PhD Dissertation

Molecular Characterization and Assessment of Viral Tumorigenesis in Breast Cancer among Women in Addis Ababa, Ethiopia

By
Endale Hadgu

Department of Biochemistry
Faculty of Medicine

July 28, 2017
Addis Ababa, Ethiopia
A Thesis Submitted to the School of Graduate Studies of Addis Ababa University in fulfillment of the requirement of the degree of Doctor of Philosophy in Biochemistry

Advisors:

1. Daniel Seifu (PhD)
   Department of Biochemistry, Faculty of Medicine, Addis Ababa University

2. Wondemagegnhu Tigeneh (MD)
   Department of Oncology and Palliative Care, Faculty of Medicine, Addis Ababa University

3. Yonas Bekuretsion (MD)
   Department of Pathology, Faculty of Medicine, Addis Ababa University

4. Abebe Bekele (MD)
   Department of Surgery, Faculty of Medicine, Addis Ababa University

5. Markos Abebe (PhD)
   Armauer Hansen Research Institute (AHRI), Ethiopia

6. Sofia D. Merajver (MD,PhD)
   University of Michigan, USA

7. Mats G Karlsson (MD, PhD)
   Orebro University, Sweden

8. Christina Karlsson(PhD)
   Orebro University, Sweden
Acknowledgement

My foremost thanks are owed to my advisors (Dr. Daniel Seifu, Dr. Wondemagegnhu Tigeneh, Dr. Yonas Bokretsion, Dr. Abebe Bekele, Dr. Markos Abebe, Prof. Mats G. Karlsson, Dr. Christina Karlsson and Prof. Sofia D. Merajver) for their instrumental role in guiding my thesis project, and meticulous review at every stage. Without their valuable advice, guidance, encouragement and kind attention, the thesis would not have been accomplished.

I would also like to express my sincere gratitude to staffs at the cancer therapy center in Tikur Anbessa Specialized Teaching Hospital (TASH), Pathology Laboratory in TASH, Pathology Laboratory in St. Paul’s Hospital Millennium Medical College, Orebro University Hospital (Sweden) and University of Michigan (U.S). My study would have not been completed without your support.

I wish to extend my special appreciation to all the staffs of the department of Biochemistry, Faculty of Medicine, Addis Ababa University for your help and encouragement throughout the entire period of my training.

My sincere appreciation and gratitude also goes to St. Paul’s Hospital Millennium medical College for sponsoring and supporting my PhD study. Without your support, the thesis would not have been accomplished.

Financial support for this work was provided by School of Graduates Studies of Addis Ababa University, the thematic research “clinico-epidemiological characterization of breast cancer in Ethiopia”, Armauer Hansen Research Institute (AHRI), Swedish International Development Agency (SIDA), University of Michigan Center for International Reproductive Health Training (CIRHT) project, and the Merajver Breast Cancer Laboratory (U.S). Thank you, without your support, the thesis would not have been accomplished.

I would also like to thank my older brother Dr. Kassaye Hadgu for your help and encouragement throughout the entire period of my training.

Finally, I would like to express my upmost gratitude to my wife, Genet Abebe, for always encouraging me to challenge myself and trusting my judgment. I would like to thank my sons (Adoniyas, Nathan and Abysmak) for your sweet smile and understanding. I love you all very much!
## Table of Contents

Acknowledgement ........................................................................................................... i

Table of contents ........................................................................................................... ii

List of Tables ................................................................................................................... v

List of Figures ................................................................................................................ vii

List of Abbreviations ..................................................................................................... ix

Abstract ........................................................................................................................ xi

1. **Chapter 1 Introduction** .............................................................................................. 1
   1.1. General Introduction ............................................................................................... 1
   1.2. Review of Literature .............................................................................................. 4
       1.2.1. An overview of cancer ...................................................................................... 4
           1.2.1.1. Epidemiology .......................................................................................... 4
           1.2.1.2. Etiology and risk factors .......................................................................... 5
           1.2.1.3. Hallmarks of cancer ................................................................................ 5
       1.2.2. Breast cancer .................................................................................................. 7
           1.2.2.1. Introduction ............................................................................................. 7
           1.2.2.2. Epidemiology of breast cancer ............................................................... 8
           1.2.2.3. Risk factors of breast cancer ................................................................... 10
           1.2.2.4. Molecular biomarkers in breast cancer .................................................. 14
           1.2.2.5. Classification of Breast Cancer ............................................................... 20
           1.2.2.6. Estrogen signaling in breast cancer ......................................................... 26
           1.2.2.7. Role of androgen receptor in breast cancer .......................................... 30
           1.2.2.8. Role of Ki67 proliferation marker in breast cancer ............................... 37
           1.2.2.9. Role of BRCA mutation in breast cancer .............................................. 40
           1.2.2.10. Role of viruses in breast cancer ............................................................ 46
       1.3. Significance of the study ....................................................................................... 50
   1.4. Hypothesis .............................................................................................................. 51
   1.5. Objectives .............................................................................................................. 51
2. **Chapter 2 Materials and Methods**

2.1. Study design and period ............................................. 52
2.2. Study area .................................................................... 52
2.3. Study subjects and tumor specimen .......................... 53
2.4. Data collection ............................................................... 53
2.5. Statistical analysis ......................................................... 54
2.6. Ethical approval ............................................................. 54
2.7. Laboratory methods ......................................................... 54
2.7.1. Hematoxylin and eosin (H&E) stain ......................... 54
2.7.2. Construction of tissue microarray (TMA) and isolation of DNA ............... 56
   2.7.2.1. Tissue microarray ................................................. 56
   2.7.2.2. Tissue core samples for PCR ................................. 59
   2.7.2.3. DNA Extraction .................................................. 59
   2.7.2.4. Digital image acquisition ...................................... 59
2.7.3. Immunohistochemistry (IHC) ................................... 60
2.7.4. Fluorescence in situ hybridization (FISH) .................. 62
2.7.5. Multiplex real time PCR ............................................. 63
2.7.6. Gene protein assay (GPA) .......................................... 65
2.8. Immunohistochemical Scoring .................................... 68
2.9. Molecular sub-typing system ....................................... 69

3. **Chapter 3 Results** .......................................................... 70

3.1. Patient and tumor characteristics ................................. 70
3.2. Molecular classification ............................................... 72
3.3. Androgen receptor status ............................................ 80
3.4. BRCA1 expression ....................................................... 83
3.5. Ki67 expression ........................................................... 86
3.6. Comparison of IHC with GPA for assessment of HER2 status .......... 89
3.7. Assessment of association between EBV, HCMV and HPV with Breast Cancer .... 91

4. **Chapter 4 Discussion** ...................................................... 93

4.1. Discussion .................................................................... 93
4.2. Conclusion ................................................................... 103
4.3. Limitation of the study.......................................................................................105
4.4. Study strengths..................................................................................................105
4.5. Recommendations & future directions..............................................................105

8. References.............................................................................................................107
Annex I Questionnaire .............................................................................................119
Annex II Consent Form (English version) .................................................................122
Annex III Consent Form (Amharic version)...............................................................123
Annex IV Material Transfer Agreement (MTA).........................................................124
Annex V Information Sheet (English version)..........................................................127
Annex VI Information Sheet (Amharic version).........................................................130
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Tables</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1. The 10-, 20-, and 30-year risk of developing breast cancer in US</td>
<td>11</td>
</tr>
<tr>
<td>1.2. Types of invasive breast carcinomas according to WHO histopathological classification 4th edition 2012</td>
<td>22</td>
</tr>
<tr>
<td>1.3. Prognosis of the molecular breast cancer subtypes</td>
<td>24</td>
</tr>
<tr>
<td>1.4. The major molecular phenotypes according to histologic or/and immunohistochemical characteristics as defined by 3 Classification Systems</td>
<td>25</td>
</tr>
<tr>
<td>1.5. Generalized systemic therapy management of early breast cancer by intrinsic molecular subtype</td>
<td>26</td>
</tr>
<tr>
<td>2.1. Sources and dilutions of primary antibodies used in the study</td>
<td>61</td>
</tr>
<tr>
<td>3.1. Age at diagnosis of the study participants</td>
<td>70</td>
</tr>
<tr>
<td>3.2. Baseline pathological characteristics of the study participants</td>
<td>71</td>
</tr>
<tr>
<td>3.3. ER, PR and HER2 status of the study participants</td>
<td>72</td>
</tr>
<tr>
<td>3.4. Association between ER and clinicopathological parameters of the study participants</td>
<td>76</td>
</tr>
<tr>
<td>3.5. Distribution of the molecular subtypes of breast cancer among the study participants</td>
<td>77</td>
</tr>
<tr>
<td>3.6. Frequency distribution of the molecular subtypes in different age ranges among the study participants.</td>
<td>78</td>
</tr>
<tr>
<td>3.7. Distribution of the clinicopathological parameters in each molecular subtypes of breast cancer among the study participants</td>
<td>79</td>
</tr>
<tr>
<td>3.8. Association between AR expression and clinicopathological parameters among the study participants</td>
<td>81</td>
</tr>
<tr>
<td>3.9. Association between AR and molecular parameters among the study participants</td>
<td>82</td>
</tr>
<tr>
<td>3.10. Distribution of clinicopathological parameters in BRCA-1 expressing and non-expressing tumors among the study participants</td>
<td>84</td>
</tr>
</tbody>
</table>
3.11. Association between BRCA-1 and molecular parameters among the study participants

3.12. Distribution of clinicopathological parameters in slowly proliferative (Ki67 low) and highly proliferative (Ki67 high) tumors among the study participants

3.13. Association between Ki67 and molecular parameters among the study participants

3.14. Concordance between the results of IHC and GPA for assessment of HER2 status in breast tumors among the study participants

3.15. EBV, HCMV and HPV detection in human breast carcinomas among the study participants
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figures</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1 Estimated world cancer incidence proportions by major sites in both sexes combined, 2012</td>
<td>4</td>
</tr>
<tr>
<td>1.2. The hallmarks of cancer</td>
<td>6</td>
</tr>
<tr>
<td>1.3. Anatomy of a normal breast</td>
<td>7</td>
</tr>
<tr>
<td>1.5. WHO cancer incidences among women in Ethiopia: GLOBOCAN Female cancer -2012</td>
<td>9</td>
</tr>
<tr>
<td>1.6. Top 10 Female cancers in Addis Ababa</td>
<td>10</td>
</tr>
<tr>
<td>1.7. cDNA based molecular classification of breast cancer</td>
<td>23</td>
</tr>
<tr>
<td>1.8. Mechanism of Estrogen action at the molecular level</td>
<td>29</td>
</tr>
<tr>
<td>1.9. Androgen Receptor (AR) signaling pathway in breast cancer and cross-talk with other signaling pathway</td>
<td>31</td>
</tr>
<tr>
<td>1.10. Associations between Estrogen Receptor (ER) and Androgen Receptor (AR) in Breast Cancer</td>
<td>33</td>
</tr>
<tr>
<td>1.11. Androgen Biosynthesis and CYP17A Inhibitors</td>
<td>36</td>
</tr>
<tr>
<td>1.12. Genes involved in hereditary predisposition to Breast Cancer</td>
<td>41</td>
</tr>
<tr>
<td>1.13. Role of BRCA 1 and BRCA 2 with other key effectors of the DNA damage response and DNA repair</td>
<td>43</td>
</tr>
<tr>
<td>2.1. Representative image of H&amp;E stained TMA cores of a specific area of a slide</td>
<td>55</td>
</tr>
<tr>
<td>2.2. Representative image of H&amp;E stained TMA of a single core</td>
<td>56</td>
</tr>
<tr>
<td>2.3. A simplified workflow of TMA preparation</td>
<td>57</td>
</tr>
<tr>
<td>2.4. Representative image of H&amp;E stained whole section slide</td>
<td>57</td>
</tr>
<tr>
<td>2.5. Representative image of TMA block</td>
<td>58</td>
</tr>
<tr>
<td>2.6. Representation of IHC reaction</td>
<td>60</td>
</tr>
</tbody>
</table>
2.7. Principles of TOCE assay design and signal generation during real-time PCR  
64
2.8. Principle of GPA for simultaneous visualization of human epidermal growth factor receptor 2 (HER2) protein, the HER2 gene, and the chromosome 17 centromere (CEN17)  
66
3.1. Representative images of ER positive immunohistochemistry in breast tumor tissue array  
71
3.2. Representative images of PR positive immunohistochemistry in breast tumor tissue array  
74
3.3. Representative images of HER2 positive immunohistochemistry in breast tumor tissue array  
75
3.4. Scoring of HER2 staining  
76
3.5 Distribution of the molecular types of breast cancer in different age ranges  
78
3.6 Representative images of AR positive immunohistochemistry in breast tumor tissue array  
80
3.7. Representative images of BRCA1 immunostaining  
83
3.8. Representative images of Ki67 immunostaining  
86
3.9. Representative images of HER2 GPA  
90
3.10. p16 Immunostaining of HPV+ breast tumors  
92
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKT</td>
<td>Ak transforming Protein Kinase B</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen Receptor</td>
</tr>
<tr>
<td>BL</td>
<td>Basal-like</td>
</tr>
<tr>
<td>BLBC</td>
<td>Basal-like breast cancer</td>
</tr>
<tr>
<td>BRCA</td>
<td>Breast Cancer Antigen</td>
</tr>
<tr>
<td>CK</td>
<td>Cytokeratins</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr Virus</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen Receptor</td>
</tr>
<tr>
<td>ERBB2</td>
<td>Erythroblastic leukemia viral oncogene homolog 2</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinases</td>
</tr>
<tr>
<td>GE</td>
<td>Genetic Expression</td>
</tr>
<tr>
<td>HCMV</td>
<td>Human Cytomegalo Virus</td>
</tr>
<tr>
<td>HER2</td>
<td>Human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>HPV</td>
<td>Human Papilloma Virus</td>
</tr>
<tr>
<td>HR</td>
<td>Homologous recombination</td>
</tr>
<tr>
<td>IGFR</td>
<td>Insulin-Like Growth Factor Receptor</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IM</td>
<td>Immunomodulatory</td>
</tr>
<tr>
<td>Ki67</td>
<td>Kiel 67</td>
</tr>
<tr>
<td>LAR</td>
<td>Luminal androgen receptor</td>
</tr>
<tr>
<td>M</td>
<td>Mesenchymal</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MSL</td>
<td>Mesenchymal stem-like</td>
</tr>
<tr>
<td>NHEJ</td>
<td>Non-homologous end joining</td>
</tr>
<tr>
<td>OS</td>
<td>Overall survival</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly ADP-Ribose Polymerase</td>
</tr>
</tbody>
</table>

ix
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PFS</td>
<td>Progression-free survival</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative PCR</td>
</tr>
<tr>
<td>RR</td>
<td>Relative Risk</td>
</tr>
<tr>
<td>TNBC</td>
<td>Triple-negative breast cancer</td>
</tr>
<tr>
<td>TNBC</td>
<td>Triple Negative Breast Cancer</td>
</tr>
<tr>
<td>TNM</td>
<td>Tumor size, Number of positive lymph nodes, Metastasis</td>
</tr>
<tr>
<td>TOCE</td>
<td>Tagging Oligonucleotide Cleavage and Extension</td>
</tr>
<tr>
<td>UNS</td>
<td>Unstable</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
</tbody>
</table>
Abstract

In this study from Tikur Anbessa Specialized Hospital, 114 breast cancer patients diagnosed between 2012 and 2015 were enrolled. Estroge receptor (ER), Progesterone Receptor (PR), Ki-67 and Human Epidermal Growth factor (HER-2) receptor status were assessed using immunohistochemistry (IHC) from tissue microarrays (TMA). Fluorescence in situ hybridization (FISH) and Gene Protein Assay (GPA) was used for assessment of gene amplification in all equivocal tumor samples and for confirmation in HER2-enriched cases. Androgen Receptor (AR) was assessed using IHC from TMA and BRCA1 was assessed using IHC from whole section. EBV, HCMV and HPV viral proteins or/and DNA were assessed using IHC or/and multiplex qPCR. The 2013 St. Gallen international panel of expert’s recommendation for classification of breast carcinoma based on IHC was applied to molecularly classify the tumors. Information obtained also included age, tumor grade, histological type, and stage of disease. In this study, the most common molecular subtypes was Luminal A (40%) followed by Luminal B (26%), TNBC (23%) and HER2-enriched (10%). ER were positive in 65% of all tumors and 43% the participants were positive for PR. There was statistically significant variation in median age at diagnosis between the different molecular subtypes (P<0.05). There was a bimodal distribution of molecular subtypes in different age ranges at diagnosis with Luminal B subtype being more common at younger ages (median=36) and Luminal A subtype being more prevalent at older ages (median=42). There were no statistically significant differences in tumor grade, histology, and stage between the molecular subtypes of breast cancer. AR was expressed in 80% of breast cancers, which is higher than the expression rates of both ER and PR. There was a statistically significant variation (P<0.05) in the proportion of AR positivity between ER-positive (93%) and ER-negative tumors (60%). There was a statistically significant variation (P<0.05) in the proportion of AR positive cases between PR-positive (98%) and PR-negative (70%) tumors as well. There was no statistically significant variation in the expression of AR between HER2-positive and HER2-negative tumors in this study (P=0.145). AR expression among TNBC was 48% which is significantly different (P<0.05) than the other molecular subtypes. No statistically significant correlation was found between the clinicopathological parameters and AR expression.
There were loss/decreased BRCA-1 protein expression in 29% of all cases. No association was found between altered nuclear BRCA1 expression and ER expression. BRCA-1 expression was not associated with AR, HER2 and Ki67. There was no statistically significant difference in BRCA-1 expression among the molecular subtypes of breast cancer in this study. No association was found with age at diagnosis, tumor grade, clinical stage and histological type of tumor.

Out of 113 participants 44 cases (39 %) had high Ki67 protein expression. High Ki67 was found to be significantly associated with median age (P<0.05). The median age at diagnosis for participants with high proliferation were 37 years compared to 42 years for participants with low proliferation. High tumor grade were associated high proliferation (P<0.05). We did find some association of Ki67 with ER; however, the association did not reach the level of statistical significance (P=0.074). There was also statistically significant variation (P<0.05) between the molecular phenotypes; Luminal B group had the highest proportion (83%) with high proliferation, followed by the TNBC (58%), then the HER2 enriched group (36%). All Luminal A subtypes had low Ki67.

The concordance rate between the results of IHC and GPA for HER2 in all cases was 97.2%. The concordance rate of IHC and GPA was high in cases that were 3+ according to IHC (100%), but slightly lower in cases that were 2+ according to IHC (91.7%).

There was no detection of HCMV and EBV in this study. 2 out of 82 (2.4%) samples were positive for HPVs. Out of the two cases positive for HPV one case had the high risk HPV16 genotype while the other were dual positive for the high risk HPV39 and low risk HPV6. Together, these studies make an important contribution to understanding the molecular characteristics of breast cancer and association of oncogenic viruses with breast cancer.

**Key words:** Breast cancer, Molecular subtypes, AR, BRCA1, Ki67, oncogenic viruses, Ethiopia, Africa
Chapter 1

Introduction

1.1. General Introduction

Breast cancer is the most common cancer in women worldwide, with nearly 1.7 million new cases diagnosed in 2012 (second most common cancer overall). This represents about 12% of all new cancer cases and 25% of all cancers in women (International Agency for Research on Cancer (IARC) and World Health Organization (WHO), 2012). It is estimated that worldwide over 522,000 women died in 2012 due to breast cancer (GLOBOCAN, 2012). Although breast cancer is thought to be a disease of the developed world, almost 50% of breast cancer cases and nearly 60% of deaths occur in lower income countries (GLOBOCAN, 2012).

There is a large variation in breast cancer survival rates around the world, with an estimated 5-year survival of 80% in high income countries to below 40% for low income countries (WHO, 2017). Low and middle income countries face resource and infrastructure constraints that challenge the goal of improving breast cancer outcomes by early detection, diagnosis and treatment (WHO, 2017).

The prevalence of breast cancer in Ethiopia is not well known due to absence of population based cancer registry. However, the study by the Addis Ababa City Cancer registry in 2014 has placed the incidence of breast cancer at number one with 34% of all cancers diagnosed in that specific year among females (African Cancer Registry Network, 2014). Breast cancer is now an emerging public health problem in most urban centers including Addis Ababa, the administrative and political center of the country with over 2.7 million residents according to the 2007 population census (Central Statistical Agency of Ethiopia, 2007).

Breast cancer is a heterogeneous disease with different subtypes that are based upon the expression level of estrogen receptor (ER), progesterone receptor (PR), and Human Epidermal Growth Factor (HER2) receptor (Bertelli et al., 2013). Different types of breast cancer vary substantially in their behavior and response to treatment.
Very few studies have been done to characterize breast cancer in Ethiopia and other African countries. Large number of studies of breast cancer in African women reported a frequency of 40–55% of triple negative breast cancer (TNBC) (Brinton et al., 2014a) which is more aggressive and has a poorer prognosis. Emerging new evidences, however, show TNBC to be low in East Africa including Ethiopia (Brinton et al., 2014a, Jiaoge et al., 2016, Kantelhardt et al., 2014, Sayed et al., 2014).

In breast cancer, a number of known and unknown mechanisms may play critical roles in carcinogenesis, progression and metastasis, which may be related to breast cancer outcomes. Some of the well known predictive or prognostic factors in breast cancer include ER and HER2 (Bertelli et al., 2013). However, it is important to identify and validate new biomarkers for better prediction and prognostication. The role of Androgen receptor (AR) in breast cancer is still uncertain but there are an increasing number of evidences supporting a role for AR in the pathogenesis and outcome of breast cancer. AR has recently emerged as a useful marker for the further refinement of breast cancer subtype classification and emerging clinical target (Pietri et al., 2016).

Approximately 5–10% of all breast cancers occur due to the inheritance of deleterious mutations in rare predisposition genes, such as BRCA1 and BRCA2 (Dutil et al., 2012, Lecarpentier et al., 2012). Many studies have shown that, the risk of cancer in a BRCA mutation carrier is significant, and knowledge of mutation status in individuals at potentially increased risk of a BRCA mutation may impact healthcare decisions to reduce risk (Kobayashi et al., 2013). Moreover, oncogenetic testing is becoming the powerful therapeutic predictive tool, as new targeted therapeutic opportunities, such as poly(ADP ribose) (PARP) inhibitors emerge and chemosensitivity to platinum-based therapy is constantly reported (Kobayashi et al., 2013). BRCA genetic analysis which is the first choice to detect mutation is cumbersome, expensive, and may be impractical as a screening method for detection in all patients. Moreover, patients with BRCA promoter methylation and other mechanisms of loss are not identified using current approaches. Immunohistochemistry for BRCA1 may be a useful initial screening test to select patients for targeted therapy and to detect patients at risk for hereditary breast and ovarian cancer syndromes (Garg et al., 2013, Roehe et al., 2012).
The absence of Ki67 protein in resting cells and its expression in all proliferating cells, whether normal or tumor cells, makes the Ki67 antibody a great tool for determining the growth fraction of any given human cell population. Ki67 is associated with the common histopathologic parameters of breast cancer, and a strong correlation exists between Ki67 expression and histological grading because both parameters are associated with proliferation.

Many studies have shown that Ki67 is an important predictive and prognostic marker of survival and tumor recurrence in breast cancer, and it may reflect the aggressiveness of this disease. High Ki67 is also associated with poor disease-free survival and overall survival (Inwald et al., 2013, Juriková et al., 2016).

