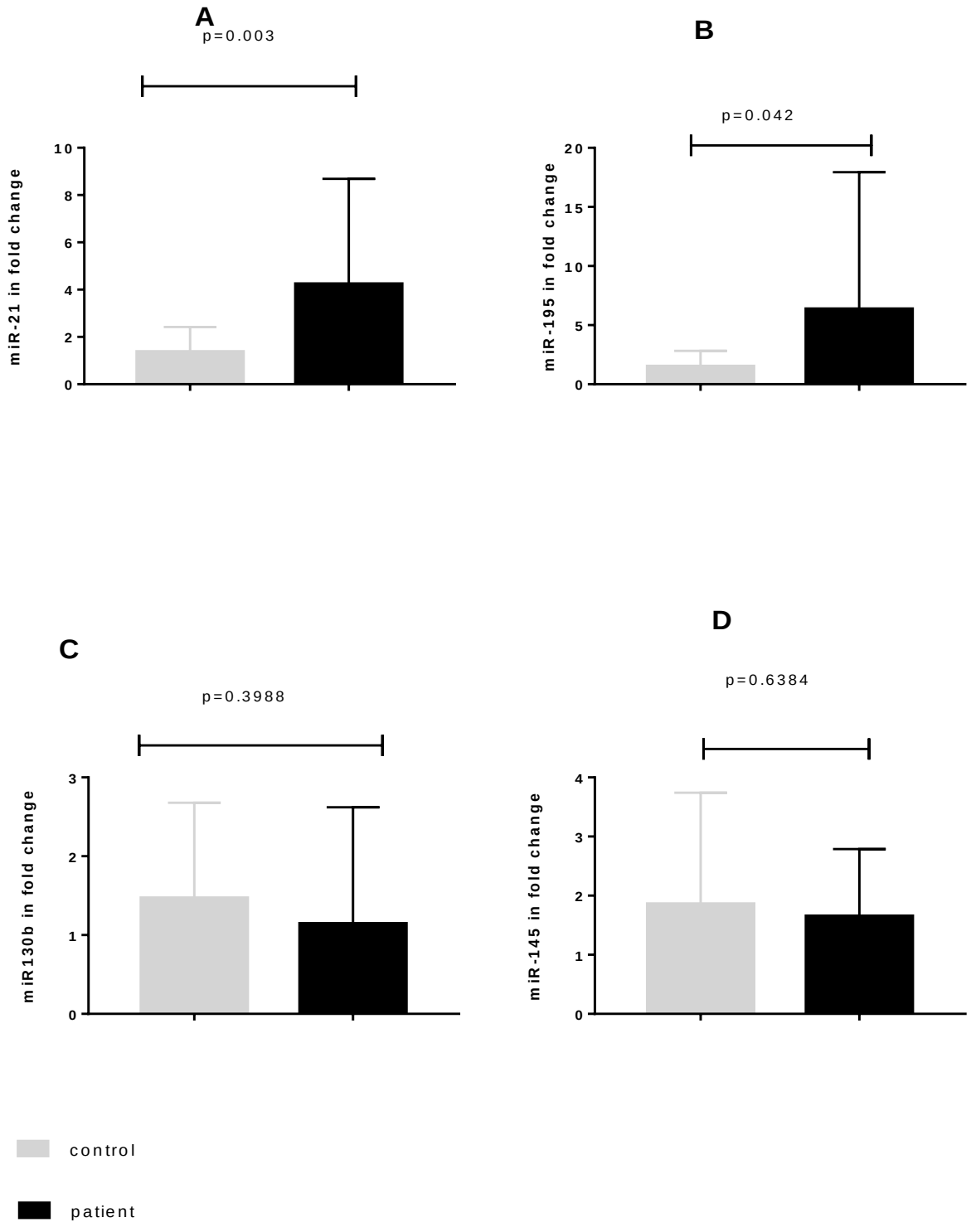


Figure 7: miR21 (A) miR195 (B), miR145(C) and miR130b (D) fold change in serum of breast cancer patients (n=25) when compared to that of healthy controls (n=25).

In the present study, expression level of miR-21 in breast cancer patients (4.237) was significantly higher than that of healthy control (1.375) (p=0.003). Receiver Operator Curve (ROC) analysis showed that the Area Under Curve (AUC) was 0.6832 (95% CI 0.53-0.80) (Figure 8 A and B).

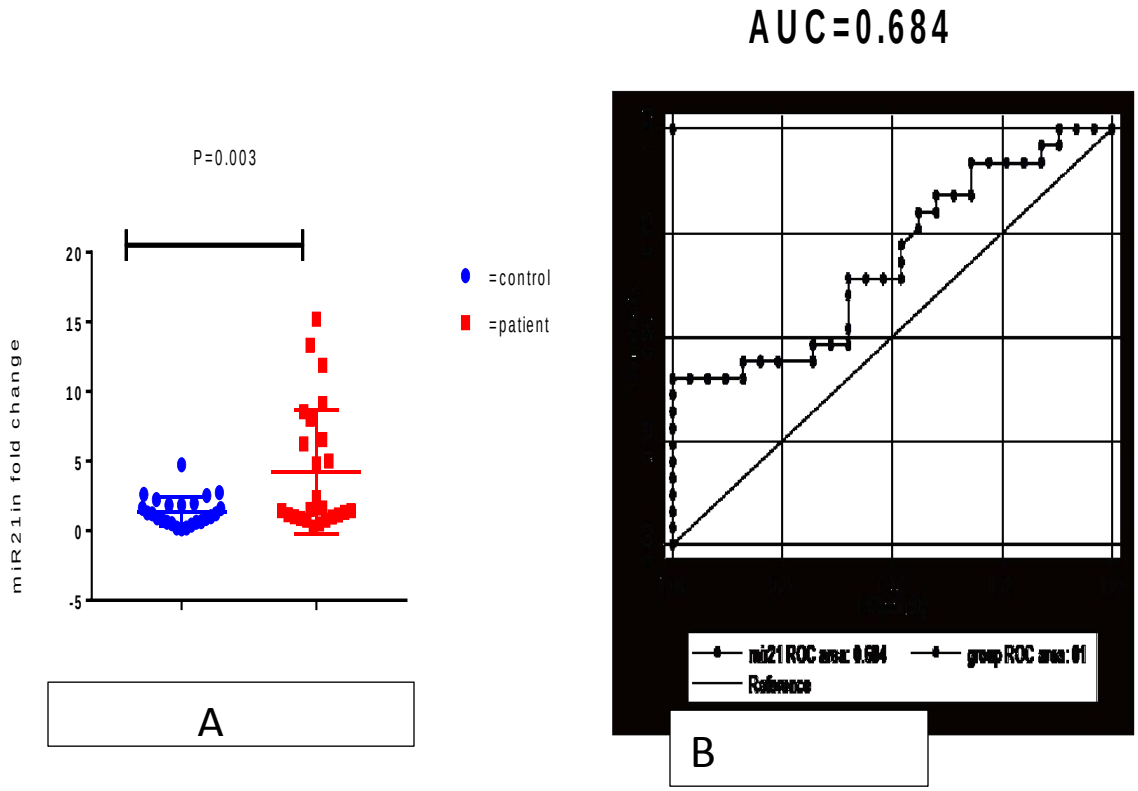


Figure 8: miR-21 expression in breast cancer patients and healthy controls (Panel A); ROC analysis of miR-21 (Panel B).

In present study, the levels of miR-195 were significantly higher, on average, in the breast cancer patients (6.364 fold) than in healthy controls (1.508 fold) (P=0.042).

Similarly, ROC analysis showed that AUC was 0.657 (95%CI 0.50.-0.81) for miR195 (Figure 9A and B).

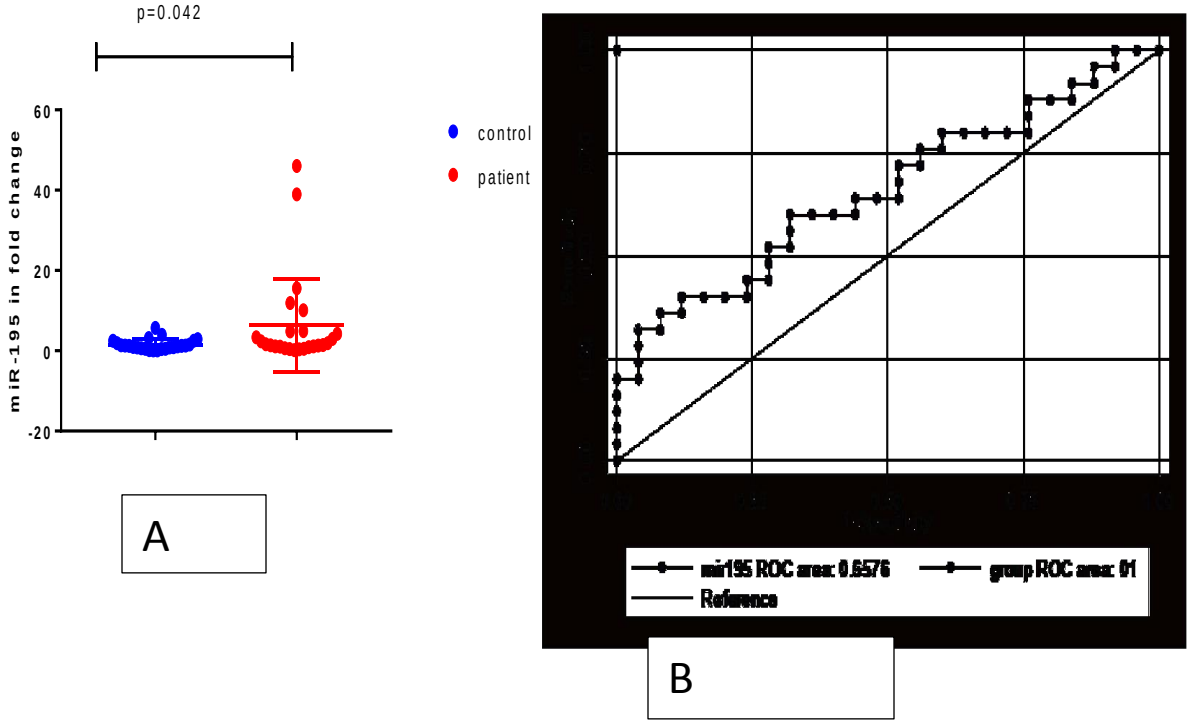


Figure 9*:* miR-195 expression in breast cancer patients and healthy controls (Panel A); ROC Analysis of miR-195 (Panel B).

