
ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES

PhD Dissertation

# Analysis of selected circulatory microRNAs in breast cancer before and after chemotherapy

**By**

**Yonas Mulugeta Balcha**

DEPARTMENT OF BIOCHEMISTRY
FACULTY OF MEDICINE

September 2018

# Analysis of selected circulatory microRNAs in breast cancer before and after chemotherapy

A thesis submitted to School of graduate studies, Addis Ababa University in fulfillment of the requirements for the Doctor of Philosophy in Biochemistry

**Yonas Mulugeta Balcha (MSc)**

**9/24/2018**

ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES
DEPARTMENT OF BIOCHEMISTRY

Addis Ababa University

School of Graduate Studies

This is to certify that the thesis prepared by Yonas Mulugeta, entitled "Analysis of selected circulatory microRNAs in breast cancer before and after chemotherapy" and submitted in fulfillment of the requirements for the Degree of Philosophy (Biochemistry) compiles with the regulations of the University and meets the accepted standards with respect to originality and quality.

Signed by examining committee:

1. Examiner: _____Signature:_____ Date:_____
2. Examiner: _____Signature:_____ Date:_____
3. Examiner: _____Signature:_____ Date:_____
4. Advisor: _____Signature:_____ Date:_____
5. Advisor:_____ Signature:_____ Date:_____

# Research Advisors

## Advisor :

**Dr. Daniel Seifu,** PhD, Associate Professor of Biochemistry
Department of Biochemistry, AAU
E-mail: abbysinia2002@gmail.com

## Co-Advisors:

**Professor Abebe Bekele**, MD, FCS
Professor of Surgery
General and Thoracic Surgeon
E-mail: abebesurg@yahoo.com

**Dr. Wondemagegnhu Tigeneh** , MD
Clinical oncologist
Department of Radiotherapy, AAU
E-mail: tigneh@yahoo.com

**Dr. Rick Kittles**, PhD, Professor
Associate Director of Health Equities,
Comprehensive Cancer Center
City of Hope, California, U.S.A
E-mail: rkittles@coh.org

**Dr. Usha Menon**, PhD, RN, FAAN, Professor
Vice Dean of Research
University of South Florida
E-mail: umenon@health.usf.edu

## Acknowledgements

First and foremost I am greatly thankful for the study participants who were willing to give blood samples and filled out questionnaires and provided me with the invaluable data.

I am deeply indebted to my adviser Dr. Daniel Seifu for his contribution of knowledge, time, and ideas; and for the uninterrupted support during my Ph.D. pursuit. I greatly value his effort in searching research collaborators for the molecular analysis of samples. I would also like to thank other research advisors who contributed immensely to this work by providing insightful comments and suggestions. I very much appreciate the efforts of Professor Abebe Bekele to make collaboration with University of Arizona possible.

Many thanks are owed to Dr. Rick Kittles, for believing in me and for being such a great support during my stay at University of Arizona. I am especially grateful for the time he dedicated for critically reviewing study methodology and for his thoughtful recommendations. My stay at his lab was particularly important not only in learning advanced molecular techniques, it was also a place to learn from and enjoy scientific discussions. I would also like to acknowledge members of his research team for their assistance and support. I am especially grateful for Ebony Shah for her skilled assistance and guidance during sample analysis.

I must thank Dr. Menon Usha, who invited me for a research visit to the University of Arizona. Her supervision, guidance and help make my scholarly

visit productive. Her enthusiasm for research was contagious and motivational. My appreciations do also extend to her colleagues and assistants. I am greatly thankful to Dr. Laura Szalacha for her help in statistics; and Diana Louise for organizing my visit and for the continuous assistance.

I thank staffs of the department of biochemistry, Addis Ababa University, for their support, suggestions and for their kind assistance at various phases of this research. I would also like to thank nurses working at the chemotherapy unit of cancer therapy center in Tikur Anbessa Specialized Teaching Hospital (TASH) for their support during sample collection.

A financial support from Addis Ababa University thematic research grant was used for sample collection and transportation. Costs for sample analysis and related expenses were covered by the University of Arizona. I gratefully acknowledge these funding sources that make my Ph. D work possible. I also express my appreciation to Arsi University for sponsoring and supporting my study.

I owe a lot to my loving and supportive wife, Alem Wondimu, who encouraged and helped me at every stage of my personal and academic career, and longed to see this achievement come true. My sons, Nolawi and Yafet, are the pride and joy of my life; I love you all. I would also like to thank my parents, family members and friends who supported me in every possible way to see the completion of this work.

# Contents

## List of figures

## List of tables

## Abbreviations

| | |
|---|---|
| ABC | Adenosine triphosphate-binding cassette |
| Ago | Argonaute protein |
| AJCC | American Joint Committee on Cancer |
| AUC | Area under the curve |
| BMI | Body mass index |
| BRCA 1 | Breast cancer susceptibility gene 1 |
| BUN | Blood urea nitrogen |
| CA | Cancer antigen |
| CEA | Carcinoembryonic antigen |
| CMF | Cyclophosphamide, methotrexate, and fluorouracil |
| DCIS | Ductal carcinoma in situ |
| DFF | DNA fragmentation factor |
| DGCR8 | DiGeorge syndrome critical region gene 8 |
| EGFR | Epidermal growth factor receptor |
| ELISA | Enzyme linked immunosorbent assay |
| ER | Estrogen receptor alpha |
| ETS | E26 transformation-specific transcription factor |
| FISH | Fluorescence in situ hybridization |
| FNAB | Fine needle aspiration biopsy |
| FOXP3 | Forkhead box P3 |
| HER2/ERBB2 | Human epidermal growth factor receptor 2 |
| IHC | Immunohistochemistry |
| LABC | Locally advanced breast cancer |

| | |
|---|---|
| miRNAs | MicroRNAs |
| MRP-1/ABCC1 | Multi-drug resistance associated protein |
| MUC1 | Mucin 1 |
| NF-κB | Nuclear factor kappa-light-chain-enhancer of B cells |
| PDCD4 | Programmed cell death 4 |
| PDK | Phosphoinositide dependent kinase |
| PI3K | Phosphatidylinositide 3-kinase |
| PIP2 | Phosphatidyl inositol 4,5-bisphosphate |
| PIP3 | Phosphatidylinositol 3,4,5-trisphosphate |
| PR | Progesterone receptor |
| pre-miRNA | Precursor miRNA |
| pri-miRNA | Primary microRNAs |
| PTEN | Phosphatase and tensin homolog |
| PUMA | p53 upregulated modulator of apoptosis |
| RCF | Relative centrifugal field |
| RISC | RNA-induced silencing complex |
| ROC | Receiver operating characteristic |
| SNP | Single-nucleotide polymorphism |
| SPRY2 | Sprouty homolog 2 |
| TNBC | Triple negative breast cancer |
| TNF | Tumor necrosis factor |
| TRBP | Tar RNA binding protein |
| UICC | International Union Against Cancer |

## Abstract

Breast cancer is the second most common cause of death from cancer in women. There is now increased prevalence of breast cancer among African women. In Ethiopia it is the leading cancer type constituting 33% of all cancer cases in women. It is a heterogeneous type of disease which comprises of many biologically different and pathologically distinct features that lead to different treatment responses.

Tests and procedures for diagnosis and prognosis of breast cancer are still limited to invasive procedures and imaging techniques. Based on their differential expression in disease and with their exceptional stability in biological fluids, microRNAs are noticeable candidates to be used as non-invasive diagnostic and prognostic biomarker. The association of change in expression level of microRNAs with clinicopathological parameters may demonstrate their potential in sorting the heterogeneous disease to specific subgroups for effective treatment options.

The purpose of this study was to investigate the differential expression of specific circulatory microRNAs in breast cancer patients and study their association with clinicopathological parameters with samples from healthy volunteers serving as control. Changes in the level of microRNA after completing chemotherapy was also assessed and checked for association with serum CA 15-3 values to see the prognostic potential of specific microRNAs.

The levels of specific circulatory microRNAs in patient and control samples were analyzed using qRT-PCR. Serum CA 15-3 values from patient samples collected after completion of chemotherapy were measured using solid phase enzyme linked immunosorbent assay.

In this study miRNA 21-5p was significantly over expressed in the serum of breast cancer patients when compared with controls ($P<0.05$). Receiver operating characteristic curve analysis shows its potential in differentiating breast cancer patients from controls with AUC value of 0.6 ($P<0.05$). A significant decrease in serum miR-326 was observed after chemotherapy ($P<0.05$) as compared to serum samples collected before chemotherapy. No correlations was found between serum CA 15-3 values and levels of circulatory microRNAs. Mean serum CA 15-3 value of patients with stage IV breast cancer was found to be high when compared with patients with other stages ($P<0.001$). Well-designed studies are required to elucidate the clinical validity of deregulated microRNAs in breast cancer to be used as diagnostic and prognostic biomarkers. Since deregulated miRNAs in the circulation are shared by several cancer types and subtypes, further studies are necessary to identify a well characterized cluster of miRNAs with discriminative ability.

# 1 CHAPTER ONE

## 1.1 INTRODUCTION

The breast is a modified skin gland enveloped in fibrousfascia. A healthy female breast is composed of different tissues, glandular tissues and stromal (supporting) tissues. Glandular tissues house the milk producing glands (lobules) and the ducts (the milk passages), while stromal tissues include fatty and fibrous connective tissues of the breast (Figure 1). Lobes and lobules are connected by milk ducts, which stores and transports milk to the nipple during lactation (Hassiotou and Geddes 2013). The cells are nourished by blood and waste products are drained by lymphatic vessels which are connected to lymph nodes.

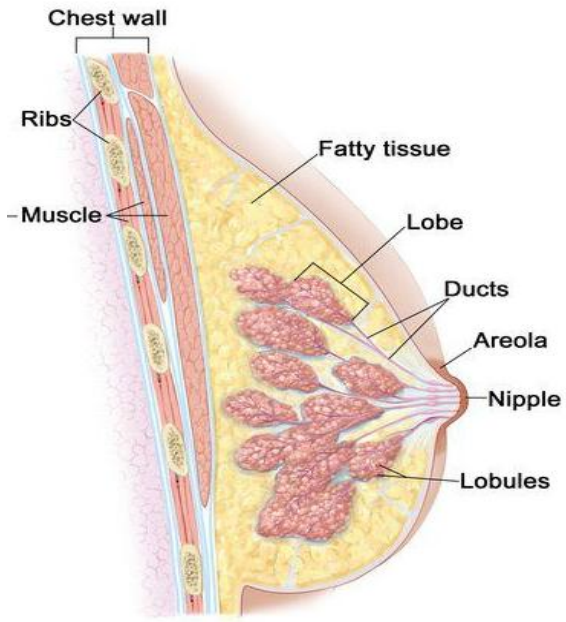



Figure 1 General illustration on anatomy of adult female breast (reprinted from https://www.ncbi.nlm.nih.gov/books/NBK65716/figure/CDR0000062970__281/)

Breast cancer is uncontrolled growth of epithelial cells originating in the ducts or breast lobules. It is rare for breast cancers to come from other structures in the breast such as fat tissues or lymphatic vessels (Sharma, Dave and Sanadya 2010). Although the exact cause of breast cancer is unknown, there are specific risk-factors which have been identified. The mechanism how these risk factors increase the chance of breast cancer development and its prognosis is not yet clear (Ataollahi et al. 2015).

Recent advances on detection and diagnosis of breast cancer has paved way for better treatment decisions and improved survival rates (Taherian, Srihari, and Ragan 2014). Diagnostic tools, which were based in the past predominantly on the anatomic extent of the disease, are shifting to the underlying biological mechanisms. Molecular analysis of the disease has led to the recognition that breast cancer is a heterogeneous disease composed of different biological subtypes; and effective genetic profiling techniques are now enabling response to chemotherapy to be predicted (Wang 2015).

## 1.2 Background and literature review

### 1.2.1 Epidemiology and Etiology of breast cancer

Being the most common invasive malignancy and the second most common cause of death from cancer in women, breast cancer has great impact in women health in all world regions (Hiatt and Brody, 2018). According to the 2014 world cancer report by the International Agency for Research on Cancer, there were

1,676,633 new cases of breast cancer among women in 2012, which composes more than a quarter of total cancer cases (Stewart and Wild 2014).

Increased prevalence of breast cancer among African women is stated elsewhere (Saghir et al. 2011), and this rise is being driven by increasing life expectancy at birth, improved control of infectious diseases, and changing lifestyle, diet and physical activity. This rapid societal, economic and lifestyle transition in some countries tends to ever-increase the impact of breast cancer.

Breast cancer among young women comprises a higher proportion of the cases in Africa, which is attributed to the low median age of African population (Akarolo-Anthony, Ogundiran, and Adebamowo 2010). There is misinterpretation of this demography driven phenomenon when it is taken as a preferential targeting of African young women with intrinsic biological significance. The dominant young age profile of cases and the high fatality rate that characterized African breast cancer cases might reflect the demographic structure of the African population (which tends to be younger) rather than intrinsic biological vulnerability as had been suggested (Azubuike et al. 2018).

The relatively more advanced stages at presentation of breast cancer among sub-Saharan patients is thought to be due to poor awareness, reduced access for health care, an absence of organized early detection programs, and poor facilities for accurate and timely diagnosis and treatment (Jedy-Agba et al. 2016). In addition to differences in detection and treatment capacity, the clinically and pathologically aggressive nature of breast cancer with rapid progression among

young women contributes much for the observed differences in mortality rates of breast cancer between African and other populations. Studies have shown strong association between poor prognostic factors with young age patients with breast cancer (Darwish et al. 2017).

A four year average incidence rate study made in Ethiopia showed breast cancer to be the leading cancer constituting 33% of the cancers in women (Solomon et al. 2018). A recently published study on incidence of cancer in Addis Ababa also found breast cancer to be the commonest type of cancer in the city (Figure 2), constituting 31% of all cancer cases in females with age standardized incidence rate of 40.6 from 100,000 females (Timotewos et al. 2018). Unlike the astonishing improvements in the survival rates and stable mortality rates in western countries, breast cancer is reported to be a fatal disease in Ethiopia, with high mortality (Dye et al. 2012).

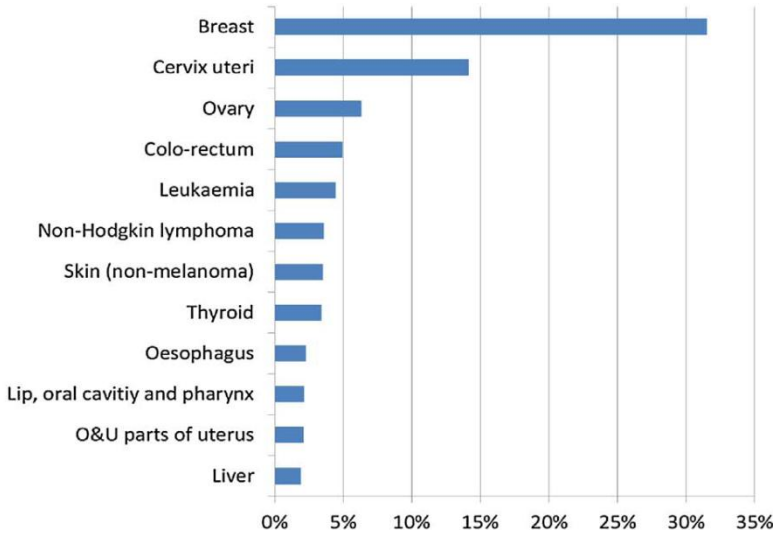



Figure 2 Proportion of common cancer cases among females in Addis Ababa(reprinted from Timotewos *et al.*, 2018)

The cause of breast cancer and the reasons for its progression is not yet fully understood. Breast cancer may occur when gradual accumulation of acquired mutation and epigenetic changes affect mammary tissue cells and their progenitors (Kleibl and Kristensen 2016). Factors including positive family history, diet, physical activity, estrogen exposure, Body Mass Index (BMI), depression and quality of life have been mentioned as breast cancer risk factors (Nasser and Pars 2011).

There are two leading models accounting for breast carcinogenesis; the clonal evolution model and the cancer stem cell model (Figure 3). According to the clonal evolution model, any breast epithelial cell can be the targets of random mutational events with advantageous genetic and epigenetic alterations are selected over time to contribute to tumor initiation and progression (Bombonati and Sgroi 2011). The fate of the accumulated alterations may end-up in impairing cell number regulation and disrupting inter-cellular interaction. Additional changes on the expanding clone contribute for the potential of the cell to invade adjacent stromal cells and spread to distant sites through lymphovascular system (Celià-terrassa and Kang 2016).

The cancer stem cell model derives from the hierarchical organization of normal breast parenchymal development pathway (Shackleton et al. 2009). The possibility of accumulating genetic and epigenetic modifications, as resident cells of the mammary tissue, and the potential of this cells to generate varied progeny of highly proliferative cells is considered to be the reason for the detected

heterogeneity of breast cancer (Peiris-Pagès et al. 2016; Bombonati and Sgroi 2011). The asymmetrical division of cancer stem cells maintains the stem cell population while at the same time differentiating into phenotypically diverse non tumorigenic cancer cells that compose the bulk of cells in a tumor. There is now accumulating evidence that cancer stem cells may also arise from restricted progenitor or differentiated cells by acquiring stem cell-like properties (Al-Ejeh et al. 2011).

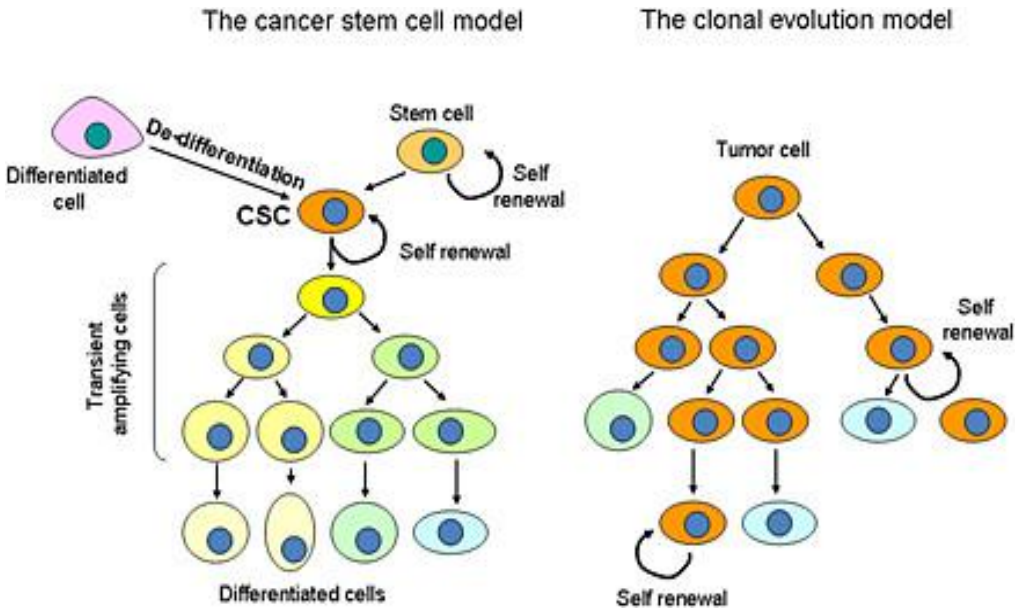



**Figure 3** The two separate and mutually exclusive models which explain the development of tumors, the cancer stem cell model and the clonal evolution model (reprinted from Carnero and Lleonart, 2016).

### 1.2.2 Diagnosis of breast cancer

Breast cancer is a heterogeneous type of disease which comprises of many biologically different and pathologically distinct features (Dai et al. 2015; Aapro 2006). Despite the significant advances in diagnosis and treatment of breast cancer, there are still challenges related to prevention, more specific diagnosis

and therapeutic resistance which requires in-depth understanding of its heterogeneity (Polyak 2007). Variation in histological grade, estrogen receptor alpha (ER), progesterone receptor (PR), epidermal growth factor receptor (EGFR) and Ki-67 status are the major features observed in both invasive and in-situ breast tumors (Lesurf et al. 2016). Based on the hormone status levels, the 2011 St Gallen International Breast Cancer Conference divide breast cancer into the following four molecular subtypes (Falck et al. 2013):

- Luminal A (ER + and/or PR+, Ki67 low (<20) and HER2-)

This is a slow-growing and most favorable prognostic subtype; the expression of hormone receptors is predictive of response to hormonal therapy.

- Luminal B (ER + and/or PR+, Ki67 high (>20) and/or HER2+)

Luminal B breast cancers tend to be higher grade and more aggressive than luminal A breast cancers. It is characterized by high Ki67, indicator of actively dividing cells. It is less endocrine sensitive and worse prognosis when compared with Luminal A (Maisonneuve et al. 2014).

- HER2-positive (ER-, PR- and HER2+)

These cancers tend to grow and spread more aggressively than other breast cancers. Targeted therapy using monoclonal antibodies, such as trastuzumab and pertuzumab, and tyrosine kinase inhibitors have proven to be effective therapeutic agents for HER2+ breast (Wang 2015).

- Triple negative breast cancer, TNBC (ER-, PR-, HER2-)

Lack of specific targeted treatment makes this subtype difficult to treat. Triple negative breast cancers have a poorer short-term prognosis than other breast cancer types. They are more common in premenopausal women and those with a breast cancer susceptibility gene 1 (BRCA1) mutation (Sood 2017).

Disproportionately high frequencies of hormone receptor poor breast cancer subtypes among Africans are reported by different researchers. TNBC and basal-like breast cancer, which is often aggressive and is associated with a higher mortality, are reported to be common in the indigenous people of West and East Africa (Brewster, Chavez-MacGregor, and Brown 2014; Vanderpuye et al. 2017; Huo et al. 2009). However, systematic studies of the disease in Africa have not confirmed the suggestion that African breast tumors are predominantly receptor poor (Akarolo-Anthony, Ogundiran, and Adebamowo 2010). Inability to apply advanced immunohistochemical (IHC) and molecular biology techniques and poor tissue preparation in low income countries is proposed to be likely reason for heightened frequency of unfavorable breast cancer subtypes reported by the studies (Makanjuola et al. 2014; Galukande et al. 2014). Large age-standardized and well-designed studies are required to accurately quantify the distribution of the various breast cancer subtypes across Africa (Eng, McCormack, and dos-Santos-Silva 2014).