Risk factors for developing breast cancer include age, genetic, environment and life style factors. Researches into breast cancer etiology have focused primarily on reproductive and other factors affecting circulating sex hormones and on genetic susceptibility. Two of the identified genetic risk factors are BRCA1 and BRCA2 mutation (Dutil et al., 2012, Lecarpentier et al., 2012). However, as identified risk factors are thought to explain the incidence in about half of all breast cancer cases only, researchers are motivated to consider other routes for disease pathogenesis. Viruses have been implicated in the development of various cancers, but they have not been much considered for breast cancer. Recent investigations have linked breast cancer to viral infections, such as Epstein–Barr virus (EBV), mouse mammary tumor virus (MMTV), Human papillomavirus (HPV) and Human cytomegalovirus (HCMV) (Akhter et al., 2014, Alibek et al., 2013). The relationship between breast cancer and viruses could be of potential importance not only for better understanding of breast cancer etiology, but also for early detection and prevention of breast cancer and treatment (Alibek et al., 2013).
1.2. Review of Literature

1.2.1. An Overview of Cancer

1.2.1.1. Cancer Epidemiology

Cancer is a chronic disease with uncontrolled growth of cells, which can invade and spread to distant sites of the body. According to the WHO world cancer report 2014, cancer is a leading cause of death worldwide (Stewart and Wild, 2014). Cancer caused over 8 million deaths worldwide in 2013 alone. This report indicates the incidence of different types of cancer in men and women are variable [Figure 1.1](Stewart and Wild, 2014). Lung, prostate, colorectal, stomach, and liver cancer are the most common types of cancer in men (Stewart and Wild, 2014). According to same report, the most common type of cancer in women are breast, colorectal, lung, uterine cervix, and stomach cancer.

![Figure 1.1 Estimated world cancer incidence proportions by major sites in both sexes combined, 2012. The figure is adapted from(Stewart and Wild, 2014)]
Although cancer is often considered to be more of a developed world issue, developing countries bear a disproportionate high burden of cancer. According to the GLOBOCAN 2012 report, 57% (8 million) of new cancer cases, 65% (5.3 million) of the cancer deaths occurred in the less developed regions (Stewart and Wild, 2014, Ferlay, 2015). Sub-Saharan Africa (SSA) is also experiencing a rapid increase in the incidence of cancer and cancer is emerging as a major public health problem because of population aging and growth, as well as increased prevalence of key risk factors, including those associated with social and economic transition (Jemal et al., 2012).

1.2.1.2. Etiology and risk factors

Cancer is the result of a number of genetic alterations (mutation) occurring in a cell. These changes alter the balance between proliferation and programmed cell death (apoptosis) mechanisms and transform the cell (Vogelstein et al., 2013). Usually, there is a long latent period from the moment of carcinogenic exposure to the neoplastic transformation in which the somatic cell is allowed to proliferate while accumulating multiple genetic mutations that might lead to a disease in an individual (Lichtenstein, 2010). Multiple factors have been associated with the oncogenic process such as environment, lifestyle, host factors, infectious agents and inheritance (Blackadar, 2016). The comparative contribution of lifestyle and environmental factors to carcinogenesis is variable in different cancers (Parkin et al., 2011). Tobacco smoking is overwhelmingly the most significant risk factor for many cancers. Diet, exercise, and alcohol use are also significant contributors to most cancer. Factors such as occupational exposures to asbestos, coal, and other substances; indoor smoke from cooking and heating; and air pollution, can also contribute to cancer. Beyond these factors, there is the role of infections in common cancers (Blackadar, 2016, Parkin et al., 2011, Swanson, 1988).

1.2.1.3. Hallmarks of Cancer

Douglas Hanahan and Robert Weinberg proposed that when cells progress towards a neoplastic state, they acquire distinctive capabilities that govern the transformation of normal cells to cancer (malignant or tumor) cells (Hanahan and Weinberg, 2000, Hanahan and Weinberg, 2011).
These were termed hallmarks of cancer and formed a useful framework in which to understand tumor pathogenesis. Weinberg and Hanahan first proposed six hallmarks in 2000 and updated their list by proposing four more new hallmarks of cancer in 2011, bringing up the list to ten hallmarks [Figure 1.2](Hanahan and Weinberg, 2000, Hanahan and Weinberg, 2011). The ten hallmarks of cancer include sustaining proliferative signaling, evading growth suppressors, avoiding immune destruction, enabling replicative immortality, resisting cell death, tumor-promoting inflammation, inducing angiogenesis, activating invasion and metastasis, genome instability and mutation and deregulating cellular energetics (Hanahan and Weinberg, 2000, Hanahan and Weinberg, 2011).

Weinberg and Hanahan also described that virtually all cancers must acquire the same ten hallmark capabilities, but their means of doing so will vary significantly, both mechanistically and chronologically (Hanahan and Weinberg, 2000, Hanahan and Weinberg, 2011). Thus, the order in which these capabilities are acquired seems likely to be quite variable across the spectrum of cancer types and subtypes. Moreover, in some tumors, a particular genetic lesion may confer several capabilities simultaneously, decreasing the number of distinct mutational steps required to complete tumorigenesis (Hanahan and Weinberg, 2000, Hanahan and Weinberg, 2011).

Figure 1.2 The Hallmarks of Cancer. The figure is adapted from (Hanahan and Weinberg, 2011)
1.2.2. Breast Cancer

1.2.2.1. Introduction

Breast cancer is a malignant tumor that starts in the cells of the breast. A malignant tumor is a group of cancer cells that can grow into surrounding tissues or spread (metastasize) to distant areas of the body. The disease occurs almost entirely in women, but men can get it, too (American Cancer Society, 2016). The normal female breast is made up mainly of lobules (milk-producing glands), ducts (tiny tubes that carry the milk from the lobules to the nipple), and stroma (fatty tissue and connective tissue surrounding the ducts and lobules, blood vessels, and lymphatic vessels) [Figure 1.3]. Most breast cancers begin in the cells that line the ducts (ductal cancers). Some begin in the cells that line the lobules (lobular cancers), while a small number start in other tissues (American Cancer Society, 2016).

![Figure 1.3 Anatomy of a Normal Breast.](image)
The figure is adapted from (American Cancer Society, 2016)
1.2.2.2. Epidemiology of Breast Cancer

Breast cancer is now the most frequently diagnosed cancer and the leading global cause of cancer death in women, accounting for 23% of cancer diagnoses (1.38 million women) and 14% of cancer deaths (458,000 women) each year (Jemal et al., 2011). According to the 2012 world health organization GLOBOCAN report, breast cancer is the leading cause of morbidity and mortality in women (Ferlay J, 2013).

Although breast cancer has a markedly higher incidence in developed countries, half of new breast cancer diagnoses and an estimated 60% of breast cancer deaths are now thought to occur in the developing world (Jemal et al., 2011). Reports are showing a continues reduction in the incidence of breast cancer in the developed countries while the incidence in developing countries is steadily increasing [Figure 1.4] (Fitzmaurice et al., 2015).

![Figure 1.4 Trends in Age-Standardized Incidence Rates for Female Breast Cancer, 1990-2013. The figure is adapted from (Fitzmaurice et al., 2015)]
According to demographic projections, Africa will face an unprecedented growth of cancer burden in the next decades. There is evidence to suggest an emerging epidemic of breast cancer in Sub-Saharan Africa (Akarolo-Anthony et al., 2010, Lingwood et al., 2008). Although the incidence is lower than in developed countries, a significant increase in breast cancer is expected in African countries as a result of urbanization, with changing patterns of risks factors and increasing life expectancy (Akarolo-Anthony et al., 2010, Lingwood et al., 2008). According to the WHO 2012 GLOBOCAN report, breast cancer is the leading cause of female cancer in Ethiopia [Figure 1.5](Ferlay, J., 2013).

Compared to developed countries, few studies have been conducted on the patient and biological characteristics of breast cancer in Sub-Saharan Africa. The few published epidemiologic studies have contradicting results. Some authors suggested that breast cancer in sub Saharan Africa exhibits specific features compared to the USA and Europe such as younger age and more aggressive features with high-grade tumors and triple-negative phenotypes [ER−/PR−/HER2−](Brinton et al., 2014a). Studies from east Africa, however, reported hormone receptor negative breast cancer to be low (Sayed et al., 2014, Jigge et al., 2016, Kantelhardt et al., 2014).

![Figure 1.5](image-url)

**Figure 1.5** WHO Cancer incidences among women in Ethiopia: GLOBOCAN Female cancer -2012. The figure is adapted from (Ferlay, J., 2013).
According to data from the Cancer Registry of Addis Ababa City (Addis Ababa City Cancer Registry), breast carcinoma was the most frequent malignant neoplasm in 2014 accounting for 33% of all cancer cases followed by cervical cancer 17% [Figure 1.6](African Cancer Registry Network, 2014).

**Figure 1.6 Top 10 Female Cancers in Addis Ababa.**

The figure is adapted from(African Cancer Registry Network, 2014).

### 1.2.2.3. Risk factors of Breast Cancer

Studies indicate known risk factors for the development of breast cancer account for only about 40% of all cases. The known risk factors are multifactorial and there is a considerable interaction between these factors. Age, reproductive factors, personal or family history of breast disease, genetic predisposition, and environmental factors have all been associated with an increased risk of developing female breast cancer.
i. Age

The risk of developing breast cancer increases with age. The probability of a woman in the United States developing breast cancer in a lifetime is 1 in 8: 1 in 202 from birth to age 39 years of age, 1 in 26 from 40-59 years, and 1 in 28 from 60-69 years (Siegel et al., 2013). In the UK in 2012-2014, on average each year almost half (48%) of cases were diagnosed in people aged 65 and over (Cancer Research UK, 2014). Table 1.1 shows the increase in 10-, 20-, and 30-year risk of developing breast cancer from different current ages in U.S (Howlader N, 2016).

Table 1.1 The 10-, 20-, and 30-year risk of developing breast cancer in U.S

<table>
<thead>
<tr>
<th>Current age (years)</th>
<th>10 years (%)</th>
<th>20 years (%)</th>
<th>30 years (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>0.44</td>
<td>1.87</td>
<td>4.05</td>
</tr>
<tr>
<td>40</td>
<td>1.44</td>
<td>3.65</td>
<td>6.80</td>
</tr>
<tr>
<td>50</td>
<td>2.28</td>
<td>5.53</td>
<td>8.75</td>
</tr>
<tr>
<td>60</td>
<td>3.46</td>
<td>6.89</td>
<td>8.89</td>
</tr>
<tr>
<td>70</td>
<td>3.89</td>
<td>6.16</td>
<td>N/A</td>
</tr>
</tbody>
</table>

ii. Genetic and Familial Factors

A family history of breast cancer in a first-degree relative is the most widely recognized breast cancer risk factor, but only 5-10% of women diagnosed with breast cancer have a known genetic predisposition. Women with a family history of breast cancer in a mother or sister have a 1.5-3 fold increase in the risk of developing breast cancer. Even in the absence of a known genetic risk factor, the presence of a family history may suggest the presence of an unknown genetic risk, or a shared environmental risk. A family history of ovarian cancer in a first-degree relative, especially if the disease occurred at an early age (<50 y), has been associated with an increased risk of breast cancer risk (Salehi et al., 2008, Center for Disease Control and Prevention (CDC), 2016, Shah et al., 2014).
Although 20-30% of women with breast cancer have at least one relative with a history of breast cancer, only 5-10% of women with breast cancer have an identifiable hereditary predisposition. \textit{BRCA1} and \textit{BRCA2} mutations are responsible for 3-8% of all cases of breast cancer and 15-20% of familial cases. Rare mutations include \textit{PTEN} and \textit{TP53} (\textit{Center for Disease Control and Prevention (CDC), 2016}).

iii. Hormonal and Reproductive Factors

Reproductive Factors

Studies support an etiologic role for estrogens in breast cancer. Several identified risk factors for breast cancer increase lifetime exposure to estrogens (\textit{Center for Disease Control and Prevention (CDC), 2016, Salehi et al., 2008, Shah et al., 2014}). These factors include early age of menarche, late onset of menopause, no full-term births, late age at first pregnancy, long-term oral contraceptive (OC) or hormone therapy use, and postmenopausal obesity (which favors conversion of androgen to estrogens in adipose tissue). Conversely, late menarche, anovulation, and early menopause (spontaneous or induced) are protective, owing to their effect on lowering endogenous estrogen levels or shortening the duration of estrogenic exposure (\textit{Center for Disease Control and Prevention (CDC), 2016, Salehi et al., 2008, Shah et al., 2014}).

Hormone Replacement Therapy

Hormone replacement therapy (HRT) is used to increase levels of estrogens around menopause when women naturally have lower endogenous estrogen content. HRT enhances risk of breast cancer among women who use it for at least 5 yr, with the risk rising by about 2.3% per year of use (\textit{Collaborative Group on Hormonal Factors in Breast, 1997}). Breast cancer risk varies by type of HRT, and was reported to be considerably higher among those using estrogen–progestin combinations compared to estrogen alone (\textit{Salehi et al., 2008}).
**Oral Contraceptive (OC) Hormones**

Compared to never-users of OC, breast cancer risk is 24% higher among current users, and 16% higher among women who ceased use within the past 10 yr. However, breast cancer risk returns to normal 10 yr or more after cessation of OC use (Salehi et al., 2008).

**Height**

A pooled analysis of seven cohort studies showed positive associations between height and breast cancer risk among postmenopausal women (Salehi et al., 2008). The association observed may be related to energy intake and nutritional status in childhood and adolescence and hormone profile during puberty (Salehi et al., 2008).

**iv. Lifestyle Factors**

**Alcohol consumption**

A study by Chen et al found that low levels of alcohol consumption were associated with a small increase in breast cancer risk; cumulative alcohol intake throughout adult life was the most consistent measure (Chen et al., 2011). Alcohol intake occurred early and late in adult life was independently associated with risk (Chen et al., 2011). The mechanism, though unclear, likely is mediated via increasing estrogen levels.

**Physical activity**

Consistent physical activity has been shown to reduce the risk of breast cancer in a dose dependent manner, with modest activity conferring a 2% decrease in risk and vigorous activity a 5% decrease in risk (Shah et al., 2014).

**Tobacco smoking**

Tobacco abuse portends a 24% higher risk of developing invasive breast cancer. Former smokers carry a 13% increased risk. Starting smoking at an earlier age has a profound impact. Compared with never smoking, beginning tobacco use prior to menarche increases breast cancer risk by 61%, and beginning tobacco use 11 or more years prior to parity carries a 45% increased risk (Gaudet et al., 2013).
**Obesity**

Obesity, specifically in postmenopausal women, has also been shown to increase a woman’s risk of breast cancer. Postmenopausal women who did not use HRT had elevated breast cancer risk with increasing weight, body mass index (BMI) and hip circumference. Studies show there is a relative risk of 1.28 for overweight women (BMI 25.0-29.9) and obese women (BMI > 30.0) compared to women in the normal weight range (Shah et al., 2014).

**Irradiation**

Radiation, particularly to the chest or in the first decade of life, profoundly increases the risk of developing breast cancer. Radiation to the chest wall for treatment of childhood cancer increases the risk of breast cancer linearly with chest radiation dose. Survivors of childhood cancers who received therapeutic radiation are at a dose dependent risk for the development of breast cancer, and those treated for Hodgkin’s disease are at highest risk (RR = 7) (Shah et al., 2014).

**1.2.2.4. Molecular Biomarkers in Breast Cancer**

The mortality rate of breast cancer is tremendously reduced in recent years while the incidence is still increasing. The reason for reduced mortality is mainly because of earlier detection through mammographic screening programs and optimization of adjuvant therapy, through improved patient selection and the discovery of new therapeutic targets (Berry et al., 2005).

The selection of patients with breast carcinoma for adjuvant treatment is based on risk stratification according to both traditional and new prognostic biomarkers. Traditional prognostic biomarkers include the parameters defining the TNM tumor stage (tumor size, number of positive lymph nodes and presence of metastatic disease), tumor grade, histologic type and expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) (Farr et al., 2013). Recent progress in molecular research introduced additional biomarkers used for diagnostic, prognostic and predictive usage. Here, the molecular biomarkers are reviewed by dividing them into two separate groups as established and emerging.
i. Established Molecular Biomarkers

**Estrogen Receptor Alpha (ER-α)**

The estrogen receptors comprise of two isoforms, encoded by different genes. The ER-α is a high affinity estrogen receptor that is responsible for the estrogen-related cellular effects in breast cancer while ER-β, which is expressed in more tissues and for which the data are limited, appears to exert an antiproliferative function in the breast. I will be using ER to denote ER-α from now onwards in this paper.

ER expression is identified in approximately 75-80% of breast cancers (Kos and Dabbs, 2016). ER expression is the major predictive marker for response to endocrine therapies which significantly have altered the natural history of breast cancer. Adjuvant endocrine therapy is the standard of care for all women diagnosed with hormone-positive breast cancer as it has been shown to reduce breast cancer–specific mortality and the risk of recurrence and of contralateral breast cancer and to increase overall survival (OS) (Burstein et al., 2014).

In ER-positive tumors, primary or acquired resistance may occur and is more often the result of ER loss, although non-genomic mechanisms such as activation of membrane bound, receptor tyrosine kinase (RTK) signaling pathways, including the AKT and MAPK kinase pathways are also involved (Giuliano et al., 2011).

**Progesterone Receptor**

Progesterone Receptor (PR) expression is regulated by the genomic transcriptional activity of ER; thus, PR positivity is usually observed in ER-positive tumors. In ER-positive tumors the status of PR expression appears of lesser importance; however, in ER-positive/PR-negative tumors there is a 28% higher relative risk for recurrence compared to the ER-positive/PR-positive tumors. Thus, PR appears to have an intrinsic positive effect on prognosis (Prat et al., 2013).

The ER-negative and PR-positive breast carcinomas are rare; they comprise approximately 1-3% of the tumors and are regarded as being either falsely negative for ER or falsely positive for PR and the recommendation is to repeat the ER examination on another tissue sample to exclude a false-negative ER result that would preclude the patient from the endocrine treatment
benefit (Patani et al., 2013). Early reports have described a modest benefit from tamoxifen or aromatase inhibitors in ER-negative/PR-positive tumors; recent studies, however, failed to confirm this (Patani et al., 2013, Davies et al., 2011). Despite the questions on the predictive role of PR, its modest prognostic effect still justifies its evaluation.

**Human Epidermal Growth Factor Receptor 2**

Human Epidermal Growth Factor Receptor 2 (HER2), also known as ERBB2 and HER2/neu, is a transmembrane receptor with tyrosine kinase function (RTK) and a member of the HER family also including HER1 (also known as EGFR), HER3 and HER4. HER2 is an orphan receptor. Ligand binding on the extracellular portion of the other family member receptors, results in formation of homodimers or heterodimers among the four members of the family, transphosphorylation of the intracellular domains of the receptors, enhancement of the kinase activity, recruitment of signaling effector proteins and activation of intracellular signaling pathways. The type of ligand, the heterodimers formed and the autophosphorylation pattern, all influence the signaling effector to be recruited, and the intracellular pathway activated. HER2 has the most potent kinase activity, activates the PI3K/Akt and MAPK pathways, and induces cell proliferation and survival, while disrupting epithelial cell organization, polarity and adhesion, thereby facilitating the formation of metastases (Rexer and Arteaga, 2012, Freudenberg et al., 2009).

In breast cancer, HER2 protein overexpression is due to amplification of the *HER2* gene, located on chromosome 17, is observed in 15-20% of breast carcinomas, and is associated with poor prognosis and high recurrence rate and mortality. Approximately 50% of HER2-overexpressing tumors correspond to the HER2-enriched subtype. The remaining HER2-positive cases fit into the luminal-B subtype which are also positive for ER. Trastuzumab, a humanized monoclonal antibody to the extracellular portion of the receptor was originally approved for metastatic breast carcinoma in combination with chemotherapy, as it was shown to significantly prolong progression-free survival (PFS) and OS; it was subsequently also approved for the use in the adjuvant setting, where it has also been found to prolong disease-free survival (DFS) and OS (Dowsett et al., 2009).
Primary or secondary resistance to trastuzumab is observed, and several preclinical studies have investigated and highlighted its possible underlying molecular mechanisms; mutations of the catalytic subunit of PI3K (PIK3CA), loss of PTEN (phosphatase and tensin homolog on chromosome 10), alterations in the status of cell cycle related proteins such as the cyclin-dependent kinase inhibitor p27 and cyclin E, signaling by other HER or non-HER RTKs, increased expression of HER ligands or other growth factors, signaling through HRs have been implicated (De et al., 2013).

ii. Emerging Molecular Biomarkers

Molecular Profiling of Breast Cancer

Breast cancer is now considered as a heterogeneous collection of different diseases that display divergent molecular features, rather than one disease with varying histological features and clinical behaviour. Genomic information can be used in conjunction with classical clinicopathological variables to determine recurrence risk and predict therapy efficacy. Gene expression profiling studies, which allow simultaneous examination of thousands of genes, have elucidated the distinct molecular subtypes of breast cancer. The seminal studies of Perou (Perou et al., 2000) led to the identification of four major intrinsic molecular subtypes: luminal-A, luminal-B, HER2-positive and basal-like (BLBC) breast cancer. Additional information about molecular classification is provided under a separate part (Part 1.2.2.5 Page 22).

Ki67 Antigen

The Ki67 antigen is a nuclear protein expressed in proliferating cells throughout all phases of the cell cycle, and is a marker of cell proliferation. Ki67 levels could discriminate within ER positive tumors, those with poor recurrence-free and disease specific survival. This formed the basis for the St Gallen panel of experts recommendation to use the Ki67 to guide treatment decisions in early breast carcinoma, and to define luminal A versus luminal B immunohistochemical surrogate subtypes (Kos and Dabbs, 2016).
A predictive role of Ki67 has also been proposed; addition of chemotherapeutic agents in the adjuvant treatment of ER-positive, high Ki67-expressing tumors have shown benefit in terms of survival, although these data require further evaluation. Furthermore, Ki67 levels assessed as a pharmacodynamic intermediate end-point during neoadjuvant endocrine therapy have been used to discriminate the sub-optimally responding patients, who need to switch to neoadjuvant chemotherapy. Low levels of Ki67 at diagnosis indicate tumors unlikely to benefit from neoadjuvant endocrine treatment, and have been used as an exclusion criterion for such treatment (Nitz et al., 2014, Kos and Dabbs, 2016). Additional information about Ki67 is provided under a separate part (Part 1.2.2.8 Page 37)

**IHC4 Prognostic Score**

The immunohistochemical (IHC) 4 prognostic score is based on four widely measured IHC markers (ER, PR, HER2, and Ki67) using FFPE tumor samples. These variables were included in a formula producing the IHC4 score which along with classical variables such as tumor status (T) according to TNM, number of involved lymph nodes, grade, and patient age, provided similar prognostic information for distant recurrence as the OncotypeDx RS, with significantly less cost(Kos and Dabbs, 2016, Helen et al., 2014). However, these results can only be obtained by standardized procedures because of the lack of reproducibility of IHC. On this point, the International Ki67 in Breast Cancer Working Group recently proposed guidelines to reduce interlaboratory variability and improve inter study comparability of Ki67, mostly using IHC with monoclonal antibody MIB1. Strengthening the analytical validity of Ki67 may enable using it for prognosis, prediction of responsiveness/resistance to endocrine therapy or chemotherapy and monitoring neoadjuvant treatment (Dowsett et al., 2011).
Gene expression prognostic tests

The most widely used genomic platforms commercially available for use in early stage breast cancer includes OncotypeDX, MammaPrint and PAM50(Helen et al., 2014). Several other tests are also marketed or are under development. Most tests provide an estimated recurrence risk and this prognostic information is considered independent of that provided by standard clinical and pathologic factors. These tests do not have “pure predictive” value, because none of these assays was specifically designed to predict which subset of patients would benefit from a specific therapeutic regimen; however, they provide some predictive information, in the sense that women in the high risk group are expected to benefit from adjuvant chemotherapy.