4.4. Association of circulating miRNAs with Clinicopathological Parameters

The expression level of miR-21 and miR-195 were higher in breast cancer study participants that underwent mastectomy as compared to breast cancer patients who did not undergo mastectomy and healthy controls (p=0.0004, p=0.0133, respectively)(Figure10).

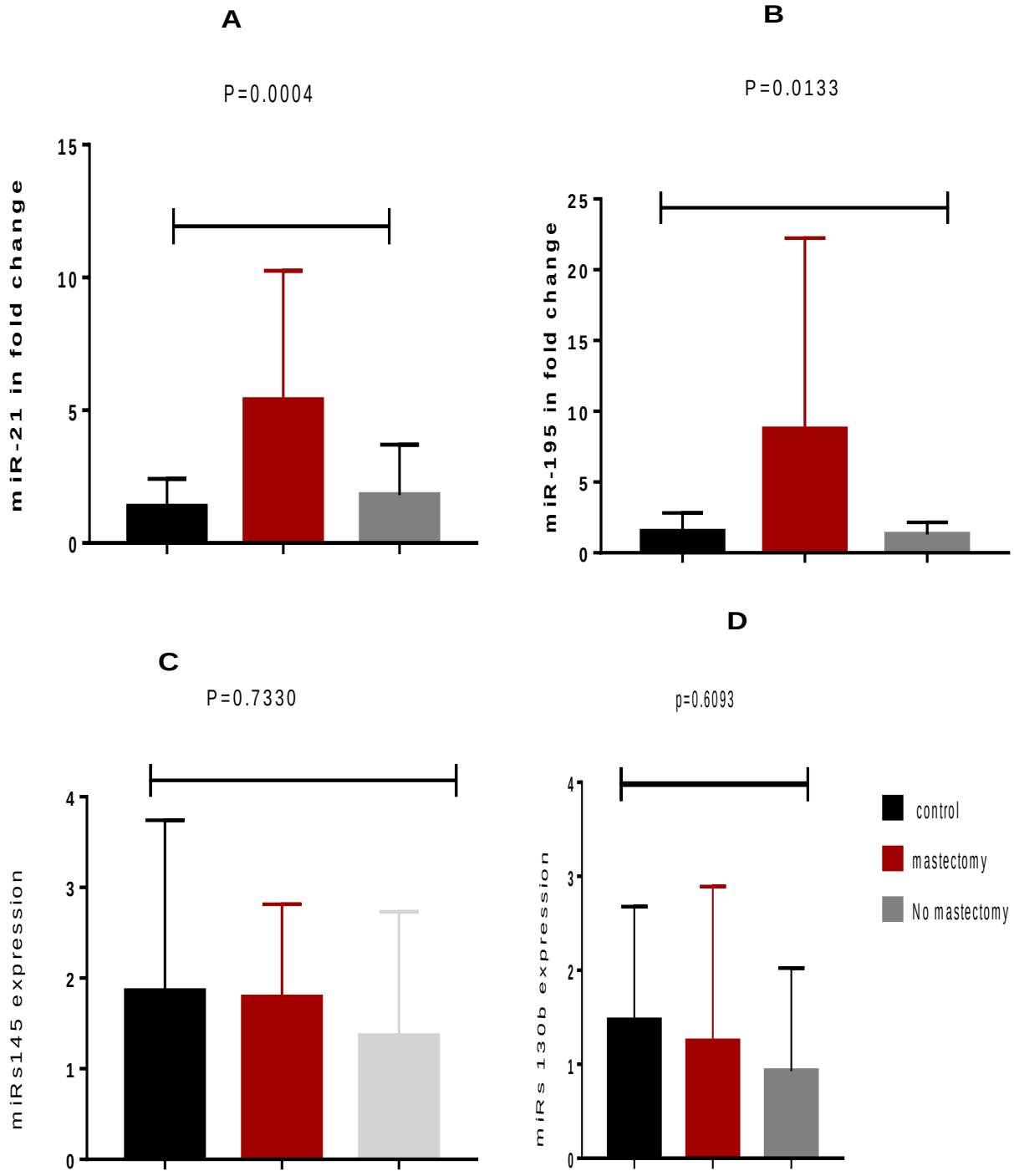


Figure 10: The expression of miR-21(A), miR-195(B), miR-145(C), miR-130b (D) with history of mastectomy.

We analyzed the expression level of miRNAs across all breast cancer stages by using the One way ANOVA test.

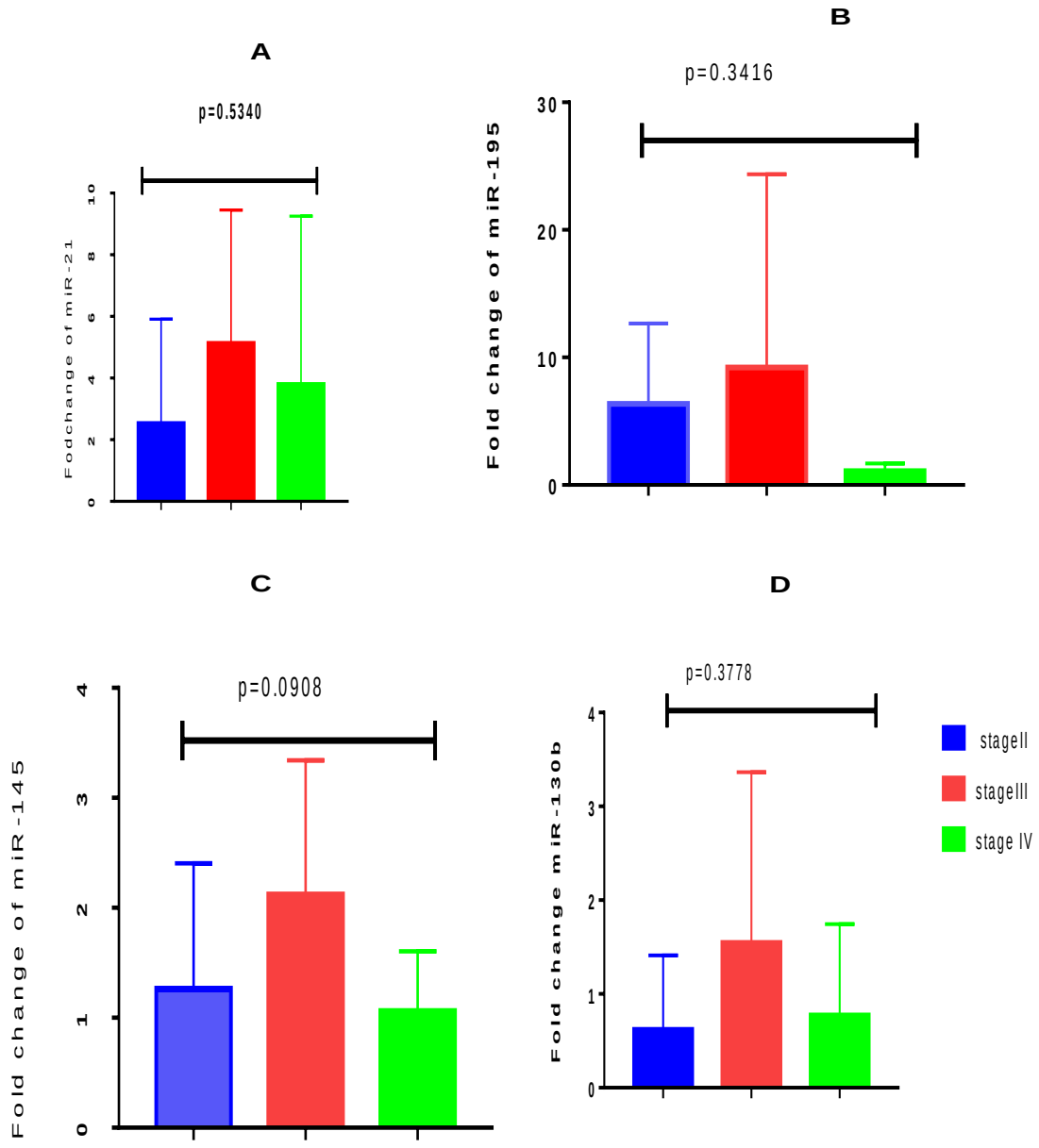


Figure 11: The expression of miR-21(A), miR-195(B), miR-145(D), miR-130b (D) across the stage of breast cancer

5. DISCUSSION

miRNAs control gene expression by targeting mRNAs and triggering either translation repression or RNA degradation (Iorio et al., 2005). On another hand microRNAs aberration in cancer tumor tissue and serum have been also demonstrated (Zhu et al., 2014). Peng and Croce(2016) stated that miRNAs expression is dysregulated in human cancer through various mechanisms, such as amplification or deletion of miRNAs genes, abnormal transcriptional control of miRNAs, dysregulated epigenetic changes and defects in the miRNAs biogenesis machinery. Evidence has shown the dual role of miRNAs in breast cancer as either oncogenes or tumor suppressors, and circulating miRNAs may correlate with disease progression, therapeutic responses and patient survival (Hamam et al., 2017).

Breast cancer is most frequently found in women around menopause and less frequently found in women below 45 years of age. As the same time the relation between age and expression of the estrogen receptor found in the examined tumor tissue .The percentage of estrogen receptor positive tumor (ER +) and estrogen receptor negative tumors (ER–) were associated with age, with higher percentages of ER+ tumors diagnosed after menopause, and high percentages of ER– tumors occurring in women less than 50 years of age (Kamińska et al., 2015).

Finding of our study indicates that the mean age of the study participant is 37.78(Table 4).This shows that breast cancers are widespread in younger women. This is in line with study conducted by Fasching (2018) where most of the breast cancer patients were diagnosed in younger than 40 years. Breast cancer in young women were also characterized by poor prognosis and high recurrence (Elkhuizen et al., 1998). Early onset of breast cancer was also related to aggressive clinicopathologic features (Lee et al., 2018). As Albain et al(1994) stated that cancers in women less than 35 years of age had high S-phase fractions and abnormal expression of p53. Mutations in p53 results in the development and progression of several neoplastic diseases including human breast cancer, and this mechanism may underlie the increased severity of breast cancer in younger patients (Allred et al., 1993). There is evidence that there is an association between age, grade of the tumor and metastasis of the tumor; distant metastasis was more prevalent among the younger age group and in the middle aged women (Nuzhat and Abouzaid, 2017). That most women in our study were young would be consistent with this possibility.

Patients with normal BMI have had longer overall survival and disease-free survival than patients with obese BMI (Berclaz et al., 2004). The mean body mass index (BMI) of our study participants lay within the normal range (18.5-24.9kg/m^2) (Table 4).