Despite the progress made in identifying risk factors and genetic markers for breast cancer, approximately 70–80% of cases occur in women without known

major predictors (Kelly et al. 2010). It has a long period of growth prior to becoming symptomatic or when it is detected during a physical exam. This period is generally between one and several years in length, depending on a woman's age and breast density (Martin et al, 2015). Early detection of breast cancer is now considered as the primary strategy for reducing breast cancer mortality. Effective early detection programs may lead to decreased staging, which increases the proportion of breast cancers detected at an early stage, when treatment is most effective (Gioia 2017). The significant decrease of mortality from breast cancer in the developed countries is now partly attributed to the advances made in detection of tumors at early stage (Warner 2011).

At present, breast cancer detection relies mostly on mammography, which has been associated with decreased breast cancer mortality (Kazarian et al. 2017). Mammography is convenient, inexpensive and has become the modality choice for an early detection of breast cancers due to its sensitivity in recognizing breast masses. However, the number of cancers escaping detection with mammography is substantial, particularly in dense-breasted women, with sensitivity as low as 30–48% (Kelly et al. 2010). To increase the specificity of its detection (and to differentiate between dense breast tissue, benign (not cancer) lumps and cancer) breast ultrasound and breast magnetic resonance imaging (MRI) may be done (Subramaniam et al. 2006).

Fine needle aspiration biopsy (FNAB) is also described as excellent technique in the diagnosis of breast cancer. The technique is described as minimally invasive, highly accurate, safe, cost-effective, and extremely benign procedure. It is primarily used for diagnosing palpable lesions; its sensitivity has been reported to be between 89% and 98% (Yu, Wei, and Liu 2012). Getting reliable FNAB cytology result depends on different factors including the skills of the aspirator, the cytopathologist and the histological type of the lesion, age of the patient, size of the lesion and method of detection (clinically detected or image-detected). Even if FNAB cytology is considered to be a powerful tool for diagnosis it has limitations in distinguishing ductal carcinoma in situ (DCIS) and invasive carcinoma (Bishop et al. 2004). The role of FNAB has been challenged lately by better overall results attained by core biopsies (Lieske, Ravichandran, and Wright 2006). Longer turn-around and patient discomfort during the core biopsy procedures are stated as drawbacks for the technique.

Recent advances in the laboratory techniques have permitted more specific diagnoses of breast cancer. The development and routine use of single-gene predictor techniques, such as IHC, fluorescence in situ hybridization (FISH), DNA microarrays, single-nucleotide polymorphism (SNP) analysis, multiplex polymerase chain reaction (PCR) and proteomics, offer the possibility of examining a very large number of genes and proteins from a single biopsy (Aapro 2006).

### 1.2.3 Staging and prognosis

Survival is known to be better for patients with localized breast tumors than for patients with more extensive disease at regional or distant sites. This is the basis for initial tumor staging categories. The staging of breast cancer is the classic and reliable indicator of prognosis that provides valuable confirmation about the current status of cancer and its therapy (Donegan 1997). Clinicopathological parameters such as tumor size, lymph node involvement, metastatic state as well as tumor receptor status are well-known prognostic markers for breast cancer (Dong et al. 2014). The well demonstrated TNM staging system is used to determine the anatomical extent of malignant disease on the basis of clinical (cTNM) and pathological (pTNM) criteria grouped under three broad headings: tumor size (T 1-4), association of lymph nodes (N 1-3) and existence of distant metastases (M 0-1). For the standardization of TNM staging, International Union Against Cancer (UICC) and American Joint Committee on Cancer (AJCC) have worked for more than 40 years to ensure proper exchange of clinical information between oncology centers with little ambiguity. With periodical modification, the TNM classification system was also designed with the aims of assisting treatment planning, providing prognostic guidance, and improving understanding of the disease process (Benson 2003). Currently, clinicians use TNM staging with other factors to make specific treatment recommendations.

### 1.2.4 Treatment options of breast cancer and response monitoring

A variety of medical therapies has been described to the treatment of breast cancer. But the types of therapy can be categorized into two major classes as local and systemic. Local therapy removes the tumor from limited area of the breast and includes surgery, radiotherapy or both. Whereas systemic therapy aims to get rid of cancer cells that may have spread from the breast to other parts of the body, and includes chemotherapy, hormonal therapy and targeted therapy.

Surgery is the oldest method of treating breast cancer which has got more advances in the recent years. The goal of this therapy is to remove the entire tumor from the breast and cancerous lymph nodes from the underarm area. There are two types of surgery to remove breast cancer, the breast conserving surgery (also called lumpectomy) and mastectomy. Breast conserving surgery refers to the removal of the tumor in the breast with a rim of normal breast tissue called a clear margin. It is usually followed by post-operative radiation therapy. It is the preferred locoregional treatment for the majority of patients with early-stage breast cancer (Clough et al. 2011). In some cases, neoadjuvant therapy can shrink a locally advanced tumor enough that lumpectomy plus radiation therapy becomes an option to mastectomy (Mandilaras et al. 2015; Tewari, Krishnamurthy, and Shukla 2009). A breast tumor can also be removed by mastectomy where the entire breast is removed. There are three types of mastectomy; partial, total and modified radical (Figure 4). In partial mastectomy the cancerous part of the breast tissue is removed along with some of the normal

tissue around it. The surgeon removes the entire breast and the lining of the chest muscle, but no other tissue in total mastectomy. The modified radical mastectomy removes the entire breast, the lining of the chest muscles and some of the lymph nodes in the underarm area (axillary nodes). The factors that influence these treatment decisions are complex and involve issues regarding molecular subtype of the disease, stage and grade, access to health care, concerns for cancer recurrence, and the impact of surgery on body image and sexuality (Bellavance and Kesmodel 2016).

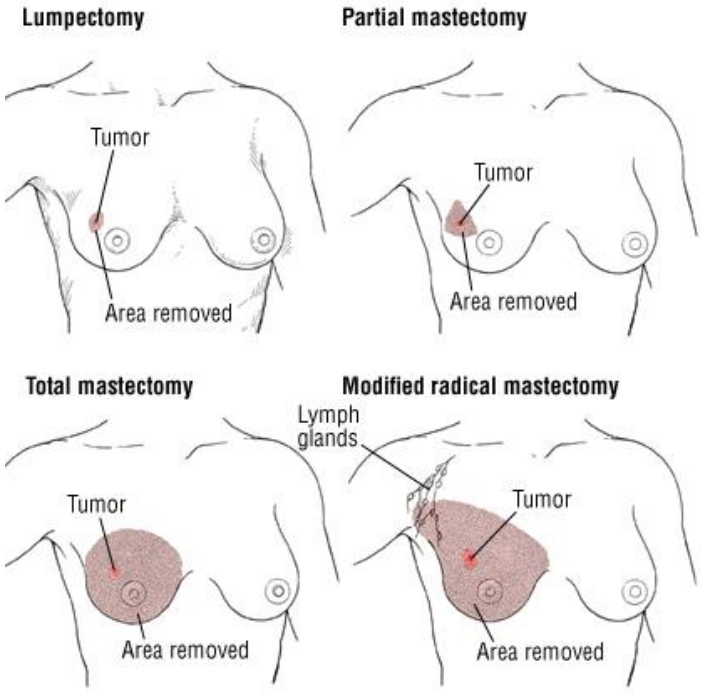



Figure 4 Surgical procedures applied to remove breast cancer, breast conserving surgery (also called lumpectomy) and mastectomy.

Radiotherapy increases both local control and overall survival after surgery. It also reduces ipsilateral breast tumor recurrence following breast conservation in

patients with a diagnosis of DCIS. Radiotherapy also affects normal cells in the area being treated, but these cells can usually recover better than cancer cells. Treatments are usually given regularly over a period of time so that they have the greatest effect on the cancer cells, while limiting the damage to normal cells.

A significant proportion of women with LABC experience relapse at distant sites due to undetected deposits of disease, or micrometastases, after locoregional treatment. If left untreated, it may develop into a life-threatening clinical recurrence. Eradicating micro-metastases by giving postoperative or adjuvant chemotherapy has proved highly effective in preventing both local and distant relapses and is essential in optimizing the chance for cure (Szabatura and Seung, 2009).

Chemotherapy is a systemic treatment which relies on drugs to inhibit the growth and progression of the tumor and to induce cancer cell death. Patients with locally advanced breast cancer (LABC) receive chemotherapy as their initial or neoadjuvant treatment modality to decrease primary tumor size, down-stage nodal involvement, and provide pathologic complete responses (Szabatura and Seung, 2009). Neoadjuvant chemotherapy also allows the in vivo assessment of tumor sensitivity to chemotherapy for optimization of available therapeutic agents (Esteva and Hortobagyi, 1998).

Clear survival benefits have been documented in using chemotherapy as a treatment option both in advanced and early stage breast cancer types.

Hundreds of randomized studies have been performed in the past on various chemotherapy regimens in an attempt to refine therapeutic strategies (Bines et al. 2014). Cyclophosphamide, methotrexate and fluorouracil (CMF) was the first adjuvant chemotherapy shown to improve outcome in breast cancer, but was generally replaced by anthracycline-containing regimens that performed better (Hassan et al, 2010). Taxanes have emerged in 1990s as the most powerful group of compounds which adds up to the combination regimens (Gradishar, 2012). Currently the standard of choice in many settings is the use of anthracyclines and taxanes (in combinations or sequentially) for improved survival and decreased progression of metastatic breast cancer (Bines et al. 2014).

Estrogen, a hormone produced by the ovaries in addition to other tissues, promotes the growth of ER-positive breast cancers. The estrogen receptor-mediated, genomic effects on proliferation and the receptor-independent, genotoxic effects of estrogen metabolites can act either in an additive or synergistic fashion to cause breast cancer (Santen and Harvey 1999). To inhibit proliferative action of estrogen, patients with ER+ breast cancer can be given hormonal therapy (also called endocrine therapy). There are two approaches in endocrine treatment of breast cancer; the first group of treatments consists of anti-estrogens which prevent estrogen from causing the cancer cells to grow by binding directly to and blocking the estrogen receptor. The second type of treatment lowers the production of estrogen by reversibly inhibiting aromatase enzyme which transforms androgen to estrogen (Seifert-Klauss, Rabe and

Krämer, 2015). Aromatase inhibitors block the synthesis of estrogen without inhibiting production of other important steroids as they inhibit the terminal step of estrogen biosynthesis (Santen and Harvey 1999). Tamoxifen is anti-estrogen which binds to the estrogen receptor and inhibit the proliferative activities of estrogen on mammary epithelium ( Fakhsheena, Nighat and Ali, 2017).

Despite the large number of systemic agents which are available to treat breast cancer, most tumors eventually become unresponsive to systemic therapy (Moiseenko et al. 2017). In recent years, several targeted agents have become available that have improved the outcomes of patients with solid tumors. One of these agents, trastuzumab (a monoclonal antibody against HER2) has proven effective in the treatment of women with HER2-positive breast cancer (Alvarez, Valero, and Hortobagyi 2010). With the approval of trastuzumab for HER2 positive breast cancer, the natural history of the disease has changed with fewer recurrences and more cures (Friend and Royce 2016).

After treatment for breast cancer, follow-up care is important to help maintain good health, to detect early relapse and to evaluate treatment response in metastatic breast cancer. The measurement of tumor marker serum levels is useful in this regard (Garcia et al. 1990). The most widely used serum tumor markers include cancer antigens (CA) CA 15-3, CA 27-29 and carcino-embryonic antigen (CEA). CA 15-3 and CA 27.29 are different epitopes on the same protein antigen product of the breast cancer-associated mucin 1 (MUC1) gene. In normal

breast tissue MUC1 is expressed in the ducts and acini, but disruption of tissue architecture during neoplastic transformation leads to shielding of MUC1 into the blood, where it can be measured (Zaretsky et al. 2006). CA 15-3 is known to be more sensitive than most candidate markers; and most recommended for clinical use to monitoring of patients with metastatic disease during active therapy (Kazarian et al. 2017). Studies demonstrated that CA 15-3 may have a role in monitoring response to chemotherapy.

## 1.3 MicroRNAs

Tumorogenesis cannot get explained exclusively by studying protein-coding genome only; non-coding ribonucleic acids (RNAs) are now being recognized as critical regulators in cancer genome (Ling et al., 2017). MicroRNAs (also named miRNAs) are well studied non-coding RNAs, which are known to play key functions in tumorogenesis.

MicroRNAs are small endogenous RNA families (21-25 nt) which are evolutionary conserved, single stranded, non-coding RNA molecules that bind target mRNA in sequence specific manner to prevent protein production. The recently released miRBase Sequence Database (release 21) contains 28645 entries representing hairpin precursor miRNAs, expressing 35828 mature miRNA products, in 223 species (http://www.mirbase.org/).

### 1.3.1 MicroRNA biogenesis and function

The miRNA pathway begins with the transcription of a stem loop primary miRNA (pri-miRNA), generally by RNA polymerase II (Figure 5). The 70-100 nt stem loop RNAs (pri-miRNA) are processed in the nucleus by a protein complex known as "the microprocessor", which is comprised of the ribonuclease Drosha and the double-stranded RNA-binding protein DiGeorge syndrome critical region gene 8 (DGCR8) to become precursor miRNA (pre-miRNA) (Ladomery, Maddocks, and Wilson 2011; Ke et al. 2003). The DGCR8 recognizes the pri-miRNA at the ssRNA-dsRNA junction and directs Drosha to a specific cleavage site ~11 base pairs from the junction on the stem where Drosha cuts to liberate a ~ 60-70 bp miRNA hairpin precursor (pre-miRNA). The pre-miRNA is exported from the nucleus in a RanGTP-dependent process after binding with exportin-5, which prevents nuclear degradation and facilitates translocation into the cytoplasm (Ladomery, Maddocks, and Wilson 2011; MacFarlane and Murphy 2010).

Once the pre-miRNAs are transported into the cytoplasm, a second ribonuclease, a multi-domain protein Dicer, trims the pre-miRNAs resulting in a 21-25 nt miRNA. The facilitation and stabilization of the dicer mediated cleavage of pre-miRNAS is made with the support of Tar RNA binding protein (TRBP) (Winter et al. 2009). After cleavage the miRNA duplex gets unwound and one strand is loaded onto Argonaute (Ago) proteins to form the RNA-induced silencing

complex (RISC), and aligns with the target mRNA. Argonaute proteins are shown to participate in the miRNA processing, stability and degradation (Winter and Diederichs 2011). The mechanisms by which miRNAs identify their gene targets to control transcription is not yet clear (Catalanotto, Cogoni, and Zardo 2016; MacFarlane and R. Murphy 2010). Bioinformatics tools are often used to predict potential miRNA and mRNA targeting interactions (Ladomery, Maddocks, and Wilson 2011).

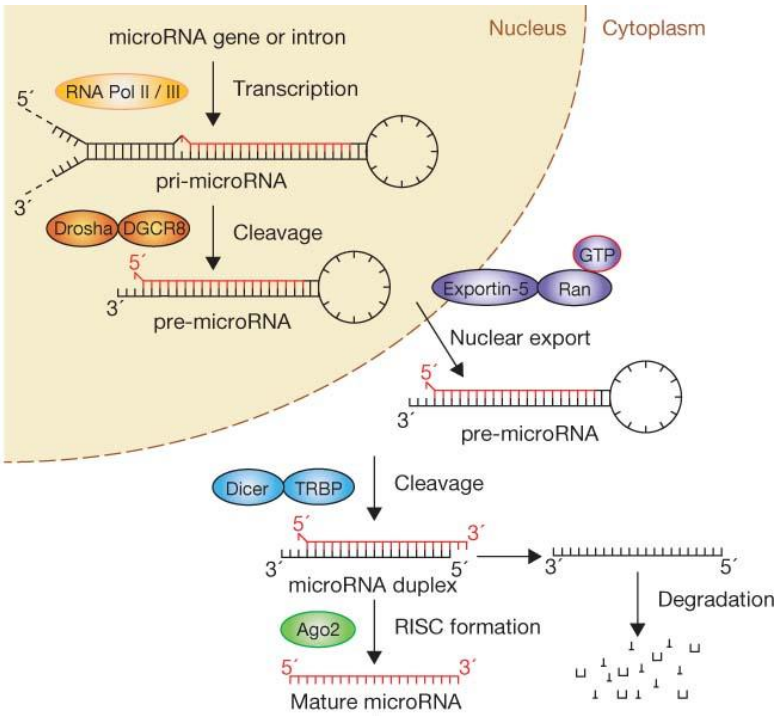



Figure 5 The biogenesis and maturation of microRNAs(reprinted from Winter *et al.*, 2009)

Cytoplasmic microRNAs are known to regulate gene expression at post transcriptional stages, through hindering translation or enhancing mRNA degradation. Watson and crick base-pairing of miRNA guides RISC to 3'-untranslated region of target mRNAs, which are degraded, destabilized or

translationally inhibited by the Ago protein (Winter and Diederichs 2011). A single miRNA guide can regulate several mRNA targets and conversely multiple miRNAs can cooperatively regulate a single mRNA target (MacFarlane and R. Murphy 2010). Recent studies are also showing specific nuclear function of microRNAs; including microRNA-guided transcriptional control of gene expression (Catalanotto, Cogoni, and Zardo 2016). Studies identified universally expressed miRNAs, and several groups of miRNAs expressed exclusively or preferentially in certain tissue types (Liang et al. 2007). The unique expression patterns of microRNAs in various cell types and in different biological processes have been implicated in cell proliferation, cell death, fat metabolism, insulin secretion, and hematopoiesis (Bartel and Chen 2004).

MiRNAs are now considered as attractive markers to study coordinated gene expression in tumorogenesis because of their capability in targeting multiple genes (Lee et al., 2015). Their deregulation in cancer may elucidate their role as oncogenes or tumor suppressor genes. Previous studies have reported differentially expressed microRNAs in solid tumors. In comparison to tissue microRNAs, the detection and differential expression of circulating miRNAs in cancer patients holds great promise for the use of microRNAs as distinctive, non-invasive cancer biomarkers.

## 1.4 Significance and outline of the thesis

Even if considerable improvement have been seen in the survival rates of breast cancer patients (due to early detection and advanced therapeutic strategies), it still causes numerous deaths every year. Challenges still prevail in the management of breast cancer patients, such as unpredictable response and development of resistance to adjuvant therapies. Therefore, more research is needed to understand molecular mechanisms of its pathogenesis, identify molecules involved in breast cancer progression and pharmacological response. Moreover, new markers to improve diagnosis and therapy of specific subtypes of breast cancer are needed.

As with many cancers, methods for diagnosis and prognosis of breast cancer are still limited to invasive procedures (such as tissue biopsies for histological examination) and imaging techniques (including mammograms, breast ultrasound, chest x-ray) (Harbeck and Gnant 2017). Literatures demonstrated that gene expression profiles has been used to develop genomic tests that may provide better predictions of clinical outcome than the traditional clinical and pathological standards (Sotiriou and Pusztai 2009).

mRNA arrays have been investigated extensively for this use, and disease-specific and prognostic signatures have been identified (Bao and Davidson 2008). However, so far these techniques have not been implemented in clinical practice, mainly because of the requirement for fresh tumor material, problems with the reproducibility when the method is applied to different platforms, complicated

bioinformatics due to massive amounts of data, and extensive costs. In contrast to mRNA expression profiling, miRNA signatures have proven to successfully distinguish tumors according to their grade of differentiation with more stability (Schuster et al. 2011).

MicroRNAs, due to their small size are relatively resistant to ribonuclease (RNase) degradation suggesting they would be eminently suitable candidates for detection in biological fluids. The exceptional stability of circulating miRNAs arises from the 40-to 100-nm lipoprotein vesicles, called exosomes, which wraps them to protect from endogenous RNase activity (Frères et al. 2016). MicroRNAs are not only found within cells but also in body fluids, including blood, saliva and urine where they can easily be detected and quantified. It has been shown that cell-free miRNAs in body fluids are stable under harsh conditions such as high temperatures, extreme pH values, repeated freeze-thaw cycles and long-term storage (Chemonges, Tung, and Fraser 2014). For these reasons miRNAs are becoming ideal candidates as non-invasive biomarkers for potentially any human disease, including cancer (Behrmann, Margue, and Kreis 2015).

Blood specimens are easy to obtain and convenient to clinical application. And the circulating miRNAs constitute a novel class of non-invasive biomarkers which show good stability under a variety of physical and chemical conditions (Han et al. 2017). Circulating microRNAs analysis may aid in risk assessment, diagnosis, prognosis, and monitoring of treatment response. Dysrgulation of

microRNA profile in cancer cells is well established; and is thought to be reflected in the extracellular space as affected cells release up-regulated miRNAs or fail to release apparently down-regulated species (Witwer 2015).

Due to the need for novel and targeted therapy for the heterogeneous breast cancer, it will be more important to correlate miRNA expression with clinical parameters. miRNA expression signatures may turn out to be an efficient tool for identifying subgroups of breast cancer patients who will benefit from alternative treatment regimens producing a desired or intended result.

In the present study, we have studied the demographic and biochemical profile of breast cancer patients undergoing chemotherapy treatment and compared their differences among stages of cancer. To propose new tools for breast cancer diagnosis and prognosis, we tested the differential expression of 6 circulating miRNAs in breast cancer patients and assessed their change before and after chemotherapy. We have also evaluated the prognostic use of CA15-3 as a monitoring tool for breast cancer patients after completing chemotherapy treatment.

# 2 CHAPTER TWO

## 2.1 Study objectives

### 2.1.1 General objective:

To investigate the use of circulatory microRNAs in breast cancer as non-invasive biomarker for diagnosis and assessment of prognosis

### 2.1.2 Specific objectives:

- Assessing the demographic and biochemical profiles of breast cancer patients and exploring associations with clinicopathological parameters
- Analysis of differential expression of specific circulatory miRNAs in breast cancer patients
- Establishing the relationship between specific miRNA expression with clinical pathology
- Assessing changes in the level of specific circulatory microRNAs during chemotherapy
- Evaluating the potential of CA 15-3 to monitor breast cancer patients during chemotherapy
- Evaluating associations between CA 15-3 values and deregulated microRNAs

# 3 CHAPTER THREE

## 3.1 Methods

### 3.1.1 Study design and participants

#### *3.1.1.1 Study design*

This study followed a longitudinal case-control procedure. It selects participants on the bases of diseases status (case and controls), and identified differentially expressed miRNA which is tested for its potential to be used as molecular diagnostic marker for breast cancer. The study also compared the expression levels of specific microRNAs before and after chemotherapy to see changes in expression level during treatment. For this, study subjects were followed until the completion of therapy and blood samples were taken from the same study subjects before and after completion of chemotherapy.