OncotypeDX Recurrence Score

OncotypeDX® measures expression of 21 genes by quantitative reverse transcriptase-PCR (qRT-PCR), from formalin-fixed paraffin-embedded (FFPE) tissues to determine a Recurrence Score (RS). This score estimates the likelihood of distant metastasis at 10 years from the date of diagnosis, and stratifies patients in to three risk groups: low, intermediate and high for RS values <18, 18–30, >30, respectively(Le Du et al., 2013). It was firstly validated as an independent prognosis marker then as predictive of tamoxifen response for ER–positive, lymph-node negative early stage breast cancer in the NSABP-B14 population(Le Du et al., 2013). Studies revealed that the 21-gene signature was better than standard clinicopathological variables at predicting recurrence. But even with these new classifiers, results remain intermediate for 22 % to 40% of the population for whom prognosis are still heterogeneous and treatment decisions still difficult(Le Du et al., 2013).

MammaPrint

Mammaprint® evaluates the expression level of 70 genes based on DNA microarray technology on fresh-frozen or FFPE tissue sample to define low or high risk of relapse. The test examines 70 genes (55 well known genes and 15 without known function) selected from a case-control study from the Netherlands Cancer Institute using archived frozen tissue from young (<55 years) node-negative breast cancer patients most of which had ER-positive tumors and did not receive adjuvant systemic therapy. The test could be performed by RT-qPCR, both in fresh frozen and
FFPE tissue, with equivalent efficacy (Le Du et al., 2013). Mammaprint is the first FDA-approved gene-expression assay to be used as a prognostic test for women with node-negative breast cancer. The test stratifies the patients in two prognostic groups: low-risk and high-risk for early metastatic recurrence (within 5 years) (Kos and Dabbs, 2016).

PAM50

The Prediction Analysis of Microarrays (PAM50) is a 50-gene set using a quantitative RT-PCR (qRT-PCR) assay that has been validated on FFPE tissue. The test measures the expression of 50 classifier genes and 5 control genes to calculate the risk of recurrence (ROR) score and classify the tumors in the 4 intrinsic subtypes of breast cancer, namely luminal-A, luminal-B, HER2-positive, and BLBC. The PAM50 test provides additional information on the biology of the tumor and quantitative data on biomarkers already used for treatment decisions such as the $ESR1$, $PGR$, and $ERBB2$ genes. It was concluded that intrinsic subtype classification by PAM50 was superior to IHC for both prognosis and prediction of benefit from adjuvant tamoxifen, in node-negative and positive disease (Nielsen et al., 2010). Retrospective studies have shown that the PAM50 assay is a predictor of survival in breast cancer independent of clinicopathologic variables, nodal status, ER and tumor grade (Chia et al., 2012).

1.2.2.5. Classification of Breast Cancer

Breast cancer is a highly heterogeneous disease under several distinct viewpoints. Indeed, different types of this neoplasm exhibit variable histopathological and biological features, different clinical outcome and different response to systemic interventions. Based on such a high degree of heterogeneity, breast cancer cannot be viewed as a single clinico-pathological entity, but it must be dissected into a number of more homogeneous entities. As a general rule, a suitable classification of any disease has to be scientifically sound, clinically useful, easily applicable and widely reproducible. Unfortunately, and despite all the efforts in the past and in more recent years, the ‘perfect’ classification of breast cancer still has not been developed.
i. Histopathological Classification

The histopathological classification of breast carcinoma is a traditional and widely used type of breast cancer classification in the clinical practice. It is based on the diversity of the morphological features of the tumors. Histopathologically breast cancer can be broadly categorized into in situ carcinoma and invasive (infiltrating) carcinoma (Malhotra et al., 2010, Viale, 2012).

Carcinoma in situ is proliferation of cancer cells within the epithelial tissue without invasion of the surrounding tissue. In contrast, invasive carcinoma invades the surrounding tissue. Breast carcinoma in situ is further sub-classified as either ductal or lobular. Ductal carcinoma in situ (DCIS) is considerably more common than its lobular carcinoma in situ (LCIS) counterpart and encompasses a heterogeneous group of tumors. DCIS has traditionally been further subclassified based on the architectural features of the tumor which has given rise to five well recognized subtypes: Comedo, Cribriform, Micropapillary, Papillary and Solid (Malhotra et al., 2010, Viale, 2012).

Similar to in situ carcinomas, invasive carcinomas are a heterogeneous group of tumors differentiated into histological subtypes. The major invasive tumor types include infiltrating ductal, invasive lobular, ductal/lobular, mucinous (colloid), tubular, medullary and papillary carcinomas. Of these, infiltrating ductal carcinoma (IDC) which is known as Invasive carcinoma of no special type (NST) according to the new WHO classification system [Table 1.2] and Invasive lobular carcinoma (ILC) are, by far, the most common subtype (Malhotra et al., 2010, Viale, 2012). A major drawback of this classification is that some 70%–80% of the all breast cancers will eventually belong to either one of the two major histopathological classes, namely invasive ductal carcinomas (IDCs) or invasive lobular carcinoma (ILC). This implies that the classification is unable to actually mirror the much wider heterogeneity of breast cancer, because it groups together, within the same class, tumors that have a very different biological and clinical profile. As a result, the histopathological classification has minimal prognostic and predictive implications, and its clinical utility is quite modest (Malhotra et al., 2010, Viale, 2012, Weigelt et al., 2010).
Table 1.2 Types of Invasive breast carcinomas according to WHO histopathological classification 4th edition 2012 (without microinvasive carcinoma and invasive papillary lesions).

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Invasive carcinoma of no special type (NST)</td>
</tr>
<tr>
<td>2</td>
<td>Invasive lobular carcinoma</td>
</tr>
<tr>
<td>3</td>
<td>Tubular carcinoma</td>
</tr>
<tr>
<td>4</td>
<td>Cribriform carcinoma</td>
</tr>
<tr>
<td>5</td>
<td>Mucinous carcinoma</td>
</tr>
<tr>
<td>6</td>
<td>Carcinoma with medullary features</td>
</tr>
<tr>
<td>7</td>
<td>Carcinoma with apocrine differentiation</td>
</tr>
<tr>
<td>8</td>
<td>Carcinoma with signet-ring-cell differentiation</td>
</tr>
<tr>
<td>9</td>
<td>Invasive micropapillary carcinoma</td>
</tr>
<tr>
<td>10</td>
<td>Metaplastic carcinoma of no special type</td>
</tr>
<tr>
<td>11</td>
<td>Rare types (Carcinoma with neuroendocrine features and Secretory carcinoma)</td>
</tr>
</tbody>
</table>

ii. Molecular Classification

Due to the limited prognostic and predictive power of the existing classifications, at the beginning of the new century, new approaches have been considered to unveil the molecular basis for heterogeneity of breast cancer. Accordingly, gene expression profiling approach and immunohistochemistry has been successfully validated to identify breast cancer subtypes and to inform the choice of the systemic treatments.

Classification based on Gene Expression

The existing histological classification systems for breast cancer are far from being accurate in predicting the prognosis or selecting the appropriate treatment of a given patient (Cleator and Ashworth, 2004). Morphologically identical tumors can display divergent clinical outcomes and responses to therapy. This can predominantly be attributed to molecular class differences that exist amongst histologically similar cancer types. Consequently, molecular classification can be more powerful than histopathology as a predictive factor for the different treatments. This would
result in less frequent use of chemotherapy with considerable advantages in reducing toxicity and costs (Zepeda-Castilla EJ, 2008).

In 2000, Perou and colleagues published the first paper classifying breast cancer into intrinsic subtypes based on gene expression profile. These data, along with the numerous subsequent contributions of different authors have changed the way researchers understand, classify and study breast cancer (Perou et al., 2000). They have led scientists and clinicians to reconsider the way to diagnose and treat patients, and ultimately, how to search for new therapeutic alternatives.

Perou’s group was the first to provide a molecular classification for breast cancer. Using a cDNA microarray of 38 breast cancer cases, the group defined a list of ‘intrinsic’ genes. The hierarchical cluster analysis revealed four molecular subtypes: luminal, HER2, basallike and normal breast. The subsequent expansion of this work in a larger cohort of patients showed that the luminal subgroup could be divided into at least two groups (luminal A and B), and that different molecular subtypes were associated with different prognoses [Table 1.3]. More recently, a new subtype classified as “claudinlow” has also been identified [Figure 1.7](Prat et al., 2010).

Figure 1.7 cDNA based Molecular Classification of Breast Cancer. The figure is adapted from (Malhotra et al., 2010)
<table>
<thead>
<tr>
<th>Molecular subtype</th>
<th>Prognosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luminal A</td>
<td>Good</td>
</tr>
<tr>
<td>Luminal B</td>
<td>Intermediate/ Poor</td>
</tr>
<tr>
<td>Basal-like</td>
<td>Poor</td>
</tr>
<tr>
<td>HER2-enriched</td>
<td>Poor</td>
</tr>
<tr>
<td>Claudin-low</td>
<td>Poor</td>
</tr>
<tr>
<td>Normal Breast Like</td>
<td>Intermediate</td>
</tr>
</tbody>
</table>

**Classification based on Immunohistochemical Characteristics**

A further advancement in the field was the use of Immunohistochemistry (IHC) as a surrogate for DNA microarray classification. Studies confirmed that it could reliably identify the major molecular classes of invasive breast carcinoma. This method represents a feasible alternative because many of the cases of breast cancer occur in places where analysis of prognostic factors needs to be economical, easy, and reproducible (Zepeda-Castilla EJ, 2008).

Three different classification systems using IHC markers or combined with histological characteristics are widely used in the scientific society (Goldhirsch et al., 2011, Nielsen et al., 2004, Sotiriou et al., 2006). The IHC markers used are Estrogen receptor (ER), progesterone receptor (PR), Human Epidermal Growth Factor Receptor (Her2/Neu), Ki67, Cytokeratin 5/6 and Epidermal growth factor receptor (EGFR) [Table 1.4].
Table 1.4 The Major Molecular Phenotypes According to Histologic or/and Immunohistochemical Characteristics as defined by 3 Classification Systems

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Nielsen 2004 (Nielsen et al., 2004)</th>
<th>St. Gallen International: Goldhirsch 2013 (Goldhirsch et al., 2011)</th>
<th>Sotiriou 2006 (Sotiriou et al., 2006)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luminal A</td>
<td>ER+, PR+/-, HER2-</td>
<td>ER+, PR +/-, HER2-; low Ki67 (&lt;20%)</td>
<td>ER+, HER2-; grade 1 and 2</td>
</tr>
<tr>
<td>Luminal B</td>
<td>ER+, PR+/-, HER2+</td>
<td>ER+, PR +/-, HER2-; high Ki67 (&gt;20 %) or ER+, PR +/-, HER2+; any Ki67</td>
<td>ER+, HER2-; grade 3</td>
</tr>
<tr>
<td>HER2+</td>
<td>ER-, PR-, HER2+</td>
<td>ER-, PR-, HER2+</td>
<td>ER+/-, HER2+</td>
</tr>
<tr>
<td>Triple-negative</td>
<td>ER-, PR-, HER2-</td>
<td>ER-, PR-, HER2-</td>
<td>ER-, PR-, HER2-</td>
</tr>
<tr>
<td>Basal-like</td>
<td>ER-, PR-, HER2- and At least 1 positive for CK5/6 or CK14 or EGFR</td>
<td>ER-, PR-, HER2-</td>
<td>ER-, PR-, HER2-</td>
</tr>
</tbody>
</table>

Identification of the molecular subtypes has reshaped understanding of breast cancer and helps to select treatment [Table 1.5]. The luminal A subtype is an indolent disease that is typically treated with hormonal therapies that either antagonize or degrade ER or inhibit aromatase, an enzyme critically involved in biosynthesis of estradiol. The luminal B subtype is associated with high recurrence, poor disease-free survival with much lower five- and ten- year survival rates than the luminal A subtype, and failure to respond consistently to any existing treatments. The HER2 subtype is treated with HER2 inhibitors such as trastuzumab. The HER2 subtype frequently metastasizes to brain, escaping further inhibition by HER2-targeting antibodies that seldom cross the blood-brain barrier due to their large size. TNBC/BLBC subtype is the most aggressive subtype of breast cancer and is associated with high mortality in women.
Table 1.5 Generalized systemic therapy management of early breast cancer by intrinsic molecular subtype. The table is modified from (Kos and Dabbs, 2016)

<table>
<thead>
<tr>
<th>Molecular Subtype</th>
<th>Typical therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luminal A</td>
<td>ET alone in most cases‡</td>
</tr>
<tr>
<td>Luminal B (HER2 negative)</td>
<td>ET + CT in most cases</td>
</tr>
<tr>
<td>Luminal B (HER2 positive)</td>
<td>CT + anti-HER2 therapy + ET</td>
</tr>
<tr>
<td>HER2-enriched</td>
<td>CT + anti-HER2 therapy</td>
</tr>
<tr>
<td>Triple negative/basal like</td>
<td>CT</td>
</tr>
</tbody>
</table>

CT, Chemotherapy; ET, endocrine therapy; ‡If gene expression signature not performed, features to consider adding CT include high tumor burden (four or more positive lymph nodes, T3 or higher) or grade 3.

1.2.2.6. Estrogen signaling in breast cancer

Estrogens are steroid hormones that play key roles to normal female physiology, reproduction and behaviour, through its effects on cellular processes including cell proliferation and cell survival. The three most common estrogens are estrone (E1), 17β-estradiol (E2) and estriol (E3). Estrone and estradiol are synthesized by the aromatization of androstenedione and testosterone, respectively (Caldon, 2014). Estriol is synthesized from estrone via a 16α-hydroxyestrone intermediate. Estradiol is the predominant estrogen during the premenopausal period. After menopause, estrone is the main estrogen. In premenopausal women, ovaries constitute the primary biosynthetic source of estrogens. Estrogen is also synthesized in extragonadal tissues including mesenchymal cells of the adipose tissue including that of the breast, osteoblasts and chondrocytes, aortic smooth muscle cells and vascular endothelium, as well as numerous parts of the brain (Caldon, 2014). Estrogen signaling is important in breast cancer because it drives proliferation in tumors that express the estrogen receptor.
Upon estrogen binding ER acts by parallel pathways to alter gene expression. ER translocates to the nucleus to activate gene targets directly or in cooperation with co-activator proteins, or it can transactivate growth receptors to boost receptor tyrosine kinase signaling. These pathways converge to promote growth and proliferation and suppress apoptosis (Musgrove and Sutherland, 2009, Marino et al., 2006).

Despite the risks associated with estrogen exposure the exact mechanisms by which estrogen contributes to the initiation and progression of breast cancer remains elusive. However, a major mechanism is potentially the induction of DNA damage as estrogen treatment leads to double stranded DNA breaks and genomic instability. Estrogen can induce DNA damage via the production of oxidative metabolites that cause DNA adducts, or other oxidative DNA damage, and this is supported by in vitro and animal model studies (Marino et al., 2006, Musgrove and Sutherland, 2009).

The second explanation for estrogen-induced DNA damage is that hyperactivated estrogen signaling provokes excessive proliferation when pathways become dysregulated, and this theory has strong support from in vitro modeling and gene signatures in breast cancer. Excessive proliferation promotes DNA damage accumulation due to insufficient timely repair leading to replication fork stalling and possibly even doubles stranded DNA breaks. It is likely that both carcinogenic estrogen metabolites and deregulated estrogen signaling contribute to estrogen-induced DNA damage. (Marino et al., 2006, Musgrove and Sutherland, 2009).

Three distinct pathways of oestrogen regulation of gene expression has been described so far (Musgrove and Sutherland, 2009)[Figure 1.8]:

i. First, in classic oestrogen signaling ligand-bound oestrogen receptor (ER) activates gene expression either through direct binding of dimeric ER to specific DNA response elements, EREs, in complexes including co-activators (CoAs) and histone acetyl transferases (HATs), or through protein–protein interactions with other transcription factors, particularly members of the activation protein 1 (Ap1) and specificity protein 1 (Sp1) families to facilitate binding to serum response elements (SREs) and activation of transcription.
ii. Second, ER can also be activated as a consequence of signalling events downstream of receptor tyrosine kinases (RTKs) such as the epidermal growth factor receptor (EGFR), HER2 and the insulin-like growth factor receptor (IGFR). Phosphorylation (P) by the Erk or Akt serine/threonine kinases leads to ligand-independent activation of the ER.

iii. Third, signalling can be mediated through non-genomic mechanisms by ER that is localized at the cell membrane or in the cytoplasm. Ligand binding induces the assembly of functional protein complexes that involve other signalling molecules and that activate intracellular signalling cascades, resulting in transcription factor (TF) activation. Two recently characterized mechanisms that ultimately activate transcription independently of ER binding to DNA are ligand-induced methylation (M) of ER and formation of an ER–PI3K–Src–focal adhesion kinase (FAK) complex that activates Akt and activation of Erk by ER–Src–PELP1 complexes.
Figure 1.8 Mechanism of Estrogen action at the molecular level. a, classic oestrogen signaling. b, signalling events downstream of receptor tyrosine kinases (RTKs). c, non-genomic mechanisms by ER that is localized at the cell membrane or in the cytoplasm (d & e are two alternatives in non genomic mechanism). The figure is adapted from (Musgrove and Sutherland, 2009)
1.2.2.7. Role of androgen receptor in breast cancer

The oncogenic role of androgen receptor (AR) in the prostate cancer is well established and its role in breast cancer is emerging recently. AR has recently emerged as a useful marker for the further refinement of breast cancer subtype classification and emerging clinical target (Pietri et al., 2016).

Indeed the argument for the importance of androgens in breast cancer has been so expanded that many authors are beginning to argue for the inclusion of AR expression to be routinely assessed in breast cancer diagnosis, as part of a quadruple panel alongside the assessment of ER, PR, and HER2 (McNamara and Sasano, 2016).

AR is expressed in approximately 80 and 60 % of primary and metastatic breast tumors, respectively. Its expression varies across the clinical subtypes, approximately 84–95 % in ER+ tumors, 50–63 % in ER−/HER2+ tumors, and 10–53 % in TNBC. The signaling effect of AR is likely to be different across breast cancer subtypes (Chia et al., 2015).

Biology of Androgen Receptor

The AR is a member of the steroid hormone receptor family that in turn belongs to the superfamily of nuclear receptors. The AR is activated by androgen (i.e., testosterone or its locally synthesized and more potent metabolite, dihydrotestosterone (DHT). The AR gene is located on the X chromosome at q11 and contains eight exons encoding for an N terminus domain (NTD), a DNA binding domain (DBD), a hinge region, and a ligand binding domain (LBD). The NTD contains the activation function 1 domain (AF-1) that retains most of the AR activity. The DBD contains two zinc finger motifs that recognize consensus androgen response elements (AREs) and anchoring of the AR to recognized sequences. The hinge region is responsible for nucleocytoplasmic shuttling of the AR and the LBD that contains the ligand binding pocket important for ligand recognition (Narayanan and Dalton, 2016, Kono et al., 2017).

The unliganded AR is maintained in an inactive complex by heat shock proteins, HSP-70 and HSP-90. Upon ligand binding, the HSPs dissociate from the AR enabling it to translocate into the nucleus and bind to DNA. Once bound to DNA, the AR recruits coactivators and general transcription factors to alter the transcription and translation of the target genes (Kono et al., 2017, Narayanan and Dalton, 2016).
AR has been investigated extensively in prostate cancer, and these studies have shown cross-talk of the AR pathway with several other key signaling pathways, including the PI3K/Akt/mTOR and MAPK pathways, and with several key proteins, including Forkhead box protein A1 (FOXA1), phosphatase and tensin homologue deleted on chromosome 10 (PTEN), PI3K, and receptor tyrosine kinases, including ERBB2 and ERBB3 (Narayanan and Dalton, 2016)[Figure 1.9].

**Figure 1.9** Androgen Receptor (AR) Signaling Pathway in Breast Cancer and cross talk with other signaling pathways. The figure is adapted from(Kono et al., 2017).
Prognostic and Predictive Value of AR by Breast Cancer Molecular Subtype

ER-Positive Breast Cancer

Approximately 70% to 90% of ER-positive tumors are also AR positive. In ER-positive AR-positive cell lines, ligand-bound AR binds to estrogen-related element in the nucleus, which leads to cell apoptosis, whereas in ER-negative AR-positive cell lines, AR binds to androgen-related element in the nucleus, leading to cell proliferation [Figure 1.10]. Several clinical studies confirmed this preclinical finding, showing that among postmenopausal women, AR expression was a more favorable prognostic factor in women with ER-positive breast cancer than in women with ER-negative breast cancer (Cochrane et al., 2014, Feng et al., 2016, Iacopetta et al., 2012, Kono et al., 2017, McNamara and Sasano, 2016).

Moreover, a prospective study of 913 patients showed that patients whose tumors had discordant ER and AR expression (ER-positive AR-negative or ER-negative AR-positive) had a worse prognosis than patients whose tumors had concordant ER and AR expression (ER positive AR-positive or ER-negative AR-negative) (Elebro et al., 2015).

This study also showed that inhibition of AR nuclear localization by enzalutamide decreased not only androgen-mediated but also estrogen-mediated tumor growth in both ER-positive AR-positive and ER-negative AR-positive breast tumors. This finding suggests the effectiveness of AR-targeted therapies for patients with ER-negative AR-positive breast cancer and also patients with ER positive AR-positive breast cancer, including the 30% to 50% of patients with ER-positive tumors that display denovo resistance to traditional endocrine therapies (Kono et al., 2017).
Figure 1.10 Associations between Estrogen Receptor (ER) and Androgen Receptor (AR) in Breast Cancer. Ligand-bound AR competes with ligand-bound ER for binding to estrogen response element (ERE) and leads to cell apoptosis in ER-positive and AR-positive cell lines. AR binds to androgen response element (ARE) and leads to cell proliferation, which is inhibited by AR agonists in ER-negative and AR-positive cell lines. DHT indicates dihydrotestosterone; FOXA1, forkhead box protein A1; the bold X’s mean “inhibit.” The figure is adapted from (Kono et al., 2017).
HER2 Positive Breast Cancer

Approximately 60% of HER2-positive breast cancers overexpress AR. In HER2-positive breast cancer, AR positivity was associated with a higher frequency of ER and progesterone receptor expression, smaller tumor size, earlier clinical stage, and lower Ki67 level compared with AR negativity. These findings suggest that HER2 and AR coexpression might be associated with less aggressive tumor subtype (Kono et al., 2017, Lin Fde et al., 2012).

In ER-negative HER2-positive breast cancer cells, binding of WNT7B to AR leads to nuclear translocation of β-catenin with androgen stimulation, and AR/FOXA1/β-catenin binding to regulatory regions of the HER2 gene induces tumor growth [Figure 1.9](Kono et al., 2017, Ni et al., 2011). This suggests that proliferation of AR-positive cells may be mediated via HER2/ERK1/2 signaling in breast cancer and that AR inhibitor may inhibit proliferation of ER-negative ERBB2-positive AR-positive breast cancers by blocking androgen-stimulated oncogenic ERBB2/ERBB3 signaling. Taken together, these findings suggest that the AR pathway is also important in ERBB2-positive breast cancer and therefore needs to be explored to support further drug development.