The study conducted by Chan et al(2014) and Kenzik et al(2018) reported that higher survival rate of breast cancer also related to normal *BMI*.In the present study the normal BMI of our study participant may be showed that they have good survival rate .

Long–term survival and treatment of breast cancers are affected by different factors, including early diagnosis and disease stage (Board, 2018). In present study 21/25 (76%) of participants were diagnosed by health professionals greater than one month after development of signs and symptoms of the disease, whereas only 4/25 (24%) of study participants were diagnosed within one month of symptom development (Table5). This result is comparable with study conducted by Ermiah et al(2012) on two hundred Libyan females with breast cancer stated that only 30.0% of patients were diagnosed within 3 months after symptoms, 14% of patients were diagnosed within 3–6 months and 56% within a period longer than 6 months. On the other hand present study agreed with scientific idea treatments for early stage breast cancer may not treat advanced stage of breast cancer effectively in which 17/25(68%) of our study participant were underwent mastectomy (Table 6).Delay in diagnosis also associated with stage of the disease (Arndt et al., 2002). At time of diagnosis, the clinical stage of our study participants were, 20% stage II, 52% stage III and 28% stage IV (Table7).This result is in line with study conducted by Ermiah et al(2012) , reporting 9% of breast cancer patients in stage I, 25.5% in stage II, 54% in stage III and 11.5% in stage IV. The study conducted by Yonas et al, 2018 unpublished data reported that study their participant were 24% in stageII,64.3% in stage III and 11.63% in stage IV which is comparable with current study.

In present study we also investigated the levels of 4 circulating target miRNAs in serum of breast cancer patients and compared with those of healthy control subjects to explore relative expression level of circulating miRNAs and we use Hs-RNU6-2 as housekeeping gene. Our results confirm that Hs-RNU6-2 as a reference control for measurement of circulating miRNAs (Li et al., 2017;Yang et al., 2013; Asaga et al., 2011;Lee et al., 2011). We found that serum Hs-RNU6-2 was consistently expressed and was not influenced by breast cancer status.

miR-21 is one of noncoding RNA molecules that significantly up regulated miRNAs in human breast cancer, and its expression has been associated with tumor progression and poor prognosis (Asaga et al., 2011).The other evidence showed that miR-21 is overexpressed in a wide variety of cancers and has been causally linked to cellular proliferation, apoptosis, and migration. Inhibition of miR-21 in MCF-7 breast cancer cells causes reduced cell growth. Tumor suppressor protein Programmed Cell Death 4 (PDCD4) is regulated by miR-21. Anti-apoptotic effect of miR-21 favor cancer cell to more proliferate and metastasis (Frankel et al., 2008).

In the present study, the expression level of miR-21 in breast cancer patients (4.237 fold) were significantly higher (p=0.003) than that of healthy controls (1.375 fold) (Table8). This result is in line with study conducted by Asaga et al(2011) and Bahrami et al(2018) reporting that the expressions of miRNAs in the serum were different between healthy controls and cancer patients. High expression of miR-21 is associated with tumor progression and poor prognosis. On the other hand Yonas and colleagues, 2018 unpublished data reported that the up regulation of miR-21 in breast cancer patients than healthy controls.

Our study is also consistent with the report of Gao et al(2013) which stated that expression of miR-21 in breast cancer (30.82) was higher than that in healthy controls (9.1). Han et al(2017) also reported that the expression of miR-21 in breast cancer is higher than that of healthy control which is in line with present study.

Our result is also in consistent with the report of Mar-Aguilar et al(2013) which stated that miR-21 expression is significantly higher in patient than healthy control. This study may be supported by the idea that report miR-21 was consistently up regulated in triple-negative breast cancer (TNBC) and MDA-MB-468 cells compared to normal tissues. Furthermore, inhibition of miR-21 by miR-21 antisense oligonucleotides decreased the proliferation, viability, and invasiveness of MDA-MB-468 cells and enhanced apoptosis .which showed the role of miR21 in BC (Fang et al., 2017; Fu et al., 2011).

miR-21 have also been identified as key factors for macrophage activation and resulted in an *in vitro* invasion of breast cancer cells. Tumor associated macrophages (TAMs) have the potential to carry out both anti- and pro-tumor activities, promoting tumor growth directly by secreting breast tumor mitogens, such as epidermal growth factor, and indirectly by stimulating tumor

angiogenesis and metastasis (Halvorsen et al., 2017). The expression level of miR-21 in current study mighty be due to miR-21 role on activation of TAMs.

The report of Hamam et al(2017) show that miR-21 is an oncogenic miRNA that inhibits several tumor-suppressor genes and thus promotes cell growth and invasion and tumor metastasis. High expression and up regulation of miR-21 is associated with tumor progression and poor prognosis Fan et al(2018)

Another researcher investigated whether suppression of miR-21 alone influences tumorigenesis, by antagonizing miR-21 in MCF-7 cells and then injected them into mammary pads of female nude mice. Tumor size with anti-miR-21 were one-half that of controls, possibly due to reduction of proliferation by anti-miR-21.These results indirectly show that miR-21 is an oncogenic miRNA that play important role in tumorigenesis. The same researcher also stated showed that antagonizing miR-21 lead to apoptosis of MCF-7 cells and low B-cell lymphoma 2 (Bcl-2) protein expression, suggesting that Bcl-2 may be an indirect target of miR-21 (O'Day and Lal, 2010; Iorio et al., 2005). Our findings of up-regulation of miR-21 in breast cancer patients mighty be consistent with this mechanism.

In our study we generated receiver operating characteristic (ROC) curve for miR-21 to show predictive power and our area under the ROC curve (AUC) was 0.6832 (95% CI 0.53-0.80) (Figure 8B).This result is comparable with Asaga et al(2011) report in which they performed ROC analysis to assess sensitivity and specificity of miR-21showed that AUC was 0.721. The present study is also in line with Yonas et al(2018) un published data report in which their ROC analysis result of miR-21 showed that AUC was 0.594(95%CI 0.499-0.69) .Another researcher on the other hand reported that sensitivity and specificity of miR-21 in the diagnosis of breast cancer were 87.6% and 87.3%, respectively with AUC of 92.9% (Gao et al., 2013).Our results agreed with Han et al(2017) result of AUC for serum miR-21 levels was 0.788((95% CI 0.681 to 0.895).

Despite the high statistical significance in the differences in levels of miR-21 between breast cancer and healthy controls, ROC analysis suggests that this test alone is unlikely to have sufficient applicability in a diagnostic test where values above 0.8 or 0.9 are attractive (Berrar

and Flach, 2012). However, it is possible, that miR-21 in combination with other miRNAs or biomarkers will have potentially more applicability in breast cancer diagnosis.

Our results also showed that there is no significance difference in miR-21 expression across the stage of the disease (Figure11). Han et al(2017) study report that across 3 stages (I, II, III), the serum levels of miR-21, showed no marked difference. This result concordant with present study.

Current study also demonstrates that miR-21 expression was higher in study participants who underwent mastectomy than those who did not undergo mastectomy and healthy control (Figure10). Consistent with our results, previous researchers have found that high miR-21 expression was related to mastectomy (p=0.005) (Lee et al., 2011).

Preliminary evidence showed miR-145 is one of the miRNAs whose expression is most commonly reduced in various human cancers, including breast cancer and their expression level is inversely related to tumor grade, size and Ki-67 proliferation index (Spizzo et al., 2010).

Ding et al(2017) reported that cell proliferation in breast cancer cells was decreased after overexpression of miR-145 and increased following miR-145 suppression**.** Overexpression of miR-145 also inhibited the expression of transforming growth factor-β1 (TGF-β1) which in-turn contributes for tumor formation. In TNBC higher cytoplasmic expression of TGF-β1 is associated with histologic grade and lymph node status, as well as reduced disease-free survival (Zhang et al., 2017).

Many reports have stated that miR-145's role as diagnostic biomarker in plasma /serum and tissue of breast cancer patients, especially those with ER+ receptor profile. However, there is debate on the mode of dysregulation (Nassar et al., 2017).

In the present study, the expression of miR-145 did not differ significantly (P=0.6384) between the breast cancer patients and healthy controls (Table8). However, the relative expression of miR-145 in breast cancer is lower than that of healthy control. The non-significance of our result mighty be due to our sample size of breast cancer study participant unlike other researcher conducted their study by using large size number of breast cancer study participants.

Different researcher reported that the lower expression of miR-145 level in breast cancer patients when compared to healthy controls (Ye et al., 2018, Iorio et al., 2005).

miR-195 plays a tumor-suppressor role in various types of cancer. Overexpression of miR-195 in different cancer cells enables it to suppress cell cycle progression and tumor cell invasion. The expression of miR-195 is inversely related to Raf-1 gene expression which in turn suppress Bcl-2 and P-glycoprotein expression in breast cancer (Yang et al., 2013).

Another study showed that highly proliferating cancer cell requires high level of de novo fatty acid synthesis than normal cell. miR-195 targets the genes of de novo lipogenesis, inhibits cell proliferation, migration, and invasion (Singh et al., 2015).

In present study, the levels of circulating miR-195 were significantly higher (P=0.042), on average, in the breast cancer patients (6.364 fold) than in healthy controls (1.508) (Table8). This result is in line with study conducted by Heneghan et al(2010) on 127 females, including 83 consecutive breast cancer patients and 44 healthy age-matched female volunteers who served as controls. They reported that the expression level of miR195 in breast cancer patients was significantly higher than that of healthy controls.

On the other hand the study conducted by Mattie et al(2006) showed that miR-195 levels were significantly higher in HER-2/ neu+ than in HER-2/neu -breast cancers. This illustrates how miR-195 may play a role in different types of breast cancer. The analysis of a cohort study done on serum from 49 breast cancer patients and 19 healthy controls showed that the level of circulating miR195 in breast cancer patients were significantly higher than that of healthy controls (Fan et al., 2018). This result is also in line with our finding. Our result were also comparable with the work of Hagrass and colleagues(2015) report that over expression of miR-195 in breast cancer patients than healthy controls.

In our study we generated receiver operating characteristic (ROC) curve for miR-195 to show predictive power and our area under the ROC curve (AUC) was0.657 (95%CI 0.50.-0.81)(Figure9B).The current result agreed with Heneghan et al(2010) report blood levels of miR-195 could detect individuals with breast cancer with 85.5% sensitivity and 100% specificity.