#### *3.1.1.2 Study area*

This study is conducted in Tikur Anbessa Specialized Hospital (TASH), which is the largest referral university hospital in the country. The teaching hospital provides specialized clinical services that are not available in other public or private institutions to the whole nation. Sample collection was carried out at the cancer center of the hospital by professionals at the spot. Sample preparation and storage was done at the core-lab of the institute; and CA 15-3 analysis is carried out at the diagnostic lab of the hospital.

Relative quantification of specific microRNAS using real time PCR were done at Dr. Rick Kittle's lab, University of Arizona. Dr. Rick Kittle's lab is focused on

identifying genetic and environmental contributions to cancer risk and treatment outcomes, including understanding the complex issues surrounding race, genetic ancestry and health disparities.

#### *3.1.1.3 Study Population*

The study participant is consisted of 157 breast cancer cases and 48 controls. The cases consist of pathologically confirmed breast cancer patients who were destined to the chemotherapy treatment during the study period. Enrollment was restricted to individuals who fulfill the inclusion criteria

**Inclusion criteria (cases)**

- Women 18 years or older
- Women with pathologically confirmed breast cancer
- Ethiopian nationality *

*MicroRNA expression in genetically diverse population will be different (Lu J. and Clarck A., 2012). It is to confine this variation, as much as possible, that we limit the study to Ethiopian subjects.

- Estimated life expectancy ≥ 12 weeks
- who are destined to chemotherapeutic treatment

**Exclusion criteria (cases)**

- Inability to understand/unwillingness to sign a written informed consent
- Women who report pregnancy or who are breast-feeding

- Major psychiatric illness that required hospitalization or medication
- Severe mental or physical disability

**Controls**

Controls were enrolled using random sampling of the community from which the cases came. Breast cancer free women 18 years old and above, with no first degree relative with breast cancer diagnosis, are included in the study.

#### *3.1.1.4 Data collection*

Data was collected when patients start their chemotherapy treatment. Major details of the study in respect of objectives of the study, study protocol, risks, confidentiality and rights of participants were explained to all potential participants. Those willing to participate signed informed consent after which questionnaires were administered by health professionals. The questionnaire was designed to gather demographic data, including age, history of breast cancer in first-degree relatives, presence of concomitant disease and delay in seeking medical attention. Information in respect of use of chat, alcohol and cigarette smoking were also obtained. Clinicopathological data and type of chemotherapy regimens were collected from patient medical records by the investigator.

Blood sample were obtained from pathologically confirmed breast cancer patients prior to chemotherapy and after completion of therapy. Blood specimen was also collected from breast cancer naïve volunteers, who are sex and age matched with the study group. In each sample collection term, 6mL of blood was

collected from study subjects and controls by using evacuated collection tubes through a puncture of intermediate arm vein. Serum was separated from the sample by allowing the blood to clot for 30 minutes and centrifuging for 15 minutes at 1000 relative centrifugal field (RCF). The serum samples were transferred to cryovials and kept at -80$^0$C until analysis.

### 3.1.2 MicroRNA analysis

Based on the involvement of target genes in tumorogenesis and treatment response we have selected six microRNAs (miR-29-3p, miR-210-3p, miR-221-3p, miR-145-5p, miR-21-5p, miR-326) to be analyzed in our study. Sequence for each of the selected microRNAs is found from miRBase database (http://www.mirbase.org).

Justification for the selection:

- miR-29-3p (42 - uagcaccaucugaaaucgguua - 63)

miR-29-3p participates in the regulation of cell proliferation, extracellular matrix composition, viability and senescence by controlling multiple overlapping pathways ( Yan X. et al. 2016). miR-29-3p can negatively regulate expression of B-Myb, which is a transcription factor associated to tumorogenesis (Zhenglong et al, 2013).

- miR-210-3p (66 - cugugcgugugacagcggcuga - 87)

Studies show that miR-210-3p regulates forkhead box P3 (FOXP3); which down regulates large number of genes which are related with the etiology and prognosis of breast cancer (Rothé et al, 2011).

- miR-221-3p (65 - agcuacauugucugcuggguuuc - 87)

One of the target transcripts for this miRNA is mRNA of p53 upregulated modulator of apoptosis (PUMA) gene (Fornari et al, 2010). PUMA is a pro-apoptotic gene which is shown to be down regulated in TNBC.

- miR-145-5p (16 - guccaguuuucccaggaaucccu - 38)

It targets DNA fragmentation factor 45 (DFF45) gene; which participate in proliferation and apoptosis (Dewi et al., 2014). The connection of this gene with processes which are highly linked with carcinogenesis makes its regulatory miRNA a candidate for the present study. Accumulating studies have also shown the differential expression of miR-145 in cancer cases.

- miR-21-5p (8 - uagcuuaucagacugauguuga - 29)

Targets Phosphatase and tensin homolog (PTEN) gene, which is one of the tumor suppressor genes through the action of phosphatase protein product. The phosphatase is involved in the regulation of cell-cycle, preventing cells from growing and dividing too rapidly (Huang *et al.*, 2009).

- miR-326 (60 - ccucugggcccuuccuccag - 79)

It targets multi-drug resistance associated protein (MRP-1/ABCC1) which transports a wide range of therapeutic agents and may play a critical role in the development of multi-drug resistance (MDR) in tumor cells (Xu et al. 2012; Liang et al. 2010).

#### *3.1.2.1 Extraction of microRNA from serum*

We have used miRNeasy Serum/Plasma Kit from QIAGEN® to extract total microRNA from serum samples. The miRNeasy Serum/Plasma Kit combines phenol/guanidine-based lysis of samples and silica-membrane–based purification of total RNA. QIAzol Lysis Reagent, included in the kit, is a monophasic solution of phenol and guanidinethiocyanate, designed to facilitate lysis, to denature protein complexes and RNases, and also to remove most of the residual DNA and proteins from the lysate by organic extraction.

QIAzol Lysis Reagent was added to serum samples. After addition of chloroform, the lysate was separated into aqueous and organic phases by centrifugation. RNA partitions to the upper, aqueous phase, while DNA partitions to the interphase and proteins to the lower, organic phase or the interphase.

The upper, aqueous phase was extracted, and ethanol was added to provide appropriate binding conditions for all RNA molecules from approximately 18 nucleotides upwards. The sample was then applied to the RNeasy MinElute spin column, where the total RNA binds to the membrane and phenol and other

contaminants were efficiently washed away. High-quality RNA was then eluted in a small volume of RNase-free water.

#### *3.1.2.2 Preparation of cDNA template*

We have used TaqMan® Advanced miRNA cDNA Synthesis Kit to modify miRNAs from total RNA by extending the 3' end of the mature transcript through poly(A) addition, and lengthening the 5′ end by adaptor ligation (Figure 6). The miRNAs then undergo universal reverse transcription followed by processes to uniformly increase miRNA cDNA (miR-Amp reaction).

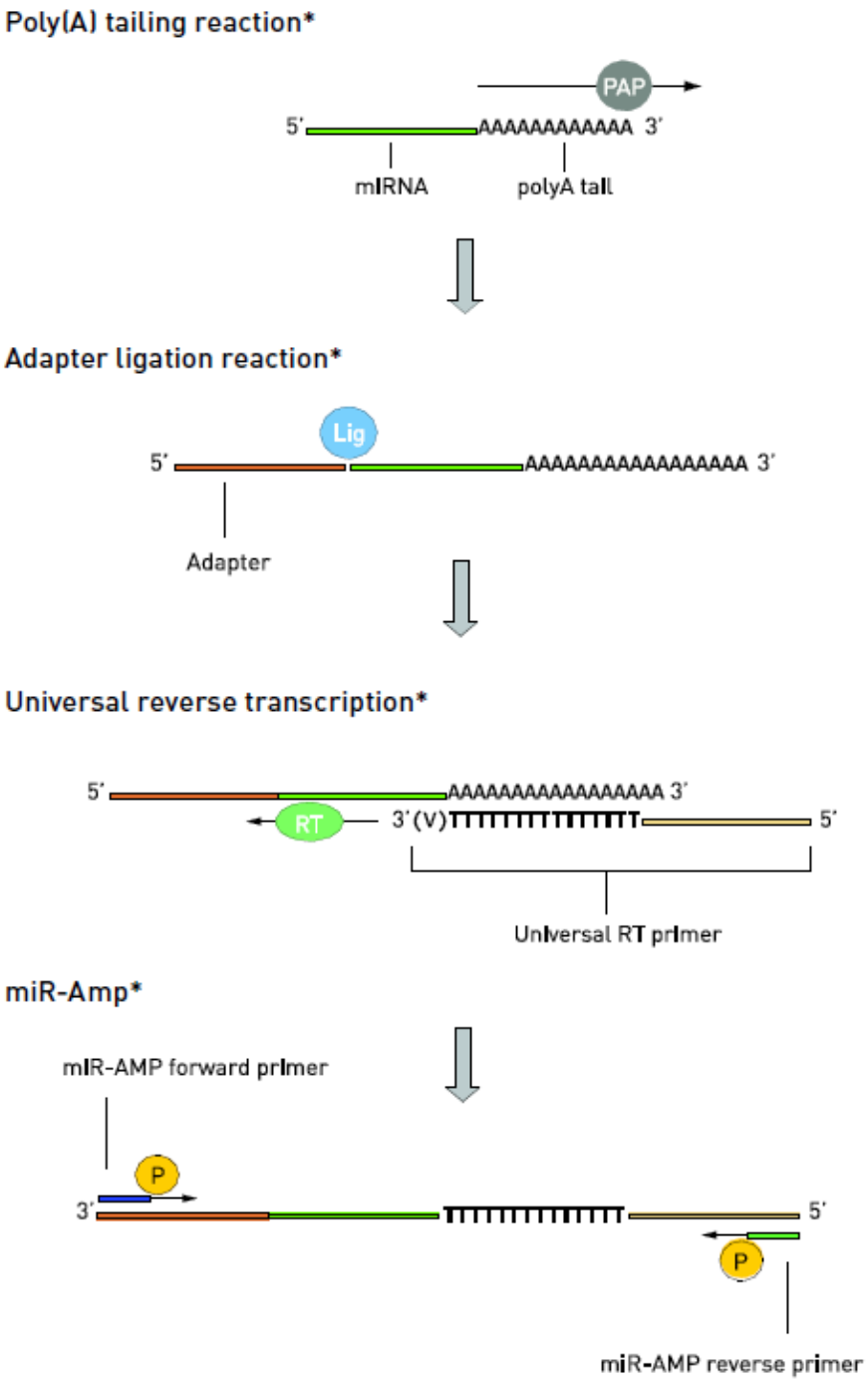



Figure 6 Poly A tailing, universal reverse transcription and pre amplification steps in microRNA analysis using Taqman ®advanced reagent (reprinted from TaqMan® Advanced miRNA manual)

### *3.1.2.3 Primer design*

We have used miRprimer software to design miRNA specific primers. The software uses an algorithm which is based on previously published techniques for manual designing of miRNA specific primers (Busk 2014). Sequences for the specified target microRNAs were also verified on online resources of Thermo Scientific®.

Table 1 Sequences of target miRNAs and primers

| | **Target MiRNAs and specific primers** | **Sequence** |
|---|---|---|
| **1** | **hsa-miR-16-5p** | **UAGCAGCACGUAAAUAUUGGCG** |
| | Forward primer | CAGTAGCAGCACGTAAATATTG |
| | Reverse primer | CAGTTTTTTTTTTTTTTCGCCAA |
| **2** | **hsa-miR-29a-3p** | **TAGCACCATCTGAAATCGGTTA** |
| | Forward primer | CGCAGTAGCACCATCTGA |
| | Reverse primer | TCCAGTTTTTTTTTTTTTTAACCGA |
| **3** | **hsa-miR-186-5p** | **CAAAGAATTCTCCTTTTGGGCT** |
| | Forward primer | CGCAGCAAAGAATTCTCCT |
| | Reverse primer | CCAGTTTTTTTTTTTTTTAGCCCAA |
| **4** | **hsa-miR-221-3p** | **AGCTACATTGTCTGCTGGGTTTC** |
| | Forward primer | GCAGAGCTACATTGTCTGCT |
| | Reverse primer | CAGTTTTTTTTTTTTTTGAAACCCA |
| **5** | **hsa-miR-210-3p** | **CTGTGCGTGTGACAGCGGCTGA** |
| | Forward primer | GCTGTGCGTGTGACA |
| | Reverse primer | GTTTTTTTTTTTTTTCAGCCGCT |
| **6** | **hsa-miR-145-5p** | **GTCCAGTTTTCCCAGGAATCCCT** |
| | Forward primer | GTCCAGTTTTCCCAGGAATC |
| | Reverse primer | AGGTCCAGTTTTTTTTTTTTTTAGG |
| **7** | **hsa-miR-21-5p** | **TAGCTTATCAGACTGATGTTGA** |
| | Forward primer | GCAGTAGCTTATCAGACTGATG |
| | Reverse primer | GGTCCAGTTTTTTTTTTTTTTCAAC |
| **8** | **hsa-miR-326** | **CCTCTGGGCCCTTCCTCCAG** |
| | Forward primer | TCTGGGCCCTTCCTC |
| | Reverse primer | GGTCCAGTTTTTTTTTTTTTTCTG |

### *3.1.2.4 qPCR amplification*

MiRNA expression was analyzed using Taqman microRNA assays (Figure 7) (Applied Biosystems, Foster City, CA). Real-time PCR was performed using the Step-One Plus (96 well) Real-time system (AppliedBiosystems, Foster City, CA, USA). Each sample was run in triplicate.

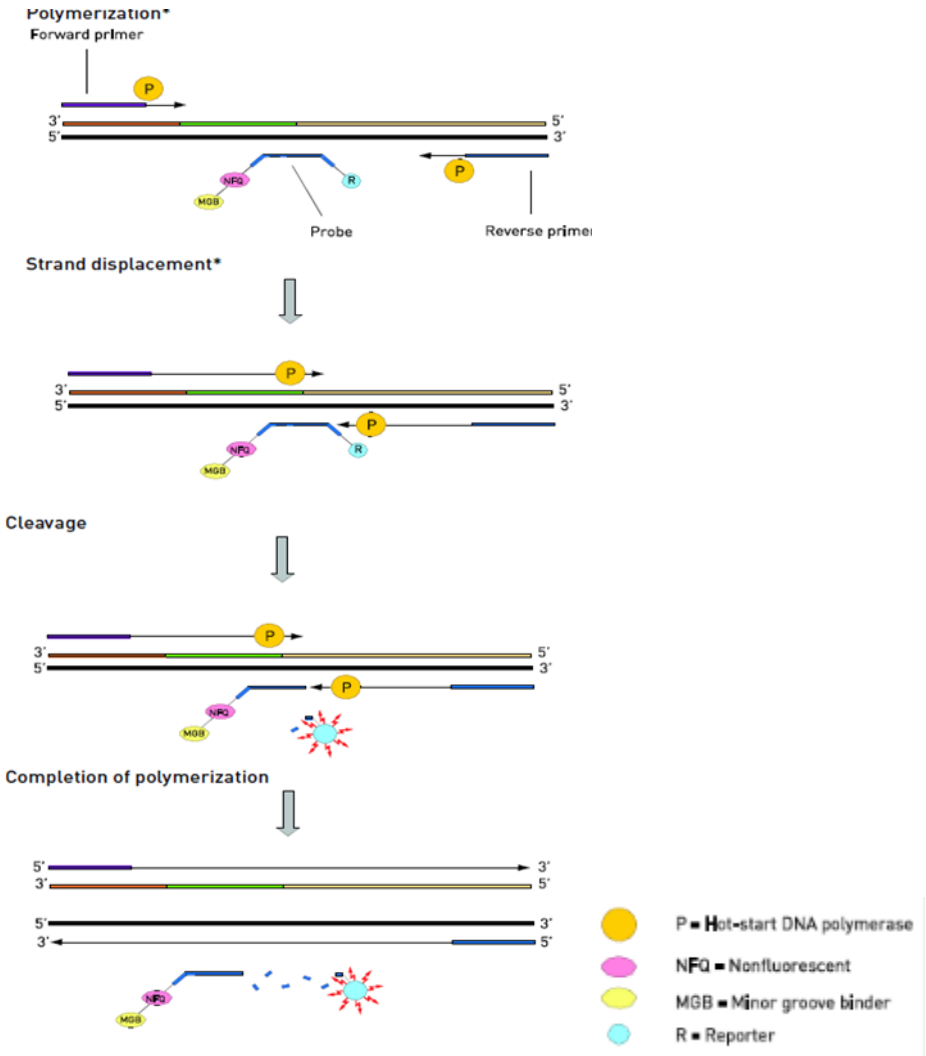



Figure 7 Fluorescence based detection of target amplification using quencher and reporter dyes (reprinted from TaqMan® Advanced miRNA manual)

When quantifying small RNA gene expression levels quantification errors may arise from variation in the amount of starting material, sample collection, RNA preparation, RNA quality and reverse transcription (RT) efficiency. Hence, proper normalization strategy is necessary to minimize this systematic and technical bias introduced at each step of miRNA quantification process (Torres et al. 2013). Incorporating an endogenous control gene into the experimental design is currently the most accurate method to correct for potential RNA input or RT efficiency biases (Kozera and Rapacz 2013). An ideal endogenous control shows gene expression that is relatively constant and moderately abundant across tissues and cell types and treatment. The issue of carefully selecting and validating endogenous control genes has not yet been addressed in relation to the relative quantitation of miRNA in breast cancer patients (Davoren et al. 2008; Das et al 2016). Based on previous studies, we have tested two recommended endogenous microRNAs for breast cancer analysis (miR-186-5p and miR-16-5p). Intergroup and intragroup analysis revealed that miR-16-5p is more stably expressed than miR-186-5p (Figure 8) in both case and control groups, which is in agreement with other studies (Lange et al. 2017; Davoren et al. 2008).

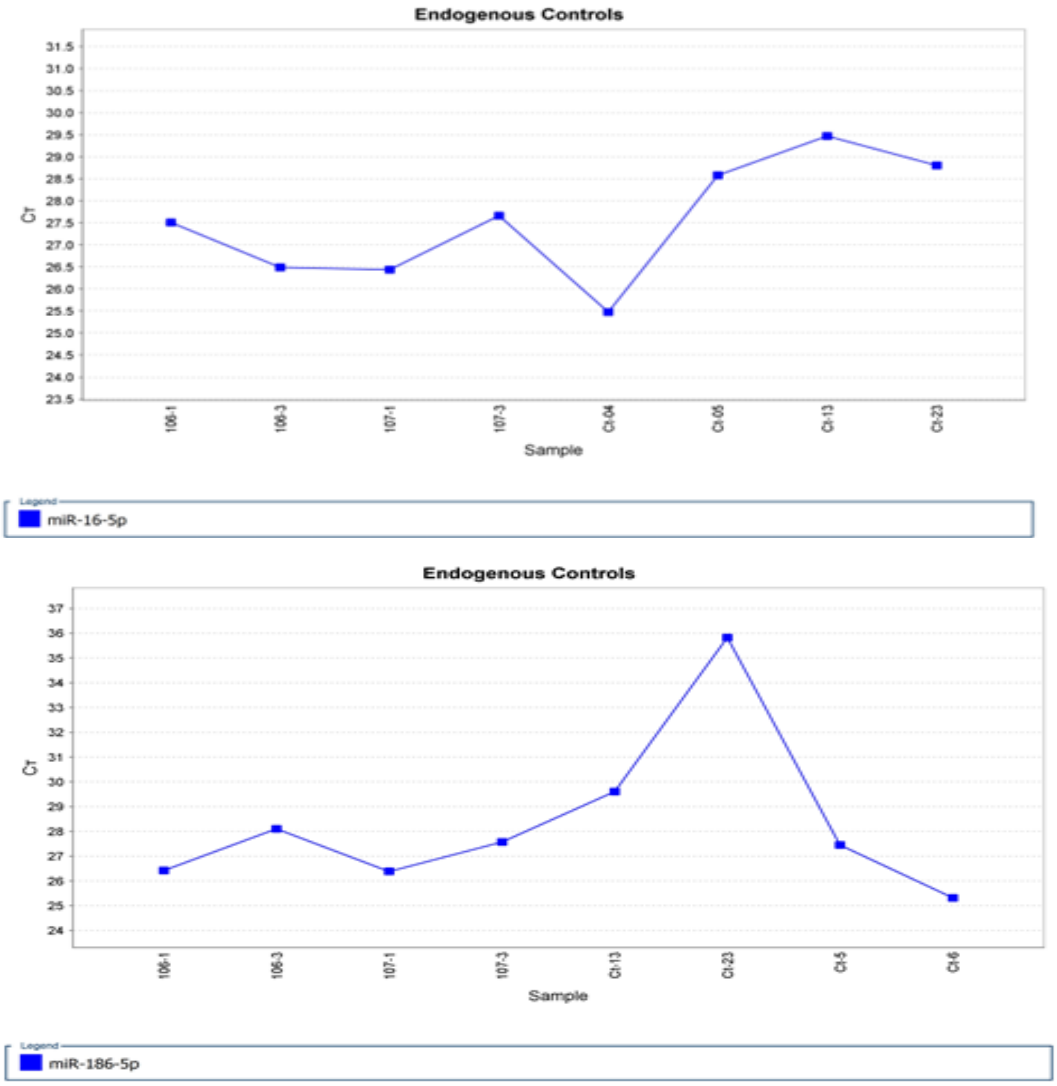



Figure 8 Intergroup and intragroup expression variation of candidate endogenous controls (miR-16-5p and miR-186-5p).

The microRNA expression level was measured using the Ct (cycle threshold) method. Ct is the fractional cycle number at which the fluorescence of each sample passes the fixed threshold.

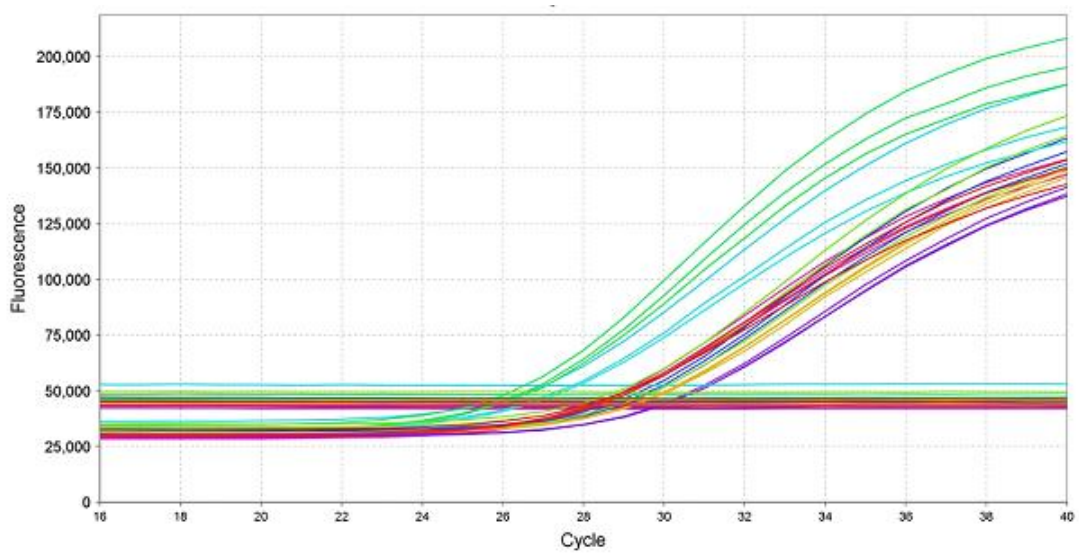



Figure 9 Sample florescence amplification plot obtained during analysis of specific microRNAs.