Triple-Negative Breast Cancer

Approximately 10% to 35% of TNBCs overexpresses AR, depending on the definition of AR positivity (McNamara and Sasano, 2016, McNamara et al., 2013). A retrospective study of 699 patients with TNBC showed that disease-free survival was significantly better in patients with AR-positive tumors than in those with AR-negative tumors(Thike et al., 2014). Another study showed that compared with AR-negative breast cancers, AR-positive breast cancers were associated with better disease-free survival and overall survival despite a significantly lower rate of pathological complete response to neoadjuvant chemotherapy which is similar to what has been observed for ER-positive compared with ER-negative breast cancers(Kono et al., 2017).

In the LAR subtype of TNBC, the level of AR was on average 9 times as great as in other subtypes of TNBC. The LAR subtype was associated with the highest overall survival rate despite having the lowest rate of pathological complete response to neoadjuvant chemotherapy (Masuda et al., 2013). Also, LAR TNBC showed ER-regulated gene transcription. Thus, AR-targeted therapy should be considered for patients with AR-positive TNBC.
AR-Targeted Therapies

AR Agonists

Selective AR modulators are a potential treatment option for breast cancer. In the cell line MDA-MB-231, TNBC cells stably expressing wild-type AR, treatment with the selective AR modulator enobosarm showed inhibition of metastasis-promoting paracrine factors such as interleukin 6 and matrix metalloproteinase 13 and subsequent migration and invasion (Narayanan et al., 2014).

AR Antagonists

The AR antagonists, which are among the therapeutic agents most commonly used to treat prostate cancer, include several categories of drugs, for example, AR inhibitors and CYP17A inhibitors. Many AR antagonists are currently being investigated in breast cancer in preclinical and clinical studies. Bicalutamide, a nonsteroidal first-generation AR antagonist, interrupts DNA-binding domain binding to the androgen-related element (Figure 2.8) (Masiello et al., 2002). However, acquired mutations in the ligand-binding domain of AR or an increase in AR protein concentration causes resistance to bicalutamide. Enzalutamide, a second-generation AR antagonist, inhibits nuclear translocation, chromatin binding, and interactions with AR coregulators [Figure 1.10]. Although enzalutamide is overall safe and well tolerated, a clinical trial in patients with prostate cancer showed that enzalutamide was associated with central nervous system adverse events, such as seizures and posterior reversible encephalopathy (Loriot et al., 2015). Several other AR antagonists including CYP17A inhibitors, such as abiraterone acetate, orteronel (TAK-700), and VT-464 (Viamet), are also currently under investigation in breast cancer. These drugs block androgen production by inhibiting 17α-hydroxylase or 17,20-lyase activity [Figure 1.11] (Toren et al., 2015).
Androgen Biosynthesis and CYP17A Inhibitors. CYP17A inhibitors block androgen production by inhibiting 17α-hydroxylase or 17,20-lyase activity. The figure is adapted from (Kono et al., 2017)
1.2.2.8. Role of Ki67 Proliferation marker in Breast Cancer

The Ki67 antigen, originally described in 1983, is a nuclear protein expressed in proliferating cells throughout all phases of the cell cycle, and is a marker of cell proliferation. The name of the protein was derived from the city of origin (Kiel/ Kiel University, Clone 67) in Germany and the number of the original clone in the 96-well plate(Gerdes et al., 1983). The expression starts in mid G1 and is maintained throughout the S, G2 and M phases of the cell cycle. In breast carcinoma, the discrimination between luminal-A and luminal-B molecular intrinsic subtypes is primarily based on the expression, in the latter group, of genes associated with proliferation. Although the ASCO 2007 guidelines stated that there was insufficient evidence to recommend routine use of K-i67 in breast carcinoma, the subsequent study by Cheang and coworkers, indicated that Ki67 levels could discriminate within ER positive tumors, those with poor recurrence-free and disease specific survival. This formed the basis for the St Gallen 2009 recommendation to use the Ki67 cutoff of 14% to guide treatment decisions in early breast carcinoma, and to define luminal A versus luminal B immunohistochemical surrogate subtypes (Agboola et al., 2013, Elkablawy et al., 2016, Haroon et al., 2013, Denkert et al., 2015, Juríková et al., 2016).

The Ki67 score or index is the percentage of positively stained cells among the total number of malignant cells scored. The use of the original anti Ki67 monoclonal antibody was restricted to fresh frozen tissue, but by using another antihuman monoclonal antibody, MIB-1, the Ki67 can be measured in formalin-fixed, paraffin-embedded sections, archived over decades(Mannell, 2016).

Function of the Ki67 protein

Although many studies have tried to determine the function of Ki67 during the cell cycle, its role still remains unclear. During interphase, Ki67 is predominantly localized in the nucleolus; however, upon onset of mitosis, it relocates to the periphery of the condensed chromosomes. Approximately 40% of the cellular pool of Ki67 is present on isolated mitotic chromosomes, which is a part of the perichromosomal layer(Juríková et al., 2016). The main components of the perichromosomal sheath include nuclear matrix proteins, nucleolar proteins, ribosomal proteins, and small nuclear RNA (snRNA). This layer covers each chromosome from one telomere to the
other, excluding centromeres, and is present on chromosomes from prophase to telophase. Ki67 is one of the first proteins that bind the perichromosomal layer in mitosis at the transition from prophase to prometaphase, most likely acting as a scaffold or platform that directs nucleolar components at the chromosome periphery to form the perichromosomal compartment (Juríková et al., 2016).

Because Ki67 is present in the cell nucleus where it only binds the perichromosomal layer in actively growing and dividing cells, it is widely used as a marker to assess cell proliferation. Therefore, changes in Ki67 expression in growing tumor cells compared to normal cells may be used as an early predictor of treatment efficacy and as a prognostic factor for long-term outcomes in patients with cancer. The role of Ki67 in cell division has been concluded from the observed arrest of cell proliferation when Ki67 is blocked either by antibodies or by inhibition of serine and threonine dephosphorylation. Another study showed its involvement in the early steps of ribosomal RNA (rRNA) synthesis. In addition, its function in stabilization and maintenance of the mitotic spindle during mitosis has also been reported. However, little is known about the other functions of the Ki67 protein. The difficulties in determining its role can be attributed to the lack of obvious homology with other proteins of known function (Juríková et al., 2016).

**Use of the Ki67 antigen**

After it was suggested that the Ki67 labeling index (LI) estimating the growth fraction could be of prognostic value in non-Hodgkin’s lymphomas (Gerdes et al., 1984), its usefulness was examined in various types of malignant neoplasms. The absence of Ki67 protein in resting cells and its expression in all proliferating cells, whether normal or Tumor cells, makes the Ki67 antibody a great tool for determining the growth fraction of any given human cell population. Ki67 is associated with the common histopathologic parameters of breast cancer, and a strong correlation exists between Ki67 expression and histologic grading because both parameters are associated with proliferation. Similarly, higher Tumor stages and nodal status are associated with a higher percentage of cells expressing Ki67. Higher Ki67 expression was found in lymph node metastases than in primary breast Tumors, which correlated with shorter survival in patients (Juríková et al., 2016, Kim et al., 2015). Based on these results, the use of Ki67 might help in the selection of appropriate treatments for certain subgroups of patients.
Another correlation was noted between steroid receptor status and Ki67 expression. Specifically, estrogen receptor (ER) status was inversely correlated with Ki67 expression, indicating that Tumors with the lowest proliferative activity have the highest rates of ER positivity (Inwald et al., 2013). Many studies have shown that Ki67 is an important predictive and prognostic marker of survival and Tumor recurrence in breast cancer, and the proliferative activity of Tumors, as determined by examining Ki67 expression, may reflect the aggressiveness of this disease (Inwald et al., 2013, Juríková et al., 2016). High Ki67 is associated with poor disease-free survival (DFS) and overall survival (OS).

Despite the apparent prognostic utility of Ki67, guidelines from the American Society of Clinical Oncology do not recommend its use in clinical practice in newly diagnosed breast cancer patients, mainly because of the lack of uniformity in sample processing and analytical validity (Harris et al., 2007). To coordinate the analytical methodology of Ki67, a reproducibility study was conducted with the goal of devising a strategy to streamline Ki67 analysis and increase scoring concordance. The inter-laboratory comparability showed substantial variability in Ki67 scoring and the analytical validity for this assay was unacceptably poor (Juríková et al., 2016).

The use of an automated analyzer seemed to standardize the Ki67 evaluation method, and the computer-assisted Ki67 scoring approach resulted in a correlation between the proliferation of Tumor cells and clinical endpoints (Juríková et al., 2016). Thus, automated Ki67 scoring may help to solve reliability concerns in Ki67 diagnostics. Despite the reservations about analytical validation, the immunohistochemical detection of Ki67 together with ER and PR, and the detection of HER2 help to define different subtypes of breast cancer in a similar manner to the results from the analysis of gene expression arrays (Juríková et al., 2016).

A Ki67 cut-off value of 14% was adopted by the 2011 St. Gallen Consensus for choosing systemic treatment in early-stage breast cancer, which recommended adjuvant endocrine therapy alone for patients with luminal A type Tumors and additional chemotherapy for patients with luminal B (HER2-negative) breast cancer (Goldhirsch et al., 2011). However, a new Ki67 cut-off of 20% was recommended by the 2013 St. Gallen Consensus to be more appropriate in defining the luminal B subtype (Goldhirsch et al., 2013).
Ki67 and racial differences in breast cancer

African American women are 40% more likely to die than white women with breast cancer in US (Guth et al., 2017), and in Africa, Nigerian women with breast cancer have higher mortality than British women (Agboola et al., 2013). Many factors may contribute to this difference, for example, delays in the diagnosis and treatment of breast cancer, which, in turn, negatively impacts on survival. While socio-economic disparities in the delivery of health care are well recognized, there is an increasing awareness of differences in tumor biology which exist between ethnic nationalities (Mannell, 2016).

A study done among young African-American, Hispanic and white women in US, with newly diagnosed invasive breast cancer seen from 2010–2015 showed breast cancer in African-American women was more likely to have a higher proliferative rate than breast cancer in white women (Guth et al., 2017). A more aggressive breast cancer phenotype in African-American women underlies the poor survival in this population group. These findings that identify racial differences in tumor biology are supported by other study from Nigeria and Nottingham, UK (Agboola et al., 2013). The levels of Ki67 were significantly higher in the breast cancer of Nigerian women than those in British women, independent of stage, grade and receptor status. Mortality was greater in the Nigerian patients. It is likely that the greater proliferative fraction, identified by high levels of Ki67, contributed to the racial differences in survival.

1.2.2.9. Role of BRCA mutation in Breast Cancer

Approximately 5-10% of breast cancers are caused by a hereditary predisposition [Figure 1.12]. Hereditary breast cancer is generally caused by mutations in BRCA1 (17q21) and BRCA2 (13q12-13). Both are tumor suppressor genes involved mainly in DNA repair. Mutation in BRCA1 and BRCA2 is associated with not only breast cancer but also with ovarian cancer causing a hereditary breast and ovarian cancer (HBOC) syndrome, which is inherited in an autosomal-dominant manner. This syndrome also increases the risk of developing pancreatic, stomach, laryngeal, fallopian tube and prostate cancer. Individuals with HBOC syndrome have a lifetime risk of developing breast cancer of 60–80%, and of 30–50% for ovarian cancer (Dutil et al., 2012, Lecarpentier et al., 2012).
Clinical Features of Breast Cancer with BRCA Mutation

i. Age of Onset

Young age of onset of breast cancer, even in the absence of family history, has been demonstrated to be a risk factor for BRCA mutations. Around 36% to 85% of patients diagnosed with breast cancers before age 30 are hereditary (Wooster et al., 1995). In several studies, BRCA mutations are independently predicted by early age at onset, being present in 6% to 10% of breast cancer cases diagnosed at ages younger than various premenopausal age cutoffs (age range, 35-50 years) (Assi et al., 2013, Wooster et al., 1995).

In cancer-prone families, the mean age of breast cancer diagnosis among women carrying BRCA1 or BRCA2 mutations is in the 40s (Ford et al., 1998). In the Ashkenazi Jewish population, 13% of patients with no known family history and diagnosed before 50 years of age had BRCA mutations (Deffenbaugh et al., 2002). Additional study indicates that early age of breast cancer diagnosis is a significant predictor of BRCA mutations in the absence of family history in this population (Hartge et al., 1999).
ii. Family history of breast or ovarian cancer

Family history of breast or ovarian cancer, particularly of early age onset, is a significant risk factor for a BRCA mutation in ethnic populations characterized by founder mutations. For example, in unaffected individuals of Ashkenazi Jewish descent, 12% to 31% will have a BRCA mutation depending on the extent and nature of the family history. Several other studies document the significant influence of family history (Heisey et al., 1999, Hartge et al., 1999).

iii. Triple Negativity

In patients with breast cancer that is “triple-negative”, there is an increased incidence of BRCA mutations. Some researchers have suggested that the physiologic pathway for development of triple-negative breast cancer is similar to that for BRCA-associated breast cancer. In 200 randomly selected patients with triple-negative breast cancer from a tertiary care center, there was a greater than 3-fold increase in the expected rate of BRCA mutations. BRCA1 mutations were found in 39.1% of patients and BRCA2 mutations in 8.7% (Gonzalez-Angulo et al., 2011). Young et al studied 54 women with high-grade, triple-negative breast cancer with no family history of breast or ovarian cancer, representing a group that previously was not recommended for BRCA testing. A total of 6 BRCA mutations, 5 BRCA1, and 1 BRCA2, were found for a mutation rate of 11% (Young et al., 2009). In another study of 77 patients with triple-negative breast cancer, 15 patients (19.5%) had BRCA mutations: 12 in BRCA1 and 3 in BRCA2 (Gonzalez-Angulo et al., 2011).

Mechanism of BRCA1 and BRCA2 genes in protecting the integrity of the genome

Maintaining genomic integrity is mediated by a cellular network of signaling events (the DDR) that are triggered in response to genotoxic stress. The DDR to DSBs involves sensors that can detect broken ends, effectors that execute repair and mediators that facilitate interactions between sensors and effectors. The DDR also includes the activation of checkpoints that delay the cell cycle before or during replication (G1/S or intra-S-phase checkpoints) or before cell division (G2/M checkpoint) to ensure that genetic errors are not transmitted to subsequent generations by allowing time for DNA repair (Roy et al., 2011).
In mammalian cells, DSBs are repaired by HR (which is mostly error-free), or by non-homologous end-joining (NHEJ; which is error-prone) [Figure 1.13]. The genome is particularly susceptible to DNA damage during replication because damage on a single strand can be converted to double-strand damage and lead to replication fork collapse. In the absence of an intact HR pathway, these replication-associated DSBs can result in chromosome rearrangements and hence genomic instability (Roy et al., 2011).

BRCA1 and BRCA2 proteins are known to function in homologous recombination (HR), a vital DNA repair process that uses the undamaged sister chromatid to carry out high-fidelity repair of predominantly replication-associated DNA double-strand breaks (DSBs). HR appears to be the major mechanism for protecting the integrity of the genome in proliferating cells, because other DSB repair pathways are error-prone and generate chromosome deletions and translocations. The loss of BRCA1 or BRCA2 function in normal cells results in growth defects, which are required, in combination with the subsequent loss of other DDR mediators, for tumor development (Roy et al., 2011).

Figure 1.13 Role of BRCA 1 and BRCA 2 with other key effectors of the DNA damage response and DNA repair. The figure is adapted from (Caldon, 2014)
Inheritance of BRCA mutations and variants of DNA damage

The cancer risk caused by BRCA1 and BRCA2 mutations are inherited in a dominant fashion even though usually only one mutated allele is directly inherited(Cui et al., 2001). A mutated BRCA gene can be inherited from either parent. Because they are inherited from the parents, they are classified as hereditary or germline mutation rather than acquired or somatic mutations. Therefore, breast cancer caused by a mutated BRCA gene is a hereditary cancer rather than a sporadic cancer(Cui et al., 2001).

BRCA mutation carriers are heterozygous for the mutation. If the functional copy is harmed, however, then the cell is forced to use alternate DNA repair mechanisms, which are more error-prone. The loss of the functional copy is called loss of heterozygosity (LOH) (Greenberg et al., 2006). There are many variations in mutation of the BRCA genes, and not all changes confer the same risks. Some variants are harmless; others are known to be very harmful. Variants are classified as follows(Lindor et al., 2013):

i. **Deleterious mutation**: The change is proven to cause significant risks.

These are DNA variants that take the form of nonsense mutations, small out-of-frame insertion or deletion mutations, larger gene rearrangements and splicing alterations that all truncate or remove important domains of the BRCA proteins. In addition, certain missense substitutions are considered “pathogenic” because they inactivate protein function. These mutations can be confidently predicted to disrupt the function of the BRCA1 or BRCA2 protein leading to increased risk of breast or ovarian cancer(Lindor et al., 2013).

ii. **Not pathogenic or low clinical significance (LCS) variants**: This group of variants includes common polymorphisms, seen in greater than 1% of alleles in the general population, and rare variants that display little or no association with breast cancer risk in families. These variants are predicted or have been shown to have no significant influence on the normal function of the BRCA1 or BRCA2 proteins(Lindor et al., 2013).
iii. Variant of unknown significance (VUS):

VUS are mainly missense substitutions that result in single amino acid changes, but also include in-frame small deletions or insertions that change only small numbers of amino acids, silent coding alterations that may influence splicing or translation, or intronic changes of unknown influence on gene splicing. These alterations have unknown functional effects on \textit{BRCA1} and \textit{BRCA2} and cannot at this time be classified as either “Pathogenic” or “Not Pathogenic/low clinical significance”. As a result individuals found to carry these variants in their DNA and members of their families cannot benefit from risk assessment measures offered to members of families known to carry \textit{BRCA1} or \textit{BRCA2} deleterious mutations (Lindor et al., 2013).

Testing for \textit{BRCA1} and \textit{BRCA2} Mutations for risk reduction and Prevention of Breast and Ovarian Cancer

Many studies have shown that, the risk of cancer in a BRCA mutation carrier is significant, and knowledge of mutation status in individuals at potentially increased risk of a BRCA mutation may impact healthcare decisions to reduce risk (Kobayashi et al., 2013). Risk-reducing options include intensive surveillance, chemoprophylaxis, prophylactic mastectomy, or prophylactic oophorectomy. Prophylactic mastectomy reduces the risk of breast cancer in high-risk women (based on family history) by 90% or more but is invasive and disfiguring. Prophylactic oophorectomy significantly reduces the risk of ovarian cancer to less than 10% and reduces the risk of breast cancer by approximately 50%. In women who have already had breast cancer, prophylactic oophorectomy reduces the risk of cancer relapse (Kobayashi et al., 2013). Moreover, oncogenetic testing is becoming the powerful therapeutic predictive tool, as new targeted therapeutic opportunities, such as poly(ADP ribose) (PARP) inhibitors emerge and chemosensitivity to platinum-based therapy is constantly reported (Kobayashi et al., 2013).

However, BRCA genetic analysis is cumbersome, expensive, and may be impractical as a screening method for detection in all patients. Moreover, patients with BRCA promoter methylation and other mechanisms of loss are not identified using current approaches. Immunohistochemistry for \textit{BRCA1} may be a useful initial screening test to select patients for targeted therapy and to detect patients at risk for hereditary breast and ovarian cancer syndromes (Garg et al., 2013, Roehe et al., 2012).
1.2.2.10 Role of Viruses in Breast Cancer

Researches into breast cancer etiology have focused primarily on reproductive and other factors affecting circulating sex hormones and on genetic susceptibility. However, as identified risk factors are thought to explain the incidence in about half of all breast cancer cases only, researchers are motivated to consider other routes for disease pathogenesis. Viruses have been implicated in the development of various cancers, but they have not been much considered for breast cancer.

Recent investigations have linked breast cancer to viral infections, such as Epstein–Barr virus (EBV), Mouse mammary tumor virus (MMTV), Human papillomavirus (HPV) and Human cytomegalovirus (HCMV)(Akhter et al., 2014, Lawson et al., 2016a, Richardson et al., 2015). The relationship between breast cancer and viruses could be of potential importance not only for better understanding of breast cancer etiology, but also for early detection and prevention of breast cancer and treatment(Alibek et al., 2013).

Human papillomavirus (HPV)

The human papillomaviruses (HPVs) are double-stranded DNA viruses in the Papovaviridae family that infect the epithelia and mucous membranes of humans. Infections by these nonenveloped viruses can lead to a wide array of clinical outcomes ranging from benign warts to cancers. HPVs are divided into five genera (the alpha-, beta-, gamma-, mu-, and nu papillomaviruses) based on shared sequence homology within the L1 capsid gene. Only the alpha- and beta papillomavirus genera are associated with cancer(Fernandes et al., 2013).

More than 120 HPV types have been cataloged, of which approximately 40 can infect the mucosa of the anogenital tract and are collectively known as mucosal HPV, which are classified based on their oncogenic potential as either low- or high-risk HPV types. The low-risk HPV (LR-HPV) are 11, 40, 42, 43, 44, 54, 61, and 70 which causes benign hyperproliferative lesions or genital warts, with a very limited tendency for malignant progression. The high-risk HPV (HR-HPV) type includes HPV 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 69, 73, and 82 which are strongly associated with premalignant and malignant lesions(Alibek et al., 2013, Fernandes et al., 2013).
The HPV cycle initiates when the virus gains access to undifferentiated cells of the basement membrane of the squamous columnar junction epithelium of the ectocervix, after these regions are exposed to mechanical or chemical trauma. The basal cells in the transformation zone retain the ability to differentiate, a property required for virion production. Cervical infection with high-risk HPV typically lasts from 12 to 18 months and in most cases is cleared spontaneously. However, in some women the immune response is insufficient to eliminate the virus, resulting in a persistent, long-term infection that may progress to a malignant lesion (Fernandes et al., 2013).

It has been proposed that the great majority of women and men have been infected with HPV at least once during their lifetime. In most cases, the infection is transient, where most of the individuals are healing, eliminating the virus without the presence of any clinical manifestation (Fernandes et al., 2013).

The HPV genome is divided between early and late genes; the products of the early genes (E1, E2, E3, E4, E5, E6, and E7), facilitate genome propagation, and the products of the late genes (L1 and L2) package HPV virions. The products of two of these early genes (E6, and E7) are the primary viral oncoproteins, most well known for their ability to extend the life span of cells by activating telomerase and to remove barriers to oncogenesis by degrading the tumor suppressors p53 and pRB (Alibek et al., 2013, Fernandes et al., 2013, Wallace and Galloway, 2015).

High risk for cancer human papilloma viruses (HPVs) have been identified in breast cancers in 30 studies conducted in 17 countries and 4 continents. HPV type 33 is the most prevalent (14%) but is mainly confined to Asian women, followed by HPV 18 (7%) and 16 (7%). In 10 case control studies, the prevalence of HPVs in breast cancers was significantly higher than in controls odds ratio (OR) = 3.60. Women with HPV-associated cervical neoplasia can later develop HPV-associated breast cancer. However, several groups have not identified HPVs in breast cancer and hence there are still disputes about the association between HPV infection and the risk of breast cancer (Bae and Kim, 2016, Lawson et al., 2016a, Lawson et al., 2015, Lawson et al., 2016b).
**Epstein-Barr virus (EBV)**

Epstein-Barr virus (Formerly designated human herpesvirus type 4 (HHV-4)) belongs to Herpesviridae family and in the subfamily alphaherpesvirinae which is best known as the cause of infectious mononucleosis (glandular fever). It is associated with a series of malignant tumors involving mostly lymphoid or epithelial cells. The former group of diseases predominantly encompasses Burkitt lymphoma (BL) and classical Hodgkin lymphoma (HL). EBV is also causative in immunodeficiency-associated lymphoproliferative disorders, such as post-transplant lymphoproliferative disease (PTLD), and non-Hodgkin lymphomas (NHL) in HIV-infected patients, such as primary central nervous system lymphoma (PCNSL), primary effusion lymphoma (PEL), and the plasmablastic lymphoma of the oral cavity. Conversely, epithelial cancers associated with EBV infection include nasopharyngeal carcinoma (NPC) and a subset of gastric and lymphoepithelioma-like carcinomas (Minarovits and Niller, 2017, Murray and Young, 2000).