From ROC analysis suggests that miR-195, like that of miR-21, is alone unlikely to have sufficient applicability in a diagnostic test (Zhu et al., 2010; Berrar and Flach, 2012). It will be important to assess whether combinations of miRNAs have greater discriminatory power.

Our results show that there is no significant difference in expression of miR-195 across the stages of breast cancer (Figure11). However our result showed that the expression of miR-195 in breast cancer patients who undergo mastectomy were significantly higher than breast cancer patients who did not undergo mastectomy (Figure10). Other researchers reported that persistently elevated postoperative levels of miR-195 were detected only in patients who developed early tumor relapse (Igglezou et al., 2014). The elevation of miR-195level in current study in surgically operated participants may be indicate that our study participants were at high risk of recurrence of the disease. Heneghan et al(2010) report that the level of circulating miR-195 were decreased significantly in two week post operation. However they did not compare between surgical operated versus not surgically operated as what we did in current study.

Depending on the types of the cancer the oncogenic and tumor suppress function of miR-130b-3p was also reported. In addition to this, the same research reported that miR130b-3p was differentially expressed in early stage non-invasive MCF-7 human breast carcinoma cells and aggressive late stage MDA-MB-231 cells (Shui et al., 2017; Yu et al., 2015).

The crucial cell cycle regulator in triple-negative breast cancer cells (TNBC) cyclic G2 (CCNG2) gene were directly repressed by miR-130b-5p (Chang et al., 2015).This shows the role of miR-130b in different breast cancer. Miao et al(2017) reported that miR-130b was up-regulated in TNBC when compared to that of normal tissue.

In the present study the expression of miR-130b did not differ significantly between the breast cancer patients and healthy controls (p value of 0.3988) (Table8).The non- significance of our study mighty be due to our BC type in which miR130b up-regulation were reported in TNBC.

6. CONCLUSION

The breast cancer in younger is more aggressive than in older age. The time at which disease diagnosed affect the stage of the disease. The characteristics of miRNAs such as their stability, tissue specificity and their role in genetic regulation make them ideal candidate as biomarkers. The present study showed that circulating miR-21 and miR-195 significantly differed between breast cancer patients and healthy controls. Although miRNAs expression level was reported to be related to clinicophatological status of breast cancer patients such as history of mastectomy, in this study we did not see significant difference in the level of miRNAs expression across different stages of breast cancer. All together, the potential use circulating miRNAs as minimally invasive biomarkers to aid in diagnosis of breast cancer is supported and further study is required to come up with minimum list of target miRNAs that can specifically and effectively differentiate breast cancer with healthy or other cancer states.

7. Strength and Limitations of the study

7.1. Strength

- To our knowledge this study is the second study in Ethiopia conducted on circulating miRNAs to use new combination of miRNAs to see their role as biomarker.
- Since this study shows that miRNAs differentiate between breast cancer patient and healthy controls this provides proof of concept for further research into an extended panel of miRNAs levels in breast cancer patients.

7.2. Limitations

- A relatively small sample size may have precluded finding statistically significant differences between breast cancer and control patients for some of the markers. Similarly, relationships with miRNAs and clinical sub categories may require larger sample sizes.
- Lack of some clinicopathological Parameters.

8. Recommendation

To further see prognostic effect of miRNAs we recommend conducting a study with a much larger sample size, with more detailed clinicopatological records of the patients, extending the number of miRNAs biomarkers, and evaluating combinations of miRNAs for improved diagnostic capacity.

We recommend to conduct this study to determine prognostic value of miRNAs for treatment response and toxicities of available drugs.

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Annex

Annex I: TNM staging system for Breast Cancer

Primary tumor (T)	
Tx	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ
Tis(DCIS)	Ductal carcinoma in situ
Tis(LCIS)	Lobular carcinoma in situ
Tis(Paget)	Paget disease of the nipple NOT associated with invasive carcinoma and/or carcinoma in situ (DCIS and/or LCIS) in the underlying breast parenchyma.
T1	Tumor ≤ 2 cm in greatest dimension
T1mic	Tumor ≤ 0.1 cm in greatest dimension
T1a	Tumor > 0.1 cm but ≤ 0.5 cm in greatest dimension
T1b	Tumor > 0.5 cm but ≤ 1 cm in greatest dimension
T1c	Tumor > 1 cm but ≤ 2 cm in greatest dimension
T2	Tumor > 2 cm but ≤ 5 cm in gre test dimension
T3	Tumor > 5 cm in greatest dimension
T4	Tumor of any size wi h direct extension to the chest wall and/or to the skin
Notes: Invasion of the dermis alone does not qualify as T4.	
T4a	Extension to chest wall, not including only pectoralis muscle adherence/invasion

T4b	Ulceration and/or ipsilateral satellite nodules and/or edema (including peau d'orange) of the skin, which do not meet the criteria for inflammatory carcinoma
T4c	Both T4a and T4b
T4d	Inflammatory carcinoma
Regional lymph nodes (N)	
Clinical:	
NX	Nearby lymph nodes cannot be assessed (for example, if they were removed previously)
N0	Cancer has not spread to nearby lymph nodes.
N1	Metastasis to movable ipsilateral axillary lymph node(s)
N2	Metastases in ipsilateral level I, II axillary lymph nodes that are clinically fixed or matted or in clinically detected ipsilateral internal mammary nodes in the *absence* of clinically evident axillary lymph node metastasis
N2a	Metastases in ipsilateral level I, II axillary lymph nodes fixed to one another (matted) or to other structures
N2b	Metastases only in clinically detected ipsilateral internal mammary nodes and in the *absence*of clinically evident level I, II axillary lymph node metastases
N3	Metastasis in ipsilateralinfraclavicular lymph node(s), or in clinically apparent ipsilateral interna mammary lymph node(s) and in the presence of clinically evident axillary lymph node metastasis; or metastasis in ipsilateral supraclavicular lymph node(s) with or without axillary or internal mammary lymph node involvement
N3a	Metastasis in ipsilateral infraclavicular lymph node(s)
N3b	Metastasis in ipsilateral internal mammary lymph node(s) and axillary lymph node(s)
N3c	Metastasis in ipsilateral supraclavicular lymph node(s)
Pathologic classification (Liao et al.):	

PNx	Regional lymph nodes cannot be assessed (not removed for pathologic study or previously removed)
pN0	No regional lymph node metastasis
pN0(i-)	No regional lymph node metastases histologically, negative IHC
pN0(i+)	Malignant cells in regional lymph node(s) ≤ 0.2 mm (detected by hematoxylin-eosin [H&E] stain or IHC, including ITC)
pN0(mol-)	No regional lymph node metastases histologically, negative molecular findings (reverse transcriptase polymerase chain reaction [RT-PCR])
pN0(mol+)	Positive molecular findings (RT-PCR) but no regional lymph node metastases detected by histology or IHC
pN1	Micrometastases; or metastases in 1-3 axillary lymph nodes and/or in internal mammary nodes, with metastases detected by sentinel lymph node biopsy but not clinically detected
pN1m	Micrometastases (> 0.2 mm and/or > 200 cells, but none > 2.0 mm)
pN1a	Metastases in 1-3 axillary lymph nodes (at least 1 metastasis > 2.0 mm
pN1b	Metastases in internal mammary nodes, with micrometastases or macrometastases detected by sentinel lymph node biopsy but not clinically detected†
pN1c	Metastases in 1-3 axillary lymph nodes and in internal mammary lymph nodes, with micrometastases or macrometastases detected by sentinel lymph node biopsy but not clinically detected†
pN2	Metastases in 4-9 axillary lymph nodes or in clinically detected‡ internal mammary lymph nodes in the absence of axillary lymph node metastases
pN2a	Metastases in 4-9 axillary lymph nodes (at least 1 tumor deposit > 2.0 mm)
pN2b	Metastases in clinically detected‡ internal mammary lymph nodes in the absence of axillary lymph node metastase

pN3	Metastases in ≥ 10 axillary lymph nodes; or in infraclavicular (level III axillary) lymph nodes; or in clinically detected ipsilateral internal mammary lymph nodes in the presence of ≥ 1 positive level I, II axillary lymph nodes; or in > 3 axillary lymph nodes and in internal mammary lymph nodes, with micrometastases or macrometastases detected by sentinel lymph node biopsy but not clinically detected†; or in ipsilateral supraclavicular lymph nodes
pN3a	Metastases in ≥ 10 axillary lymph nodes (at least 1 tumor deposit > 0.2 cm); or metastases to the infraclavicular (level III axillary lymph) nodes
pN3b	Metastases in clinically detected ipsilateral internal mammary lymph nodes in the presence of ≥ 1 positive axillary lymph nodes; or in > 3 axillary lymph nodes and in internal mammary lymph nodes, with micrometastases or macrometastases detected by sentinel lymph node biopsy but not clinically detected
pN3c	Metastases in ipsilateral supraclavicular lymph nodes
Distant metastasis (M)	
Mx	Distant spread (metastasis) cannot be assessed
M0	No distant spread is found on x-rays (or other imaging procedures) or by physical exam
M1	Cancer has spread to distant organs (the most common sites are bone, lung, brain, and liver)

Information Source

1. NCCN Clinical Practice Guidelines in Oncology: Breast Cancer. V 3.2013. National Comprehensive Cancer Network. Available at *http://bit.ly/jOuSUf*. September 5, 2013.
2. TNM Classification for Breast Cancer from the American Joint Committee on Cancer(AJCC) *Cancer Staging Manual*, 7th Edition provided by the American Cancer Society 200

Annex II: English Information Sheet and Consent form for patients

Information Sheet: For patient

Title of the project: Circulating Breast cancer-associated microRNA Expression in Serum as Biomarker for Breast Cancer Detection.

Dear participant,

We would like to ask your Volunteer participation for the medical research entitled ”**Circulating Breast cancer-associated microRNA expression in Serum as Biomarker for Breast Cancer Detection”** in Tikur Anbessa Specialized University Hospital, Addis Ababa, Ethiopia**.** This study have been approved by the Ethical Clearance Committee of College of Health Science, School of medicine Department of medical biochemistry, Addis Ababa University, the science and ethical review office of AHRI and permission (letter of support) from the Tikur Anbessa Specialized University Hospital. We will explain the purpose of the study and the activities that are going to be done in the study. You can raise questions that you want to be explained and clarified for you. After carefully listening and reading, the explanations regarding the medical research, if you volunteer to participate in the study you can put your signature in the space provided.

Why do we need to conduct the study?