The ΔΔCT method for relative quantitation of gene expression was used to determine the target microRNA expression levels. The ΔCT was calculated by subtracting the Ct of endogenous control from the Ct of the target microRNAs. The ΔΔCT was calculated by subtracting the ΔCT of the reference sample from the ΔCT of each sample. Fold change was calculated using the equation $2^{-\Delta\Delta CT}$.

### 3.1.3 Chemotherapy response

Tumor response to chemotherapy in advanced breast cancer offers prognostic information and may be used as a surrogate marker for evaluating treatment efficacy. We have measured serum CA 15-3 level of study subjects (after completion of chemotherapy) to evaluate its prognostic use after chemotherapy. Serum CA 15-3 was measured by CA-15 Enzyme Immunoassay Kit based on the principle of a solid phase enzyme linked immunosorbent assay (ELISA) by two step immunoassay sandwich method with a final fluorescent detection. The test

consists of antibody coated solid phase receptacles (SPR) and ready to use reagent strips (Figure 10). All of the assay steps are performed automatically by Mini Vidas multiparametric immunoanalyzer from Biomerieux®, France.

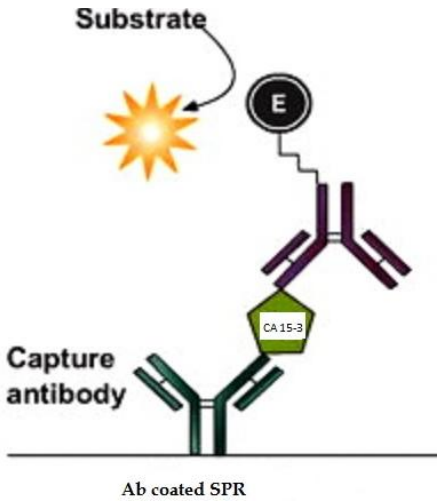



Figure 10 Assay principle for CA 15-3 quantitation

### 3.2 Statistical analysis

Descriptive comparisons of study variables between cases and controls used the Chi-squared test for categorical data, and the Student's t-test for continuous data. Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) version 22.0 (SPSS, Inc., Chicago, IL, USA) and $P<0.05$ was considered to indicate a statistically significant difference.

The raw Ct data of microRNAs were exported to the Expression Suite software (v.1. 1, Life Technologies) for normalization and quality control. Threshold and baseline were automatically calculated for each assay using miR-16-5p as endogenous control. MicroRNA discrimination potential was analyzed by

computing receiver operating characteristic (ROC) curves and calculating areas under the curves (AUC) with corresponding 95% confidence intervals (CI).

### 3.3 Ethics statement

This study was approved by the Institutional and National review boards. Only eligible cases, which fulfill the inclusion criteria of the study, were taken as a study subject. Participants were fully informed about the study, and only those who give their free consent (Annex V) were included in the study. Subjects were having the right to withdraw from the study at any time without affecting their further medical care. Confidentiality of records obtained from subjects was reserved by limiting access to the data and by replacing patient identification with code numbers. Material transfer agreement was signed between University of Arizona (UA) and Addis Ababa University (AAU) (Annex VI) to transfer biological samples for analysis.

# 4 CHAPTER FOUR

## 4.1 RESULTS

### 4.1.1 Sample profile and breast cancer characteristics

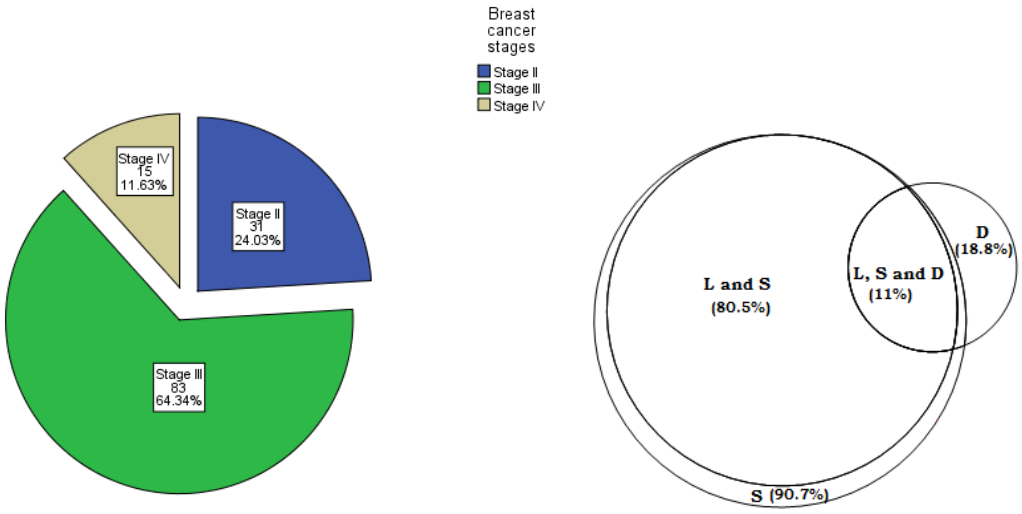
The risk factors for breast cancer includes, but not limited to, age, family history, reproductive factors, smoking, overweightness, drinking alcohol and previous treatment using radiation therapy. In this study the age of study subjects ranged between 20 and 70 years, and the mean age was 41.4 years. Of the 157 breast cancer patients only 40 (28.7%) were found to be overweight. The mean age, weight, height and BMI of study subjects is summarized in the following table.

Table 2 Mean age, weight, height and BMI of study subjects

| | Age | Weight | Height | BMI |
|---|---|---|---|---|
| N | 153 | 157 | 157 | 157 |
| Mean | 41.38 | 59.52 | 1.58 | 23.63 |
| Std. Dev. | 11.44 | 12.21 | .063 | 4.44 |

To measure the frequency of everyday habits, which are considered as risk factors, study subjects were asked whether they drink alcohol, smoke cigarettes or chew chat. Only few study subjects (2(1.3%) for alcohol, 1(0.6%) for smoking, 6(3.8%) for chat chewing) replied “yes”.

The majority of the study subjects were in Stage III (n=83, 64.3%) (Figure 11 (a)). Patients were motivated to seek health care due to a lump in their breast (n=95, 80.5%), swelling of the breast (n=107, 90.7%), and a few had a discharge (n=22, 18.8%) (Figure 11 (b)).




(a) (b)

Figure 11 (a) Percentages of study subjects found to be at the different stages of breast cancer, (b) Percentages of study subjects recognizing the different symptoms of breast cancer;

L-lump, S-swelling and D-discharge

Of the 157 breast cancer patients included in our study 18 (11.3%) have confirmed the occurrence of breast cancer in their first degree relatives. A small number had concomitant disease (n=24, 15.2%) and the majority had mastectomy (n=137, 86.7%).

We found prolonged delay in time interval between symptom recognition by the study subjects to first visit to a health care centers. Significant number of the

study subjects (n=60, 37%) show total delay of more than a year from symptom recognition to presentation (Figure 12). We also found significant correlation between delay in seeking medical attention after recognizing symptoms and stage of breast cancer during presentation (P=0.026).

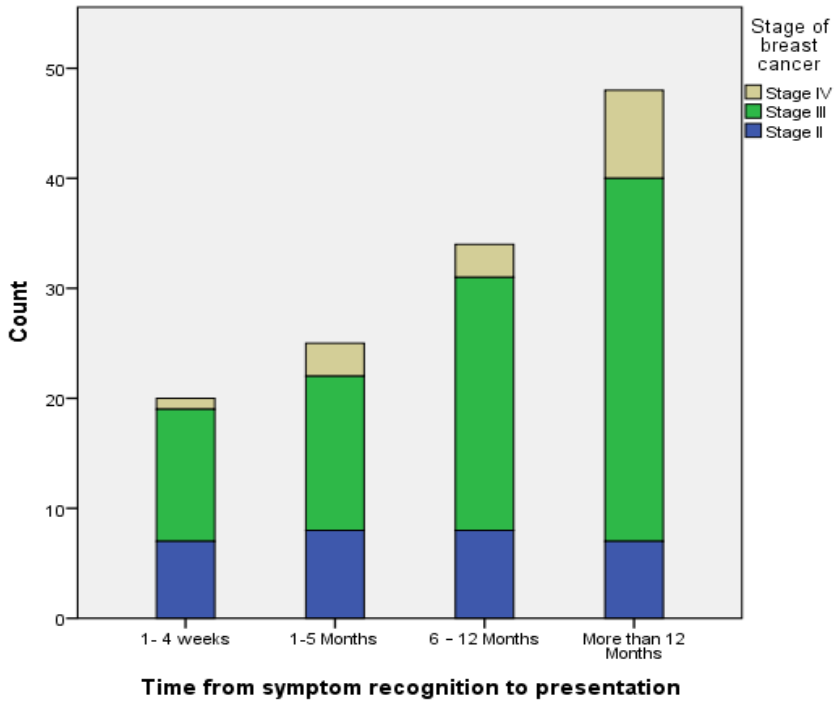



Figure 12 Stacked bar chart showing distribution of stages of breast cancer, with different period of delay (form recognition of symptoms to seeking medical attention)

### 4.1.2 Biochemical tests

Initiating chemotherapy is a multifactorial process that includes a detailed review of the patient's cancer, comorbidities, and potential risk factors for side effects. Complete blood counts and organ function tests are routinely performed for breast cancer patients to minimize the level of chemotherapy side effects and

to check if the cancer is metastasized to body organs. Mean biochemical profiles of study subjects prior to initiating chemotherapy are summarized in Table 3.

Table 3 Mean biochemical profiles of study subjects prior to initiating chemotherapy

| | WBC | Neutrophil | Platelet | AST | ALT | Serum Cr | BUN |
|---|---|---|---|---|---|---|---|
| N | 152 | 150 | 153 | 153 | 152 | 149 | 140 |
| Mean | 7.07 | 4.28 | 311.80 | 31.69 | 26.11 | 0.91 | 19.8 |
| Std. Dev | 2.67 | 5.15 | 101.39 | 28.42 | 23.38 | 0.18 | 8.42 |

The mean liver function results were found to fall in the normal range with Aspartate transaminase (AST) M = 31.7 U/L, SD = 28.43 and Alanine transaminase (ALT) M = 26.11 U/L, SD = 23.39. Only in seven study subjects (4.57%) both ALT and AST values were found to be high.

Blood urea nitrogen (BUN) and creatinine provides a rough measurement of the glomerular filtration rate, which is used to measure kidney function. Even if mean Serum Creatinine (M = .91mg/dL, SD = .18) and mean BUN (M = 19.8mg/dL, SD = 8.42) values of the study subjects were in the normal range; BUN was found to be high in 56 (36.6%) of breast cancer patients. There was marginally significant difference in BUN (P=.050) between patients at stage 4 (n = 13, M = 24.15mg/dL, SD = 10.45), who have significantly higher BUN levels

(Figure 13), when compared with other stages (n = 127, M = 19.35mg/dL, SD = 8.11).

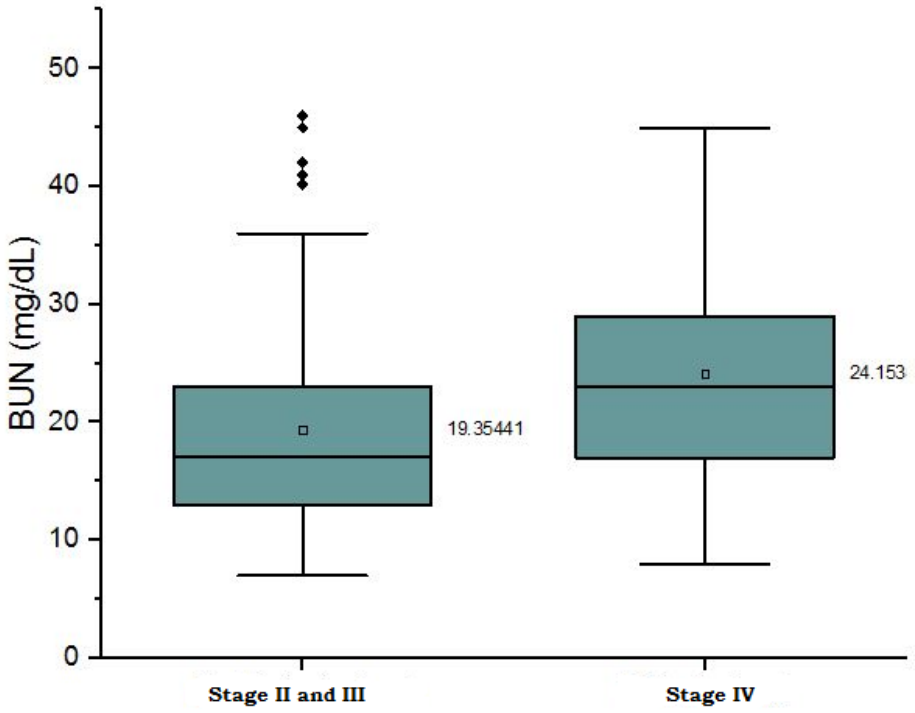



Figure 13 Differences in mean BUN level between stage IV breast cancer with others stages

Low blood cell counts can be a serious complication during cancer treatment. There were two significant differences in blood cell count profile by stage of disease at diagnosis among those in stages II, III, or IV. Those in stage IV had significantly higher white blood cell counts ($P<0.05$, M = 8.85, SE = .65) than those in stage II (M = 6.99, SE = .46) or in stage III (M = 6.88, SE = .29) (Figure 14 (a)); those in stage IV also had significantly higher platelet count ($P<0.05$, M = 369.53, SE = 25.00) than those in stage II (M = 290.41, SE = 17.98) or in stage III (M = 303.40, SE = 10.83) (Figure 14 (b)).

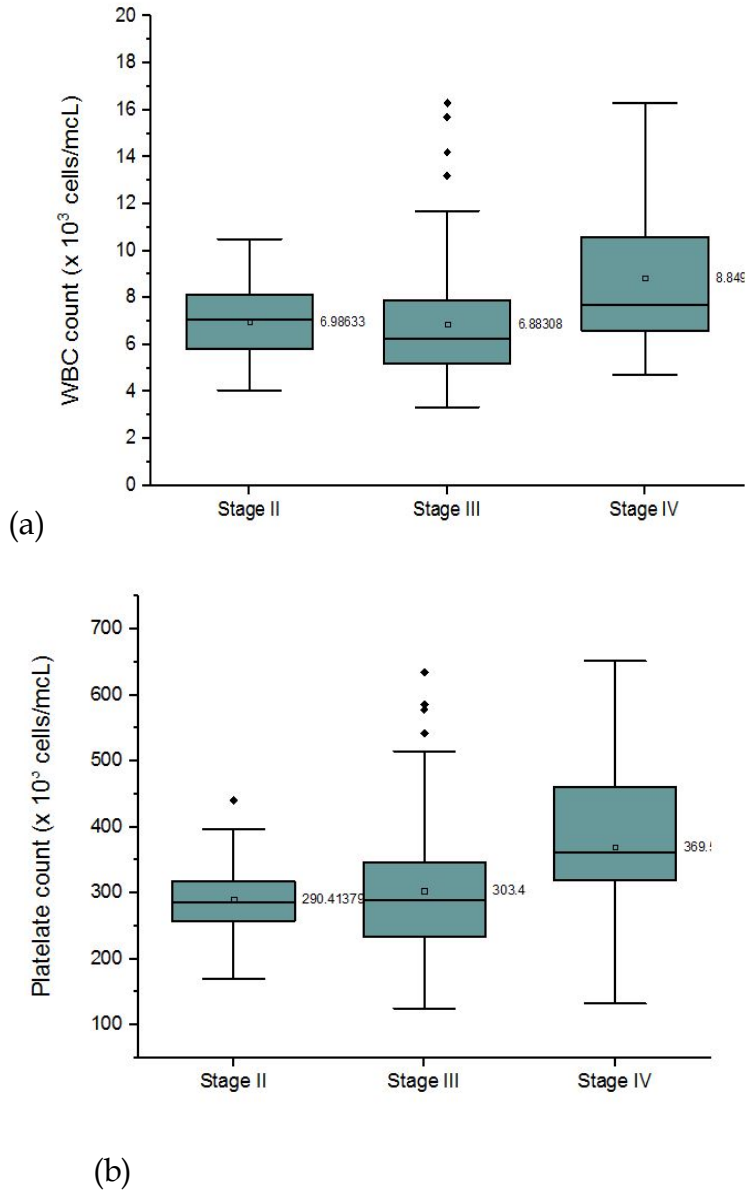



Figure 14 (a) Differences in mean WBC count among the different stages of breast cancer, (b) Differences in mean platelet count among different stages of breast cancer

### 4.1.3 Difference in serum microRNA levels between BC patients and controls

To identify differentially expressed circulatory microRNA in breast cancer patients, the expression levels of six target microRNAs (Mir-21-5p, Mir-29-3p, Mir-145-5p, Mir-210-3p, Mir-221-3p and Mir-326) were compared between 124 breast cancer cases (cases with before and after chemotherapy samples) and 44

controls. Our data shows a trend of increased expression for the five microRNA types in breast cancer cases when compared with controls (Table 4 and Figure 15) and this difference were found to be statistically significant for miR-21-5p ($P<0.05$). MiR-210-3p was found to be down regulated in breast cancer cases, even if the difference was not statistically significant.

Table 4 Relative fold change of circulatory microRNA in breast cancer patients as compared to controls

| | MicroRNA | Breast cancer vs. controls | |
|---|---|---|---|
| | | Fold change | p-value |
| Before chemotherapy | Mir-21-5p | 1.43 | 0.044 * |
| | Mir-29-3p | 1.14 | 0.376 |
| | Mir-145-5p | 1.37 | 0.104 |
| | Mir-210-3p | 0.57 | 0.187 |
| | Mir-221-3p | 1.31 | 0.222 |
| | Mir-326 | 1.58 | 0.133 |
| After chemotherapy | Mir-21-5p | 1.27 | 0.164 |
| | Mir-29-3p | 1.07 | 0.555 |
| | Mir-145-5p | 1.19 | 0.387 |
| | Mir-210-3p | 0.76 | 0.499 |
| | Mir-221-3p | 1.22 | 0.375 |
| | Mir-326 | 1.09 | 0.821 |

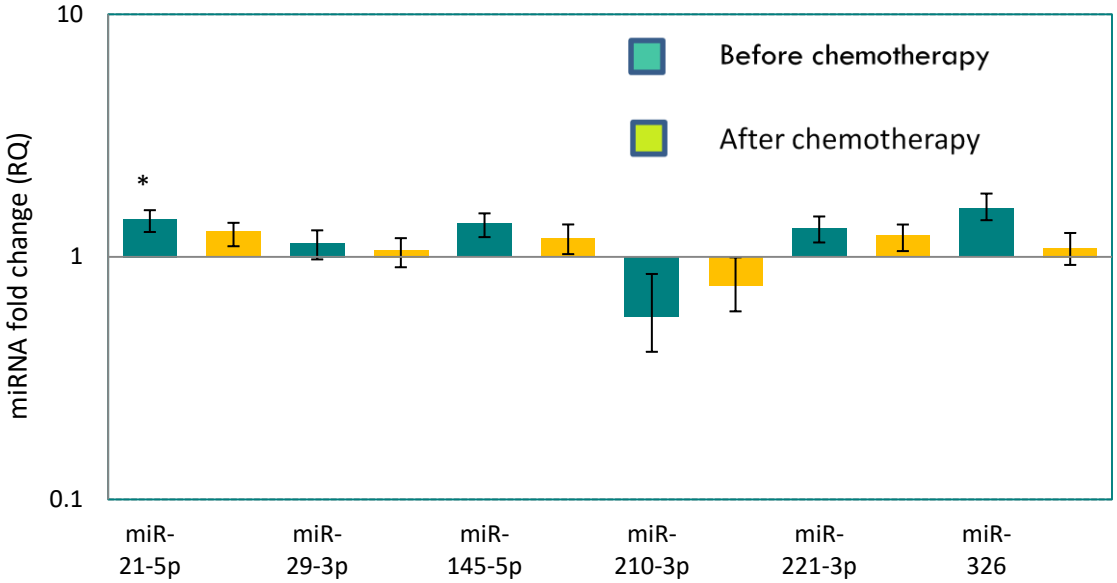
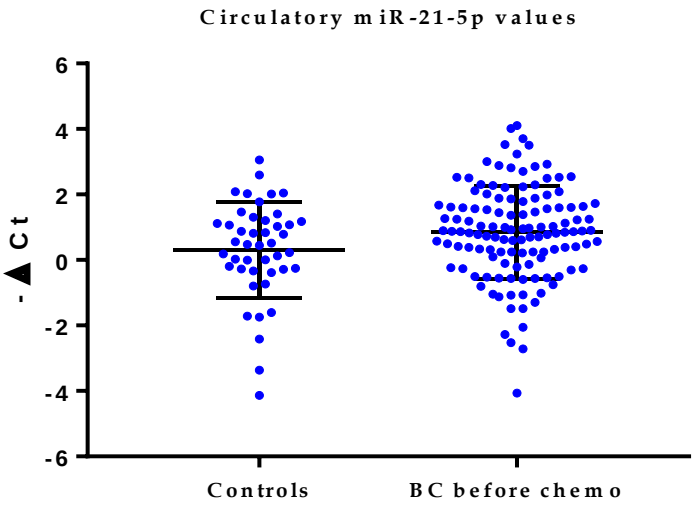
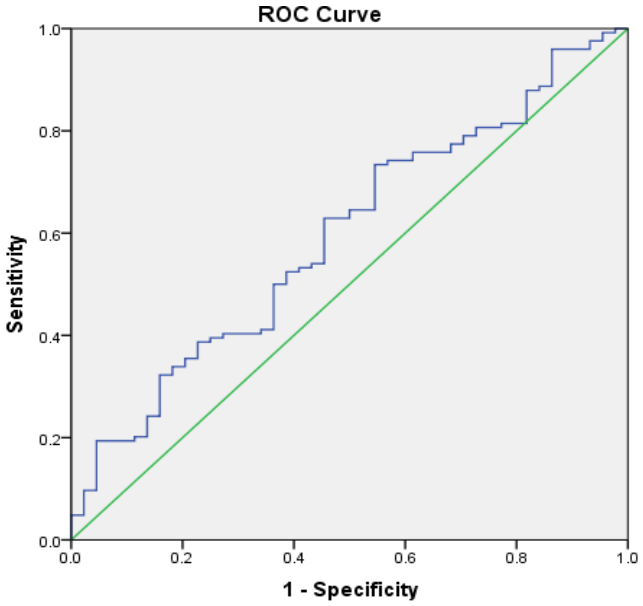



Figure 15 Log 2 transformed relative fold change of circulatory microRNAs before and after chemotherapy as compared to controls, with miR-16-5p used as endogenous control

As shown on Figure-15 the mean -ΔCt for circulatory miR-21-5p in breast cancer patients is significantly differentiated when compared with controls (Figure 16 (a)). To evaluate the diagnostic efficiency of mir-21-5p in discriminating breast cancer patients from healthy individuals, receiver-operator characteristic (ROC) analyses were applied (Figure 16 (b)). The area under the curve (AUC) was 0.594 (95%CI 0.499-0.690). We searched differences in the expression levels of miR-21-5p among different stages of breast cancer; we found no statistically significant difference.