Detection of Epstein-Barr virus (EBV) in the neoplastic tissues of breast cancer cases has been reported by some authors. A possible association of EBV with breast cancer was proposed as a consequence of the high incidence of male breast cancers, which was reported in Mediterranean countries, an area endemic for EBV; also, the occurrence of some EBV associated lymphomas in the breast, and the morphological similarities between medullary carcinoma of the breast and nasopharyngeal carcinoma (NPC). EBV sequences were found in breast tissues and milk, and transfection of p31 fragment of EBV DNA has been shown to immortalize epithelial cells including mammary epithelial cells (Akhter et al., 2014, Al Moustafa et al., 2016, Hu et al., Richardson et al., 2015, Zekri et al., 2012).

**Human Cytomegalovirus (HCMV)**

Large spectrums of clinical problems are associated with human cytomegalovirus (HCMV), including birth defects, atherosclerosis, cardiovascular disease, organ transplant failure, and cancer. CMV is the prototypical betaherpesvirus, a subfamily of herpesviruses characterized by slow replication and strict tropism for host species but broad tropism for the cell types infected within the host. CMV is the largest of the human herpesviruses, with a linear dsDNA genome (Akhter et al., 2014, Contreras et al., 2014, Goodrum, 2016).
HCMV asymptomatically infects a large majority of the world’s population and typically causes disease only in the absence of adequate cellular immunity. The prevalence of HCMV ranges from 40% to 99% of the world’s population, with higher seroprevalence in developing countries (Goodrum, 2016).

Asymptomatic long-term virus shedding in urine, saliva, and genital secretions usually marks the primary infection in healthy individuals. Following this, HCMV establishes lifelong persistence, during which it may replicate chronically or reactivate from latency sporadically, and likely frequently, in response to normal biological processes, including the differentiation of monocytes into macrophages or lactation. These reactivation events are typically controlled by the immune system and rarely result in clinical presentation, although they likely contribute to transmission of the virus. However, reactivation from latency in a host with inadequate or compromised T cell immunity poses a serious disease risk (Goodrum, 2016, Kaminski and Fishman, 2016, Spector, 2015).

Emerging evidence demonstrates that HCMV proteins and nucleic acids are frequently detected in tissue specimens in very high prevalence in patients with cancers of different origin, including colon, breast, prostate mucoepidermoid salivary gland tumors, medulloblastomas, neuroblastoma, glioblastoma, and rhabdomyosarcoma. Because of its high prevalence in cancer, HCMV may play an important but not yet well-defined role in the establishment of several cancer forms. HCMV proteins are known to interfere with cellular and immunologic functions that may affect tumor biology in a complex manner (Akhter et al., 2014, Alibek et al., 2013, Cobbs, 2013, Herbein and Kumar, 2014, Richardson et al., 2015, Taher et al., 2014).
1.3. **Significance of the study**

Prevalence of breast cancer in Ethiopia is not well studied but the recent study by the Addis Ababa City Cancer registry in 2014 edition has placed the incidence of breast cancer at number one with 34 % of all cancers diagnosed in that specific year among females(African Cancer Registry Network, 2014). Breast cancer is now an emerging public health problem in most urban centers including Addis Ababa, the administrative and political center of the country with over 2.7 million residents.

Breast cancer is a heterogeneous disease with different subtypes that are based upon the expression level of estrogen receptor (ER), progesterone receptor (PR), and HER-2 receptor(American Cancer Society, 2016). Different types of breast cancer vary substantially in their behavior and response to treatment.

Very few studies have been done to characterize breast cancer in Africa. Large number of studies of breast cancer in African women reported a frequency of 40–55% of triple negative breast cancer (TNBC) which is more aggressive and has a poorer prognosis. Emerging new evidences, however, show TNBC to be low in East Africa including Ethiopia(Brinton et al., 2014a, Jiagge et al., 2016, Kantelhardt et al., 2014, Sayed et al., 2014).

Researches into breast cancer etiology have focused primarily on reproductive and other factors affecting circulating sex hormones and on genetic susceptibility. However, as identified risk factors are thought to explain the incidence in about half of all breast cancer cases only, researchers are motivated to consider other routes for disease pathogenesis. Viruses have been implicated in the development of various cancers, but they have not been much considered for breast cancer. Recent investigations have linked breast cancer to viral infections, such as Epstein–Barr virus (EBV), mouse mammary tumor virus (MMTV), Human papillomavirus (HPV) and Human cytomegalovirus (HCMV)(Akhter et al., 2014, Alibek et al., 2013).

Previous studies from Ethiopia except one study on molecular characterization are based on assessment of ER and PR, which has limitations in revealing the distribution of molecular subtypes in our patients. No previous study has assessed the AR expression, Ki67 expression and BRCA1 expression in breast cancer among Ethiopian women. No other study has compared IHC with gene amplification technology for assessment of HER2 status in Ethiopia. No other study so far has also assessed association of oncogenic viruses with breast cancer in Ethiopia.
Therefore, this study was undertaken to assess the molecular classification of breast cancer among Ethiopian women using ER, PR, HER2 and Ki67. In addition the study was undertaken to assess the expression of AR, Ki67 and BRCA1 in breast cancer. Additionally, the study was conducted to compare IHC with GPA for assessment of HER2 status in breast cancer. The study was also conducted to assess the role of some oncogenic viruses in breast tumorigenesis. Results obtained from this study will also be used by health care providers for health service planning and can serve as a base line data to other related studies.

1.4. Hypothesis

The molecular classification of breast cancer and expression of AR, Ki67 and BRCA1 will be similar to studies done in other countries in Africa and elsewhere. The concordance between IHC and GPA will be similar to other studies done previously. There will be role of viruses in breast carcinogenesis.

1.5. Objectives

The overall aim of this doctoral thesis was to determine the molecular characteristics of breast cancer among Ethiopian women and assess the association of some oncogenic viruses in breast cancer.

The specific objectives have been:

1. Evaluation of ER, PR, HER2 and Ki67 protein biomarkers using Immunohistochemistry for molecular classification of breast cancer
2. Evaluation of AR status among the molecular subtypes of breast cancer
3. Evaluation of Ki67 proliferation marker among the molecular subtypes of breast cancer
4. Evaluation of BRCA-1 protein expression in breast cancer
5. Comparison of Immunohistochemistry (IHC) with Gene Protein Assay (GPA) for Evaluation of Human Epidermal Growth Factor Receptor 2 (HER2) Status
6. Evaluation of the role of HPV, CMV and EBV in Breast Cancer
Chapter 2

Materials and Methods

2.1. Study design and period

It was a cross sectional, retrospective study of women diagnosed with breast cancer that were treated at the Oncology Centre in Tikur Anbessa Specialized Hospital (TASH) between August 2015 and September 2016.

2.2. Study area

Ethiopia is situated in East Africa, bordered by Eritrea, Sudan, Kenya, Somalia and Djibouti. The land area of Ethiopia is estimated at about 1.1 million square kilometers and the current population is approximately 74 million, of which 84% live in rural areas (CSA, 2007). Addis Ababa is the capital city of Ethiopia with an area of 530 km$^2$ and a total population of 2.7 million according to 2007 census report of the Central Statistical Agency of Ethiopia [Central Statistical Agency of Ethiopia 2007]. It has 10 sub-cities. The health service coverage of Addis Ababa is 71%. There are 5 Governmental hospitals, 24 health centers, 32 health posts & more than 500 private health institutions providing different health services. The study was conducted in Tikur Anbessa Specialized Hospital (TASH), which is the largest tertiary and teaching referral hospital in the country. TASH was established in 1965 E. C. and has 1,262 rooms and run by Addis Ababa University. As a referral hospital, it provides various health services, including maternal and child health services. The four main departments which provide major services in the hospital are Internal Medicine, Surgery, Pediatrics, and Gyneacology/Obstetrics departments using 558 beds.
2.3. Study Subjects and Tumor Specimen

The patients enrolled in the study consisted of women with available archived surgical specimen at TASH or St. Paul’s Hospital Millennium Medical College (SPHMMC) which is also a referral hospital in the capital providing surgical service to breast cancer patients in the country. Information about demographic data and tumor characteristics were obtained from the patient medical records at TASH. The collected variables were age, tumor type, grade and stage of disease. Only patients having undergone surgery between 2012 and 2015 were included in the study because biopsy specimens collected from patients before this period were assessed to be missing at the hospitals.

Patients and tumor specimen used in this study were obtained twice during the study period. Study participants were enrolled in the first round between June 2014 and July 2015. The second rounds of patient enrollment were done between March and July 2016 to increase the sample size. Patients and tumor specimens used in the study are described as follows:

120 patients were initially included in the first round; however 31 cases were excluded because formalin-fixed paraffin-embedded (FFPE) tissue was not available in the pathology departments. The first round of enrollment consisted of 89 breast cancer patients. In second round of enrollment, 37 patients were included. Participants from the two study cohorts consisted of 126 breast cancer patients. All archived FFPE blocks were sectioned and H&E stained and examined by a pathologist to confirm for presence of invasive tumors. Out of the 126 blocks obtained from all the participants 12 were rejected because there were no invasive tumors in the biopsy sample and the final cohort for the study consisted of 114 patients.

2.4. Data Collection

Data collection for this study took place from June 2014 to July 2016. Data were drawn from patient medical records by trained physicians during participants visit to the TASH for regular follow-up or chemo/radiotherapy. Questionnaires were completed by the attending physician and collected data includes information on socio-demographic and clinicopathological characteristics.
2.5. Statistical Analysis

All statistical analyses were conducted using SPSS, version 21. Statistical significance was defined as $P$-value less than 0.05, and all statistical tests were two-sided. The distribution of variables was examined, and the presence of missing data, impossible and extreme values for all variables was evaluated for each objective. For categorical measures, frequencies and percentages were examined. For continuous measures, measures of central tendency and distributional properties were examined (mean, standard deviation, median, range). Chi-square test, and one way ANOVA were used to determine correlations.

2.6. Ethical Approval

The study was first approved by the ethical review committee of the department of Biochemistry and then approved by Institutional Review Board (IRB) of College of Health Science, Addis Ababa University. Ethical approval was also obtained from SPHMMC to collect archived FFPE tissue samples from enrolled patients. The study is also approved by the National Research Ethics Review Committee at the Ethiopian ministry of Science and Technology. Written and informed consent was obtained from every patient.

2.7. Laboratory Methods

2.7.1. Hematoxylin & Eosin Stain

Hematoxylin and Eosin (H&E) stain for whole slide sections and TMA slides in this study were done at Orebro University Hospital pathology laboratory using Tissue-Tek Prisma autostainer and coverslipper according to the manufacturer’s protocol (Sakura Finetek USA, Inc., Torrance, CA). Figure 2.1 and 2.2 shows representative image of H&E stained TMA cores of a specific area of a slide.

Principle:

Hematoxylin and Eosin (H & E) staining uses a combination of two dyes, Hematoxylin and Eosin used for demonstration of nucleus and cytoplasmic inclusions. Haematoxylin alone is not technically a dye, and will not directly stain tissues. It therefore needs to be used in combination with a “mordant” – a compound that helps it link to the tissue. The mordant used is typically a metal cation, such as aluminium. Haematoxylin in complex with aluminium salts is cationic and
acts as a basic dye. It is positively charged and can react with negatively charged, basophilic cell components, such as nucleic acids in the nucleus. These stain blue as a result. Eosin is anionic and acts as an acidic dye. It is negatively charged and can react with positively charged, acidophilic components in the tissue, such as amino groups in proteins in the cytoplasm. These stain pink as a result.

Figure 2.1 Representative image of H&E stained TMA cores of a specific area of a slide. Each row in this figure represents triplicates of cores for five different patients (A-E).
2.7.2. Construction of Tissue Microarray and isolation of DNA

2.7.2.1. Tissue Microarray

Tissue Microarray (TMA) was constructed using the TMA grand master automated system according to the manufacturer’s protocol (3DHISTECH Ltd., Budapest, Hungary). A breasts pathologist at the Orebro University Hospital marked representative parts of the individual invasive tumor for sampling of tissue microarray (TMA). 0.6 millimeter punch biopsies corresponding to the marked area were taken from donor paraffin blocks and merged into TMA recipient paraffin blocks that contained 135 to 240 punch biopsy cores. For each patient three biopsy was performed on the same tumor. In total, 447 biopsy cores were taken and pooled into three TMA blocks. Each TMA block included 1 control tissue (Liver) to assure proper identification of the individual cores. Figure 2.3 shows a simplified workflow of TMA preparation procedure. Figure 2.4 shows representative image of H&E stained whole section slide to be used for selecting areas to be used for constructing the TMA. Figure 2.5 shows a representative image of a TMA block constructed from 45 patients tumor block.
Figure 2.3 A Simplified workflow of TMA preparation

Figure 2.4 Representative image of H&E stained whole section slide. Encircled regions are selected by pathologist to take biopsy for constructing TMA and PCR purpose. The three circles without tail are areas to be used for constructing TMA; the circle with the tail is an area to take sample for PCR purpose.
Figure 2.5 Representative image of a TMA block. Each three circles represent one patient sample from different region in the biopsy. This TMA block contains biopsies from 45 patients and 1 control tissue (The 3 circles present at the top) to assure proper identification of the individual cores.
2.7.2.2. Tissue core samples for PCR

TMA grand master automated system (3DHISTECH Ltd., Budapest, Hungary) was used to sample cores for PCR analysis according to the manufacturer’s protocol. A pathologist marked representative parts of the individual invasive tumor with percentage of tumor cells for sampling of tissue for PCR. 0.6 millimeter punch biopsies corresponding to the marked area were taken from paraffin blocks. For each patient two biopsy cores were taken on the same tumor.

2.7.2.3. DNA Extraction

DNA was isolated from tissue core samples of each tumor specimens. The presence of malignant cells was assessed in all samples by evaluation of slides stained with Hematoxylin and eosin (H&E). Genomic DNA (gDNA) was isolated from the tumor cores using the QIAamp DNA Mini Kit (Qiagen), including proteinase K treatment (Qiagen) followed by purification using the QIAcube automated system (Qiagen). The DNA concentration was measured using a Nanodrop ND-1000 (Nanodrop Technologies). The gDNA samples were stored at 4°C.

2.7.2.4. Digital image acquisition

Digital images for constructing TMA were taken from slides scanned on a Pannoramic 250 digital scanner (3D HISTECH Ltd., Budapest, Hungary) and representative areas selected from images using the software program ‘Case viewer’ (3D HISTECH Ltd., Budapest, Hungary). All slides stained using IHC and Gene protein Assay (GPA) were also scanned on a Pannoramic 250 digital scanner (3D HISTECH Ltd., Budapest, Hungary) and images scored using the software program ‘Case viewer’ (3D HISTECH Ltd., Budapest, Hungary).
2.7.3. Immunohistochemistry

Immunohistochemistry (IHC) analysis for this study was done at the Orebro University Hospital (ORUH) in Sweden except the BRCA1 staining which was done at the University of Michigan (UM) in the United States. Both laboratories use the Dako Autostainer Link hence same principle and procedure is applied.

**Principle:**

The principle of the IHC method lies in binding primary antibodies that recognize the specific antigen in biological tissues. The antibody-antigen binding can be visualized in different manners. Enzymes, such as Horseradish Peroxidase (HRP) or Alkaline Phosphatase (AP), are commonly used to catalyze a color-producing reaction [Figure 2.6].

![Image of IHC reaction]

*Figure 2.6* Representation of IHC reaction. *DAB: diaminobenzidine; HRP: horseradish peroxidase.* The figure is adapted from (Wolf et al., 2015)
**IHC protocol**

Immunohistochemistry (IHC) was done in automated system using the Dako Autostainer Link (Agilent, USA) according to the manufacturer’s procedure. Briefly, Formalin fixed, paraffin sections were cut at 5 microns and rehydrated to water. Heat induced epitope retrieval was performed with FLEX TRS High pH (pH 9) retrieval buffer for 20 minutes. After peroxidase blocking, the specific monoclonal antibodies was applied at a dilution specified in table 2.1 at room temperature for 20 minutes. The Dako FLEX + Rabbit EnVision System was used for detection. DAB chromogen was then applied for 10 minutes. Slides were counterstained with Harris Hematoxylin for 5 seconds and then dehydrated and coverslipped. A positive sample was set up as a positive control and PBS, instead of primary antibody, was used as a negative control.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Manufacturer</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER</td>
<td>EP1</td>
<td>Agilent Dako</td>
<td>RTU</td>
</tr>
<tr>
<td>PR</td>
<td>PgR1294</td>
<td>Agilent Dako</td>
<td>RTU</td>
</tr>
<tr>
<td>Ki67</td>
<td>Mib-1</td>
<td>Agilent Dako</td>
<td>RTU</td>
</tr>
<tr>
<td>AR</td>
<td>EP120</td>
<td>Epitomics</td>
<td>1/100</td>
</tr>
<tr>
<td>HER2</td>
<td>Herceptest</td>
<td>Agilent Dako</td>
<td>RTU</td>
</tr>
<tr>
<td>p16</td>
<td>G175-405</td>
<td>BD</td>
<td>1/25</td>
</tr>
<tr>
<td>EBV</td>
<td>CS.1-4</td>
<td>Agilent Dako</td>
<td>RTU</td>
</tr>
<tr>
<td>CMV</td>
<td>CCH2+DDG9</td>
<td>Agilent Dako</td>
<td>RTU</td>
</tr>
<tr>
<td>BRCA-1</td>
<td>MS110</td>
<td>Calbiochem</td>
<td>1:150</td>
</tr>
</tbody>
</table>

RTU=Ready to use

---

*Table 2.1 Sources and dilutions of primary antibodies used in the study*
2.7.4. Fluorescence in situ hybridization

Fluorescence in situ hybridization (FISH) for this study was done at Orebro University Hospital (ORUH) using Vysis automated system (Abbott Laboratories Inc., Des Plaines, IL) according to the manufacturer’s recommendation with the PathVysion DNA Probe Kit (HER2 SpectrumOrange/CEP 17 SpectrumGreen Vysis/Abbott Molecular)

**Principle:**

FISH involves the precise annealing of a single-stranded, fluorescently labeled DNA probe to complementary target sequences. The hybridization of the probe with the cellular DNA site is visible by direct detection using fluorescence microscopy. The Locus Specific Identifier (LSI) HER2/neu DNA probe is a 226 Kb SpectrumOrange directly-labeled, fluorescent DNA probe specific for the HER2/neu gene locus (17q11.2-q12). The Chromosome Enumeration Probe (CEP) 17 DNA probe is a 5.4 Kb SpectrumGreen directly-labeled, fluorescent DNA probe specific for the alpha satellite DNA sequence at the centromeric region of chromosome 17 (17p11.1-q11.1). The probes are pre-mixed and pre-denatured in hybridization buffer for ease of use. Unlabeled blocking DNA is also included with the probes to suppress sequences contained within the target loci that are common to other chromosomes. Formalin-fixed, paraffin-embedded tissue specimens are placed on slides. The DNA is denatured to single-stranded form and subsequently allowed to hybridize with the PathVysion probes. Following hybridization, the unbound probe is removed by a series of washes and the nuclei are counterstained with DAPI (4,6 diamidino-2-phenylindole), a DNA-specific stain that fluoresces blue. Enumeration of the LSI HER2/neu and CEP 17 signals is conducted by microscopic examination of the nucleus, which yields a ratio of the HER2/neu gene to chromosome 17 copy number.
2.7.5. Multiplex real time PCR

The multiplex quantitative real time PCR in this study was done at the Orebro University Hospital. Real-time PCR amplification was performed using the Anyplex™ II HPV-28 Detection Assay (Seegene, Seoul, Korea), in accordance with the manufacturer’s protocol, in a CFX96 real-time thermocycler (Bio-Rad, Hercules, CA, USA). The Anyplex™ II HPV-28 Detection is a novel multiplex real-time PCR assay that permits the simultaneous amplification, detection and differentiation of target nucleic acids of 28 HPV types and Internal Control (IC). The Anyplex™ II PCR System detects 19 high-risk HPV types (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 69, 73, 82) and 9 low-risk HPV types (6, 11, 40, 42, 43, 44, 54, 61, 70).

Principle:
Anyplex™ II HPV-18 Detection Assay is based on a newly developed TOCE™ (Tagging Oligonucleotide Cleavage and Extension) technology, which enables detection of multiple targets in a single fluorescence channel through melting temperature analysis of a series of artificial templates on real-time PCR instruments. TOCE is a novel approach to real-time PCR and has several unique oligonucleotide components [Figure 2.7]. The key components for TOCE technology are dual priming oligonucleotide (DPO™) primer pairs, “Pitchers” and “Catchers.” The DPO is target-specific primer design and provides highly specific amplification of the target region. The Pitcher is a tagging oligonucleotide that hybridizes specifically to the target region. The Catcher is a fluorescently labeled artificial template. Melting curve analysis is done to look at the dissociation-characteristics (Tm) of the specific catcher double-stranded DNA during heating. As the temperature is raised, the double strand begins to dissociate leading to a rise in the absorbance intensity. Cyclic-CMTA (Cyclic-Catcher Melting Temperature Analysis) method is used to semiquantitatively detect HPV at cycle 30 (+++), cycle 40 (++) and cycle 50 (+).
**Figure 2.7** Principles of TOCE assay design and signal generation during real-time PCR. (A) TOCE assay is initiated with hybridization of upstream and downstream primers (DPO) and Pitcher to the selected target sequence. (B) Taq polymerase-mediated extension of primers. (C) Taq polymerase having a 5’ nuclease activity encounters the target-bound Pitcher, and cleaves the Pitcher, releasing the tagging portion. The sequence of released tagging portion is complementary to capturing portion of the Catcher. (D) As the tagging portion is fully extended on the Catcher to create the duplex Catcher, quenching is diminished and the fluorescent signal can be detected.
### 2.7.6. Gene Protein Assay

Gene Protein Assay (GPA) for this study was performed at Falun Hospital (Sweden) using the BenchMark XT platform (Ventana Medical Systems, Tucson, AZ) according to the manufacturer’s recommendation. The gene and protein detection combines PATHWAY anti-HER2/neu (4B5) rabbit monoclonal primary antibody (Ventana Medical Systems) for IHC and the INFORM HER2 Dual ISH DNA probe cocktail (Ventana Medical Systems) for ISH on a single slide.

HER2 status is usually assessed by IHC for HER2 protein overexpression or by fluorescence in situ hybridization (FISH) for HER2 gene amplification. Both methods were approved by the US Food and Drug Administration (FDA). IHC is used primarily and FISH is used as a reflex test on IHC 2+ cases. FISH results have been accepted as a gold standard in the assessment of HER2 status. However, FISH uses dark-field fluorescence microscopy and lacks morphology details. Furthermore, the fluorescent signals fade over time; therefore, the slides cannot be stored for a long time. The bright-field in situ hybridization (BISH) methods, including chromogenic in situ hybridization, silver in situ hybridization, and dual in situ hybridization, have been developed to overcome some of the limitations of FISH. The gene protein assay (GPA) described in this study merges HER2 IHC and BISH to assess HER2 status in tumor cells by correlating with tissue morphology. Comparative studies comparing GPA with IHC alone and FISH alone have shown concordance exceeding 90% by using tissue microarray (TMA) slides on both breast cancer and gastric cancer cases (Hirschmann et al., 2012, Nitta et al., 2012).