Breast cancer is a major global health threat among women worldwide. Althoughit is highly curable if diagnosed and treated appropriately at an early stage, there are challenges to the clinician in the management of breast cancer. In the present study, we will be looking into the circulating breast cancer-associated microRNA**,** which affect gene regulation, to see the expression level in breast cancer patients. If the expression level of miRNAs increase in the patients regarding to healthy control, it will be a good markers for breast cancer detection in a minimally invasive manner, sensitive enough to detect early presence of tumours. Therefore, these molecules as significant novel markers with potential use as diagnostic, prognostic and therapeutic tools.

Sampling Procedure

If you are voluntary to participate in the study, 5ml (equivalent of 1 to 2 tea spoonful) of blood will be drown by trained laboratory personnel or clinician under aseptic conditions. Then, serum will be prepared and miRNAs will be extracted, which will then be reverse transcriptase by specific primers. Finally, they will be analyzed using real time polymerase chain reaction.

Risks and discomfort

There is no serious health hazard will be caused due to your participation in the study rather than which should be taken for the sake of your diagnosis. The procedure used in blood collection is minimally invasive, if you may result to some minor discomfort around the needle entrance area; it is easily controlled through medical practice. This however is a situation which may occur during routine practice not a risk that could happen due to enrolment into this study.

Role of participant in the study

If you are volunteer to participate in the study, we would like to obtain a total of 5ml blood for clinical and molecular analysis. You will also be requested to give answers for the structured questions during the interview.

Incentives and Payment

You will be paid 50 birr due to your participation as compensation for your time you spend with as for this study. If the results of the laboratory examinations do have a direct benefit in the health of the study participants, you can discuss with the physician and get your results and treatment as required. However, this study will benefit at large the community in minimizing the burden of breast cancer.

Participant's right and Withdrawal from the Study

Your participation in the study is entirely dependent in your voluntary willing. Even after giving your consent to participate in the study, you have a full right to withdraw from the study at any time. If you with draw from the study there is no consequences on the service you get from the institute.

Confidentiality

The Information you gave for this study only according to your permission. We will not use your information for purposes other than this study. We assure that the information and data obtained from the laboratory results are confidential. After doing the study the results will be sent for publication without exposing your identity.

Agreement

After reading and listening about the study procedures and other related issues done in the study, you will be kindly requested to put your signature of agreement. Your signature indicates that your participation is only based on your volunteer participation.

Assurance of Principal Investigator

I put my signature below to confirm you that I take over the responsibility for the scientific ethical and technical conduct of the research project and for provision of progress reports for all stakeholders of the research project.

Tariku Sime (PI)

Signature: _____ Date: _____

Contact

In case you might have any questions, unclear ideas and doubt about the study, you should feel free to ask any questions anytime throughout the study by using this address:

PI Address: Tariku Sime

Phone Number; **+251921195218**

Email:**tarikusime2@gmail.com**

Biochemistry Department school of Medicine, Addis Ababa University

Email:**biochemistry.som@aau.edu.et**

Tel: +251-011-550-52-48

Armauer Hansen Research Institute (AHRI). **Tel: +251- 011-348-12-89**

Informed Consent form for participants

I have read or, had read to me this information sheet. I have understood the information above and I have had the opportunity to ask any questions and all my questions have been answered to my satisfaction. I freely and voluntarily agree to participate in this research study by giving blood sample for Laboratory examination and I understand I may withdraw from further participation in the study at any time without consequence, that I might cost some time to respond all questions when I give answers during the activity and that I can wait until to finish it. I have received a copy of this agreement form to keep.

I_____ hereby give my consent for giving of the requested information and specimens as the medical doctor and the researcher find best for me.

ParticipantNameSignature: Date:_____

Investigator _____Signature: Date_____

Witness _____Signature _____Date_____

We thank you for consenting to take part in the study !!

Annex III: Amharic Information and Consent form for patients

የጥናቱ ማብራሪያ ቅፅ ለጥናቱ ተሳታፊዎች

የጥናቱርእስ፡ በደም ዉስጥ ከጡት ካንሰር ጋር ግኙኝነት ያላቸዉ የሚይክሮ አር ኤን ኤ ቅንጣቶች የጡት ካንሰርን የመጠቆም ችሎታቸዉ

የተከበሩ ተሣታፊ፡

ከላይ በተጠቀሰው ርዕስ ላይ ለሚደረገው ጥናት እንዲሳተፉልን በአክብሮት ልንጠይቅዎት እንወዳለን:: የጥናቱ ሁኔታ በተመለከተም ማብራሪያ እንሰጥዎታለን:: እናም ጥያቄ ካልዎት ግልፅ እናደርግሎታለን፡፡በዚህ ጥናት መሳተፍ ግድ የሌለብዎት ሲሆን ለመሳተፍ ፈቃደኛ ከሆኑ ብቻ ይህን ቅፅ አንብበዉ ወይ ተነቦልሁ በቅጹ መጨረሻ ላይ ይፈርማሉ፡፡

ይህ ጥናት ለምን አስፈለገ?

የጡት ካንሰር በአሁኑ ጊዜ በአለማችን ላይ አደገኛ ከሚባሉት በሽታዎች ተርታ አንዱነው፡፡ምንም እንኳን የጡት ካንሰርን ገና ከጅማሬዉ መኖሩን ካረጋገጥን ና ከታከምን በቀላሉ ማዳን ቢቻልም በሽታዉን ግን መቆጣጠር በጣም አስቸጋሪ ሆኗል፡፡እናም የእኛጥናት ዋና ዓላማ የሚያተኩረዉ የጂን ለዉጦችን የሚያመለክቱ በደም ዉስጥ የሚገኙ *የማይክሮ አርኤን ኤቅንጣቶች ለይቶ ማጥናት ይሆናል፡ጸ*ዚህ ጥናት ውጤትም የጡት ካንሰር *ቅድመ-ምርመራ ለማድረግ፤ የ*በሽታዉን ሁኔታ ለመረዳት ና ተገቢዉን ህክምና ለመዉሰድየሚያስፈልገውን ነገርሊጠቁመንይችላል፡፡

በጥናቱ ወቅት ምንምን የምርመራ ዓይነቶች ይሠራሉ?

እርስዎ በጥናቱ ተሳታፊ ከሆኑ በመጀመሪያ ከዚህ ጥናትጋር የተያያዙ እርስዎን የተመለከቱ መረጃዎችና የእርስዎ የጤና ሁኔታን የሚያትቱ ጥያቄዎች እንጠይቅዎታለን፡፡ከዛም 5 ሚሊ ሊትር (ከ 1 -2 የሻይማንኪያ) የደምናሙና ወስደን የደምዉሃን ከደምሴሎች ከለየን ቦሃላ በአርማወርሀንሰን የምርምር ኢንስቲትዩት የማይክሮ አርኤንኤ ቅንጣቶችን በሚመርምር ዘመናዊ መሳሪያ እንመረምራለን፡፡

የጎንዮሽ ጉዳት

በዚህ ጥናት በመሳተፋችሁ በሰዉነታችሁ ናበጤናችሁ ላይ ምንም ችግር እንደማያመጣ በጥንቃቄ ታይቶ በአዲስ አባባ ዩኒቨርስቲ እና በአርማወርሀንሰን የምርምር ኢንስቲትዩት የጥናትና ምርምር ስነ_ምግባር ገምጋሚ ኮሚቴዎች ፍቃድ ተሰቶታል፡፡የደም ናሙና ስንወስድ በሰዉነትዎ ላይ ምንም ጉዳት አያደርስም፡፡ነገር ግን መጠነኛ ህመም መርፊ በሚገባበት ቦታ ዓለ፡፡

የተሣታፊዎችአስተዋፅኦ

በዚህጥናትለመሳተፍፈቃደኛከሆኑ፤እርስዎ5ሚሊሊትርየደምናሙናይሰጣሉ፡፡በተጨማሪምከዚህጥናትየተያየዙእርስዎንየተመ ለከቱመረጃዎችናየእርስዎየጤናሁኔታንየሚያትቱጥያቄዎችይመልስሉናል፡፡

ይህጥናትለተሳታፊዎችያለውእንደምታምንድነው?

እርስዎ በዚህ ጥናት በመሳተፋቹ 50ብር የሚከፈልዎትመሆኑን እናሳውቃለን:: ይህምላጠፉት ሰአት ማካካሻ ነዉ::

የምርመራ ዉጤትዎ አስፈላጊ ከሆነ ከህኪም ጋር በመነጋገር አስፈላጊዉን ሕክምና ይደረግልዎታል፡፡ ይሁን እንጂ ይህ ጥናት በህብረተሰባችንበተለይምበሴቶችላይየጡትካንሰርየሚያደርስዉንጉዳትለመቀነስ ያለዉ እንደምታ ትልቅ ስለሆነ በዚህ ጥናት በመሳተፍ የበኩልዎትን አስተዋፅኦያደርጋሉ፡፡

የተሣታፊዎች መብት

በጥናቱ የሚሳተፉት የፍቃደኝነት ማረጋገጫ ሲሰጡን ብቻ ነው፡፡በፈለጉትጊዜስምምነቱንሊያቆርጡይችላሉ:: በጥናቱ ሲሳተፉግልፅያልሆኑ ነገሮች የመጠየቅ ሙሉ መብት አለዎት፡፡ እኛም የተነሱትን ጥያቄዎች ግልፅእናደርግልዎታለን፡፡በጥናቱ ላይም ለመሳተፍ ካል ተስማሙም የህክምና አገልግሎት ሙሉ በሙሉ የማግኘት ይችላሉ፡፡

የጥናቱሚስጥራዊነት

እርስዎየሰጡንመረጃናየመርመራዉጤትዎከተጠቀሰውከእርሶየተወሰደመረጃከምርምርስራውጭለሌነገርአይውልም::በመጨረሻም የጥናቱውጤት የእርስዎን ሚስጥራዊንት በጠበቀሁኔታ ለህትመትይላካል፡፡

ስምምነት

ጥናቱን በተመለከተ በቂ መረጃ ካገኙ በሃላየስምምነት ፊርማ ይፈርማሉ፡፡ይህፊርማ የእርስዎን ፈቃደኝነትን ያረጋግጣል፡፡

መረጃ ስለ ማግኘት

ስለዚህጥናትማንኛውምጥያቄካለዎትበማንኛውምጊዜማነጋገርቢያስፈልግዎየጥናቱመአከልለማግኘትእንደሚከተለውነው፡፡

ዋናተመራማሪ

ታሪኩስሜ

ኢ-ሜይል፣tarikusime2@gmail.com

ሞባይል፤ +251921195218

ባዮኬሚስትሪ ት/ክፍል ህክምና ት/ቤት አዲስ አበባ ዩኒቨርሲቲ

ኢ-ሜይል: **biochemistry.som@aau.edu.et**

ስልክ፡**+251-011-550-52-48**

በአርማወርሀንሰን የምርምር ኢንስቲትዩት የጥናትና ምርምር ስነ_ምግባር ገምጋሚ ኮሚቴ(AHRI)