(a)




(b)

Figure 16 (a) Mean -ΔCt values of circulatory miR-21-5p between breast cancer patients (before chemotherapy) and controls, (b) ROC curve analysis for miR-21-5p in distinguishing breast cancer cases from controls

### 4.1.4 Differentially expressed miRNAs in serum samples before and after Chemotherapy

In this study, we determined the impact of chemotherapy on circulatory microRNA (miRNA) expression in breast cancer patients. Most of the study subjects (n=113, 72%) were using CMF (cyclophosphamide, methotrexate and flurouracil) regimen, while 44 (28%) where destined AC-Taxol ( Adriamycin, Cyclophosphamide followed by Taxol) regimen. Our data showed that miRNA expression profiles in serum change in the course of treatment of breast cancer patients (Table 5).

Table 5 Relative expression and paired sample correlations of circulatory microRNA in breast cancer patient samples obtained before and after chemotherapy

| MicroRNA | Paired sample t-test for mean -ΔCt (mean ± SD) | | | Paired samples correlation | |
|---|---|---|---|---|---|
| | Before chemotherapy | After chemotherapy | p-value | R-value | p-value |
| Mir-21-5p | 0.84 ± 1.42 | 0.65 ± 1.26 | 0.27 | 0.05 | 0.57 |
| Mir-29-3p | 3.41 ± 1.67 | 3.29 ± 1.31 | 0.46 | 0.23 | 0.02* |
| Mir-145-5p | 0.67 ± 1.58 | 0.49 ± 1.89 | 0.419 | 0.96 | 0.30 |
| Mir-210-3p | 4.70 ± 2.86 | 5.05 ± 2.80 | 0.34 | 0.33 | 0.001*** |
| Mir-221-3p | -1.96 ± 2.00 | -2.22 ± 1.70 | 0.20 | 0.36 | 0.000*** |
| Mir-326 | 3.91 ± 2.61 | 3.29 ± 1.79 | 0.03* | 0.14 | 0.15 |

In general, lower levels of up-regulated microRNAs and higher levels of down-regulated microRNAs were found in the serum taken after chemotherapy compared to serum before chemotherapy; and this difference were found to be statistically significant for miR-326 (P=0.03) (Figure 17).

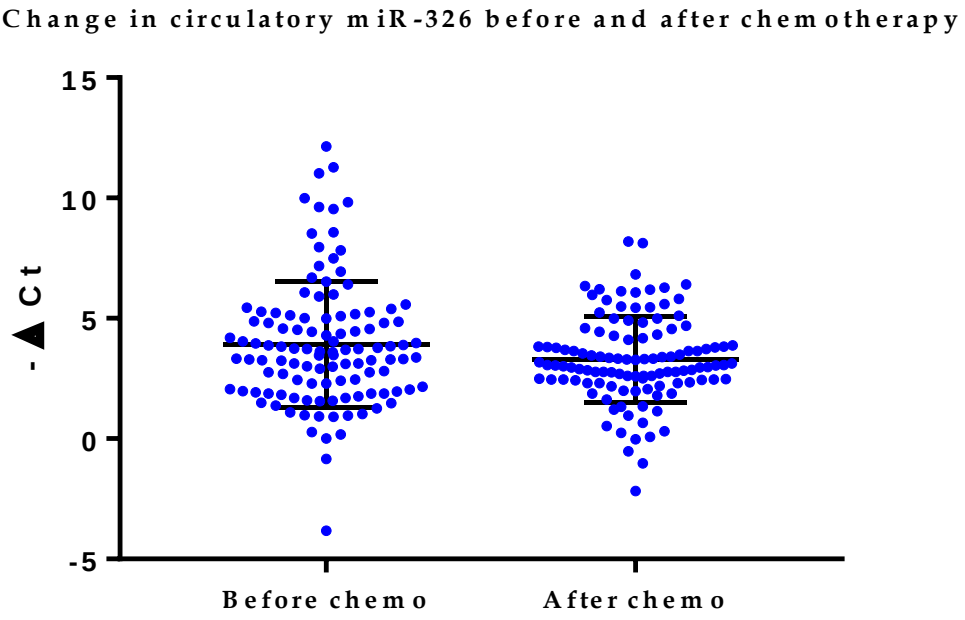



Figure 17 Difference in the level of mean circulatory miR-326 before and after chemotherapy

We found strong paired sample correlation between before chemotherapy and after chemotherapy samples for miR-29-3p, miR-210 and miR-221-3p (Figure 18).

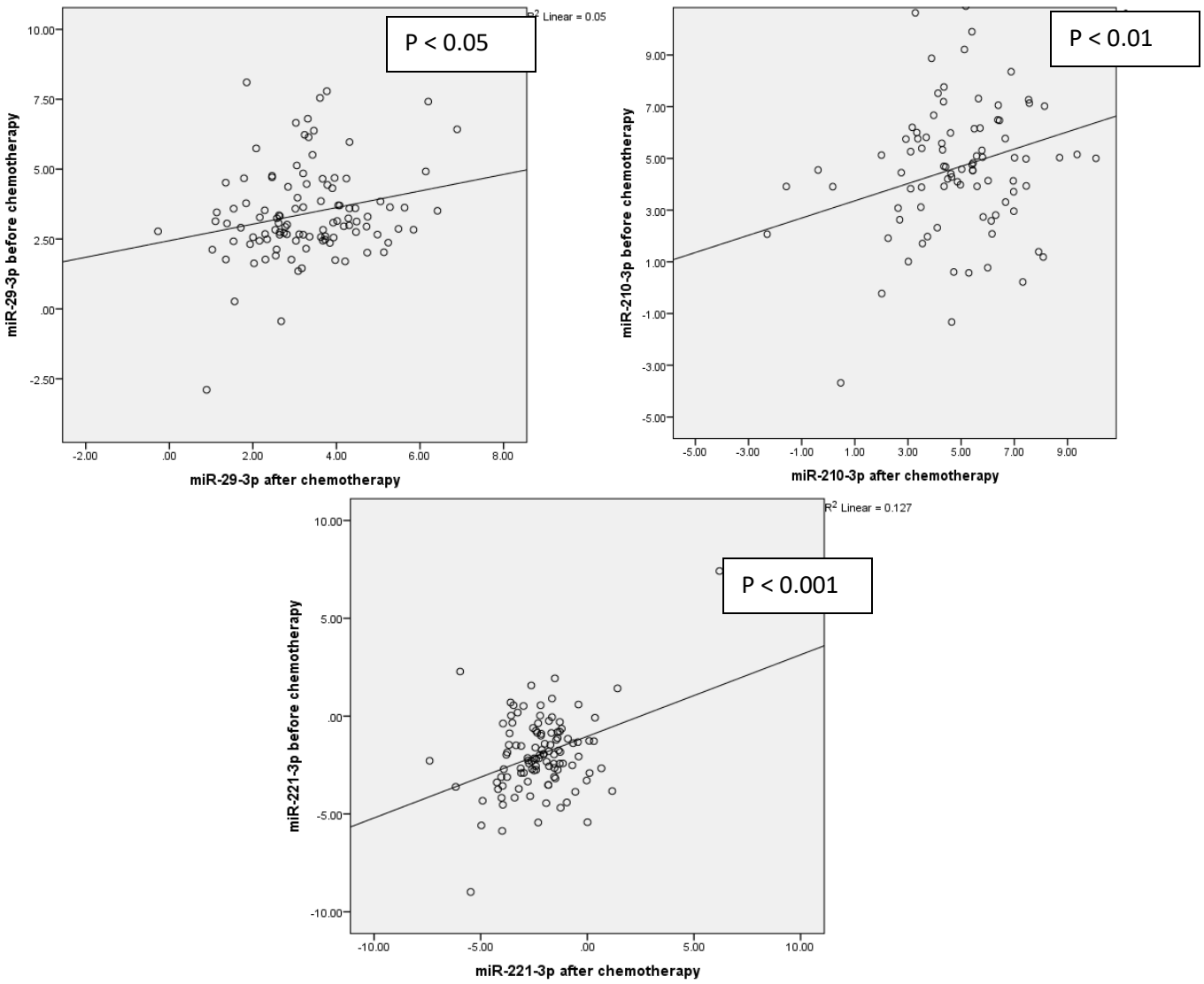



Figure 18 Paired sample correlation of circulatory miR-29-3p, miR-210-3p and miR-221-3p in breast cancer patient samples obtained before and after chemotherapy.

### 4.1.5 CA 15-3

CA 15-3 serum levels were evaluated for only for 63 of the study subjects (due to the limited amount of reagent we got from local suppliers) after completion of chemotherapy. The majority of our study subjects (n=55, 87.3%) were found to have normal CA 15-3 value (i.e. < 30 U/ml) and the mean CA 15-3 value was 27.6 U/ml. No statistically significant difference in serum CA 15-3 values was observed among breast cancer patients with different delay periods in seeking medical attention.

Elevation of CA 15-3 value in the group of patients with stage IV breast cancer was statistically significant as compared with the stage II (P=0.001) and stage III (P<0.001) (Figure 19). We didn't find significant correlation of serum CA 15-3 value with levels of circulatory miR 21-5p.

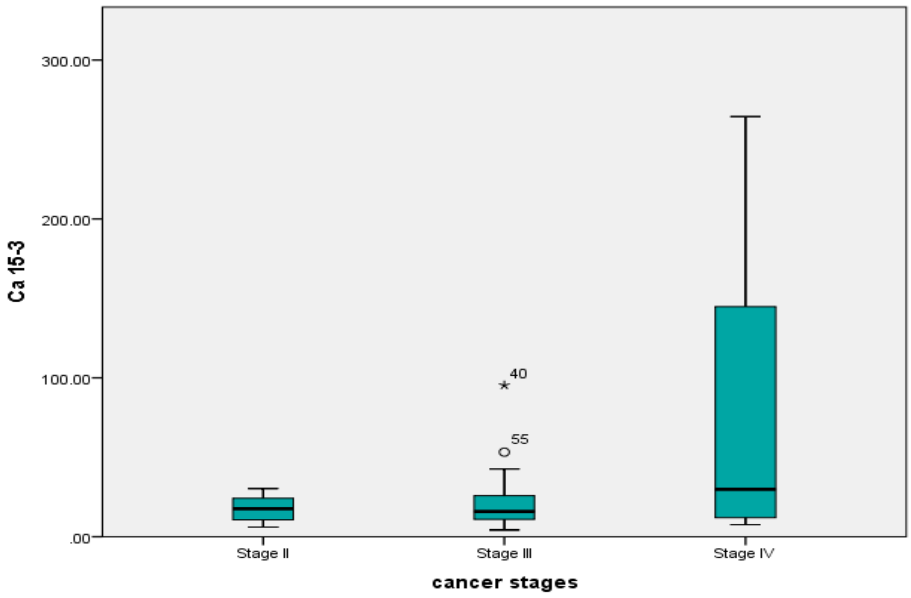



Figure 19 Observed mean CA 15-3 differences among stages of breast cancer after completing six courses of chemotherapy

# 5 CHAPTER FIVE

## 5.1 DISCUSSION

Breast cancer is the most commonly diagnosed cancer in Sub-Saharan Africa, and is also the second leading cause of death from cancer in women. A recent shift on the commonly diagnosed cancer type in women, from cervical cancer to breast cancer, across the region is confirmed by accumulating studies. Even if different factors have been suggested for bringing this shift, the particular reasons are unknown (Jemal et al 2012).

Our data suggests profound prevalence of breast cancer in young and middle age women (mean age 41.38). This is in line with previous studies made in the country (Abate et al. 2016; Kantelhardt et al. 2014). Breast cancer at younger age is characterized by late stage presentation and aggressive clinical behavior with poor prognosis (Deus, Carvalho, and Bacchi 2015). Studies have showed a positive association between breast cancer diagnosis at young age and high breast cancer specific mortality (Brandt et al. 2015).

As it is discussed elsewhere on researches made in developing countries (Pakseresht et al. 2014; Ezeome 2010), our study also found a significant delay in seeking care after noticing symptoms. Most patients 94 (75.9%) are presented at the late stage of the disease (III and IV), which is a bit higher than the percentage reported by Akinbami et al. 2012 in Nigeria (71%) (Akinbami et al. 2012). A similar study in the India has also showed advanced stage of cancer at

presentation (88.9 %) (Pakseresht et al. 2014).This late stage diagnosis may also be one of the factors for the poor prognosis of breast cancer in the country as reported by Timothy D. (Dye et al. 2012).

Our study has demonstrated that 133 (84.2 %) of patients delay in getting medical consultation for more than a month after recognizing symptoms. And we also found that delay in getting medical attention is significantly correlated with stages of the diseases. In line with our result studies performed in the developed countries also show an association of delays between symptom discovery and treatment start with advanced clinical stage of breast cancer (Rivera-Franco and Leon-Rodriguez 2018). Even if patient delay in seeking medical attention is considered to be the major reason, delays in the health care system, the number and type of health care providers contacted before diagnosis, delayed referrals, misdiagnosis, delays in obtaining diagnostic confirmation, and in starting treatment, lack of periodic mammographic examination for women at risk of breast cancer may also account for the significant delays seen in developing countries (Espina, McKenzie, and dos-Santos-Silva 2017).

Even if most breast cancer cases are known to be sporadic, 5-10 % of the cases are attributed to hereditary predisposition (Tazzite et al., 2004). Genetic alteration in the tumor suppressor genes (BRCA1 and BRCA2) are well established risk factors for breast cancer. Studies confirm that having one first degree relative with breast cancer approximately doubles a woman's risk and having two first

degree relatives increases her risk 5 fold (Deshpande, Pandey and Shyama, 2017). In our study 18 (11%) of the study subjects have reported to have first degree relative with breast cancer, which is lower than the previously reported percentage by Phipps A. et al (15%).

Lump and swelling of the breast are found to be the major primary symptom of breast cancer noticed by most participants of the study. A clinical study made by Dye et al. (2012) to identify symptoms of breast cancer in the same center also reported lump as typically recognized symptom among most breast patients with some dismissing the symptom for several years (Dye et al. 2012).

Biochemical profile helps in assessing the functional capacity of vital organs of the body or general health. To check the proper functionality of liver and kidney in breast cancer patients, who are destined to chemotherapy treatment, we have analyzed the blood urea nitrogen (BUN), Aspartate transaminase (AST), Alanine transaminase (ALT) and serum creatinine values. Irrespective of the parameters tested, mean values were found to fall in the normal reference ranges. The significant increase in the level of blood urea nitrogen in late stage of the disease shows the association of the disease with renal abnormalities. There are multifactorial mechanisms which lead to renal disorders in breast cancer patients and the bidirectional connection between late stage cancer and chronic kidney disease is well established (Jhaveri and Salahudeen 2015; Humphreys 2004).

Before initiating chemotherapy it is also a basic procedure to check whether the blood has normal amounts of various types of blood cells. This is used to determine whether the patient has another medical condition that needs to be addressed first. It is also well documented that the cancer itself or treatments may derange blood cell levels which are necessary for proper functioning of the body. Study subjects with stage IV breast cancer have showed an elevated total WBC count when compared with stage II and III in our study. A similar study made by Akinbami et al (2012) in Lagos, showed doubling of total WBC count in stage IV breast cancer patients when compared with stage II (Akinbami et al. 2012). Increased circulating WBC is also associated with poor prognosis in similar studies; and this association may relate to the activation of innate immunity during tumor progression (Wen et al. 2015).

Increased platelet activation is a common feature in breast cancer patients, and its aggregation correlates with metastatic potential of tumor cells. Our study has also showed significant correlation of platelet count with stages of breast cancer ($P<0.05$). Accumulating studies have tried to see the underlying mechanisms of this association of platelets with disease stage and prognosis. The list of advantages by tumor cell-induced platelet aggregation for survival of tumor cells and its metastasis includes: a tumor cell covered with coats of platelets acquires the ability to evade the body's immune system, platelets may shield cancerous cells from high shear forces seen in flowing blood that could potentially damage

the tumor cell and easy extravasation of microvasculature by tumor-platelet aggregates to invade new site (Jurasz, Alonso-Escolano, and Radomski 2004). Platelets are also sources for angiogenesis regulating factors which are required for metastatic stage of cancer (Lal, Dittus, and Holmes 2013).

Circulating miRNA levels differ according to physiological and pathological states at different diseases, suggesting that miRNAs may be useful biomarkers for the diagnosis and clinical progression of various diseases. In this study, we demonstrated that upregulation of circulatory miR-21-5p is associated with breast cancer and it is found to be significantly modulated in patients compared to controls. Even if its discrimination potential is low (with AUC of 0.6, P=0.04), the ROC analysis showed that miR-21-5p can help to differentiate breast cancer patients from controls.

Increased expression of miR-21 is shown to increase cancer cell migration, invasion, proliferation, and anti-apoptotic properties (Kelly et al., 2017). Signaling pathways and mRNAs which can be targeted by miR-21-5p for its oncogenic effect includes PTEN/PI3K/Akt pathway, targeting programmed cell death 4 (PDCD4) and NF-κB (Figure 20).

Phosphatidylinositide 3-kinase (PI3K)/Akt is a common survival pathway. PI3K catalyses phosphatidyl inositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-trisphosphate (PIP3), which recruits Akt and phosphoinositide dependent kinase (PDK) to allow PDK to phosphorylate and activate Akt. Activated Akt

increases cell proliferation and decreases cell apoptosis via a broad range of downstream target proteins. PTEN is a negative regulate of PI3K/Akt pathway by turning PIP3 into PIP2 (Chen, Xu, and Chen 2015)(Figure 20).

Loss of PTEN function has been shown experimentally to cause many disturbances in cell and organism physiology; commonly linked to increases in cell growth and proliferation (Leslie and Longy 2016). It is frequently inactivated by mutation in a number of cancers. Studies have demonstrated that miR-21-5p indirectly targets PTEN and down-regulates its expression via the down-regulation of the Sprouty homolog 2 (SPRY2) level (Cui et al. 2017).

Tumor suppressor gene, programmed cell death 4 (PDCD4), is a direct target gene to miR-21-5p, which is leading to cell proliferation and inhibition of apoptosis and regulating cancer invasion and metastasis in breast cancer (Zhu et al. 2007; Peralta-Zaragoza et al. 2016; Asangani et al. 2008).

The overexpression of miR-21 and increased activation of NF-κB in breast cancer is reported before, indicating the interplay of miR-21 and NF-κB. NF-κB is known to induce miR-21-5p transcription, which participates in feedback loops with the transcription factor that regulate its transcription (Badr 2016). NF-kB is also known to promote cancer metastasis through up-regulating the expression of cytokines, such as Interleukin 6 (IL-6) and tumor necrosis factor (TNF), which elicit a robust inflammatory response favoring tumor invasion (Niu et al. 2012).

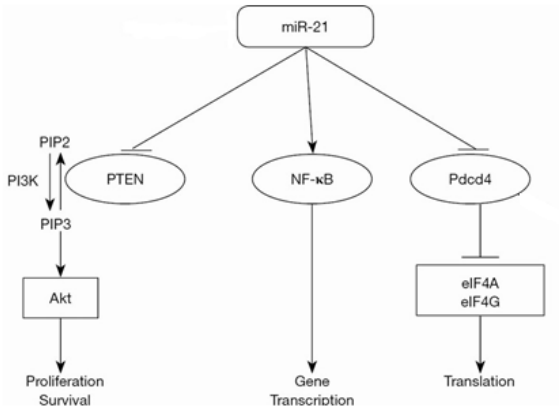



Figure 20 miR-21-5p targeted pathways for its oncogenic action (reprinted from Chen, Xu and Chen, 2015)

Even if the differential expression of miR-21-5p in breast cancer patients is also supported by accumulating studies, recognizing single microRNA as diagnostic marker for the heterogenic cancer might be difficult. Studies on gene expression profile of miRNA in tumor tissues and plasma also suggests that miR-21-5p is associated with many types of cancer which makes it imprecise in diagnosing specific cancer types (Zhang et al. 2016; Matamala et al. 2015). The candidacy of miR-21-5p as a specific non-invasive biomarker in showing presence of tumor is also challenged by its association with immune responses. Its role in promoting proliferation of lymphocytes and its association with inflammation, affecting wound healing, is demonstrated (Yan et al. 2017; Barnett et al. 2016).

Even if there are reports for the prognostic potential of miR-21-5p in other diseases (Makiguchi et al. 2016) we didn't found significant differences among

stages of breast cancer, depicting its insignificant potential for prognostic use in breast cancer.

A number of studies have reported fluctuations in circulating miRNA levels in response to chemotherapy (Hummel et al. 2011; Hamam et al. 2017). To identify blood plasma miRNAs showing a response to chemotherapy and in order to assess their candidacy as minimally invasive tools for therapy monitoring, we compared miRNA levels in samples of blood plasma from breast cancer patients prior to chemotherapy and after the completion of chemotherapy.