**Principle:**

GPA simultaneously assesses HER2 gene copy number and protein on a single slide using bright-field microscopy [Figure 2.8]. It is tricolor visualization of HER2 protein, the HER2 gene, and CEN17. First, HER2 protein is localized through immunohistochemical staining (IHC) with a rabbit monoclonal anti-HER2 antibody and a conventional 3,3'-diaminobenzidine (DAB)-based detection method. Then, the HER2 gene and CEN17 are localized by brightfield in situ hybridization (BISH) with a cocktail of 2,4-dinitrophenyl (DNP)-labeled HER2 probe and digoxigenin (DIG)-labeled CEN17 probe. The HER2 gene and CEN17 signals are visualized
with silver (a silver acetate, hydroquinone, and hydrogen peroxide reaction) and fast red (a fast red and naphthol phosphate reaction), respectively.

Figure 2.8 Principle of GPA for simultaneous visualization of human epidermal growth factor receptor 2 (HER2) protein, the HER2 gene, and the chromosome 17 centromere (CEN17) using a novel HER2 gene-protein assay. The figure is adapted from (Nitta et al., 2012)

Protocol

GPA was performed on the BenchMark XT platform (Ventana Medical Systems, Tucson, AZ) according to the manufacturer’s recommendation. The gene and protein detection combines PATHWAY anti-HER2/neu (4B5) rabbit monoclonal primary antibody (Ventana Medical Systems) for IHC and the INFORM HER2 Dual ISH DNA probe cocktail (Ventana Medical Systems) for ISH on a single slide. As recommended, HER2 IHC was performed first using the
iVIEW DAB IHC Detection Kit (Ventana Medical Systems). Thereafter, hybridization was performed using a cocktail of the 2,4 dinitrophenyl (DNP)–labeled HER2 probe and digoxigenin (DIG)–labeled chromosome 17 centromere (CEN17) probe. The HER2 gene and CEN17 signals were detected using the ultraView Silver ISH DNP Detection Kit and ultraView Red ISH DIG Detection Kit (Ventana Medical Systems), respectively. The HER2 gene signals were detected prior to CEN17 detection. The slides were counterstained with Hematoxylin II (Ventana Medical Systems).

**Signal Visualization**

Signal visualization was performed after scanning slides on a Pannoramic 250 digital scanner (3D HISTECH Ltd., Budapest, Hungary) and images scored using the software program ‘Case viewer’ (3D HISTECH Ltd., Budapest, Hungary) in which the HER2 gene appeared as discrete black signals and CEN17 as red signals in the nuclei, and the HER2 protein showed brown staining in the cell membranes. GPA slides were interpreted for both HER2 IHC and HER2 ISH according to the 2013 HER2 ASCO/CAP guidelines. Light microscope was used to decide on some cases.
2.8. Immunohistochemical Scoring

ER and PR
Tumors were considered positive for ER and PR when at least 1% of the tumor cells showed nuclear staining irrespective of intensity according to ASCO/CAP 2013 guidelines (Deyarmin et al., 2013).

HER2
HER2 was graded based on recommendations from Allred scoring system as described in the American College of Pathologists “Template for Reporting Results of Biomarker Testing of Specimens From Patients With Carcinoma of the Breast” by Fitzgibbons et al. (Fitzgibbons et al., 2014). Grading is based on the degree of membrane staining for HER2, on a scale of 0-3+. Grades of 0-1+ are considered negative, a grade of 2+ is equivocal, and a grade of 3+ is considered positive for HER2 labeling.

AR
Tumors were considered positive for AR when at least 1% of the tumor cells showed nuclear staining irrespective of intensity as described in (Asano et al., 2017, Asano et al., 2016)

Ki67
Malignant cells with nuclear staining were considered to be positive. Ki67 grading was based on the percentage of positively stained tumor cells among the total number of malignant cells assessed irrespective of staining intensity (Urruticoechea et al., 2005). A minimum of 500 cells were counted in hot spots. A Ki67 cut-off point of 20 % was defined as high according to the St. Gallen international panel of experts’ recommendation (Goldhirsch et al., 2013).

BRCA-1
Nuclear labeling of 10% or more of tumor cells irrespective of staining intensity was considered as positive expression (Kim et al., 2011, Vaz et al., 2007). Cytoplasmic only labeling was considered as negative.
**EBV**
Tumors were considered positive for EBV when the tumor cells showed membrane and cytoplasmic staining irrespective of intensity as described in (Agilent/Dako, 2012)

**HCMV**
Tumors were considered positive for HCMV when the tumor cells showed nuclear and cytoplasmic staining irrespective of intensity as described in(Agilent/Dako, 2012)

**P16**
Tumors were considered positive for p16 when the tumor cells showed nuclear and/or cytoplasmic staining irrespective of intensity as described in(BD Biosciences, 2017)

2.9. Molecular Sub-typing system

Breast carcinoma was classified into the following four sub-types according to St. Gallen international expert’s consensus 2013(Goldhirsch et al., 2013): luminal A (ER and/or PR-positive, HER2-negative and Ki67<20%), luminal B (ER and/or PR-positive, HER2-positive OR ER and/or PR-positive, HER2-negative and Ki67≥20%), HER2-enriched (ER and PR-negative, HER2 positive) and triple-negative (ER-negative, PR-negative and HER2-negative).
Chapter 3

Results

3.1. Patients and Tumor Specimen

There were 114 participants in this study. Mean age at diagnosis was 43 years (SD 13) and median age was 40 (range 22–75). Most of the study participants (40%) were < 40 years. About 31% of the study participants were ≥ 50 years and only 19 % were 40-49 years old. Table 3.1 and Table 3.2 show age distribution and pathological characteristics of the study subjects respectively.

Table 3.1 Age at diagnosis of the study participants (n=114)

<table>
<thead>
<tr>
<th>Age</th>
<th>Cases, n</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;30</td>
<td>16</td>
<td>14</td>
</tr>
<tr>
<td>30-39</td>
<td>30</td>
<td>26</td>
</tr>
<tr>
<td>40-49</td>
<td>22</td>
<td>19</td>
</tr>
<tr>
<td>50-59</td>
<td>19</td>
<td>17</td>
</tr>
<tr>
<td>&gt;=60</td>
<td>16</td>
<td>14</td>
</tr>
<tr>
<td>Missing</td>
<td>11</td>
<td>10</td>
</tr>
</tbody>
</table>
Table 3.2 Baseline Pathological characteristics of the study participants (n=114)

<table>
<thead>
<tr>
<th>Variables</th>
<th>N(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Histological Grade</strong></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>7(6)</td>
</tr>
<tr>
<td>II</td>
<td>32(28)</td>
</tr>
<tr>
<td>III</td>
<td>39(34)</td>
</tr>
<tr>
<td>Missing</td>
<td>36(32)</td>
</tr>
<tr>
<td><strong>Histological Type</strong></td>
<td></td>
</tr>
<tr>
<td>Infiltrating Ductal</td>
<td>67(59)</td>
</tr>
<tr>
<td>Lobular</td>
<td>6(5)</td>
</tr>
<tr>
<td>Others/Not classified</td>
<td>25(22)</td>
</tr>
<tr>
<td>Missing</td>
<td>16(14)</td>
</tr>
<tr>
<td><strong>Stage</strong></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>19(17)</td>
</tr>
<tr>
<td>II</td>
<td>37(32)</td>
</tr>
<tr>
<td>III</td>
<td>36(31)</td>
</tr>
<tr>
<td>IV</td>
<td>4(4)</td>
</tr>
<tr>
<td>Missing</td>
<td>18(16)</td>
</tr>
</tbody>
</table>
3.2. Molecular Classification

3.2.1. Result

ER, PR and HER2 status

Table 3.3 shows the ER, PR and HER2 status of the tumor specimen. ER positive tumors represent 66% of the cases. There was only one case which was PR + ER-. Figures 3.1, 3.2 and 3.3 shows representative image of tumors IHC positive for ER, PR and HER2. Figure 3.4 shows HER2 staining with scores of 0, 1+, 2+ and 3+.

<table>
<thead>
<tr>
<th>Variables</th>
<th>N(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Estrogen Receptor</strong></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>74(65)</td>
</tr>
<tr>
<td>Negative</td>
<td>40(35)</td>
</tr>
<tr>
<td><strong>Progesterone Receptor</strong></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>49(43)</td>
</tr>
<tr>
<td>Negative</td>
<td>64(56)</td>
</tr>
<tr>
<td>Missing</td>
<td>1(1)</td>
</tr>
<tr>
<td><strong>HER2</strong></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>26(23)</td>
</tr>
<tr>
<td>Negative</td>
<td>87(76)</td>
</tr>
<tr>
<td>Missing</td>
<td>1(1)</td>
</tr>
</tbody>
</table>
Figure 3.1 Representative images of ER positive immunohistochemistry in breast tumor tissue array. A, H&E staining (10X); B, immunostaining (10X); C, immunostaining of a specific area (40X)
Figure 3.2 Representative images of PR positive immunohistochemistry in breast tumor tissue array. A, H&E staining (10X); B, immunostaining (10X); C, immunostaining of a specific area (40X)
Figure 3.3 Representative images of HER2 positive immunohistochemistry in breast tumor tissue array. A, H&E staining (10X); B, immunostaining (10X); C, immunostaining of a specific area (40X)
**Figure 3.4** Scoring of HER2 staining. HER2 labeling was reported as Negative (0, no staining, A, 40X; and 1, weak staining, B, 40X), Equivocal 2 (moderate staining, C, 40X) and Positive 3 (strong staining, D, 40X).

Table 3.4 present the distribution of clinicopathological features in ER-positive and ER-negative tumors. There was no statistically significant difference in clinicopathological features between the two groups.

**Table 3.4** Association between ER and clinicopathological parameters of the study participants (n=114).

<table>
<thead>
<tr>
<th>Clinico-pathological parameters</th>
<th>ER Positive</th>
<th>ER Negative</th>
<th>Total</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Median age at Diagnosis(min-max)</strong></td>
<td>42(22-75)</td>
<td>41(27-65)</td>
<td>0.284</td>
<td></td>
</tr>
<tr>
<td><strong>Tumor Grade, n (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>5(10)</td>
<td>2(7)</td>
<td>7(9)</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>18(36)</td>
<td>14(50)</td>
<td>32(41)</td>
<td>0.480</td>
</tr>
<tr>
<td>III</td>
<td>27(54)</td>
<td>12(43)</td>
<td>39(50)</td>
<td></td>
</tr>
</tbody>
</table>
Molecular subtypes

A total of 112 cases had complete data concerning immunohistochemistry. These cases were classified according to St. Gallen international classification system 2013 (Goldhirsch et al., 2013). Table 3.5 shows distribution of the molecular subtypes. The most frequent molecular subtype was Luminal A (40%). Triple negative breast cancer represents 23% of all cases.

**Table 3.5** Distribution of the Molecular Subtypes of breast cancer among the study participants (n=114)
Table 3.6 and Figure 3.5 shows an earlier onset of luminal B compared to the other molecular subtypes and statistically significant variation in mean age at diagnosis between the different subtypes (P<0.05). It also reveals a decline in luminal B subtypes as patients age increase with the incidence of luminal A exceeding it at the age ranges >=50.

Table 3.6 Frequency distribution of the molecular subtypes in different age ranges among the study participants.

<table>
<thead>
<tr>
<th>Age at diagnosis</th>
<th>Molecular Subtypes</th>
<th>Total</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Luminal A</td>
<td>Luminal B</td>
<td>HER2-enriched</td>
</tr>
<tr>
<td>&lt;40</td>
<td>16</td>
<td>19</td>
<td>4</td>
</tr>
<tr>
<td>40-49</td>
<td>5</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>&gt;=50</td>
<td>18</td>
<td>4</td>
<td>2</td>
</tr>
</tbody>
</table>

Figure 3.5 Distribution of the molecular types of breast cancer in different age ranges. It shows a Luminal B peak in <40 age range and Luminal A peak in the age range >=50.
Table 3.7 shows the distribution of clinicopathological parameters in each molecular subtype. There was a statistically significant variation between median ages across the molecular subtypes. There was no statistically significant difference in the distribution of the other clinicopathological parameters between the different molecular subtypes of the tumors.

Table 3.7 Distribution of the Clinicopathological parameters in each molecular subtypes of breast cancer among the study participants.

<table>
<thead>
<tr>
<th>Clinicopathological Parameters</th>
<th>Luminal A (min-max)</th>
<th>Luminal B (min-max)</th>
<th>HER2-enriched (min-max)</th>
<th>Triple Negative (min-max)</th>
<th>Total (min-max)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age at diagnosis</td>
<td>47(22-75)</td>
<td>35(22-53)</td>
<td>41(27-65)</td>
<td>46(29-60)</td>
<td></td>
<td>0.009</td>
</tr>
<tr>
<td>Tumor grade, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>5(18)</td>
<td>0(0)</td>
<td>1(13)</td>
<td>1(6)</td>
<td>7(9)</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>11(39)</td>
<td>8(36)</td>
<td>5(63)</td>
<td>7(39)</td>
<td>31(41)</td>
<td>0.243</td>
</tr>
<tr>
<td>III</td>
<td>12(43)</td>
<td>14(64)</td>
<td>2(25)</td>
<td>10(55)</td>
<td>38(50)</td>
<td></td>
</tr>
<tr>
<td>Stage, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>9(24)</td>
<td>5(19)</td>
<td>1(11)</td>
<td>4(18)</td>
<td>19(20)</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>13(35)</td>
<td>13(48)</td>
<td>1(11)</td>
<td>9(41)</td>
<td>36(38)</td>
<td>0.632</td>
</tr>
<tr>
<td>III</td>
<td>14(38)</td>
<td>8(30)</td>
<td>6(67)</td>
<td>8(36)</td>
<td>36(38)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>1(3)</td>
<td>1(4)</td>
<td>1(11)</td>
<td>1(5)</td>
<td>4(4)</td>
<td></td>
</tr>
<tr>
<td>Histological type, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ductal</td>
<td>29(70)</td>
<td>15(60)</td>
<td>7(80)</td>
<td>17(80)</td>
<td>66(69)</td>
<td>0.708</td>
</tr>
<tr>
<td>Lobular</td>
<td>2(10)</td>
<td>4(10)</td>
<td>0(0)</td>
<td>0(0)</td>
<td>6(6)</td>
<td></td>
</tr>
<tr>
<td>Others/Not classified</td>
<td>9(20)</td>
<td>8(30)</td>
<td>2(20)</td>
<td>5(20)</td>
<td>24(25)</td>
<td></td>
</tr>
</tbody>
</table>
3.3. Androgen receptor Status

Result
A total of 112 cases had complete data concerning AR immunostaining. These cases were classified as positive and negative as previously done by (Asano et al., 2017). Out of 112 participants, 91 (81%) were positive for AR expression. The remaining 20 participants (19%) were negative for AR expression. Figure 3.6 shows representative image of tumors IHC positive for AR.

Figure 3.6 Representative images of AR positive immunohistochemistry in breast tumor tissue array. A, H&E staining (10X); B, immunostaining (10X); C, immunostaining of a specific area (40X)
Table 3.8 and 3.9 shows the distribution of clinicopathological and molecular parameters in AR expressing and non expressing tumors. There was no statistically significant difference in the distribution of the clinicopathological parameters between AR expressing and non expressing tumors.

**Table 3.8** Association between AR expression and clinicopathological parameters among the study participants

<table>
<thead>
<tr>
<th>Clinico-pathological parameters</th>
<th>AR Negative</th>
<th>AR Positive</th>
<th>Total</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age at Diagnosis(min-max)</td>
<td>44(29-70)</td>
<td>40(22-75)</td>
<td></td>
<td>0.363</td>
</tr>
<tr>
<td>Tumor Grade, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>1(6)</td>
<td>6(10)</td>
<td>7(9)</td>
<td>0.774</td>
</tr>
<tr>
<td>II</td>
<td>8(47)</td>
<td>23(39)</td>
<td>31(39)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>8(47)</td>
<td>30(51)</td>
<td>38(48)</td>
<td></td>
</tr>
<tr>
<td>Stage, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>3(16)</td>
<td>16(21)</td>
<td>19(20)</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>5(26)</td>
<td>30(41)</td>
<td>35(38)</td>
<td>0.507</td>
</tr>
<tr>
<td>III</td>
<td>10(53)</td>
<td>26(34)</td>
<td>36(38)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>1(3)</td>
<td>3(4)</td>
<td>4(4)</td>
<td></td>
</tr>
<tr>
<td>Histological Type, n (%)</td>
<td></td>
<td></td>
<td></td>
<td>0.169</td>
</tr>
<tr>
<td>Infiltrating ductal</td>
<td>17(85)</td>
<td>49(65)</td>
<td>66(69)</td>
<td></td>
</tr>
<tr>
<td>Lobular</td>
<td>0(0)</td>
<td>6(8)</td>
<td>6(6)</td>
<td></td>
</tr>
<tr>
<td>Others/Unknown</td>
<td>3(15)</td>
<td>21(27)</td>
<td>24(25)</td>
<td></td>
</tr>
</tbody>
</table>
Table 3.9 Association between AR and molecular parameters among the study participants

<table>
<thead>
<tr>
<th>Molecular parameters</th>
<th>AR Negative</th>
<th>AR Positive</th>
<th>Total</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>15(75)</td>
<td>23(25)</td>
<td>38(34)</td>
<td>0.000*</td>
</tr>
<tr>
<td>Positive</td>
<td>5(25)</td>
<td>68(75)</td>
<td>73(66)</td>
<td></td>
</tr>
<tr>
<td>PR, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>19(95)</td>
<td>44(48)</td>
<td>63(53)</td>
<td>0.000*</td>
</tr>
<tr>
<td>Positive</td>
<td>1(5)</td>
<td>47(52)</td>
<td>48(43)</td>
<td></td>
</tr>
<tr>
<td>HER2, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>17(89)</td>
<td>67(74)</td>
<td>84(76)</td>
<td>0.145</td>
</tr>
<tr>
<td>Positive</td>
<td>2(11)</td>
<td>24(26)</td>
<td>26(24)</td>
<td></td>
</tr>
<tr>
<td>Molecular Subtype, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Luminal A</td>
<td>4(21)</td>
<td>41(45)</td>
<td>45(41)</td>
<td></td>
</tr>
<tr>
<td>Luminal B</td>
<td>1(5)</td>
<td>28(31)</td>
<td>29(26)</td>
<td>0.000*</td>
</tr>
<tr>
<td>HER2-enriched</td>
<td>1(5)</td>
<td>10(11)</td>
<td>11(10)</td>
<td></td>
</tr>
<tr>
<td>TNBC/basal-like</td>
<td>13(68)</td>
<td>12(13)</td>
<td>25(23)</td>
<td></td>
</tr>
</tbody>
</table>

*P≤0.05
3.4. BRCA-1 Expression

Result
A total of 77 cases had complete data concerning BRCA-1 immunostaining. These cases were classified as normal (positive expression) and abnormal (loss of expression) according to (Kim et al., 2011, Vaz et al., 2007). Out of 77 participants, 22 (29%) were negative for BRCA1 expression. The remaining 50 participants (71%) were positive for BRCA1 expression. Figure 3.7 shows representative image of tumors IHC stained for BRCA-1.

Figure 3.7 Representative images of BRCA1 immunostaining (no staining, A, 20X), (weak staining, B, 20X), (moderate staining, C, 20X) and (strong staining, D, 40X).
Table 3.10 and 3.11 shows the distribution of clinicopathological and molecular parameters in BRCA-1 expressing and non expressing tumors. There was no statistically significant difference in the distribution of the clinicopathological parameters between BRCA-1 expressing and non expressing tumors.

Table 3.10 Distribution of clinicopathological parameters in BRCA-1 expressing (normal) and non-expressing (abnormal) tumors among the study participants.

<table>
<thead>
<tr>
<th>Clinico-pathological parameters</th>
<th>BRCA-1 Normal</th>
<th>BRCA-1 Abnormal</th>
<th>Total</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age at Diagnosis(min-max)</td>
<td>41(24-75)</td>
<td>40(22-65)</td>
<td></td>
<td>0.492</td>
</tr>
<tr>
<td><strong>Tumor Grade, n (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>4(9)</td>
<td>0(0)</td>
<td>4(7)</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>18(42)</td>
<td>7(64)</td>
<td>25(46)</td>
<td>0.330</td>
</tr>
<tr>
<td>III</td>
<td>21(49)</td>
<td>14(36)</td>
<td>25(46)</td>
<td></td>
</tr>
<tr>
<td><strong>Stage, n (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>11(23)</td>
<td>7(47)</td>
<td>18(29)</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>19(40)</td>
<td>3(20)</td>
<td>22(36)</td>
<td>0.224</td>
</tr>
<tr>
<td>III</td>
<td>16(34)</td>
<td>4(27)</td>
<td>20(32)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>1(2)</td>
<td>1(7)</td>
<td>2(3)</td>
<td></td>
</tr>
<tr>
<td><strong>Histological Type, n (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td>0.763</td>
</tr>
<tr>
<td>Infiltrating ductal</td>
<td>33(67)</td>
<td>11(73)</td>
<td>44(69)</td>
<td></td>
</tr>
<tr>
<td>Lobular</td>
<td>2(4)</td>
<td>1(7)</td>
<td>3(5)</td>
<td></td>
</tr>
<tr>
<td>Others/Unknown</td>
<td>14(29)</td>
<td>3(20)</td>
<td>17(27)</td>
<td></td>
</tr>
</tbody>
</table>
Table 3.11 Association between BRCA-1 and molecular parameters among the study participants.

<table>
<thead>
<tr>
<th>Molecular parameters</th>
<th>BRCA-1 Normal</th>
<th>BRCA-1 Abnormal</th>
<th>Total</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ER, n (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>18(33)</td>
<td>10(45)</td>
<td>28(36)</td>
<td>0.294</td>
</tr>
<tr>
<td>Positive</td>
<td>37(67)</td>
<td>12(55)</td>
<td>49(64)</td>
<td></td>
</tr>
<tr>
<td><strong>PR, n (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>30(56)</td>
<td>14(64)</td>
<td>44(58)</td>
<td>0.518</td>
</tr>
<tr>
<td>Positive</td>
<td>24(44)</td>
<td>8(36)</td>
<td>32(42)</td>
<td></td>
</tr>
<tr>
<td><strong>HER2, n (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>43(80)</td>
<td>15(73)</td>
<td>58(78)</td>
<td>0.287</td>
</tr>
<tr>
<td>Positive</td>
<td>11(20)</td>
<td>7(27)</td>
<td>18(22)</td>
<td></td>
</tr>
<tr>
<td><strong>Ki67, n (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>30(56)</td>
<td>16(73)</td>
<td>46(61)</td>
<td>0.165</td>
</tr>
<tr>
<td>High</td>
<td>24(44)</td>
<td>6(27)</td>
<td>30(39)</td>
<td></td>
</tr>
<tr>
<td><strong>AR, n (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>12(22)</td>
<td>4(19)</td>
<td>16(21)</td>
<td>0.763</td>
</tr>
<tr>
<td>Positive</td>
<td>42(78)</td>
<td>17(81)</td>
<td>59(79)</td>
<td></td>
</tr>
<tr>
<td><strong>Molecular Subtype, n (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Luminal A</td>
<td>22(42)</td>
<td>8(36)</td>
<td>30(40)</td>
<td></td>
</tr>
<tr>
<td>Luminal B</td>
<td>14(26)</td>
<td>6(27)</td>
<td>20(27)</td>
<td>0.862</td>
</tr>
<tr>
<td>HER2-enriched</td>
<td>4(8)</td>
<td>3(14)</td>
<td>7(9)</td>
<td></td>
</tr>
<tr>
<td>TNBC/basal-like</td>
<td>13(25)</td>
<td>5(23)</td>
<td>18(24)</td>
<td></td>
</tr>
</tbody>
</table>
3.5. Ki67 Expression

Result
A total of 113 cases had complete data concerning Ki67 immunostaining. These cases were classified as low proliferation (Ki67 < 20%) and high proliferation (Ki67 ≥ 20%) according to the St. Gallen international experts recommendation (Goldhirsch et al., 2013). Out of these 113 participants, 69 (61%) had low proliferation (Ki67 < 20%). The remaining 44 participants (39%) had high proliferation (Ki67 < 20%). Figure 3.8 shows representative image of tumors IHC stained for Ki67.