ስልክ፡+251- 011-348-12-89

የፈቃደኝነትማረጋገጫቅጽ

ስለጥናቱአስፈላጊየሆኑትንመረጃዎችበመረጃቅጹላይአንብቤወይምተነበልኝከጥናቱጋርየተያያዙጥያቄዎችንምጠይቄማብራሪያዎችምተሰጥተዉኛል፡፡በጥናቱላይመሳተፌንለመሰረዝከፈለኩበማንኛዉምሰዓትመሰረዝእንደምችልናይህምበጤናእንክብካቤዬላይምንምዓይነትተጽእኖእንደማይኖረዉተረድቻለሁ፡፡ስለሆነምሁኔታዉንበሚገባበማጤንበፈቃደኝነትበምርምሩላይለመሳተፍለተመራማሪዉፈቃዴንሰጥቻለሁ፡፡እናምለጥናቱአስፈላጊየሆኑመረጃዎችንናለምርመራየሚያገለግሉናሙናዎችንለመርማሪዉሀኪም ናለተመራማሪዉለመስጠትፈቃደኛነኝ፡፡

እኔ _____

የተባልኩግለሰብለምርምሩየሚሆኑመረጃዎችንእናየደምናሙናለመስጠትመስማማቴንበፊርማየአረጋግጣለሁ፡፡

የተሳታፊዉስም----------ፊርማ----------ቀን----------

የተመራማሪዉስም----------ፊርማ----------ቀን----------

የምስክሮችስም----------ፊርማ----------ቀን----------

Annex IV: English Information Sheet and Consent form for Healthy participants

Information Sheet: For Healthy participants

Title of the project: Circulating Breast cancer-associated microRNA Expression in Serum as Biomarker for Breast Cancer Detection.

Dear participant,

As we are developing country we are conducting different research to come up with solution of problem that affects our development. From the major problem that influences our development health problem is one .So Medical research considered as major part of solution to solve health problem. For this purpose we also conduct medical research to put our foot in health development. In this research we need healthy person like you for study purpose. So we would like to ask your volunteer participation for the medical research entitled"**CirculatingBreast cancer-associated microRNA expression in Serum as Biomarker for Breast Cancer Detection**" in Tikur Anbessa Specialized University Hospital, Addis Ababa, Ethiopia. This study have been approved by the Ethical Clearance Committee of College of Health Science, School of medicine Department of medical biochemistry, Addis Ababa University, the science and ethical review office of AHRI and permission (letter of support) from the Tikur Anbessa Specialized University Hospital. We will explain the purpose of the study and the activities that are going to be done in the study. You can raise questions that you want to be explained and clarified for you. After carefully listening and reading, the explanations regarding the medical research, if you volunteer to participate in the study you can put your signature in the space provided.

Why do we need to conduct the study?

Breast cancer is a major global health threat among women worldwide. Althoughit is highly curable if diagnosed and treated appropriately at an early stage, there are challenges to the clinician in the management of breast cancer. In the present study, we will be looking into the circulating breast cancer-associated microRNA**,** which affect gene regulation, to see the expression level in breast cancer patients. If the expression level of miRNAs increase in the patients regarding to healthy control, it will be a good markers for breast cancer detection in a

minimally invasive manner, sensitive enough to detect early presence of tumours. Therefore, these molecules as significant novel markers with potential use as diagnostic, prognostic and therapeutic tools.

Sampling Procedure

If you are voluntary to participate in the study, 5ml (equivalent of 1 to 2 tea spoonful) of blood will be drown by trained laboratory personnel or clinician under aseptic conditions. Then, serum will be prepared and miRNAs will be extracted, which will then be reverse transcriptase by specific primers. Finally, they will be analyzed using real time polymerase chain reaction.

Risks and discomfort

There is no serious health hazard will be caused due to your participation in the study rather than which should be taken for the sake of your diagnosis. The procedure used in blood collection is minimally invasive, if you may result to some minor discomfort around the needle entrance area; it is easily controlled through medical practice. Beyond this there is no other risk that affect your healthy.

Role of participant in the study

If you are volunteer to participate in the study, we would like to obtain a total of 5ml blood for clinical and molecular analysis. You will also be requested to give answers for the structured questions during the interview.

Incentives and Payment

You will be paid 50 birr due to your participation as compensation of your time you spend with as for this study. If the results of the laboratory examinations do have a direct benefit in the health of the study participants, you can discuss with the physician and get your results and treatment as required. However, this study will benefit at large the community in minimizing the burden of breast cancer.

Participant's right and Withdrawal from the Study

Your participation in the study is entirely dependent in your voluntary willing. Even after giving your consent to participate in the study, you have a full right to withdraw from the study at any time. If you with draw from the study there is no consequences on the service you get from the institute at any time.

Confidentiality

The Information you gave for this study only according to your permission. We will not use your information for purposes other than this study. We assure that the information and data obtained from the laboratory results are confidential. After doing the study the results will be sent for publication without exposing your identity.

Agreement

After reading and listening about the study procedures and other related issues done in the study, you will be kindly requested to put your signature of agreement. Your signature indicates that your participation is only based on your volunteer participation.

Assurance of Principal Investigator

I put my signature below to confirm you that I take over the responsibility for the scientific ethical and technical conduct of the research project and for provision of progress reports for all stakeholders of the research project.

Tariku Sime (PI)

Signature: _____ Date: _____

Contact

In case you might have any questions, unclear ideas and doubt about the study, you should feel free to ask any questions anytime throughout the study by using this address:

PI Address: Tariku Sime

Phone Number; **+251921195218**

Email:**tarikusime2@gmail.com**

Biochemistry Department school of Medicine, Addis Ababa University

Email:**biochemistry.som@aau.edu.et**

Tel: +251-011-550-52-48

Armauer Hansen Research Institute (AHRI).

Tel: +251- 011-348-12-89

We thank you for consenting to take part in the study !!

Informed Consent form for Healthy participants

I have read or, had read to me this information sheet. I have understood the information above and I have had the opportunity to ask any questions and all my questions have been answered to my satisfaction. I freely and voluntarily agree to participate in this research study by giving blood sample for Laboratory examination and I understand I may withdraw from further participation in the study at any time without consequence, that I might cost some time to respond all questions when I give answers during the activity and that I can wait until to finish it. I have received a copy of this agreement form to keep.

I_____ hereby give my consent for giving of the requested information and specimens as the medical doctor and the researcher find best for me.

ParticipantNameSignature: Date:_____

Investigator _____Signature: Date_____

Witness _____Signature _____Date_____

Annex v: Amharic Information and Consent form for Healthy participant

የጥናቱ ማብራሪያ ቅፅ ለጥናቱ ተሳታፊዎች

የጥናቱርእስ፡ በደም ዉስጥ ከጡት ካንሰር ጋር ግኙኝነት ያላቸዉ የሚይክሮ አር ኤን ኤ ቅንጣቶች የጡት ካንሰርን የመጠቆም ችሎታቸዉ

የተከበሩ ተሣታፊ፡

እንደሚታወቀዉ አገራችን እድገት ላይ ካሉ አገር ትመደባለች፡፡ለዝም የተያዩ ችግር ፈቺ የምርምር ስራዎች እየተሰሩ ይገኛሉ፤የጤና ችግር የአገሪቱን እድገት ከሚገቱት አንዱ በመሆኑ የተነሳ የተለያዩ ምርምር እዝ ላይ እየተሰሩ ነዉ፡፡እኛም የበኩላችን ለማበርከት በጤና ላይ ምርምር እየሰራን እንገኛለን፤ለዝ ምርምር ደግሞ ጤነኛ የሆኑትንም ሰዎች ያስፈልጉናል፡፡ስለዝህም ከላይ በተጠቀሰው ርዕስ ላይ ለሚደረገው ጥናት እንዲሳተፉልን በአክብሮት ልንጠይቅዎት እንወዳለን:: የጥናቱ ሁኔታ በተመለከተም ማብራሪያ እንሰጥዎታለን:: እናም ጥያቄ ካልዎት ግልፅ እናደርግሎታለን፡፡በዚህ ጥናት መሳተፍ ግድ የሌለብዎት ሲሆን ለመሳተፍ ፈቃደኛ ከሆኑ ብቻ ይህን ቅፅ አንብበዉ ወይ ተነቦልሁ በቅጹ መጨረሻ ላይ ይፈርማሉ፡፡

ይህ ጥናት ለምን አስፈለገ?

የጡት ካንሰር በአሁኑ ጊዜ በአለማችን ላይ አደገኛ ከሚባሉት በሽታዎች ተርታ አንዱነው፡፡ምንም እንኳን የጡት ካንሰርን ገና ከጅማሬዉ መኖሩን ካረጋገጥንና ከታከምን በቀላሉ ማዳን ቢቻልም በሽታዉን ግን መቆጣጠር በጣም አስቸጋሪ ሆኗል ፡፡ እናም የእኛ ጥናት ዋና ዓላማ የሚያተኩረዉ የጂን ለዉጦችን የሚያመለክቱ በደም ዉስጥ የሚገኙ *የማይክሮ አር ኤን ኤ ቅንጣቶች ለይቶ ማጥናት ይሆናል ፡፡* የዚህ ጥናት ውጤትም የጡት ካንሰር *ቅድመ-ምርመራ ለማድረግ ፤የበሽታዉን ሁኔታ* ለመረዳትና ተገቢዉን ህክምና ለመወሰድ የሚያስፈልገውን ነገር ሊጠቁመንይችላል፡፡

በጥናቱ ወቅት ምንምን የምርመራ ዓይነቶች ይሠራሉ?