Our data show that miRNA expression profiles in serum change in the course of treatment. Even if the change is not statistically significant for most microRNAs, we found a decreasing fold change in the level of circulatory microRNAs after chemotherapy. Expression level of miR-326 in matched pre-treatment and post-treatment samples shows a significant decrease ($P<0.05$) after chemotherapy. In line with our result, Diener et al showed a general decrease in the level of circulatory microRNAs in patients with hematologic malignancies after chemotherapy (Diener et al. 2015). Currently there is no clear understanding whether these effects are tumor driven or due to an effect of non-tumor processes that regulate the miRNA appearance in the blood (Jo et al. 2017).

The differential expression of miR-326 in different disease conditions and its involvement in chemotherapy resistance is well documented (Liang *et al.*, 2010, Wang et al., 2016). It is found to get up-regulated in the circulation of untreated

relapsing–remitting multiple sclerosis (Gandhi, 2015). miR-326 is negatively correlated and is known to regulate expression level of multidrug resistance-associated protein (MRP-1) , which is an adenosine triphosphate-binding cassette (ABC) transporter that decreases intracellular drug accumulation due to active efflux (Kjersem et al. 2014; Liang et al. 2010). Increased expression of this transporter is associated with multidrug resistance (Morrow et al. 2006). Its down-regulation is linked with the pathogenesis of cervical carcinoma where it plays tumor suppressor role by targeting ELK1 mRNA, which functions as a transcription factor that mediates transcriptional regulation using a conserved E26 transformation-specific (ETS) DNA-binding domain (Zhang et al. 2017). High expression of circulatory miR-326 before onset of chemotherapy was associated with reduced progression free survival and overall survival (Kjersem et al. 2014).

The observed change in the level of circulatory miR-326 after chemotherapy in our study subjects may arise from the effect of chemotherapy on boosting T cell activation. Studies have confirmed that chemotherapy benefits the immune response of breast cancer patients by expanding tumor-specific T cells in peripheral blood (Bernal-Estévez et al. 2016). Dewi et al (2014) have showed that miR-326 expression is down regulated during T cell activation. The prognostic value of miR-326 requires confirmation using an independent patient cohort that includes clinical follow-up data. Kjersem et al (2014) found the prognostic

potential of miR-326 as it associate with shortened progression free survival and overall survival (Kjersem et al 2014).

The strong paired correlation in the level of circulatory microRNAs between pretreatment and post treatment samples for miR-29-3p ($P<0.05$), miR-210 ($P<0.01$) and miR-221-3p ($P<0.001$) in our data validates the consistence pattern of change induced by chemotherapy for those microRNAs.

CA 15-3 is one of the first prognostic breast cancer factor relying on circulating tumor cells and when determined individually it has prognostic value. It is a mucin belonging to a large family of glycoproteins encoded by MUC 1 gene. Accumulating studies show that in preoperative breast cancer CA 15-3 is significantly elevated in the circulation ( Valić *et al.*, 2017). Elevated preoperative CA 15-3 levels is also directly related to tumor burden such as larger tumor size, more lymph node metastases, and advanced stage (Park et al. 2008). CA 15-3 can also be used in conjunction with diagnostic imaging, history and physical examination in evaluating response to treatment (Mohd, Trivedi and Atara, 2016). High levels of CA15-3 predict poor response to chemotherapy and persistently elevated levels post chemotherapy indicate a reduced disease-free survival in locally advanced breast cancer (Al-Azawi *et al.*, 2006; Cidon, 2017). Here we show that most of our study subjects (87%) have post chemotherapy CA 15-3 value which falls under the normal range. Even if post therapy CA 15-3 values are known to show response to therapy (Ebeling et al. 2002), in the follow-

up of the patients increasing and decreasing serum CA 15-3 values have more clinical significance than values remaining in the reference range (Kallioniemi et al. 1988; Vaibhav et al. 2015). As the stage IV breast cancer study subjects do not pass through local therapies (surgery or radiotherapy), they were found to have significantly higher CA 15-3 value when compared with both, stage II and III. Studies which compare pre and post-surgery CA 15-3 values have confirmed a significant decline in the level of the marker after surgery (Mohd, Trivedi and Atara, 2016; Ebeling et al. 2002).

# 6 CHAPTER SIX

## 6.1 CONCLUSION

Prolonged patient delay (time from onset of first symptoms to first consultation of a doctor) is known risk factor in breast cancer patients for advanced stage presentation and shorter survival. We found significant delay in seeking medical attention after onset of symptoms in our study subjects. Lump and swelling of breast were found to be the major symptoms recognized by patients for seeking medical care. There was significant derangement of biochemical profile at the advanced stage of the disease, which signifies the interplay between breast cancer and defective organ functions.

Circulating miRNAs hold a great promise as diagnostic, prognostic or predictive biomarkers in the clinical management of patients with breast cancer. Despite this growing evidence, their use as diagnostic tools in clinical practice remains sparse. By comparing differential expression of specific microRNAs in breast cancer, our study demonstrated the potential of miR-21-5p as diagnostic circulatory biomarker for breast cancer. To increase its diagnostic potential, further investigation on its association to clinicopathological parameters on large number of samples will help. It is also expected that a systemic approach on clustered deregulated microRNAs, rather than single microRNA, will be more informative to specifically stratify the heterogeneous disease into specific groups for optimization of treatment efficacy. Our study also confirmed that level of specific circulatory microRNAs change through the courses of chemotherapy

(miR-326), which requires further study to signify the prognostic potential of circulatory microRNAs.

## 6.2 Limitations of the study

Limitation of the study includes relatively small number of samples in the control group, lack of reliable housekeeping circulating miRNAs for normalization of expression levels and the incomplete CA 15-3 data (before chemotherapy) to monitor chemotherapy response of study subjects. It was also good if we were having hormone status level of study subjects to see variations in circulatory microRNAs among the different molecular subtypes of breast cancer.

## 6.3 Recommendations and future directions

Even if extrapolating our results to the general population needs caution (as we recruited study subjects who are destined to chemotherapy), our study found significant delay in seeking medical care after recognizing symptoms. As treatment success in breast cancer is related to how quickly treatment is initiated, it is necessary to identify and address major reasons behind the decision of symptomatic women delay in seeking health care. Lack of screening programs, delays in health care system and limitations in specific detection using mammograms in younger patients may contribute for the observed delay in addition to the personal delays driven by fear and denial. Women may benefit from greater awareness on the advantages of early detection and expansion of diagnostic and treatment facilities in the country for breast cancer.

Exceptional stability in body fluids, their evolutionary conserved sequence, specific pathologic profile and highly sensitive and specific detection using real time PCR makes circulatory microRNAs an attractive non-invasive candidate for use in diagnostic and prognostic testing, and in personalizing or targeting therapies. However, the low amount of circulatory microRNA compromises its quality and exact quantification. Lack of standardized endogenous control, which can be used for normalizing variances on the amount of starting material and miRNA extraction, is also a major challenge which needs to be addressed.

Since breast cancer is a heterogeneous disease, systematically stratified panels of microRNAs may provide greater accuracy than a single microRNA. Future studies may systematically associate deregulations in panels of microRNAs with clinicopathological features and therapy responses for optimized treatment.

# 7 References


Aapro M. 2006. "Breast Cancer: Not a Single Disease." *Eur J Cancer Suppl* 4 (4):1–3

Abate S., Yilma Z., Assefa M., and Tigeneh W. 2016. "Trends of Breast Cancer in Ethiopia." *International Journal of Cancer Research and Molecular Mechanisms* 2.1: 2–6.

Akarolo-Anthony., Sally N., Ogundiran T., and Adebamowo C. 2010. "Emerging Breast Cancer Epidemic: Evidence from Africa." *Breast Cancer Research* 12 (SUPPL. 4). doi:10.1186/bcr2737.

Akinbami A., Popoola A., Adediran A., Dosunmu A., Oshinaike O., Adebola P. and Ajibola S. 2012. "Full Blood Count Pattern of Pre-Chemotherapy Breast Cancer Patients in Lagos, Nigeria." *Caspian Journal of Internal Medicine* 4 (1): 574–79.

Al-Azawi D., Kelly G., Myers E., McDermott E., Hill A., Duffy M. and Higgins N. 2006. "CA 15-3 Is Predictive of Response and Disease Recurrence Following Treatment in Locally Advanced Breast Cancer." *BMC Cancer* 6: 3–9. doi:10.1186/1471-2407-6-220.

Al-Ejeh F., Smart C., Morrison B., Chenevix-Trench G., López A., Lakhani S., Brown M., and Khanna K. 2011. "Breast Cancer Stem Cells: Treatment Resistance and Therapeutic Opportunities." *Carcinogenesis* 32 (5): 650–58. doi:10.1093/carcin/bgr028.

Alvarez H., Valero V., and Hortobagyi G. 2010. "Emerging Targeted Therapies for Breast Cancer." *Journal of Clinical Oncology* 28 (20): 3366–79. doi:10.1200/JCO.2009.25.4011.

Asangani A., Rasheed S., Nikolova D., Leupold J., Colburn N., Post S. and Allgayer H. 2008. "MicroRNA-21 (miR-21) Post-Transcriptionally Downregulates Tumor Suppressor Pdcd4 and Stimulates Invasion,

Intravasation and Metastasis in Colorectal Cancer." *Oncogene* 27 (15): 2128–36. doi:10.1038/sj.onc.1210856.

Ataollahi M., Sharifi J., Paknahad M., and Paknahad A. 2015. "Breast Cancer and Associated Factors: A Review." *Journal of Medicine & Life* 8 (Spec Iss 4): 6–11.

Azubuike, Samuel O., Colin M., Louise H., and Richard M. 2018. "Rising Global Burden of Breast Cancer: The Case of Sub-Saharan Africa (with Emphasis on Nigeria) and Implications for Regional Development: A Review." *World Journal of Surgical Oncology* 16 (1):1–13. doi:10.1186/s12957-018-1345-2.

Badr F. 2016 "Potential Role of miR-21 in Breast Cancer Diagnosis and Therapy" *JSM Biotechnology & Biomedical Engineering* 3(5):1068

Badr M. 2016. "Potential Role of miR-21 in Breast Cancer Diagnosis and Therapy" 49 (Tmem 49): 1–8.

Bao T. and Davidson N. 2008. "Gene Expression Profiling of Breast Cancer." *National Institutes of Health* 42: 249–60. doi:10.1016/j.yasu.2008.03.002.Gene.

Barnett E., Conklin D., Ryan L., Keskey C., Ramjee V., Sepulveda E., Srivastava S., Bhatnagar A. and Cheadle W. 2016. "Anti-Inflammatory Effects of miR-21 in the Macrophage Response to Peritonitis." *Journal of Leukocyte Biology* 99 (2): 361–71. doi:10.1189/jlb.4A1014-489R.

Bartel P., and Chen C.. 2004. "Micromanagers of Gene Expression: The Potentially Widespread Influence of Metazoan microRNAs." *Nature Reviews. Genetics* 5 (5): 396–400. doi:10.1038/nrg1328.

Behrmann I., Margue C. and Kreis S. 2015. "Circulating microRNAs as Candidate Biomarkers for the Surveillance of Melanoma Patients." *EBioMedicine* 2 (7): 625–26. doi:10.1016/j.ebiom.2015.07.015.

Bellavance C. and Kesmodel S. 2016. "Decision-Making in the Surgical Treatment

of Breast Cancer: Factors Influencing Women's Choices for Mastectomy and Breast Conserving Surgery." *Frontiers in Oncology* 6 (March): 1–7. doi:10.3389/fonc.2016.00074.

Benson J. 2003. "The TNM Staging System and Breast Cancer." *The Lancet Oncology* 4 (1): 56–60. doi:10.1016/S1470-2045(03)00961-6.

Bernal-Estévez D., Sánchez R., Tejada R. and Parra-López C. 2016. "Chemotherapy and Radiation Therapy Elicits Tumor Specific T Cell Responses in a Breast Cancer Patient." *BMC Cancer* 16 (1): 1–13. doi:10.1186/s12885-016-2625-2.

Bines J., Earl H., Buzaid A. and Saad E. 2014. "Anthracyclines and Taxanes in the Neo/adjuvant Treatment of Breast Cancer: Does the Sequence Matter?" *Annals of Oncology* 25 (6): 1079–85. doi:10.1093/annonc/mdu007.

Bishop J., Coleman M., Cooke B. and Davies R. 2004. "Breast Fine Needle Aspiration Cytology and Core Biopsy: A Guide for Practice." *Breast*, www.nbcc.org.au

Bombonati A. and Sgroi D. 2011. "The Molecular Pathology of Breast Cancer Progression." *The Journal of Pathology* 223: 307–17. doi:10.1002/path.2808.

Brandt J., Garne J., Tengrup I. and Manjer J. 2015. "Age at Diagnosis in Relation to Survival Following Breast Cancer: A Cohort Study." *World Journal of Surgical Oncology* 13 (1): 33. doi:10.1186/s12957-014-0429-x

Brewster A., Chavez-MacGregor M. and Brown P. 2014. "Epidemiology, Biology, and Treatment of Triple-Negative Breast Cancer in Women of African Ancestry." *The Lancet Oncology* 15 (13). Elsevier Ltd: e625–34. doi:10.1016/S1470-2045(14)70364-X.

Carnero A., and Lleonart M. 2016. "The Hypoxic Microenvironment: A

Determinant of Cancer Stem Cell Evolution." *BioEssays* 38: S65–74. doi:10.1002/bies.201670911.

Catalanotto C., Cogoni C., and Zardo G. 2016. "MicroRNA in Control of Gene Expression: An Overview of Nuclear Functions." *International Journal of Molecular Sciences* 17 (10). doi:10.3390/ijms17101712.

Celià-terrassa T. and Kang Y. 2016. "Distinctive Properties of Metastasis-Initiating Cells." *Genes and Development*, 892–908. doi:10.1101/gad.277681.116.892.

Chemonges S., Tung J. and Fraser J. 2014. "Proteogenomics of Selective Susceptibility to Endotoxin Using Circulating Acute Phase Biomarkers and Bioassay Development in Sheep: A Review." *Proteome Science* 12 (1). doi:10.1186/1477-5956-12-12.

Chen J., Xu T. and Chen C. 2015. "The Critical Roles of miR-21 in Anti-Cancer Effects of Curcumin." *Annals of Translational Medicine* 3 (21): 330. doi:10.3978/j.issn.2305-5839.2015.09.20.

Cidon U. 2017. "A Brief Overview of the Role of CA 15-3 in Breast Cancer." WebmedCentral *Breast* 8(6): 55–57.

Clough K., Kaufman G., Nos C., Buccimazza I. and Sarfati I. 2011. "Reply to Comments on: Improving Breast Cancer Surgery: A Classification and Quadrant per Quadrant Atlas for Oncoplastic Surgery." *Annals of Surgical Oncology* 18 (S3): 259–60. doi:10.1245/s10434-010-1302-y.

Cui M., Liu W., Zhang L., Guo F., Liu Y., Chen F., Liu T., Ma R. and Wu R. 2017. "Clinicopathological Parameters and Prognostic Relevance of miR-21 and PTEN Expression in Wilms' Tumor." *Journal of Pediatric Surgery* 52 (8): 1348–54. doi:10.1016/j.jpedsurg.2016.12.005.

Dai X., Li T., Bai Z., YangY., Liu X., Zhan J. and Shi B. 2015. "Breast Cancer Intrinsic Subtype Classification , Clinical Use and Future Trends" *Am J Cancer Res* 5 (10): 2929–43.

Darwish A., Helal A., Aly El-din N., Solaiman L., Amin A. 2017. "Breast cancer inwomen aging 35 years old and younger: The Egyptian National Cancer Institute (NCI) experience" *The Breast* 31: 1-8

Das M., Andreassen R., Haugen T. and Furu K. 2016. "Identification of Endogenous Controls for Use in miRNA Quantification in Human Cancer Cell Lines" *Cancer Genomics & Proteomics* 13: 63-68

Davoren P., McNeill R., Lowery A., Kerin M. and Miller N. 2008. "Identification of Suitable Endogenous Control Genes for microRNA Gene Expression Analysis in Human Breast Cancer." *BMC Molecular Biology* 9 (1): 76. doi:10.1186/1471-2199-9-76.

Deshpande T., Pandey A. and Shyama S. 2017. "Review: Breast cancer and etiology" *Trends Med. 17: 1-7*

Deus R., Carvalho F. and Bacchi C. 2015. "Breast Cancer in Very Young Women: Clinicopathological Study of 149 Patients ≤25 Years Old." *The Breast* 24 (4). 461–67. doi:10.1016/j.breast.2015.04.002.

Dewi I., Gidlof O., Ivars F. and ohman J. 2014 "The Alteration of MiR-326 and MiR-142-3p Expressions during Immune Activation Is Positively Correlated with Graft-protective Cytokine TGF-beta" *Journal of heart and lung transplant* 33(4): S253–S254 doi: 10.1016/j.healun.2014.01.664

Diener Y., Walenda T., Jost E., Brümmendorf T., Bosio A., Wagner W. and Bissels U. 2015. "MicroRNA Expression Profiles of Serum from Patients before and after Chemotherapy." *Genomics Data* 6: 125–27. doi:10.1016/j.gdata.2015.08.018.

doi: 10.18203/2349-2902.isj20162735.

Donegan W., 1997. "Tumor-Related Prognostic Factors for Breast Cancer." *CA: A Cancer Journal for Clinicians* 47 (1): 28–51. doi:10.3322/canjclin.47.1.28

Dong G., Wang D., Liang X., Gao H., Wang L., Yu X. and Liu J. 2014. "Factors Related to Survival Rates for Breast Cancer Patients." *International Journal of Clinical and Experimental Medicine* 7 (10): 3719–24.

Dye T., Bogale S., Hobden C., Tilahun Y., Deressa T., and Reeler A. 2012. "Experience of Initial Symptoms of Breast Cancer and Triggers for Action in Ethiopia." *International Journal of Breast Cancer* 2012: 908547. doi:10.1155/2012/908547.

Ebeling G., Stieber P., Untch M., Nagel D., Konecny G., Schmitt U., Fateh-Moghadam A. and Seidel D. 2002. "Serum CEA and CA 15-3 as Prognostic Factors in Primary Breast Cancer." *British Journal of Cancer* 86 (8): 1217–22. doi:10.1038/sj.bjc.6600248.

Eng A., McCormack V. and Dos-Santos-Silva I. 2014. "Receptor-Defined Subtypes of Breast Cancer in Indigenous Populations in Africa: A Systematic Review and Meta-Analysis." *PLoS Medicine* 11 (9). e1001720 doi:10.1371/journal.pmed.1001720.

Espina C., McKenzie F. and dos-Santos-Silva I. 2017. "Delayed Presentation and Diagnosis of Breast Cancer in African Women: A Systematic Review." *Annals of Epidemiology* 27 (10). 659–671.e7. doi:10.1016/j.annepidem.2017.09.007.

Esteva J. and Hortobagyi G. 1998. "Integration of Systemic Chemotherapy in the Management of Primary Breast Cancer." *Oncologist*.4586: 5–313.

Ezeome R., 2010. "Delays in Presentation and Treatment of Breast Cancer in

Enugu, Nigeria." *Nigerian Journal of Clinical Practice* 13 (3): 311–16.

Fakhsheena A., Nighat R. and Ali M. 2017. "Breast Cancer Therapy : A Mini Review" *MOJ Drug Design Development & Therapy Breast* (2): 2–5. doi:10.15406/mojddt.2017.01.00006.

Falck A., Fernö M., Bendahl P. and Rydén L. 2013. "St Gallen Molecular Subtypes in Primary Breast Cancer and Matched Lymph Node Metastases--Aspects on Distribution and Prognosis for Patients with Luminal A Tumours: Results from a Prospective Randomised Trial." *BMC Cancer* 13: 558. doi:10.1186/1471-2407-13-558.

Fornari F., Milazzo M., Galassi M., Callegari E., Veronese A., Miyaaki H., Sabbioni S., et al 2010. "p53/mdm2 Feedback Loop Sustains miR-221 Expression and Dictates the Response to Anticancer Treatments in Hepatocellular Carcinoma" *Molecular Cancer Research* 12(2):203 doi: 10.1158/1541-7786.MCR-13-0312-T

Frères P., Wenric S., Boukerroucha M., Fasquelle C., Thiry J., Bovy N., Struman I. et al. 2016. "Circulating microRNA-Based Screening Tool for Breast Cancer." *Oncotarget* 7 (5): 5416–28. doi:10.18632/oncotarget.6786.

Friend S. and Royce M.. 2016. "The Changing Landscape of Breast Cancer: How Biology Drives Therapy." *Medicines* 3 (1): 2. doi:10.3390/medicines3010002.

Galukande M., Wabinga H., Mirembe F., Karamagi C. and Asea A. 2014. "Molecular Breast Cancer Subtypes Prevalence in an Indigenous Sub Saharan African Population." *The Pan African Medical Journal* 17: 249. doi:10.11604/pamj.2014.17.249.330.

Gandhi R. 2015. "miRNA in multiple sclerosis: search for novel biomarkers" *Multiple Sclerosis Journal* 21(9): 1095 –1103 doi: 10.1177/ 135245851557877

Garcia B., Blankenstein M., van der Wall E., Nortier J., Schornagel J. and Thijssen J. 1990. "Comparison of Breast Cancer Mucin (BCM) and CA 15-3 in Human Breast Cancer." *Breast Cancer Research and Treatment* 17 (2): 69–76. doi:10.1007/BF01806286.

Gioia S. 2017. "Why Is Breast Cancer Early Detection Important?" *Mastology* 27 (3): 173–75. doi:10.5327/Z259453942017EDIT273.

Gradishar J., 2012. "Taxanes for the Treatment of Metastatic Breast Cancer." *Breast Cancer: Basic and Clinical Research*, 159(6): 159–171 doi:10.4137/BCBCR.S8205.

Han Y., Liu M., Wang Z., Huang M., Xu N. and Wu L. 2017. "Serum MicroRNAs Related with Chemoradiotherapy Resistance in Advanced-Stage Cervical Squamous Cell Carcinoma." *Translational Oncology* 10 (3): 378–84. doi:10.1016/j.tranon.2017.03.005.

Harbeck N. and Gnant M. 2017. "Breast Cancer." *The Lancet* 389 (10074). 1134–50. doi:10.1016/S0140-6736(16)31891-8.

Hassan M., Ansari J., Spooner D. and Hussain S. 2010. "Chemotherapy for breast cancer (Review)" Oncology Reports 24: 1121-1131

Hassiotou F., and Donna G. 2013. "Anatomy of the Human Mammary Gland: Current Status of Knowledge." *Clinical Anatomy* 26 (1): 29–48. doi:10.1002/ca.22165.