Figure 3.8 Representative images of Ki67 immunostaining (Low proliferation, A, 10X), (Low proliferation, B, 40X), (High proliferation, C, 10X) and (High proliferation, D, 40X).
Table 3.12 shows the distribution of clinicopathological parameters in highly proliferating (Ki67 high) and slowly proliferating (low Ki67) tumors. There was statistically significant difference in the median age at diagnosis between highly proliferating and slowly proliferating tumors. There was also statistically significant difference in tumor grade between highly proliferating and slowly proliferating tumors. There was no statistically significant difference in the distribution of the other clinicopathological parameters between highly proliferating and slowly proliferating tumors.

Table 3.12 Distribution of clinicopathological parameters in slowly proliferative (Ki67 low) and highly proliferative (Ki67 high) tumors among the study participants.

<table>
<thead>
<tr>
<th>Clinico-pathological parameters</th>
<th>Ki67 low</th>
<th>Ki67 high</th>
<th>Total</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age at Diagnosis (min-max)</td>
<td>42(22-75)</td>
<td>37(25-58)</td>
<td></td>
<td>0.034</td>
</tr>
<tr>
<td>Tumor Grade, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>7(16)</td>
<td>0(0)</td>
<td>7(9)</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>20(44)</td>
<td>12(38)</td>
<td>32(42)</td>
<td>0.029</td>
</tr>
<tr>
<td>III</td>
<td>18(40)</td>
<td>20(62)</td>
<td>38(49)</td>
<td></td>
</tr>
<tr>
<td>Stage, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>12(21)</td>
<td>7(18)</td>
<td>19(20)</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>22(39)</td>
<td>14(37)</td>
<td>36(38)</td>
<td>0.959</td>
</tr>
<tr>
<td>III</td>
<td>21(37)</td>
<td>15(39)</td>
<td>36(38)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>2(4)</td>
<td>2(5)</td>
<td>4(4)</td>
<td></td>
</tr>
<tr>
<td>Histological Type, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infiltrating ductal</td>
<td>33(67)</td>
<td>11(73)</td>
<td>44(69)</td>
<td>0.763</td>
</tr>
<tr>
<td>Lobular</td>
<td>2(4)</td>
<td>1(7)</td>
<td>3(5)</td>
<td></td>
</tr>
<tr>
<td>Others/Unknown</td>
<td>14(29)</td>
<td>3(20)</td>
<td>17(27)</td>
<td></td>
</tr>
</tbody>
</table>
Table 3.13 shows the distribution of molecular characteristics in highly proliferating (Ki67 high) and slowly proliferating (low Ki67) tumors. There was statistically significant difference in the distribution of molecular subtypes between highly proliferating and slowly proliferating tumors. The association between ER status and Ki67 did not reach the level of statistical significance (0.074). There was no statistically significant difference in the distribution of the other molecular parameters between highly proliferating and slowly proliferating tumors.

Table 3.13 Association between Ki67 and molecular parameters among the study participants.

<table>
<thead>
<tr>
<th>Molecular parameters</th>
<th>Ki67 low</th>
<th>Ki67 high</th>
<th>Total</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ER, n (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>20(29)</td>
<td>20(45)</td>
<td>40(35)</td>
<td>0.074</td>
</tr>
<tr>
<td>Positive</td>
<td>49(71)</td>
<td>24(55)</td>
<td>73(65)</td>
<td></td>
</tr>
<tr>
<td><strong>PR, n (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>37(54)</td>
<td>27(61)</td>
<td>64(57)</td>
<td>0.418</td>
</tr>
<tr>
<td>Positive</td>
<td>32(46)</td>
<td>17(39)</td>
<td>49(43)</td>
<td></td>
</tr>
<tr>
<td><strong>HER2, n (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>53(78)</td>
<td>33(75)</td>
<td>86(77)</td>
<td>0.719</td>
</tr>
<tr>
<td>Positive</td>
<td>15(22)</td>
<td>11(25)</td>
<td>26(23)</td>
<td></td>
</tr>
<tr>
<td><strong>AR, n (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>12(18)</td>
<td>9(21)</td>
<td>21(19)</td>
<td>0.667</td>
</tr>
<tr>
<td>Positive</td>
<td>56(82)</td>
<td>34(79)</td>
<td>90(81)</td>
<td></td>
</tr>
<tr>
<td><strong>Molecular Subtype, n (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Luminal A</td>
<td>45(42)</td>
<td>0(36)</td>
<td>45(40)</td>
<td></td>
</tr>
<tr>
<td>Luminal B</td>
<td>5(26)</td>
<td>25(27)</td>
<td>30(27)</td>
<td></td>
</tr>
<tr>
<td>HER2-enriched</td>
<td>7(8)</td>
<td>4(14)</td>
<td>11(9)</td>
<td></td>
</tr>
<tr>
<td>TNBC/basal-like</td>
<td>11(25)</td>
<td>15(23)</td>
<td>26(24)</td>
<td></td>
</tr>
</tbody>
</table>

*P<0.05
3.6. Comparison of Immunohistochemistry with Gene Protein Assay (GPA) for assessment of HER2 status

Result

A total of 108 patients fulfilled all criteria for this study. These cases were classified as per recommendations by ASCO/CAP. We found that, using IHC, in 73 cases (68%) the HER2 expression was at level 0 or 1+, in 12 cases (11%) the HER2 expression was at level 2+ and in 23 cases (21%) the HER2 expression was at level 3+, respectively. Twenty six of the 108 cases (24.1%) were judged as positive by the GPA test (95% CI: 16.4–33.3%). The concordance rate between the results of IHC and GPA in all cases was 97.2% (95% CI: 92.1–99.4%). Table 4.11 shows the concordance of HER2 IHC assessment with GPA. Figure 3.9 shows representative image of tumors positive for protein and gene of HER2 using the GPA.

Table 3.14 Concordance between the results of IHC and GPA in breast tumors among the study participants.

<table>
<thead>
<tr>
<th>HER2 IHC Score</th>
<th>HER2 GPA</th>
<th>Concordance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-amplified</td>
<td>Amplified</td>
</tr>
<tr>
<td>0 or 1+ (n=73)</td>
<td>71</td>
<td>2</td>
</tr>
<tr>
<td>2+ (n=12)</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>3+ (n=23)</td>
<td>0</td>
<td>23</td>
</tr>
<tr>
<td>Total (n=108)</td>
<td>82</td>
<td>26</td>
</tr>
</tbody>
</table>
Figure 3.9 Representative images of HER2 GPA (Gene amplified and protein expressed, A, 10X), (Gene amplified and protein expressed, B, 20X), (Gene amplified and protein expressed, C, 40X) and (Gene amplified and protein expressed, D, 100X).
3.7. Assessment of Association between EBV, HCMV and HPV with Breast Cancer

A total of 114 patients fulfilled all criteria for the EBV study. All of the 114 cases (100%) were judged as negative for EBV viral protein by the IHC test. Similarly a total of 114 patients fulfilled all criteria for the HCMV study and all of the 114 cases (100%) were judged as negative for HCMV viral protein by the IHC test. Out of 114 cases, a total of 82 cases had valid result for HPV and in the remaining were rejected because of undetected internal control. Out of the 82 valid tests 2 were found to be positive (2.4%). One of the cases was positive for high risk HPV16 genotype while the other was positive both for high risk HPV39 and low risk HPV6. P16 protein staining in the two HPV positive cases was positive for the patient with HPV16 detection and weakly positive for case with HPV39 and HPV6 co-infection (Figure 2). Table 4.12 shows the distribution of the viral detection. Figure 3.10 shows IHC staining for p16 in the two cases positive for HPV DNA.

<table>
<thead>
<tr>
<th>Type of Virus</th>
<th>No of cases</th>
<th>Sample</th>
<th>Detection Method</th>
<th>Negative</th>
<th>Positive</th>
<th>Percentage positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBV</td>
<td>114</td>
<td>FFPE</td>
<td>IHC</td>
<td>114</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HCMV</td>
<td>114</td>
<td>FFPE</td>
<td>IHC</td>
<td>114</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HPV</td>
<td>82</td>
<td>DNA</td>
<td>qPCR</td>
<td>80</td>
<td>2</td>
<td>2.4</td>
</tr>
</tbody>
</table>

Table 3.15 EBV, HCMV and HPVs detection in human breast carcinomas among the study participants.
Figure 3.10 p16 Immunostaining of HPV+ breast tumors. A, H&E staining of the HPV39 & HPV6 positive case. B, Faint; diffuse nuclear and cytoplasmic p16 immunostaining of the HPV39 & HPV6 positive case. C, H&E staining of the HPV16 positive case. A, Strong; diffuse nuclear and cytoplasmic p16 immunostaining of the HPV16 positive case.
Chapter 4
Discussion

4.1. Discussion

Molecular Classification

Progress in molecular research have led to the classification of breast cancers into distinct subgroups (luminal, normal breast-like, Her2/neu-positive and basal-like subtypes) based on mRNA expression profiles (Badve et al., 2011). However, gene expression profiling using cDNA microarray or RNA seq technology is not currently feasible in clinical settings due to its high cost and technical complexity. Therefore, IHC markers have been validated and used as surrogates for cDNA microarray in molecular subtyping of breast cancer (Gruver et al., 2011).

In the present study, we found that luminal A subtype was the most prevalent followed by luminal B subtype, Triple Negative, and HER2-enriched derived exclusively. Our findings, from TMA IHC, are in contrast to most other studies performed in standard paraffin sections, in tissues from Sub-Saharan Africa particularly in west and central African countries where triple negative phenotype was reported to be the most common molecular subtype (43-82%) of breast cancer (Brinton et al., 2014b). In western countries, 30-70% of breast cancers are luminal A tumors and our result (40%) is comparable to the distribution seen in the western societies (Komen, 2016). However, Luminal B which is the second most common molecular subtype in our study (26%) is present at higher proportion among our study participants when compared to the rate seen in the Western high resource regions (10-20%) (Komen, 2016).

A previous study among Ethiopian women carried out by Kantelhardt (Kantelhardt et al., 2014) in 2014 compared ER negativity in 352 patients out of 1208 consecutive patients treated at Addis Ababa-University Hospital, Ethiopia, from June 2005 through December 2010. They reported ER negative tumors to be around 35% in agreement with our present results (Kantelhardt et al., 2014). A comparable result was also reported in a recent study done by groups at the University of Michigan in 2016 from one of the referral hospitals in Addis Ababa where they found ER negative tumors to be 26.5% (Jiagge et al., 2016).
A study among women of East African origin in US, in which a majority of the subjects (74%) were Ethiopians living in the United States, reported estrogen receptor negative tumors at 22% (Jemal and Fedewa, 2012). Similarly, a recent study in Kenya reported estrogen receptor negative tumor at 27.2% (Sayed et al., 2014). These few studies among East Africans, as well as our present study, suggest the existence of geographical diversity in the distribution of molecular subtypes of breast cancer in Sub-Saharan Africa. The findings indicate that the frequency of ER negative tumors in East Africa may not be different from that found in the Western countries which is between 20 to 30% (Yersal and Barutca, 2014).

Similarly the frequencies of triple negative tumors in these East African studies were lower than reported in west and central Africa. TNBC in the Kenyan study was reported in 20.2% of all cases (Sayed et al., 2014) and TNBC in the Michigan study among Ethiopian women was reported to be around 15% which is lower than our finding (23%), but all of these studies indicate TNBC tumors to be less common than reported elsewhere in Africa. Hence, taken together, the data from East Africa suggest that it would not be accurate to associate African ancestry with increased probability of diagnosis with ER negative or TNBC tumors, as the Sub-Saharan African populations are known to be themselves highly heterogeneous in lifestyles, exposures, and genetic admixtures.

Present study has shown HER2 positivity at a slightly lower proportion than the rate seen among Ethiopians in the Michigan study (Jiagge et al., 2016) study (33%), the only other study (to our knowledge) which has incorporated HER2 assessment for molecular phenotyping of breast cancer from Ethiopia. However, HER2 positivity in our study (23%) is comparable to white Americans, African Americans, and West Africans which is about 17%, 19% and 20% respectively(Jiagge et al., 2016).

Our result shows an earlier onset of luminal B compared to the other molecular subtypes with statistically significant difference in median age at diagnosis (P<0.05). The median age at diagnosis in our study also reveals a decline in luminal B subtypes as patients’ age increase with the incidence of luminal A exceeding that of luminal B at age ranges >=50. This finding is comparable to studies in the west where a bimodal age distribution at diagnosis is seen where incidence of the more aggressive phenotype luminal B peaks at earlier ages whereas luminal A type peaks at older ages(Sweeney et al., 2014).
The median age at diagnosis in our study was comparable to most previous studies in Africa (Brinton et al., 2014a) and the few studies done among breast cancer patients in Ethiopia (Kantelhardt et al., 2014, Jiagge et al., 2016). However, it is much lower than the median age at diagnosis in most Western countries which is 55-60 years (Leong et al., 2010). The difference could be explained by the fact that African nations have younger population pyramids and the proportions reported in this and earlier studies are not age adjusted so the distributions are expected to always be shifted to younger ages in African cases compared to the cases in western countries; only 5% of African population is older than 60 years as compared to 24% of the population of Europe (Nations, 2015) being > 60 years old. More extensive population based registries in Africa, with active registration of cases, are urgently needed to understand the burden of cancer and to aid in cancer control program. Gaining a better understanding of environmental and lifestyle risk factors is crucial, as the incidence of breast cancer appears to be increasing at all ages in all African regions.

There was no statistically significant difference between median age at diagnosis and the ER status of breast cancer in this study. In this study, invasive ductal carcinoma was the predominant histological type (60%), which is comparable to the study by Kantelhardt in 2014 (Kantelhardt et al., 2014). No statistically significant correlation was found between the molecular subtypes of breast cancer and histological type of breast cancer in this study. High tumor grade (Grade III) was reported in 34% of our study participants which is comparable with the study done by Kantelhardt (Kantelhardt et al., 2014). The increased rate of high grade tumors observed in this study is in part due to late diagnosis of breast cancer. No statistically significant correlation was found between the molecular subtypes of breast cancer and tumor grade in this study. The numbers of cases in each category were likely too small to appreciate differences, if they exist.
Androgen Receptor Status

In breast cancer, a number of known and unknown mechanisms may play critical roles in carcinogenesis, progression and metastasis, which may be related to breast cancer outcomes. Some of the well known predictive or prognostic factors in breast cancer include ER and HER2 (Bertelli et al., 2013). However, it is important to identify and validate new biomarkers for better prediction and prognostication. The role of AR in breast cancer is still uncertain but there are an increasing number of evidences supporting a role for AR in the pathogenesis and outcome of breast cancer. Approximately 70% to 90% of ER-positive tumors are also AR positive (Collins et al., 2011, Hu et al., 2011). It has been documented that AR expression is related to positive prognostic factors, including smaller tumor size, lack of lymph node metastasis, lower histologic grade and that it serves as a prognostic and predictive factor in breast cancer in women with ER-positive breast cancer (Collins et al., 2011, Iacopetta et al., 2012).

In HER2-positive breast cancer, AR positivity was associated with a higher frequency of ER and progesterone receptor expression, smaller tumor size, earlier clinical stage, and lower Ki67 level compared with AR negativity. Approximately 60% of HER2-positive breast cancers overexpress AR. These findings suggest that HER2 and AR coexpression might be associated with less aggressive tumor subtype (Kono et al., 2017, Lin Fde et al., 2012). Approximately 10% to 35% of TNBCs overexpresses AR (McNamara and Sasano, 2016, McNamara et al., 2013, Asano et al., 2017). Studies show, among TNBC patients, disease-free survival was significantly better in patients with AR-positive tumors than in those with AR-negative tumors and has been indicated that AR might have a role as a prognostic marker and a therapeutic target in this subgroup (Thike et al., 2014, Kono et al., 2017).

In our study, AR was expressed in 80% of breast cancers, which is higher than the expression rates of both ER and PR. This result is comparable to previous studies conducted in other parts of the world (70-90%) (Collins et al., 2011, Hu et al., 2011). Our study also showed 93% of ER positive breast cancers express AR. There was a statistically significant variation (P<0.05) in the proportion of AR positivity between ER-positive (93%) and ER-negative tumors (60%). This result is in line with previous observations and indicates majority of ER positive tumors in this cohort co-express AR which according to accumulating evidences has beneficiary role (Hu et al., 2011, Collins et al., 2011). There was a statistically significant variation (P<0.05) in the
proportion of AR positive cases between PR-positive (98%) and PR-negative (70%) tumors as well. This is also in line with previous observations (Hu et al., 2011).

There was no statistically significant variation in the expression of AR between HER2-positive and HER2-negative tumors in this study (P=0.145). About 83% of HER2+/ER- tumors were positive for AR in this study which is higher than previous studies (around 60%) (Kono et al., 2017, Thike et al., 2014). The reason for this difference could be due to small number of HER2+/ER- tumors (only 12) in our study. The expression of AR among TNBC in our study was 48% which is slightly higher than previous reports (McNamara et al., 2013, Asano et al., 2017) but significantly different (P<0.05) than the other molecular subtypes. No statistically significant correlation was found between the clinicopathological parameters and AR expression.

**BRCA1 Expression**

Approximately 5–10% of all breast cancers occur due to the inheritance of deleterious mutations in rare predisposition genes, such as BRCA1 and BRCA2(Dutil et al., 2012, Lecarpentier et al., 2012). Many studies have shown that, the risk of cancer in a BRCA mutation carrier is significant, and knowledge of mutation status in individuals at potentially increased risk of a BRCA mutation may impact healthcare decisions to reduce risk(Kobayashi et al., 2013). Moreover, oncogenetic testing is becoming the powerful therapeutic predictive tool, as new targeted therapeutic opportunities, such as poly(ADP ribose) (PARP) inhibitors emerge and chemosensitivity to platinum-based therapy is constantly reported(Kobayashi et al., 2013).

BRCA genetic analysis which is the first choice to detect mutation is cumbersome, expensive, and may be impractical as a screening method for detection in all patients. Moreover, patients with BRCA promoter methylation and other mechanisms of loss are not identified using current approaches. Immunohistochemistry for BRCA1 may be a useful initial screening test to select patients for targeted therapy and to detect patients at risk for hereditary breast and ovarian cancer syndromes(Garg et al., 2013, Roehe et al., 2012).
In our study, we investigated the immunohistochemical expression of BRCA1 proteins in invasive breast cancer. We found that out of 77 participants 22 cases (29 %) had decreased or loss of BRCA-1 protein expression. There is no published African study to our knowledge to compare to our results but other studies done elsewhere reported 30-59% loss of BRCA-1 protein expression in invasive breast carcinoma(Kim et al., 2011, Rakha et al., 2008, Hedau et al., 2015).

To evaluate the relation between BRCA1 IHC expression and molecular parameters in breast cancer, we assessed its expression in the different categories based on ER, HER2, AR, Ki67 and molecular classification (as define based on ER, PR, HER2 and Ki67 as per recommendation from the St. Gallen expert panels (Goldhirsch et al., 2013)). No association was found between altered nuclear BRCA1 expression and ER expression. However, even though not statistically significant (P=0.294) 10 out of 28 cases in ER negative tumors (36% ) showed altered BRCA1 nuclear expression while 12 out of 49 ER positive tumors (25%) showed altered BRCA1 nuclear expression. Our results showed that BRCA-1 expression was not associated with AR, HER2 and Ki67. There was no statistically significant difference in BRCA-1 expression among the molecular subtypes of breast cancer in this study. No association was found with age at diagnosis, Tumor grade, clinical stage and histological type of tumor.

**Ki67 Expression**

The absence of Ki67 protein in resting cells and its expression in all proliferating cells, whether normal or tumor cells, makes the Ki67 antibody a great tool for determining the growth fraction of any given human cell population. Ki67 is associated with the common histopathologic parameters of breast cancer, and a strong correlation exists between Ki67 expression and histological grading because both parameters are associated with proliferation. Higher Ki67 expression was found in lymph node metastases than in primary breast tumors, which correlated with shorter survival in patients(Juríková et al., 2016, Kim et al., 2015).
Another correlation was noted between ER and Ki67 expression. ER status was inversely correlated with Ki67 expression, indicating that tumors with the lowest proliferative activity have the highest rates of ER positivity (Inwald et al., 2013). Many studies have shown that Ki67 is an important predictive and prognostic marker of survival and tumor recurrence in breast cancer, and it may reflect the aggressiveness of this disease. High Ki67 is also associated with poor disease-free survival and overall survival (Inwald et al., 2013, Juríková et al., 2016).

In this study, we investigated the immunohistochemical expression of Ki67 proteins in invasive breast cancer. We found that out of 113 participants 44 cases (39%) had high Ki67 protein expression. This is different with studies from other parts of Africa and the Middle East (Agboola et al., 2013, Elkablawy et al., 2016, Ermiah et al., 2012). The study from Nigeria have reported a high Ki67 in 82.6% of cases, Libyan group reported high Ki67 in 76% patients and a group from Saudi reported around 74% high Ki67. The difference can be due to true biological difference between our patients and the others; however the difference can also partially be explained due to variation in the cut-off points used to dichotomize patients as high proliferation and low proliferation in the different studies. We used a 20% cut-off point; however the Nigerian and the Libyan groups used 10% cut-off to dichotomize patients as high proliferation and low proliferation. The Saudi study used 25% to divide the patients as low and high Ki67 expression. Our finding however is comparable to Caucasians and Hispanic in Guth et al. (Guth et al., 2017) study. They compared Ki67 expression among Caucasians, African-Americans, Asian and Hispanic and found high Ki67 (defined as ≥10% positive nuclei) in 45%, 64%, 62% and 48% respectively (Guth et al., 2017).

In the present high Ki67 was found to be significantly associated with median age (P<0.05). The median age at diagnosis for participants with high proliferation were 37 years compared to 42 years for participants with low proliferation. High tumor grade were associated with high proliferation (P<0.05). The association of age and tumor grade is consistent with findings of many previous studies regarding the predictive and prognostic role of Ki67 in Breast cancer (Elkablawy et al., 2016, Inwald et al., 2013, Juríková et al., 2016).
A number of previous studies have also reported positive association between high Ki67 and ER status (Pathmanathan et al., 2014). In the present study, we did find some association of Ki67 with ER; however, the association did not reach the level of statistical significance (P=0.074). This may be because of small sample size and sampling bias in our study. There was also statistically significant variation between the molecular phenotypes; we found that the Luminal B group had the highest (83%) proliferation, followed by the TNBC (58%), then the HER2 enriched group (36%). Whereas all Luminal A subtypes had low Ki67. This finding of high Ki67 in the luminal B group and TNBC with lowest proliferation in the Luminal A group has also been confirmed by other studies (Stathopoulos et al., 2014).