እርስዎበጥናቱተሳታፊከሆኑበመጀመሪያከዚህጥናትጋርየተያያዙእርስዎንየተመለከቱመረጃዎችናየእርስዎየጤናሁኔታንየሚያትቱ ጥያቄዎችእንጠይቅዎታለን፡፡ከዛም 5 ሚሊሊት (ከ1-2 የሻይ ማንኪያ) የደም ናሙና ወስደን የደም ዉሃን ከደም ሴሎች ከለየን ቦሃላ በአርማወርህንሰንየምርምርንስቲትዩትየማይክሮአርኤንኤቅንጣቶችንበሚመርምርዘመናዊመሳሪያእንመረምራለን፡፡

የጎንዮሽ ጉዳት

በዚህ ጥናት በመሳተፋችሁ በሰዉነታችሁ ናበጤንነታቹ ላይ ምንም ችግር እንደማያመጣ በጥንቃቄ ታይቶ በአዲስ አባባ ዩኒቨርስቲ እና በአርማወርህንሰን የምርምር ኢንስቲትዩት የጥናትና ምርምር ስነ_ምግባር ገምጋሚ ኮሚቴዎች ፍቃድ ተሰቶታል፡፡የደም ናሙና ስንወስድ በሰዉነትዎ ላይ ምንም ጉዳት አያደርስም፡፡ነገር ግን መጠነኛ ህመም ደም ሲቀዳ መርፊ በሚገባ በት ቦታ ዓለ፡፡ከዝህ ሌላ በጤናቹ ላይ የሚያመጣ ጉዳት ዬለም፡፡

የተሣታፊዎች አስተዋፅኦ

በዚህጥናትለመሳተፍፈቃደኛከሆኑ፤እርስዎ 5 ሚሊሊትር የደምናሙናይሰጣሉ፡፡ በተጨማሪም ከዚህ ጥናት የተያየዙ እርስዎን የተመለከቱ መረጃዎች ና የእርስዎየጤናሁኔታንየሚያካትቱጥያቄዎችይመልሱልናል፡፡

ይህጥናትለተሳታፊዎችያለውእንደምታምንድነው?

እርስዎ በዚህ ጥናት በመሳተፋቹ 50ብር የሚከፈልዎትመሆኑን እናሳውቃለን፡፡ ይህም ለእኛ ቢሎ ላባከኑት ማካካሻ ነዉ፡፡ የምርመራ ዉጤትዎ አስፈላጊ ከሆነ ከሀኪም ጋር በመነጋገር አስፈላጊዉን ሕክምና ይደረግልዎታል፡፡ ይሁን እንጂ ይህ ጥናት በህብረተሰባችንበተለይምበሴቶችላይየጡትካንሰርየሚያደርስዉንጉዳትለመቀነስ ያለዉ እንደምታ ትልቅ ስለሆነ በዚህ ጥናት በመሳተፍ የበኩልዎትን አስተዋፅኦያደርጋሉ፡፡

የተሣታፊዎች መብት

በጥናቱ የሚሳተፉት የፍቃደኝነት ማረጋገጫ ሲሰጡን ብቻ ነው፡፡በፈለጉትጊዜስምምነቱንሊያቆርጡይችላሉ:: በጥናቱ ሲሳተፉግልፅያልሆኑ ነገሮች የመጠየቅ ሙሉ መብት አለዎት፡፡ እኛም የተነሱትን ጥያቄዎች ግልፅእናደርግልዎታለን፡፡በጥናቱ ላይም ለመሳተፍ ካል ተስማሙም የህክምና ኣገልግሎት ሙሉ በሙሉ የማግኘት ይችላሉ፡፡

የጥናቱሚስጥራዊነት

እርስዎ የሰጡን መረጃና የመርመራ ዉጤትዎ ከተጠቀሰው ከእርሶ የተወሰደ መረጃ ከምርምር ስራ ውጭ ለሌላ ነገር ኣይውልም::በመጨረሻም የጥናቱውጤት የእርስዎን ሚስጥራዊንት በጠበቀሁኔታ ለህትመትይላካል፡፡

ስምምነት

ጥናቱን በተመለከተ በቂ መረጃ ካገኙ በሃላየስምምነት ፊርማ ይፈርማሉ፡፡ይህፊርማ የእርስዎን ፈቃደኝነትን ያረጋግጣል፡፡

መረጃ ስለ ማግኘት

ስለዚህጥናትማንኛውምጥያቄካለዎትበማንኛውምጊዜማነጋገርቢያስፈልግዎየጥናቱመአከልለማግኘትእንደሚከተለውነው፡፡

ዋናተመራማሪ

ታሪኩስሜ

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በአርማወርሀንሰን የምርምር ኢንስቲትዩት የጥናትና ምርምር ስነ_ምግባር ገምጋሚ ኮሚቴ(AHRI)

ስልክ፡+251- 011-348-12-89

የፈቃደኝነትማረጋገጫቅጽ

ስለጥናቱአስፈላጊየሆኑትንመረጃዎችበመረጃቅጹላይአንብቤወይምተነበልኝከጥናቱጋርየተያያዙጥያቄዎችንምጠይቄማብራሪያዎችምተሰጥተዉኛል፡፡በጥናቱላይመሳተፌንለመሰረዝከፈለኩበማንኛዉምሰዓትመሰረዝእንደምችልናይህምበጤናእንክብካቤዬላይምንምዓይነትተጽእኖእንደማይኖረዉተረድቻለሁ፡፡ስለሆነምሁኔታዉንበሚገባበማጤንበፈቃደኝነትበምርምሩላይለመሳተፍለተመራማሪዉፈቃዴንሰጥቻለሁ፡፡እናምለጥናቱአስፈላጊየሆኑመረጃዎችንናለምርመራየሚያገለግሉናሙናዎችንለመርማሪዉሀኪም ናለተመራማሪዉለመስጠትፈቃደኛነኝ፡፡

እኔ _____

የተባልኩግለሰብለምርምሩየሚሆኑመረጃዎችንእናየደምናሙናለመስጠትመስማማቴንበፊርማየአረጋግጣለሁ፡፡

የተሳታፊዉስም----------ፊርማ----------ቀን----------

የተመራማሪዉስም----------ፊርማ----------ቀን----------

የምስክሮችስም----------ፊርማ----------ቀን----------

Annex VI: Data collection Sheet

Participant Data

Admission Date __/___/___

Participant name_____

Participant Id:

Medical Record Number (MRN): _____

Hospital name: _____

City/Town_____

Region/sub city: _____

Woreda _____

Keble_____

Part I. Socio-demographic characteristics

1. Sex: Female ☐
2. Age:_____
3. Marital status Single ☐ Married ☐ Divorced ☐
   Widow ☐ Cohabit ☐
1. Body Mass Index Calculation
   4.1. Height (Meter) ☐☐.☐☐
   4. 2. Weight (Kg) ☐☐.☐☐
   4.3. Body Mass Index (Kg/m^2) ☐☐.☐☐
2. Occupation: Farmer ☐ Government worker ☐ Self employee ☐
   Student ☐ Retire ☐ Merchant/Business man ☐
   Others (specify) ☐
3. Education level: can't read and write ☐ Can read and write/no formal education ☐
   Primary education ☐ Secondary education ☐ College / University ☐
4. Residence: Urban ☐ Rural ☐

Part II. Participant Medical History

1. Do you have a smoking habit? Yes ☐ No ☐
2. Do you have habit of consuming alcohol? Yes ☐ No ☐
3. Was there a family history of malignancy? Yes ☐ No ☐

   3.1. If yes, which type of malignancy? Breast ☐ ovarian ☐ Blood ☐

   Brain ☐ Lung ☐ Colon Endometrial ☐

   Others ☐
4. Did you receive blood? Yes ☐ No ☐
   If yes when? _____
5. Do you have any other chronic disease? Yes ☐ No ☐

   5.1. If yes, what is that? Diabetes mellitus ☐ Hypertension ☐

   Renal disease ☐ Respiratory disease ☐ others (specify) ☐
6. Are you taking medication now? (*The interviewer should look the records / sample drugs*)
   Yes ☐ No ☐

6.1. If yes, what type? Chemotherapy, ☐ analgesic drugs/steroids ☐ Antibiotics ☐

7. Menstruation status? Regular menstruation cycle ☐ Irregular Menopause ☐

   7.1. If you are menopause, when 40-45 ☐ 46-50 ☐ 51-55 ☐ ›55 ☐

   7.2. When did you start menstruation (age of first menstruation cycle you menarchy)

   8-10 ☐ 11- 13 ☐ 14-15 ☐ > 15 ☐
8. Do you mean history of pregnancy? Yes ☐ No ☐
9. Did you give birth? Yes ☐ No ☐

   If yes, did you well fed your breast? Yes ☐ No ☐
10. Do/did you use Contraceptive? Yes ☐ No ☐
11. Did you receive hormonal therapy? Yes ☐ No ☐

If yes for how money months/years?

12. Did you have a history of exposure to Radation? Yes ☐ No ☐

If yes when?_____

Part III. Breast Cancer status

1. When do you know you had BC? _____
2. How do you know you had BC? _____

   Self-examination ❑ Clinical examination ❑incidentally❑

3. When did your symptoms start? _____
4. Do you have the following symptoms on your breast?

Symptoms	**Yes**	**No**
A lump or thickening in an area of your breast		
A change in the size or shape of one or both breasts		
Dimpling of the skin of your breasts		
A change in the shape of your nipple (turns in, sinks into the breast, or has an irregular shape)		
A blood stained discharge from the nipple		
A rash on a nipple or surrounding area		
A swelling or lump in your armpit		
Pain in your breasts not related to your period		

5. Any therapeutic prior to admission this hospital?Yes❑No❑

   If yes, therapy details: Surgery ☐ Radiotherapy ☐Chemotherapy ☐

   Hormonal therapyother ☐ ☐

   Remarks:

Annex VII: Amharic Data collection sheet

የተሳታፊዉመረጃመሰብሰቢያቅፅ

የተሳታፊዉ መረጃ	
ቀን (ቀን/ወር/ዓ.ም) የተሳታፊዉ ስም_____ የተሳታፊዉ መለያ ቁጥር : የህክምና መዝገብ ቁጥር _____ የጤና ተቋሙስም _____	
ከተማ_____ ወረዳ _____	ክልል/ክ/ከተማ _____ ቀበሌ_____

ክፍልአንድ፡አጠቃላይመረጃ

1. ጾታ፡ሴት☐
2. እድሜ:_____
3. የጋብቻሁኔታ፡ያላገባች ☐ያገባች☐አግብታየፈታች ☐

   ባል የሞተባት ☐ ሳይጋቡ አብረው የሚኖሩ☐
4. ክብደትበቁመትሲለካ
   4.1. ቁመት(ሜትር)☐☐.☐☐
   4.2. ክብደት(ኪ..ግ) ☐☐.☐☐
   4.3. ክብደትበቁመትሲለካ(ኪ..ግ/ሜ$^{2)}$☐☐.☐☐
5. ስራ: ገበሬ☐የመንግስትሰራተኛ☐የግል☐ ተማሪ☐ጡረተኛ☐ነጋዴ☐ሌላካለይገለጽ---------
   -----
6. የትምህርትደረጃ፡ማንበብና መጻፍ የማይችል☐ማንበብና መጻፍ የሚይችል☐የመጀመሪያ ደረጃ ት/ት
   የተማረ☐ሁለተኛ ደረጃ ት/ት የተማረ ☐ኮሌጅወይምዩኒቨርስቲደ☐
7. የመኖሪያአድራሻ፡ከተማ☐ገጠር ☐