Hiatt A., and Brody G. 2018. "Environmental Determinants of Breast Cancer." *Annu Rev Public Health*. 39:113–33

Huang Y., Liu J., Deatherage D., Luo J, Mutch D., Goodfellow P., Miller D., Huang T. 2009. "Epigenetic repression of microRNA-129-2 leads to overexpression of SOX4 oncogene in endometrial cancer." *Cancer research*

69(23): 9038-46 doi: 10.1158/0008-5472.CAN-09-1499

Hummel R., Wang T., Watson D., Michael M., Hoek M., Haier J. and Hussey D. 2011. "Chemotherapy-Induced Modification of microRNA Expression in Esophageal Cancer." *Oncology Reports* 26 (4): 1011–17. doi:10.3892/or.2011.1381.

Humphreys D. 2004. "Renal Failure Associated with Cancer and Its Treatment: An Update." *Journal of the American Society of Nephrology* 16 (1): 151–61. doi:10.1681/ASN.2004100843.

Huo D., Ikpatt F., Khramtsov A., Dangou J., Nanda R., Dignam J., Zhang B., et al. 2009. "Population Differences in Breast Cancer: Survey in Indigenous African Women Reveals over-Representation of Triple-Negative Breast Cancer." *Journal of Clinical Oncology* 27 (27): 4515–21. doi:10.1200/JCO.2008.19.6873.

Jedy-Agba E., Valerie M., Adebamowo C., and Isabel D. 2016. "Stage at Diagnosis of Breast Cancer in Sub-Saharan Africa: A Systematic Review and Meta-Analysis." *The Lancet Global Health* 4 (12): e923–35. doi:10.1016/S2214-109X(16)30259-5.

Jemal A., Bray F., Forman D., O,Brien M.,Ferlay J. and Parkin M. 2012 "Cancer Burden in Africa and Opportunities for Prevention" *Cancer* doi: 10.1002/cncr.27410

Jhaveri D. and Salahudeen A. 2015. "Onconephrology: Cancer, Chemotherapy and the Kidney." *Onconephrology: Cancer, Chemotherapy and the Kidney*, 25–34. doi:10.1007/978-1-4939-2659-6.

Jo P., Azizian A., Salendo J., Kramer F., Bernhardt M., Wolff H., Gruber J., et al. 2017. "Changes of Microrna Levels in Plasma of Patients with Rectal Cancer during Chemoradiotherapy." *International Journal of Molecular Sciences* 18

(6):1140-1153 doi:10.3390/ijms18061140.

Jurasz P., Alonso-Escolano D. and Radomski M. 2004. "Platelet-Cancer Interactions: Mechanisms and Pharmacology of Tumour Cell-Induced Platelet Aggregation." *British Journal of Pharmacology* 143 (7): 819–26. doi:10.1038/sj.bjp.0706013.

Kallioniemi P., Oksa H., Aaran R., Hietanen T., Lehtinen M., and Koivula T. 1988. "Serum ca 15-3 Assay in the Diagnosis and Follow-up of Breast Cancer." *British Journal of Cancer* 58 (2): 213–15. doi:10.1038/bjc.1988.196.

Kantelhardt J., Zerche P., Mathewos A., Trocchi P., Addissie A., Aynalem A., Wondemagegnehu T., et al. 2014. "Breast Cancer Survival in Ethiopia: A Cohort Study of 1,070 Women." *International Journal of Cancer* 135 (3): 702–9. doi:10.1002/ijc.28691.

Kazarian A., Blyuss O., Metodieva G., Gentry-Maharaj A., Ryan A., Kiseleva E., Prytomanova O., et al. 2017. "Testing Breast Cancer Serum Biomarkers for Early Detection and Prognosis in Pre-Diagnosis Samples." *British Journal of Cancer* 116 (4). Nature Publishing Group: 501–8. doi:10.1038/bjc.2016.433.

Kazarian A., Blyuss O., Metodieva G., Gentry-Maharaj A., Ryan A., Kiseleva E., Prytomanova O., et al. 2017. "Testing Breast Cancer Serum Biomarkers for Early Detection and Prognosis in Pre-Diagnosis Samples." *British Journal of Cancer* 116 (4). Nature Publishing Group: 501–8. doi:10.1038/bjc.2016.433.

Ke S., Liu C., Liu P., and Liang C. 2003. "MicroRNAs: Key Participants in Gene Regulatory Networks." *Current Opinion in Chemical Biology* 7 (4): 516–23. doi:10.1016/S1367-5931(03)00075-9.

Kelly G., Gibier J., Pottier N., Hémon B., Seuningen I., Glowacki F., Leroy X., Cauffiez C., Gnemmi V., Aubert S. and Perrais M. 2017. "Targeting miR-21 decreases expression of multi-drug resistant genes and promotes

chemosensitivity of renal carcinoma." *Tumor Biology*.39(7): 1-10 doi:10.1177/1010428317707372

Kelly K., Dean J., Comulada S. and Lee S. 2010. "Breast Cancer Detection Using Automated Whole Breast Ultrasound and Mammography in Radiographically Dense Breasts." *European Radiology* 20 (3): 734–42. doi:10.1007/s00330-009-1588-y.

Kjersem B., Ikdahl T., Lingjaerde O., Guren T., Tveit K., and Kure E. 2014. "Plasma microRNAs Predicting Clinical Outcome in Metastatic Colorectal Cancer Patients Receiving First-Line Oxaliplatin-Based Treatment." *Molecular Oncology* 8 (1): 59–67. doi:10.1016/j.molonc.2013.09.001.

Kleibl Z., and Kristensen V. 2016. "Women at High Risk of Breast Cancer: Molecular Characteristics, Clinical Presentation and Management." *The Breast* 28. Elsevier Ltd: 136–44. doi:10.1016/j.breast.2016.05.006

Kozera B. and Rapacz M. 2013. "Reference Genes in Real-Time PCR." *Journal of Applied Genetics* 54 (4): 391–406. doi:10.1007/s13353-013-0173-x.

Ladomery R., Maddocks D., and Wilson I. 2011. "MicroRNAs: Their Discovery, Biogenesis, Function and Potential Use as Biomarkers in Non-Invasive Prenatal Diagnostics." *International Journal of Molecular Epidemiology and Genetics* 2 (3): 253–60.

Lal I., Dittus K. and Holmes C. 2013. "Platelets, Coagulation and Fibrinolysis in Breast Cancer Progression." *Breast Cancer Research : BCR* 15 (4): 207. doi:10.1186/bcr3425.

Lee H., Han S., Rhee H., Park J., Lee J., Oh Y., Choi S., Shin S. and Kim W. 2015. "Differential expression of microRNAs and their target genes in non-small-cell lung cancer" *Molecular Medicine Reports* 11: 2034-2040

Leslie R. and Longy M. 2016. "Inherited PTEN Mutations and the Prediction of Phenotype." *Seminars in Cell and Developmental Biology* 52: 30–38. doi:10.1016/j.semcdb.2016.01.030.

Lesurf R., Aure M., Mørk H., Vitelli V., Lundgren S., Børresen-Dale A., Kristensen V., et al. 2016. "Molecular Features of Subtype-Specific Progression from Ductal Carcinoma In Situ to Invasive Breast Cancer." *Cell Reports* 16 (4): 1166–79. doi:10.1016/j.celrep.2016.06.051.

Liang Y., Ridzon,D., Wong L. and Chen C.. 2007. "Characterization of microRNA Expression Profiles in Normal Human Tissues." *BMC Genomics* 8: 1–20. doi:10.1186/1471-2164-8-166.

Liang Z., Wu H., Xia J., Li Y., Zhang Y., Huang K., Wagar N., et al. 2010. "Involvement of miR-326 in Chemotherapy Resistance of Breast Cancer through Modulating Expression of Multidrug Resistance- Associated Protein 1." *Biochemical Pharmacology* 79 (6): 817–24. doi:10.1016/j.bcp.2009.10.017.

Lieske B., Ravichandran D. and Wright D. 2006. "Role of Fine-Needle Aspiration Cytology and Core Biopsy in the Preoperative Diagnosis of Screen-Detected Breast Carcinoma." *British Journal of Cancer* 95 (1): 62–66. doi:10.1038/sj.bjc.6603211.

Ling H., Girnita L., Buda O. and Calin G. 2017. "Non-coding RNAs: the cancer genome dark matter that matters!" *Clin Chem Lab Med* 55(5): 705–714

MacFarlane L. and Murphy P. 2010. "MicroRNA: Biogenesis, Function and Role in Cancer." *Current Genomics* 11 (7): 537–61. doi:10.2174/138920210793175895.

Maisonneuve P., Disalvatore D., Rotmensz N., Curigliano G., Colleoni M., Dellapasqua S., Pruneri G., et al. 2014. "Proposed New Clinicopathological Surrogate Definitions of Luminal A and Luminal B (HER2-Negative)

Intrinsic Breast Cancer Subtypes." *Breast Cancer Research* 16 (3): R65. doi:10.1186/bcr3679

Makanjuola A., Javid F., Obafunwa J., Oludara M. and Popoola A.. 2014. "Breast Cancer Receptor Status Assessment and Clinicopathological Association in Nigerian Women: A Retrospective Analysis." *Journal of Cancer Research & Therapy* 2 (8): 122–27. doi:10.14312/2052-4994.2014-19.

Makiguchi T., Yamada M., Yoshioka Y., Sugiura H., Koarai A., Chiba S., Fujino N., et al. 2016. "Serum Extracellular Vesicular miR-21-5p Is a Predictor of the Prognosis in Idiopathic Pulmonary Fibrosis." *Respiratory Research* 17 (1). 1–15. doi:10.1186/s12931-016-0427-3.

Mandilaras V., Bouganim N., Spayne J., Dent R., Arnaout A., Boileau J., Brackstone M., Meterissian S. and Clemons M. 2015. "Concurrent Chemoradiotherapy for Locally Advanced Breast Cancer; time for a New Paradigm?" *Current Oncology* 22 (1): 25–32. doi:10.3747/co.21.2043.

Martin Y., Jong R., Etta D., Pritchard K., Smith R. 2015. "Earlier Detection and Diagnosis of Breast Cancer:" *Canadian Breast Cancer Foundation*. 1-39

Matamala N., Vargas M., González-Cámpora R., Miñambres R., Arias J., Menéndez P., Andrés-León E., et al. 2015. "Tumor MicroRNA Expression Profiling Identifies Circulating MicroRNAs for Early Breast Cancer Detection." *Clinical Chemistry* 61 (8): 1098–1106. doi:10.1373/clinchem.2015.238691.

Mohd K., Trivedi H., Atara A. 2016. "Analysis of Tumour Marker CA 15-3 in Breast Cancer Following Surgery." *International Surgery Journal* 3 (3): 1491–94.

Moiseenko F., Volkov N., Bogdanov A., Dubina M. and Moiseyenko V. 2017. "Resistance Mechanisms to Drug Therapy in Breast Cancer and Other Solid

Tumors: An Opinion." *F1000Research* 6 (0): 288. doi:10.12688/f1000research.10992.1.

Morrow S., Peklak-Scott C., Bishwokarma B., Kute T., Smitherman P. and Townsend J. 2006. "Multidrug Resistance Protein 1 (MRP1, ABCC1) Mediates Resistance to Mitoxantrone via Glutathione-Dependent Drug Efflux." *Molecular Pharmacology* 69 (4): 1499–1505. doi:10.1124/mol.105.017988.1999.

Nasser S., and Pars M. 2011. "Breast Cancer from Molecular Point of View: Pathogenesis and Biomarkers." *Breast Cancer - Focusing Tumor Microenvironment, Stem Cells and Metastasis*. 103-126 doi:10.5772/24940.

Niu J., Shi Y., Tan G., Yang C., Fan M., Pfeffer L. and Wu Z. 2012. "DNA Damage Induces NF-κB-Dependent MicroRNA-21 up-Regulation and Promotes Breast Cancer Cell Invasion." *Journal of Biological Chemistry* 287 (26): 21783–95. doi:10.1074/jbc.M112.355495.

Pakseresht S., Ingle G., Garg S. and Sarafraz N. 2014. "Stage at Diagnosis and Delay in Seeking Medical Care among Women with Breast Cancer, Delhi, India." *Iranian Red Crescent Medical Journal* 16 (12): e14490. doi:10.5812/ircmj.14490.

Park W., Oh J., Kim J., Park S., Kim K., Kim J. and Lee K. 2008. "Preoperative CA 15-3 and CEA Serum Levels as Predictor for Breast Cancer Outcomes." *Annals of Oncology* 19 (4): 675–81. doi:10.1093/annonc/mdm538.

Peiris-Pagès M., Martinez-Outschoorn U., Pestell R., Sotgia F. and Lisanti M. 2016. "Cancer Stem Cell Metabolism." *Breast Cancer Research* 18 (1). Breast Cancer Research: 55. doi:10.1186/s13058-016-0712-6.

Peralta-Zaragoza O., Deas J., Meneses-Acosta A., De la O-Gómez F., Fernández-Tilapa G., Gómez-Cerón C., Benítez-Boijseauneau O., et al. 2016. "Relevance

of miR-21 in Regulation of Tumor Suppressor Gene PTEN in Human Cervical Cancer Cells." *BMC Cancer* 16 (1): 1–16. doi:10.1186/s12885-016-2231-3.

Polyak K. 2007. "Science in Medicine Breast Cancer : Origins and Evolution." *Cell* 117 (11): 3155–63. doi:10.1172/JCI33295.group.

Rivera-Franco M. and Leon-Rodriguez E. 2018. "Delays in Breast Cancer Detection and Treatment in Developing Countries." *Breast Cancer: Basic and Clinical Research* 12:1-5 doi:10.1177/1178223417752677

Rothé F., Ignatiadis M., Chaboteaux C., Haibe-Kains B., Kheddoumi N., Majjaj S., et al. (2011) Global MicroRNA Expression Profiling Identifies MiR-210 Associated with Tumor Proliferation, Invasion and Poor Clinical Outcome in Breast Cancer. *Plos one* 6(6): e20980. doi:10.1371/journal.pone.002098

Saghir E., Nagi S., Adebamowo C., Anderson B., Carlson R., Bird P., Corbex M., Badwe R., et al. 2011. "Breast Cancer Management in Low Resource Countries (LRCs): Consensus Statement from the Breast Health Global Initiative." *Breast* 20 (SUPPL. 2). Elsevier Ltd: S3–11. doi:10.1016/S0960-9776(11)70012-1.

Santen J. and Harvey H. 1999. "Use of Aromatase Inhibitors in Breast Carcinoma." *Endocrine-Related Cancer* 6 (1): 75–92. doi:10.1677/erc.0.0060075.

Schuster C., Budczies J., Faber C., Kirchner T. and Hlubek F. 2011. "MicroRNA Expression Profiling of Specific Cells in Complex Archival Tissue Stained by Immunohistochemistry." *Laboratory Investigation* 91 (1). 157–65. doi:10.1038/labinvest.2010.134.

Seifert-Klauss R., Krämer A. and Schneeweis A. 2015. "Update on Aromatase Inhibitors" *J Reproduktionsmed Endokrinol_Online* 12 (4): 353–9.

Shackleton M., Quintana E., Fearon E. and Morrison S. 2009. "Heterogeneity in Cancer: Cancer Stem Cells versus Clonal Evolution." *Cell* 138 (5): 822–29. doi:10.1016/j.cell.2009.08.017.

Sharma G., Dave R., Sanadya J., Sharma P. and Sharma K. 2010. "Various types and management of breast cancer: an overview" *J. Adv. Pharm. Tech. Res.* 1 (2): 109–26.

Solomon T., Mahlet K., Mathewos A., Biniyam T. et al. 2018. "Estimates of Cancer Incidence in Ethiopia in 2015 Using Population-Based Registry Data." *Journal of Global Oncology* 4: 1-12 doi: 10.1200/JGO.17.00175

Sood A. 2017. "Triple Negative Breast Cancer: A Unique Type of Breast Cancer." *Anaplastology* 6 (1): 1–3. doi:10.4172/2161-1173.1000164.

Sotiriou C. and Pusztai L. 2009. "Gene-Expression Signatures in Breast Cancer." *New England Journal of Medicine* 360 (8): 790–800. doi:10.1056/NEJMra0801289.

Stewart W., and Wild C. 2014. "World Cancer Report 2014." *World Health Organization*, 362–376. doi:9283204298.

Subramaniam E., Liung T., Mashor M., Ashidi N. and Isa M. 2006. "Breast Cancer Diagnosis Systems : A Review." *International Journal of The Computer, the Internet and Management* 14 (2): 24–35.

Szabatura A. and Seung A. 2009. "Early and Locally Advanced Breast Cancer Learning Objectives." *Pharmacotherapy Self-Assessment Program, 6th Edition*, 1–9.

Taherian F., Atefeh, Sriganesh S., and Mark R. 2014. "Breast Cancer Classification: Linking Molecular Mechanisms to Disease Prognosis." *Briefings in Bioinformatics* 16 (3): 461–74. doi:10.1093/bib/bbu020.

Tazzite A., Jouhadi H., Nadifi S., Aretini P., Falaschi E., Collavoli A., Benider A., Caligo M. 2012. " BRCA1 and BRCA2 germline mutations in Moroccan breast/ovarian cancer families: Novel mutations and unclassified variants" *Gynecologic Oncology* 125: 687–692

Tewari M., Krishnamurthy A. and Shukla H. 2009. "Breast Conservation in Locally Advanced Breast Cancer in Developing Countries: Wise or Waste." *Surgical Oncology* 18 (1): 3–13. doi:10.1016/j.suronc.2008.07.004.

Timotewos G., Solomon A., Mathewos A., Addissie A., Bogale S., Wondemagegnehu T., Aynalem A., et al. 2018. "First Data from a Population Based Cancer Registry in Ethiopia." *Cancer Epidemiology* 53 (March). Elsevier: 93–98. doi:10.1016/j.canep.2018.01.008.

Torres A., Torres K., Wdowiak P., Paszkowski T. and MacIejewski R. 2013. "Selection and Validation of Endogenous Controls for microRNA Expression Studies in Endometrioid Endometrial Cancer Tissues." *Gynecologic Oncology* 130 (3). 588–94. doi:10.1016/j.ygyno.2013.06.026.

Vaibhav S., Arvind G., Deepak M., Shikha G. and Mahajan S. 2015. "A Prospective Study on Analysis of Ca15-3 in Breast Cancer Patient as a Prognostic Marker." *IOSR Journal of Dental and Medical Sciences Ver. I* 14 (12): 2279–2861. doi:10.9790/0853-141210508.

Valić A., Milas I., Mayer L., Šetić M., Matijević V., and Stanec M. 2017. "Prognostic Significance Of Ca 15-3 Tumor Marker In Breast Cancer Patients." *Libri Oncol*. 45(1):1–8

Vanderpuye G., Hammad N., PoojaPrabhakar, Simonds H., Olopade F. and Stefan D. 2017. "An Update on the Management of Breast Cancer in Africa." *Infectious Agents and Cancer* 12 (1). Infectious Agents and Cancer: 13. doi:10.1186/s13027-017-0124-y.

Wang H., Shi J., Li B., Zhou Q., Kong X. and Bei Y. 2017. "MicroRNA Expression Signature in Human Calcific Aortic Valve Disease." *BioMed Research International* 2017. doi:10.1155/2017/4820275

Wang Z. 2015. "Personalized Medicine for HER2- Positive Breast Cancer" *Breast Cancer Management* 4: 237–40.

Warner E. 2011. "Breast-Cancer Screening," *The new england journal o f medicine clinical practice* 1025–32. doi:10.1056/NEJMcp1101540.

Wen J., Ye F., Huang X., Li S., Yang L., Xiao X. and Xie X. 2015. "Prognostic Significance of Preoperative Circulating Monocyte Count in Patients with Breast Cancer Based on a Large Cohort Study." *Medicine (United States)* 94 (49): 1–7. doi:10.1097/MD.0000000000002266.

Winter J. and Diederichs S. 2011. "Argonaute Proteins Regulate microRNA Stability: Increased microRNA Abundance by Argonaute Proteins Is due to microRNA Stabilization." *RNA Biology* 8 (6): 1149–57. doi:10.4161/rna.8.6.17665.

Winter J., Jung S., Keller S., Gregory R. and Diederichs S. 2009. "Many Roads to Maturity: microRNA Biogenesis Pathways and Their Regulation." *Nature Cell Biology* 11 (3): 228–34. doi:10.1038/ncb0309-228.

Witwer K. 2015. "Circulating MicroRNA Biomarker Studies: Pitfalls and Potential Solutions." *Clinical Chemistry* 61 (1): 56–63. doi:10.1373/clinchem.2014.221341.

Xu K., Liang X., Shen K., Cui D., Zheng Y., Xu J., Fan Z., et al. 2012. "miR-297 Modulates Multidrug Resistance in Human Colorectal Carcinoma by down-Regulating MRP-2." *Biochemical Journal* 446 (2): 291–300. doi:10.1042/BJ20120386.

Yan X., Serre C., Bergeron L., Mur L., Busuttil V., Botto J. and Domloge N. 2016. "Modulation of a Specific Pattern of microRNAs , Including miR-29a , miR-30a and miR-34a , in Cultured Human Skin Fibroblasts , in Response to the Application of a Biofunctional Ingredient That Protects against Cellular Senescence in Vitro," *Journal of Cosmetics, Dermatological Sciences and Applications*, 5, 332-342 doi:10.4236/jcdsa.2015.54040.

Yan Y., Deng X., Ning X., Li F. and Cao J. 2017. "Pathogenic Mechanism of miR-21 in Autoimmune Lymphoid Hyperplasia Syndrome." *Oncology Letters* 13 (6): 4734–40. doi:10.3892/ol.2017.6039.

Yu Y., Wei W., and Liu J. 2012. "Diagnostic Value of Fine-Needle Aspiration Biopsy for Breast Mass: A Systematic Review and Meta-Analysis." *BMC Cancer* 12 (1). 1-14 doi:10.1186/1471-2407-12-41.

Zaretsky Z., Barnea I., Aylon Y., Gorivodsky M., Wreschner D. and Keydar I. 2006. "MUC1 Gene Overexpressed in Breast Cancer: Structure and Transcriptional Activity of the MUC1 Promoter and Role of Estrogen Receptor Alpha (ERα) in Regulation of the MUC1 Gene Expression." *Molecular Cancer* 5: 1–14. doi:10.1186/1476-4598-5-57.

Zhang C., Liu K., Li T., Fang J., Ding Y., Sun L., Tu T., et al. 2016. "MiR-21: A Gene of Dual Regulation in Breast Cancer." *International Journal of Oncology* 48 (1): 161–72. doi:10.3892/ijo.2015.3232.