ክፍልሁለት፡-የታካሚሁኔታ /የህክምናመረጃ

1. ሲጋራያጨሳሉ? አዎአላጨስም☐

2. የአልኮልመጠጥይጠጣሉ? አዎአልጠጣም☐

3. ከቤተሰብዎአባል/ የዘርግንድየካንሰርወይምእጢያለበትአለ; አዎ☐የለም☐

3.1. አዎካሉ የትገኛዉ የካንሰር ዓይነት ነበር? የጡት ☐ የደም ☐ የአንጀት☐

የማህፀን ☐ የአንጎል ☐ የሳንባ ☐ ሌላ ካለ ☐

4. ደምተለግሰሁያዉቻሉ? አዎአላዉቅም ☐
አዎ ካሉ መቼ? ☐

5. ለረጅምጊዜየቆየህመምአለብዎት;(*የታካሚማህደርይታይ)አለ*☐*የለም* ☐

5.1. ካለብዎትምንድንነው; የስኳርበሽታ☐የደምግፊት☐የኩላሊትህመም☐የመተንፈሻአካል ☐
ሌላካለይጠቀስ----------

6. አሁንየሚዎስዱትመድሃኒት ፡አለ☐የለም☐

6.1. ካለ ምንአይነትነው (*መድሃኒቱወይምየታካሚማህደርይታይ)*

ኬሞፈዉስ☐ለበሽታአምጭተህዋሲያንየሚሰጥ/የህመም ማስታገሻ☐

7. የወር አበባ ሁኔታ? ዑደቱን የጠበቀ☐ያልተበቀ☐አርጫለሁ ☐

7.1. አርጫለሁ ካሉ በስንት ዓመትዎ? 40-45 ☐ 46-50 ☐ 51-55 ☐ ›55 ☐

7.2. *መጀመሪያ* የወር አበባ ሲያዩ የስንት ዓመት ነበሩ?

8-10☐ 11- 13 ☐ 14-15 ☐> 15 ☐

7. የእርግዝና ሁኔታ፡ እርጉዝ ነኝ ☐አይደለሁም ☐
8. ልጅ ወልደዋል? አዎ ☐አልወለድኩም ☐
አዎ ካሉ አጥብተዋል?አዎ ☐አላጠባሁም ☐

9. የእርግዝናመከላከያ ይጠቀማሉ?አዎ☐አልጠቀምም☐

አዎ ካሉ ለስንት ወራት/ አመት?

10. የሆርሞን መቀየር ህክምና አድርገዋል? አዎ☐አላደረኩም ☐

11. የጨረር ህክምና ታክመዉ ያዉቃሉ? አዎ☐አላዉቅም☐

ክፍልሶስት፡የጡት ካንሰርንበተመለከተ

1. የጡት ካንሰርን እንዳለብዎት መቼ አወቁ? _____

2. እንዴት አወቁ? _____

   ራስ በመመርመር ❑በሀኪም❑በድንገት❑

3. የህመም ስሜት መቼ ጀመረሁ? _____

4. የሚከተሉትየጡት ህመም ምልክቶች አለብዎት?

ምልክቶች	**አለ**	**የለም**
በጡትዎት አካባቢ እብተት መታየት		
በአንዱ ወይም በሁለቱም ጡትዎት ላይ የቅርፅና የመጠን ለዉጥ መኖር		
የጡትዎ ቆዳ ላይ ጎድጎድ ያለ ምልክትመታየት		
በጡትዎት ጫፍ ላይ የቅርፅ ለዉጥ መኖር (ወደ ዉስጥ መሰርጎድና ከወትሮዉ የተለየ የቅርፅ ለዉጥ)		
ደም የቀላቀለ ፈሳሽ በጡት ጫፍ መፍሰስ		
የጡትዎት ጫፍ ወይም ዙሪያዉ ቀልቶ መታየት		
በብብትዎት አካባቢ እብተት መታየት		
ከወር አበባ ወይም ሌሎች የተፈጥሮ ለዉጦች ጋር ያልተገናኘ ህመም በጡትዎት ላይ ሲሰማዎት		

5. እዚህሆስፒታልከመጎጣትዎትበፊትሌላህክምናወስደዋል?አዎ❑አልወሰድኩም❑

አዎካሉቢገልጹልን

ቀዶ ህክምና ❑ጨረር ❑ኬሞፈዉስ ❑የሆርሞን ህክምና ❑ሌላ ካለ ❑

አስተያየት ካለዎት፡

_____

Annex VIII:Standard Operation Procuder (Sop)

Sop For Extraction of miRNA From Serum

Equipment and Material

- MiRNeasy serum/plasma kit
- Ethanol(96-100%)
- QIAzol Lysis
- miRNeasy Serum/Plasma Spike-In Control
- chloroform
- RNeasy MinElute spin column
- Buffer of RPE
- Buffer of RWT
- RNase-free water

PROTOCOL

1)Prepare 200µl serum or thaw frozen samples.

2) Add 5 volumes QIAzol Lysis Reagent to the sample(add 1ml QIAzol Lysis Reagent).Mix by vortexing.

3)Incubate the homogenate at room temperature(15-25°C) for 5 minute.

4)Add 3.5 µl miRNeasy Serum/Plasma Spike-In Control (1.6 x 108 copies/µl working solution). and mix thoroughly.
5) Add200µl chloroform. Vortex or shake vigorously for 15 s.

6)Incubate at room temperature for 2-3 minute.

7)Centrifuge for 15 min at 12,000 x g at 4°C.

8)Transfer the upper aqueous phase to a new collection tube (not supplied). Avoid transfer of any interphase. Add 1.5 volumes of 100% ethanol (e.g., for 600µl aqueous phase, add 900µl ethanol).Mix thoroughly by pipetting.

9)Pipet up to700µl Buffer RWT to the RNeasy ,including any precipitate, into an RNeasy MinElute spin column in a 2ml collection tube. Close the lid and centrifuge at12000 x g for 15 s at room temperature. Discard the flow-through.

10)Repeat step 9 using the remainder of the sample.

11)Add 700µl Buffer RWT to the RNeasy MinElute spin column. Close the lid, and centrifuge for 15 s at12000 x g .Discard the flow-through.

12) Pipet 500 µl Buffer RPE onto the RNeasy MinElute spin column. Closethe lid gently and centrifuge for 15 s at12000 x g to wash the column. Discard the flow-through

13) Add 500 µl of 80% ethanol onto the RNeasy MinElute spin column.Close the lid gently and centrifuge for 2 min at12000 x g to wash the spin column membrane. Discard the collection tube with the flow-through

14)Place the RNeasy MinElute spin column into a new 2 ml collection tube (supplied). Open the lid of the spin column, and centrifuge at full speed for 5 min to dry the membrane. Discard the collection tube with the flow-through.

15)Place the RNeasy MinElute spin column in a new 1.5 ml collection tube (supplied). Add 14 µl RNase-free water directly to the center of the spin column membrane. Close the lid gently, and centrifuge for 1 min at full speed to elute the RNA.

SOP FOR RERVERSETRANSCRIPTION: miRNA TO COMPLEMENTARY DNA (cDNA)

EQUIPMENT AND MATERIAL

- miScript II RT Kit
- 10x miScript Nucleics Mix
- 5x miScript HiSpec Buffer
- 5x miScript HiFlex Buffer
- miScript SYBR Green PCR Kit,
- snRNA RNU6 as endogenous control
- PCR tubes(for 20 µl reactions)
- Ice
- Microcentrifuge

PROCEDURE FOR cDNA

1)Thaw template RNA on ice. Thaw 10x miScript Nucleics Mix, RNase freewater, and 5x miScript HiSpec Buffer(for selective conversion of mature miRNAs into cDNA)at room temperature (15–25ºC).

Mix each solution by flicking the tubes. Centrifuge briefly to collect residual liquid from the sides of the tubes and then store on ice.

2)Prepare the reverse transcription master mix on ice.We prepare for 20µl volume of reaction.(it was prepared using 4 µl of 5x miScript HiSpec Buffer,2 µl of 10x miScript Nucleics Mix,7 µl of RNase-free water,2 µl of RTmix).

3)Add5µl template of RNAto each tube containing reverse transcription master mix. Gently mix, briefly centrifuge, and then store on ice.

4) Incubate for 60 min at 37ºC.

5) Incubate for 5 min at 95ºC to inactivate miScript Reverse Transcriptase Mix and place on ice.

6) stored at -20 ºC until use.

SOP FOR REAL-TIME PCR FOR DETECTION OF MATUREmiRNA

PROCEDURE

1)Thaw 2x QuantiTect SYBR Green PCR Master Mix, 10x miScript Universal Primer, 10x miScript Primer Assay, template cDNA, andRNase-free water at room temperature (15–25ºC). Mix the individual solutions.

2)Prepare a reaction mix.The reaction performed in total volume of 20 µl containg 10 µl of SYBR Green Mix,2 µl Universal primer,2 µl miRNA specific primer,4 µl RNase free water PCR reaction mix

3)Dispense 2 µltemplate cDNA into the individual plate/Rotor-Disc wells.

4)Mix the reaction mix thoroughly but gently, and dispense appropriate volumes into the tubes

5) Carefully, tightly seal the plate or disc with caps.

6) Program the real-time cycler accordingly.The cycling program start at 95ºC for 15 min, followed by 40 cycles of 94ºC for15 sec,55ºC for 30 sec and 70ºC for 30sec.

7. Place the plate/Rotor-Disc in the real-time cycler and start the cycling program.

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Declaration

I, the undersigned, hereby declare that this MSc thesis is my original work and has not been presented for a degree in any other university, and that all sources of materials used for the thesis have been duly acknowledged.

Name of candidate:	signature	date
Tariku Sime	_____	_____
Advisors		
Dr.Daniel Seifu	_____	_____
Co-Advisors		
Dr.Markos Abebe	_____	_____
Mr.Dawit Hailu	_____	_____
Dr.Rawleigh Howe	_____	_____
Dr.Yonas Mulugeta	_____	_____
Mrs.Fregenet Tesfaye	_____	_____