Zhang P., Kong F., Deng X., Yu Y., Hou C., Liang T. and Zhu L. 2017. "MicroRNA-326 Suppresses the Proliferation, Migration and Invasion of Cervical Cancer Cells by Targeting ELK1." *Oncology Letters* 13 (5): 2949–56. doi:10.3892/ol.2017.5852.

Zhenglong W. Huang X., Huang X., Zou Q. and Guo Y. 2013 "The inhibitory role of Mir-29 in growth of breast cancer cells" *Journal of Experimental & Clinical*

*Cancer Research* 32:98 doi:10.1186/1756-9966-32-98

Zhu S., Si M., Wu H. and Yuan Y. 2007. "MicroRNA-21 Targets the Tumor Suppressor Gene Tropomyosin 1 (TPM1)." *Journal of Biological Chemistry* 282 (19): 14328–36. doi:10.1074/jbc.M611393200.

## Annex I: **Socio-demographic and Clinical data recording Format**

**Admission Date** _____/_____/_____
(To be filled by the data/sample Collector nurse)

Section I. Review on General Information

1.1. Initials (code) _____

1.2. Age (in years) _____

1.3. Sex Male ☐ Female ☐

1.4. Religion Orthodox/ Muslim /Catholic/ Protestant/

Other (Specify): _____

1.5. Ethnicity _____

1.6. Current weight (Kg) _____

1.7. Height (m)

_____

1.8. BSA _____

1.9. BMI (kg/$m^2$) _____

1.10. Blood pressure _____

1. 11. Sero status (HIV) Négative ☐ Positive ☐

Section II. Breast cancer related information

- Karnofsky Performance status <70 ☐ >70 ☐
- Menopausal status Premenopausal ☐ Postmenopausal ☐
- Histologic type of the cancer:
  - Infiltrating ductal_____
  - Intraductal_____
  - Lobular_____
- Tumor size at diagnosis _____
- Degree of differentiation:
  - Well differentiated_____
  - Moderately differentiated_____
  - Poorly differentiated_____

- Nodal involvement
  - Number of lymph nodes positive_____
  - Number of lymph nodes removed_____
- Stage of breast cancer _____
  - Which LN?

2.1.1.1. Axillary_____

2.1.1.2. Supraclavicular_____

27.2. TNM stage of the tumor _____

2.2. Site of tumor?: on Left breast ☐ on Right breast ☐

2.3. Location on the breast

2.3.1. Upper Outer quadrant_____

2.3.2. Upper inner quadrant_____

2.3.3. Lower outer quadrant_____

2.3.4. Lower inner quadrant_____

2.10 Patient Habits of smoking Yes ☐ No ☐

2.11. Chat chewing status Yes ☐ No ☐

2.12. Alcohol drink status Yes ☐ No ☐

2.13 Presence of severe concomitant disease Yes ☐ No ☐

Section III. Treatment related information

3.1 Type of the current chemotherapy setting (Protocol)

Neo-adjuvant ☐

Protocol CMF / AC / FEC / FAC / (others specify_____)

Adjuvant ☐

Protocol CMF / AC / FEC / FAC / (others specify_____)

Metastatic ☐

Protocol CMF / AC / FEC / FAC / (others specify_____)

3.2. For patients on adjuvant chemotherapy (in Q3.1), was the patient put on primary (neo-adjuvant) therapy previously?

Yes ☐ No ☐ date _____

3.3. For patients on adjuvant chemotherapy (in Q3.1), was surgery done before this chemotherapy?

Yes ☐ No ☐

3.4: type of surgery

BCS

3.5 What is the Current CPA based regimen

_____

_____

3.6. Current CPA based

dosage_____

_____

3.7 Co-medications (if any, including premedications) (Name, dose, etc)

_____

---

3.8. Is the patient on other medical conditions [if any]?

| | |
|---|---|
| Age related | on birth control, menopause, and osteoporosis _____ |
| Arthritis | NSAIDS _____ |
| Cardiovascular | Hypertension, arrhythmias _____ |
| On anticoagulants | Warfarin _____ |

Endocrine Diabetes, hyperlipidemia

_____

Epilepsy Phenytoin, carbamazepine

_____

HIV/AIDS NRTIs, PIs_____

SSRIs _____

Others (please specify) _____

3.9. Did the mother, father, sister or brother have breast cancer? Yes_____ No_____

3.10. Do you have a family history of ovarian cancer? Yes_____ No_____

Others (please specify) _____

3.11. Did you give birth? Yes_____ No _____

If yes, did you well fed your breast? Yes_____ No _____

3.12. Have you had radiation treatment to your chest? Yes_____ No_____

3.13 Did you receive blood? Yes_____ No_____

3.14 Approximately how long did you have health concern(s) or symptom(s) before contacting a doctor?

a. 1- 4 weeks b. 1-5 Months

c. 6 – 12 Months d. More than 12 Months

3.15. Have you experienced any of the following health concerns or symptoms before diagnosis?

Changes in the appearance of the breast, e.g.,

The nipple _____

The skin of the breast_____

The shape of the breast _____

The size of the breast _____

Nipple discharge including bleeding_____

Lump swelling or thickening in breast or armpit_____

Unexplained weight loss _____

3.16 Have you had any of the following treatments for your cancer yet? If so, please can you estimate the date this treatment started? Please tick all that apply.

a) Surgery Yes ____ No_____ Date of first treatment _____
b) Chemotherapy Yes ____ No_____ Date of first treatment _____
c) Others / Please specify _____

3.17 Which panel of chemotherapy regimen is prescribed?

CMF: cyclophosphamide, methotrexate, and 5-fluorouracil _____

FAC: 5-fluorouracil, doxorubicin, cyclophosphamide _____

TC: Taxotere (docetaxel) and cyclophosphamide _____

AC: Adriamycin (doxorubicin) and cyclophosphamide _____

Others specify…….

IV. Clinical and/or Lab parameters

4.1 CPA infusion: Start time_____End_____

4.2. Time of blood sample taking

T1 _____ T3_____

T2 _____ T4_____

4.3 Serum creatinine (SCr) _____

4.4. CrCl (ml/min) _____

4.5. WBC count _____

4.6. Absolute neutrophil count _____

4.7. Platelet count _____

4.8. Total bilirubin _____

2.9. Liver function test AST_____ ALT_____

Other tests (please include) _____

2.10. Kidney function test _____

Completed by: Approved by:

Name _____ Name:_____

Qualification _____ Qualification_____

Signature _____ Signature_____

## Annex II: Information Sheet (English version)

**Addis Ababa University**

**Faculty of Medicine**

**Department of Biochemistry**

**Subject information sheet**

**Title of the project:** Analysis of selected circulatory microRNAs in breast cancer before and after chemotherapy.

**Background of the study:** According to the technical report from Addis Ababa city cancer registry (AACCR) office, breast cancer is the second most common type of cancer after cervical carcinoma in our country. Its incidence is also shown to rank highest in the city in contrary to cervical carcinoma at national level. Unlike the experience in western world, previous studies on breast cancer in Addis Ababa shows late stage diagnosis (3/4) and having more aggressive breast cancer type at early age (premonoposal) for most of the patients; which leads to ineffective therapy.

Even if the diagnostic tools which are currently used in the country for diagnosis and prognosis of breast cancer are important and necessary; Complexity of sample taking procedures, lack of precision on repeated analysis, lack of early and specific diagnosis and need for specialized personal in the area of invasive diagnosis necessitates biomarkers that can be measured repeatedly and non-invasively. Such markers would need to be sensitive and specific enough to aid in the detection of breast cancer at an early stage, would monitor progression of the disease, and could predict the individual patient's response to treatment.

Circulatory microRNAs are good candidates as a biomarker, their exceptional stability formulates them to be easily detectable and quantifiable in the circulation.

**Objectives:**

**General objective:**

To investigate the use of circulatory microRNAs in breast cancer as non-invasive biomarker for diagnosis and assessment of prognosis

**Specific objectives:**

- Analysis of differential expression of specific circulatory miRNAs in breast cancer patients
- Establishing the relationship between specific miRNA expression with clinical pathology
- Assessing changes in circulatory microRNA during chemotherapy
- Evaluating the potential of CA 15-3 to monitor breast cancer patients during chemotherapy
- Evaluating associations between CA 15-3 values and deregulated microRNAs
- **Significance of the study:**

This study will present more effective diagnostic and prognostic biomarkers for early and non-invasive diagnosis of breast cancer. The possible identification of predictive circulatory miRNAs for chemotherapy effectiveness will disentangle the chemotherapy resistance and cytotoxicity which usually occurs with cancer treatment. The possible identification of molecular circulatory biomarkers will be supportive for classification of patients into additional taxonomic classes in implementing guided individualized treatment.

- In addition to providing the above clinical purpose, the study will add-up knowledge in genetic and epigenetic areas of breast cancer types which are common in the country. The results that will be obtained after the completion of

the study will be disseminated in the form of journal articles, public lectures, paper presentation, thesis write-up etc.

**Study site and period of the study:**

This study will be conducted in Addis Ababa University, school of health sciences, with the participation of different departments of the school and collaborating institutes. It will be carried out from December 2013-September 2015.

**Study procedure:**

This study will follow longitudinal case-control procedures. It will involve selecting participants on the bases of diseases status (case and controls), and will identify differentially expressed miRNA which might be candidate molecular diagnostic marker for breast cancer subtypes and predictor of response to therapy. 2mm biopsies will be taken from morphologically representative areas of formalin-fixed and paraffin-embedded tumor tissue. Blood sample will be obtained from pathologically confirmed breast cancer patients prior to chemotherapy, after 6 weeks of chemotherapy and after completion of therapy. In each sample collection term, 6ml of blood will be collected from study subjects by using evacuated collection tubes through a puncture of intermediate arm vein.

**Potential risk:**

Collection of blood from the study subjects through vein puncture might lead to mild pain.

**Annex III: Information Sheet (Amharic Version):**

**□መረጃ ቅ□**

**<u>የጥናቱ ርዕስ፡-በ□ም □ስ□ □ሚ□ኙ □ማ□□ሮ RNA ቅንጣቶች የጡት ካንሰርን አይነትና የህክምና ውጤትበማመላከት ላይ ያላቸውን አስተዋጽዎ መገምገም</u>**

**የጥናቱ መነሻ፡- ከአዲስ አበባ የካንሰር ምዝገባ ቢሮ በተገኘ□ መረጃ መሰረት የጡት ካንሰር ከማህጸን ካንሰር ቀጥሎበሃገሪቱ ውስጥ በብዛት የሚገኝ የካንሰር አይነት ነው። ካደጉት አገሮች ልምድ በተጻራሪ በሃገራችን ውስጥ የሚገኙ የጡት ካንሰር ታካሚዎች ወደ ህክምና የሚመጡት ዘግይተውና በሽታው ከተባባሰ በኋላ መሆኑ የህክምናውን ውጠ ,ታማነትእንደሚቀንሱትይታወቃል።በአሁኑ ጊዜ በአገራችን ውስጥ የሚገኙ የጡት ካንሰርመመርመርያመንገዶችናመሳርያዎችአስፈላጊና ጠቃሚ ቢሆንምየናሙናአወሳሰድመንገድ ውስብስብነት፣የተከታታይ ምርመራዎችየውጤት መለያየት፣ የአመላካቾቹ የትንበያአቅም ማነስናልዩነቶችንበግልጽ ያለማሰየት ችግር ይታያል። ይህም በቀላሉ መለካትየሚያስችሉናይበልጥ ጠቃሚ የሆኑ አመላካቾችን አስፈላጊነትያሳያል።ከዚህአንጻርበደም ውስጥ በተለያየ መጠን የሚገኙትንየማይክሮRNAቅንጣቶች ካላችው ባህርይ አንጻርየተሻሉ ጠቋሚዎችይሆናሉ ተብሎይገመታል።**

**<u>የጥናቱ ዓላማ</u>**

**<u>ዋና ዓላማ</u>፡- በደም ውስጥ የሚገኙ የማይክሮRNAቅንጣቶችን የጡት ካንሰር ዓይነቶችንበመለየትናየህክምና ውጤቶችንምበማመላከትረገድያላቸውንአስተዋጽዎ መገምገም**

**<u>የተመረጡ ዓላማዎች</u>**

- **በታካሚዎችመካከል የሚታዩ የደም ውስጥ የማይክሮ RNAቅንጣቶችንልዩነት ማየት**
- **የቅንጣቶቹንየህክምና ውጤት አመላካችነትመመርመር**
- **በተመረጡ የማይክሮRNAቅንጣቶችመኖርና በበሽታው መካከልያለውንግንኙነትመለየት**

**<u>ከጥናቱ የሚገኙ ጥቅሞች</u>**

- **በደም ውስጥ የሚገኙትቅንጣቶች የጡት ካንሰር ዓይነቶችንናየህክምናውን ውጠ ,ታማነትበማመላከትበኩልያላቸውንአስተዋጽዎ ያሳያል።በዚህም ታካሚዎችባለችው የጡት ካንሰር ዓይነትተለይተውየተሻለና ውጤታማ ህክምና የሚገኙበትንመንገድ ይጠቁማል። ከዚህምበተጨማሪ የጥናቱ ውጤት በዚህዙርያለሚደረጉ ምርምሮችመረጃ በመሆንያገለግላል።**

**<u>የጥናቱ ቦታናጊዜ</u>**

**ጥናቱ በአዲስአበባ ዩንቨርሲቲ ህክምና ትምህርት ቤትከተለያዩ የትምህርትክፍሎች ጋር በመተባበርከህዳር** 2006 **እስከመስከረም** 2008 **ይካሄዳል።**

**<u>የጥናቱ አካሄድ</u>**

**ጥናቱ የጡት ካንሰር ታካሚዎች ህክምናንበመከታተልና ከጡት ካንሰርነጻከሆኑ ሴቶች ጋር በማነጻጸር ይካሄዳል።ለዚህም ከታካሚዎችበቀዶ ህክምና ወቅትከሚወገደው የጡት አካል ላይ አነስተኛ** (2 **ሚሜ**) **ናሙና የሚወሰድሲሆን ታካሚዎችየ**chemotherapy**ህክምናከመጀመራቸው በፊት፣ ከጀመሩ ከስድስትሳምንታትበኋላና ሲጨርሱ የደም ናሙና በመውሰድይሰራል።**

**<u>የሚያስከትለውጉዳት</u>**

**ደምበመውሰድ ሂደትጊዜያዊና መጠነኛ ህመምሊሰማ ይችላል።**

## Annex IV: Informed consent form (English Version)

**Addis Ababa University**

**School of health sciences**

**Informed Consent Form**

**Code number** _____

**Name of study subject** _____

I have been informed about a study that plans to investigate the use of distinctive circulatory microRNAs as non-invasive biomarker in breast cancer patients. For this tissue sample and blood needs to be taken from me. The aims of the study and the possible risks, including mild pain were explained to me. I am also informed that all the information contained in the questionnaire is to be kept confidential. Moreover, I have also been well informed of my right to keep hold of information, decline to cooperate and make myself withdraw from the study.

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

I have read and understood the conditions stated above and I am willing to participate in the study

**No**_____ **Yes**_____

**Name of the participant** _____

**Signature** _____

**Name of researcher** _____

**Signature** _____

**Name of witness** _____

**Signature** _____

**Date** _____

**Contact Person:** **Yonas Mulugeta**

**Mob: 0911375676, e-mail: yonimulu@yahoo.com**

**Annex V: Consent Form (Amharic version)**

## የስምምነት ቅጽ

የሚስጥር ቁጥር_____

የተሳፊው ሙሉ ስም_____

እኔ ስሜ ከላይ የተጠቀሰው ውል ተቀባይ በጠት ካንሰር ምርመራ ላይ ሊደረግ ለታሰበው ጥናት መረጃ አግኝቻለሁ። ለዚህ ይረዳ ዘንድ ከእኔ ላይ የጡትና የደም ናሙና እንደሚፈለግ ተረድቻለሁ። ስለጥናቱ አላማ፣እንዲሁም ናሙና ሊወሰድ በእኔ ላይ መጠነኛ የህመም ስሜት ሊያስከትል እንደሚችል ከውል ሰጪው ገለጻ ተረድቻለሁ።

በተጨማሪም መጠይቁ ውስጥ በተካተቱት ጥያቄች መሰረት የምሰጣቸው መረጃች በጠቅላላ በሚስጥር እንደሚጠበቁ ተገልጾልኛል። እንዲሁም እኔን በተመለከተ የምጠየቀውን መረጃ ያለመስጠት፣ በጥናቱ ያለመተባበርና ከጥናቱ በማንኛውም ጊዜ ራሴን የማግለል መብቴ የተጠበቀ መሆኑ ተገልጾልኛል።

ስለዚህ ለውል ሰጪው መረጃ እና የስምምነት ቃሌን የሰጠሁት በአጠቃለይ ሁኔውን በመረዳትና በፍጹም ፈቃደኝነት ነው። ከእኔ የሚወሰደው ናሙና ለምርምር እንደሚውል ተረድቻለሁ። በተጨማሪም ጥያቄ ለመጠየቅ ተፈቅዶልኝ ለማወቅ የፈለኩትን ያህል ማብራሪያ አግኝቻለሁ።

የውል ተቀባይ ፊርማ _____ የውል ሰጪ ፊርማ_____

ቀን_____

ለተጨማሪ መረጃ: ዮናስሙሉጌታ

ሞባይል-0911375676, ኢሜል-yonimulu@yahoo.com

# Annex VI: Signed material transfer agreement

**Ministry of Science and Technology, Federal Democratic Republic of Ethiopia**
**Federal Health Research Ethics Review Committee**

Address: Tel. 251-011-4674353 P.O. Box 2490 Fax 251-011-4660241
E-mail most@ethionet.et Addis Ababa –Ethiopia

## Material Transfer Agreement

This Material Transfer Agreement (MTA) has been prepared for use by **Department of Biochemistry, Addis Ababa University ("Provider")** and The Arizona Board of Regents on behalf of the **University of Arizona ("Recipient")** in the transfer of Provider research material (samples, derivatives, and specimens) to the Recipient related to the protocol: **"Distinctive expression of circulatory microRNAs in breast cancer patients for prediction of chemotherapeutic response and differentiation of subtypes"**

**Provider scientist: Dr. Daniel Seifu, Department of Biochemistry**

**Recipient scientist: Dr. Rick Kittles, Associate director of cancer disparities, University of Arizona**

1. Provider agrees to transfer to Recipient's Dr. Rick Kittles the following research materials /specimen ("Material"):

   **Serum samples from 157 breast cancer patients and 60 controls.**

   The Material will only be used for research purposes as described in the protocol by Recipient's investigator in designated laboratory for the research project described below, under suitable containment conditions. This research material will not be used for commercial purposes such as screening, production or sale for which a commercialization license may be required. Recipient agrees to comply with all National and International guidelines rules and regulations applicable to the Research Project and the handling of the Material.

   a) Are the research materials of human origin?

      Yes ☑ No ☐

   b) If yes, are they collected according to the details in the protocol and in adherence to National Health Research Ethics Review Committee (NERC) and Addis Ababa University, faculty of Medicine Ethics Review Committee recommendations and their approval.

      Yes ☑ No ☐

2. This Material will be used by Recipient's investigator solely in connection with the following research project ("Research Project") described with specificity as follows **"Distinctive expression of circulatory microRNAs in breast cancer patients for prediction of chemotherapeutic response and differentiation of subtypes."**
3. In all presentations or written publications concerning the research project, recipient shall acknowledge Provider's contribution of the Material unless requested otherwise.
4. The Material represents a significant contribution on the part of Provider and is considered proprietary to Provider. Recipient therefore agrees to retain control over this research Material and further agrees not to transfer the Material to other people not under its direct supervision without advance written approval of

Provider. The Material will be disposed of as agreed upon per protocol at the end of completion of the project.

5. The Provider does not take any responsibility for loss, damage, wastage or spoilage of the Material during or after shipment to the address provided by the Recipient under conditions agreed to in the protocol on shipment of the samples. This Material is provided as a service to the research community. IT IS BEING SUPPLIED TO RECIPIENT WITH NO WARRANTIES, EXPRESS OR IMPLIED, INCLUDING ANY WARRANTY OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE. Provider makes no representations that the use of the Material will not infringe any patent or proprietary right of third parties.
6. The Recipient shall notify the Provider in witting of any improvement, modification discovery or development to the Material or the information made by Recipient or parties, collaborating with Recipient, here in after referred to as "invention". Nothing in this agreement shall, however, be construed as conveying to the Provider any rights under any patents or other intellectual property to such invention, other than as explicitly provided herein. At its option the Provider shall be entitled to receive sample of any materials derived from the Materials for its own research and evaluation purposes only.
7. The under signed Provider and Recipient expressly certify and affirm that the contents of any statements made herein are truthful and accurate.
8. Intentionally blank.
9. The Provider maintains, ownership right of the Material unless stated otherwise.
10. The Provider will retain a copy of every sample sent abroad as much as possible for local research needs.
11. The Recipient shall be bound by applicable state and federal rules governing Equal Employment Opportunity and Nondiscrimination and Immigration.
12. This MTA is subject to cancellation under Arizona Revised Statutes section 38-511 regarding conflict of interest on the part of individuals negotiating contracts on behalf of the State of Arizona.

The rest of this page is intentionally left blank.

**Material Transfer Agreement**
**Signature page**

For Recipient:
Duly Authorized

Stephen G. Harsy, PhD
Director
Contract & Research Support Program
The University of Arizona

Signature Stamp

Date 5/3/16

Mailing Address for Material:
**Research Programs & Global Advances**
**1305 N. Martin Ave.**
**Tucson, AZ 85721**
Tel: 520 626-3628
Fax

Mailing Address for Notices:
**Contracting & Research Support Programs (CRSP)**
**888 N. Euclid Ave., #515**
**Tucson, AZ 85719**
Tel: 520 626-3050
Fax:

Acknowledged by Recipient Principal Investigator:

Rick Kittles, M.D.

For Provider:
Provider's Investigator

Daniel Seifu (PhD)

Signature: ----------
Date 25/04/2016

Mailing Address
Department of Biochemistry
College of Health Sciences
Addis Ababa University
P.O. Box: 9086
Tel: +251911232754
Fax: _____

Duly Authorized

**Dean School of Medicine**

Signature: ----------
Date 25/04/2016

Mailing Address for Notices:
School of medicine
College of Health Sciences
Addis Ababa University
P.O. Box: 9086
Tel: +251911242550
Fax: _____