**Comparison of IHC with GPA for assessment of HER2 status**

HER2 status is usually assessed by immunohistochemistry (IHC) for HER2 protein overexpression or by fluorescence in situ hybridization (FISH) for HER2 gene amplification. Both methods are approved by the US Food and Drug Administration (FDA). IHC is used primarily because it is easier to perform and relatively inexpensive. FISH is used as a reflex test on IHC 2+ cases by most laboratories as per the 2013 ASCO/CAP recommendation (Wolff et al., 2013). FISH results have been accepted as a gold standard in the assessment of HER2 status. However, FISH uses dark-field fluorescence microscopy and lacks morphology details. Furthermore, the fluorescent signals fade over time; therefore, the slides cannot be stored for a long time. The bright-field in situ hybridization (BISH) methods, including chromogenic in situ hybridization (CISH) and silver in situ hybridization (SISH) have been developed to overcome some of the limitations of FISH. Studies show high level of agreement between CISH and FISH genetic testing with respect to assay performance which makes the two techniques equivalent, in a technical perspective (Poulsen et al., 2013).
The gene protein assay (GPA) described in this study merges HER2 IHC and BISH to assess HER2 status in tumor cells by correlating with tissue morphology. A few comparative studies comparing GPA with IHC alone and FISH alone have shown concordance exceeding 90% by using tissue microarray (TMA) slides (Hirschmann et al., 2012, Nitta et al., 2012). In this study, HER2 protein overexpression was demonstrated in 21.3% of formalin-fixed paraffin-embedded specimens of surgically resected breast cancers in TMA slides, and HER2 gene amplification was demonstrated in 24%. The concordance rate between the results of IHC and GPA in all cases was 97.2%. The concordance rate of IHC and GPA was high in cases that were 3+ according to IHC (100%), but slightly lower in cases that were 2+ according to IHC (91.7%). Overall, our result shows a strong concordance between IHC and GPA. Our finding is comparable to previous studies (Hou et al., 2017, Nitta et al., 2012).

**Assessment of Association between EBV, HCMV and HPV with Breast Cancer**

Researches into breast cancer etiology have focused primarily on reproductive and other factors affecting circulating sex hormones and on genetic susceptibility. However, as identified risk factors are thought to explain the incidence in about half of all breast cancer cases only, researchers are motivated to consider other routes for disease pathogenesis. Viruses have been implicated in the development of various cancers, but they have not been much considered for breast cancer. Recent investigations have linked breast cancer to viral infections, such as Epstein–Barr virus (EBV), mouse mammary tumor virus (MMTV), Human papillomavirus (HPV) and Human cytomegalovirus (HCMV)(Akhter et al., 2014, Lawson et al., 2016a, Richardson et al., 2015). The relationship between breast cancer and viruses could be of potential importance not only for better understanding of breast cancer etiology, but also for early detection and prevention of breast cancer and treatment(Alibek et al., 2013).

In this study, we explored the presence of EBV, HCMV and 19 high-risk HPV types (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 69, 73, 82) and 9 low-risk HPV types(6, 11, 40, 42, 43, 44, 54, 61, 70). Multiplex qPCR for HPV genotyping and tissue microarray methodologies using specific monoclonal antibodies were used for detection of EBV and HCMV. There was no detection of HCMV and EBV in this study. The role of EBV and CMV in breast cancer is controversial. A recent meta-analysis done by Richardson et al., looked at 11
different studies on CMV and showed 9 out of 11 to (82%) report positivity for CMV between 2 to 50% (Richardson et al., 2015). Similarly, these authors reviewed 54 different studies for EBV and found out 12 out of 54 (22.2%) studies across all assay types detecting positivity. In our study, 2 out of 82 (2.4%) samples were positive for HPVs. Out of the two cases positive for HPV one case had the high risk HPV16 genotype while the other were dual positive for the high risk HPV39 and low risk HPV6.

Like HCMV and EBV, the association of HPV with breast cancer is controversial and is an area of continues research. A review of 24 different case-control studies by Lowsen et al. indicate that HPVs are detected from 2 to 74% of all case (Lawson et al., 2016b). High risk HPV16 and HPV18 are more commonly detected than the other genotypes. High risk HPV39 which is detected in our study was not found in any of these studies (Lawson et al., 2016b). The tumor with HPV16 was also strongly positive for p16. There is evidence that indicates p16 (cell cycle protein) expression is associated with HPV biological activity and high expression of p16 can be a surrogate for indication of transcriptionally active HPV (Lawson et al., 2015). However, in breast tissues, low expression of p16 is present in normal ductal epithelial tissues with a progressive increase of p16 expression in benign and malignant breast lesions (Lawson et al., 2015).
4.2. Conclusion

Our study confirms the findings from Ethiopia and other East African countries that hormone receptor negative tumors are not the most common molecular subtypes of breast cancer in this particular part of Africa, unlike West and central Africa. Hence, majority of breast cancer cases in this population may benefit from hormonal therapy and/or anti-HER2 or other targeted therapies. Additionally, our findings confirm the geographic variability in the distribution of the molecular subtypes of breast cancer in Africa and hence have important clinical and policy implications for breast cancer control. Furthermore, Ethiopian breast cancer patients exhibit highly proliferative Luminal B tumors at young ages. Future research should examine currently recognized as well as novel genetic and environmental factors that may contribute to the differences in the distribution of the molecular subtypes between different populations in Africa.

AR is expressed in a significant number of most types of breast cancers and is more frequently expressed than ER and PR. Our study shows AR expression is significantly high among ER+ breast cancer patient. In addition, AR is expressed in a significant number of triple-negative breast cancers, which indicates that our patients with triple-negative cancers could benefit from AR targeted treatments which are under clinical trials. Since AR expression has important consequences on the prognosis and treatment of breast cancer, further studies with an increased sample is necessary to confirm our reports.

Our result shows around one third of all breast cancer patients have reduced BRCA1 expression using IHC. Given the high cost of BRCA1 genetic testing, IHC for BRCA1 may be a useful initial screening test to select patients for targeted therapy and to detect patients at risk for hereditary breast and ovarian cancer syndromes. Since the availability of low cost diagnostic is very critical to resource limited countries like Ethiopia, further large confirmatory studies are warranted to determine the potential use of IHC expression in our setting.

In this study, the high Ki67 is significantly lower compared with rates reported in other African countries but comparable with the western patients and were highest in the luminal-B and TNBC molecular subtypes. Since Ki67 is emerging as a valuable addition to the histopathological assessment of breast cancers that is likely to assist with prognostic evaluation and refinement in treatment, additional research aimed at standardizing methodology to ensure that Ki67 results are reproducible and clinically relevant is important.
Our study found that the IHC shows good concordance compared to GPA technique/method. Realizing the possible differences between the results of IHC and GPA in HER2 equivocal cases, even though IHC is an important and valuable tool in the daily routine of pathology laboratories, it is valuable to use GPA when the result of IHC is inconclusive. Genetic evaluation by GPA seems to be easy to interpret and reliable way to ensure proper treatment of patients with breast cancer HER2 overexpressing tumors.

Our study found no viral proteins of HCMV and EBV in breast carcinoma. These adds to other findings with no detection of these viruses in breast carcinoma; however does not rule out association of these viruses with breast cancer due to limitation of the technique used in the study as well as possible other mechanisms of oncogenesis as suggested by other researchers. However, we found high risk HPV viral DNA in a small percentage of our patients and hence suggest a possible association of breast cancer with high risk HPV. Hence, it is our conclusion that cervical cancer prevention strategies may also help protection of breast cancer in small groups of patients.
4.3. Limitation of the Study

Perhaps the most important limitation of the thesis project was that we could not obtain complete socio-demographic and clinicopathological data (including age at diagnosis, tumor grade, histological type and clinical stage). Another limitation of the thesis was the small number of participants included in the study. While our results are important starting point, to determine the molecular characteristics of breast cancer and assess role of oncogenic viruses in breast cancer, the investigation of this same questions in multiple institutions and at a national level is needed.

4.4. Study Strengths

This thesis project has a number of notable strengths. While many studies from Ethiopia have compared ER status of breast cancer to look only at hormone receptor status, we investigated all of the established markers (ER, PR, HER2 and Ki67) to molecularly classify and characterize the tumors. Another major strength of this study is the attempt for the first time to look into additional prognostic/predictive biomarker (AR) in breast cancer, as well as investigate methodological issues (BRCA-1 and GPA for HER2). Furthermore, our analyses which demonstrated that HPV may have role in breast cancer addresses a new issue in breast cancer prevention. Additionally, the analysis of all experiments was done in research laboratories in US or Europe ensure all tests were accurately done.

4.5. Recommendations and future directions

This thesis project provides evidence regarding the molecular characteristics of breast cancer and the results suggest that most breast cancer patients in Ethiopia may benefit from hormonal therapy. Future research should include larger samples of women to be able to confirm our finding in large patient cohorts and include women from all regions to address different ethnic backgrounds. Along with our study molecular all previously studied used IHC but not gene expression profiling to classify tumors from Ethiopian patients. Therefore, it would be interesting to test tumors from Ethiopian patients using gene expression technologies.
This thesis project also provides an important contribution to by comparing and confirming the diagnostic efficacy of IHC with a GPA. The GPA technique which is bright-field microscopy based technology comparable to FISH is also proven to be easy for interpretation without the need for fluorescent microscope. Hence, we suggest evaluating this technique using large number of samples to use it for assessment of gene amplification in HER2 equivocal cases. Additionally, this thesis project provided evidence for variable AR distribution among the molecular subtypes of breast cancer in Ethiopian patients. The results revealed AR is highly expressed in ER-positive tumors than ER-negatives. The expression of AR in TNBC patients indicate that they may benefit from anti-AR targeted therapies which are under evaluation for safety and efficacy.

This thesis project also provided evidence for possible detection of BRCA1 loss of expression in breast tumor using IHC. Given the high rate of negativity and high cost of the genetic testing for BRCA1, the IHC test can be potentially important to be used as a screening test in women at high risk for familial breast cancer. However, studies with larger samples of women and using genetic testing along with the IHC is needed to confirm our findings.

In addition, this thesis project confirmed that Ki67 proliferation marker in Ethiopian patients can be used for prognostic purpose along with traditional biomarkers. However, studies assessing inter observer and inter-laboratory variability in our setting are recommended.

Finally, this project also provided the preliminary evidence in Ethiopia of the association between high risk HPV and the risk to breast cancer in small group of patients. These findings may be important in preventing breast cancer in this group of women by promoting HPV vaccinations.

Taken together, the studies that form this thesis contribute significantly to the body of knowledge regarding the molecular characteristics of breast cancer and the role of some oncogenic viruses among Ethiopian women. In future work, several aspects of the current work can be explored further.
5. References:


LINDOR, N. M., GOLDGAR, D. E., TAVTIGIAN, S. V., PLON, S. E. & COUCH, F. J. 2013. BRCA1/2 Sequence Variants of Uncertain Significance: A Primer for Providers to Assist in Discussions and in Medical Management. The Oncologist, 18, 518-524.


The prevalence of BRCA1 mutations among young women with triple-negative breast cancer.  
*BMC Cancer*, 9, 86.  
Annex I

Questionnaire

Code NO________________                                     Biopsy Number:____________________

Part 1: Socio-Demographic characteristics

1.1. Age_______________
1.2. Region _____________
1.3. Residence area: Rural__________Urban______________
1.4. Education level: Illiterate _____High school or less_____college or above______
1.5. Socioeconomic status: High____Middle________Low_______
1.6. Marital status: Single_____Married_____Widowed________
1.7. Height__________    Weight__________   BMI__________

Part 2: Clinico-pathological characteristics

2.1. Duration of symptom in month ______________
2.2. Duration of illness _________________
2.3. Breast lump/mass_______________
2.4. Family history

2.4.1. Family history of breast cancer: Yes______ No________
   • If yes, 1st degree relative_____2nd degree relative_____3rd degree relative__________

2.4.2. Family history of ovarian cancer: Yes______No________
   • If yes, 1st degree relative_____2nd degree relative_____3rd degree relative__________

2.4.3. Family history of other type of cancer: Yes______No________
   Please specify: ___________________

2.5. How was the lump found

   • Self accidentally____________
• Self as part of regular physical examination
• Routine physical examination
• Mammographic screening
• Unknown

2.6. Physical finding at presentation

• Nipple retraction
• Bloody discharge
• Palpable axillary lump
• Fixation to overlying skin
• Fixation to the underlying muscle
• Erythema of skin
• Peau d’ orange
• Satellite nodules

2.7. History of benign breast disease

2.8. Prior mammographic screening

2.9. Alcohol consumption

2.10. Smoking Status

2.11. Oral contraceptive use

2.12. Reproductive history

2.12.1. Age at menarche

2.12.2. Menstrual status: Pre Post

2.12.3. Age at menopause

2.12.4. Child birth:

• Never
• Have (Number)

2.12.4.1. Age at full term pregnancy
2.13. Ever been breast feeding any child: Yes_______No____________

2.14. Tumor size__________

2.15. Histology of the cancer:
- Infiltrating ductal________
- Intraductal________
- Lobular____________

2.16. Degree of differentiation:
- Well differentiated________
- Moderately differentiated______
- Poorly differentiated________

2.17. Lymph node involvement
- Number of lymph nodes positive________
- Number of lymph nodes removed________
- Which LN?
  - ✓ Axillary________
  - ✓ Supraclavicular________

2.18. Location
- Upper Outer quadrant________
- Upper inner quadrant________
- Lower outer quadrant________
- Lower inner quadrant________
- Central________

2.19. Stage of disease at diagnosis: I____II____III____IV____

2.20. Site: Right _________ Left __________
Annex II
Addis Ababa University, Medical Faculty, Department of Biochemistry
Graduate Study Program
Consent Form

For participation as a volunteer in the research undertaking
Code number_____________________________________________________
Name of study subject_____________________________________________

I have been informed about a study that plans to determine the molecular and genetic characteristics of triple negative breast cancer in Ethiopia. For this purpose, Breast tissue and Blood needs to be taken from me. The aims of the study and the possible risks, including mild pain during blood collection were explained to me.
I am also informed that all the information contained within the questionnaire is to be kept confidential. Moreover, I have also been well informed of my right to keep hold of information, decline to cooperate and make myself withdraw from the study.
It is, therefore, with full understanding of the situation that I gave the informed consent voluntarily to the researcher to use the breast tissue and blood taken from me for the investigation. Moreover, I have had the opportunity to ask questions about it and received clarification to my satisfaction. I have also been informed that the nature of the questionnaire is private.

Signature ___________________________ Signature ___________________________
(Participant) (Investigator) Date

If you have any questions about the study, please contact
Endale Hadgu, Tel. 0911612817, E-mail= endalehadgu@yahoo.com
Institutional Review board, Tel. 0115538743, E-mail= aaumfirb@yahoo.com
Annex III
አዱስ እና በባ ያሳካቸው ይታረክ ይታ䲢 ይች ይምርምር ይታሸ ከወ

የስር ይግባኝ ይናስ

የሚስጥር ይስማና ይተግባኝ

ማጠየቅ ይሸነ

ማጠየቅ ይሸነ

እንዱሁም ይስማና ይተግባኝ

እንዱህ ከውሌ ይስማና ይተጠበቁ

የተጠበቁ ይስማና ይተጠበቁ

ማጠየቅ ይሸነ

ማጠየቅ ይሸነ

ማጠየቅ ይሸነ
Annex IV:

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

This Material Transfer Agreement (MTA) has been prepared for use by

Department of Biochemistry and ________________ in all transfer of research material (samples, derivatives, and specimens) related to the protocol: “Molecular and Genetic Characterization of Triple Negative Breast Cancer in Ethiopian Women”

Provider: Department of Biochemistry, Faculty of Medicine, Addis Ababa University

Recipient: ________________

1. Provider agrees to transfer to recipient’s designated (human biological sample) the following research materials (specimen).

   **Human Breast Tissue and Human Blood (Plasma)**

   The research 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 Research Material.

   a) Are the Research materials of human origin?

      Yes [X] No 

   b) If yes, will they be 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 [X] No 

2. This research material and its derivatives will be used by recipient’s investigator solely in connection with the following research project (“Research Project”) described with specificity as follows “Molecular and Genetic Characterization of Triple Negative Breast Cancer in Ethiopian Women”.

3. In all presentations or written publications concerning the research projects, recipient will seek agreement of provider and acknowledge provider’s contribution of this research material unless requested otherwise.

4. This research 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 research Material to other people not under her/his direct supervision without advance written approval of provider. The research 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 research 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 Research Material is provided as a service to 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 research material will not infringe any patent or proprietary right or third parties.

6. The recipient shall notify the provider in writing of any intention, improvement, modification discovery or development to the material or the information made by Recipient or parities, collaborating with Recipient, herein after referred to an “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 any affirm that the contents of any statements made herein are truthful and accurate.

8. Any additional terms (use an attached page if necessary):

9. The provider maintains, ownership right of the research material and its derivatives unless stated otherwise. The provider will retain a copy (aliquot) of every sample sent abroad as much as possible for local research needs.
Material Transfer Agreement

Signature page

For Recipient:

Recipient’s Investigator

____________________

Duly Authorized

____________________

Signature

____________________

Signature/Stamp

____________________

____________________

____________________

____________________

Date __________

Date __________

Mailing Address

____________________

Mailing Address for Notices:

____________________

____________________

____________________

____________________

Tel: ____________________

Tel: ____________________

Fax: ____________________

Fax: ____________________

For Provider:

Provider’s Investigator

____________________

Duly Authorized

____________________

Signature

____________________

Signature

____________________

____________________

Date ________________

Date ________________

Mailing Address

P.O. Box _________________

P.O. Box ________________

Tel: ____________________

Tel: ____________________

Fax: ____________________

Fax: ____________________
Annex V Information sheet (English Version)

Information sheet for participants of the study entitled “Molecular Characterization and Assessment of Viral Tumorigenesis in Breast Cancer among women in Addis Ababa, Ethiopia”

Addis Ababa University, Faculty of Medicine, Department of Biochemistry
Addis Ababa, Ethiopia

Principal Investigator: Endale Hadgu

Advisors: Dr. Daniel Seifu, Dr. Wondemagegnhu Tigeneh, Dr. Yonas Bokretsion

Name of the sponsor: Addis Ababa University & Armauer Hansen Research Institute (AHRI)

This information sheet is prepared by a group of researchers at AAU for a project with the aim of assessing the Molecular and genetic characteristics of Triple Negative Breast Cancer among Ethiopian Women.

1. Aim of the Study

Prevalence of breast cancer in Ethiopia is not well studied but the recent study by the Addis Ababa City Cancer registry in 2012 edition has placed the incidence of breast cancer at number one with 34 % of all cancers diagnosed in that specific year among females. Breast cancer is now an emerging public health problem in most urban centers including Addis Ababa, the administrative and political center of the country with over 2.7 million residents.

Breast cancer is a heterogeneous disease with different subtypes that are based upon the expression level of estrogen receptor (ER), progesterone receptor (PR), and HER-2 receptor. Different types of breast cancer vary substantially in their behavior and response to treatment.

Very few studies have been done to characterize breast cancer in Africa. Large number of studies of breast cancer in African women reported a frequency of 40–55% of triple negative breast cancer (TNBC) which is more aggressive and has a poorer prognosis. Emerging new evidences, however, show TNBC to be low in East Africa including Ethiopia.

Researches into breast cancer etiology have focused primarily on reproductive and other factors affecting circulating sex hormones and on genetic susceptibility. However, as identified risk factors are thought to explain the incidence in about half of all breast cancer cases only,
researchers are motivated to consider other routes for disease pathogenesis. Viruses have been implicated in the development of various cancers, but they have not been much considered for breast cancer. Recent investigations have linked breast cancer to viral infections, such as Epstein–Barr virus (EBV), mouse mammary tumor virus (MMTV), Human papillomavirus (HPV) and Human cytomegalovirus (HCMV).

The aim of this study is to determine the molecular characteristics of breast cancer among Ethiopian women and assess the role of some oncogenic viruses in breast tumorigenesis. Results obtained from this study will also be used by health care providers for health service planning and can serve as a baseline data to other related studies.

2. Study Design and Procedure

If you agree to take part in the study, one of the investigators or a health worker will give you verbal and/or written information about the study and you will be given the consent form to sign. The physician or health professional will ask you some questions about your general health and perform a complete medical examination and assess whether you qualify to participate in the study. If you are fit for the study breast tissue samples in your pathology laboratory will be collected for molecular characterization and assessment of viral pathogenesis in breast cancer.

3. Risk and discomfort

Participating in this project will not cause more discomfort than is required you could go through for routine examination. But, there could be minor pain and change in color of your skin following the blood drawing and which would disappear in short duration. If you have any discomfort, you can contact any of the investigators in this project.

4. Benefits and incentives

You will not be provided with any direct incentives for your participation in the research. But the result from this study will help scientists and health workers to find a better way of treating breast cancer.

5. Confidentiality

All information about the patients will be kept confidential. Logbooks used in the laboratory will have no names but codes. The information sheet that links the coded number to patient name will be locked inside a box and it will not be revealed to anyone except your physician and the principal investigator.
6. Right to refuse or withdraw

You have full right to refuse or withdraw from participating in this study at any time before and after consent without explaining the reason. Your decision will not affect your right to get health service you are supposed to get otherwise.

7. Whom to contact

This study protocol is reviewed and approved by Faculty of Medicine Research and Publication Committee at Addis Ababa University. The purpose of the review by the committee is to make sure that research participants are protected from harm. For more information you can contact the chairman of the committee____________________________________________

To know more information about the study you can contact any of the following individuals:

1. Mr Endale Hadгу (mobile tel.No.0911-61-28-17)
2. Dr. Daniel Seifu (mobile tel. No. 0911-23-27-54)
3. Dr. Wondemagegnhu Tigeneh (mobile tel.No.0911-89-73-56)
4. Dr. Yonas Bokretsion (mobile tel.No.0911-40-58-99)
5. Dr. Abebe Bekele (mobile tel.No.0911-40-58-99)
6. Institutional Review board, Tel. 0115538743
Annex VI Information sheet (Amharic version)
አዱስ አበባ የላጉ ይጣራጋ ምአት ነው ከorable ወረጋገጥ

አዱስ አስቀል አገልግሎት

ቀzeweb: - ከወ/መ ከ61-28-17/ እቶ. 0911-

አማር አጭለት:

©/C የእጋ ከ8/  እቶ. 0911-23-27-54/
©/C የወስደማሮ ከ9/  እቶ. 0911-89-73-56/
©/C የስሪ ከ9/ እቶ. 0911-40-58-99/
©/C አህ ከ8/ እቶ. 0913-89-20-35/

ስደሚሹ የሚያከሹ ያለበት: - ከወ/መ ከ8/ ያስ጗ር ያሃል እቶ.

የአማር ያስ጗ር ያስ጗ር እቶ.

የጥናት መረጃ የሚያካሂዉ ያተምራ ጭክርነት እቶ.

1. ያደሩ ዯግባኝ:
ወ/መ ከስር የስገም ከ8/ እቶ. ከወ/መ የስገም ከ8/ እቶ.

2. ያደሩ ዯግባኝ በ10 እቶ.

3. ያደሩ ዯግባኝ በ12 እቶ.
4. ይቅሞችና ባካካሻ በዚህ ይብት የተሣっこ ይገኝም፡፡ ይህ ቤት ከተሠረጥ መረጃ የነት ከንብቱ ከምሩም ከተለሽና ያስፈርษል፡፡

5. ይስጥር ሁለወ የሰጡን መሌሶች በሚስጥር ያገኝለ፡፡ በቤተ明珠 የሚቀመጠው መዝገብ የሚገኝ መረጃ ያህክምና ይንክባካቤን ያሻሸሊሌ፡፡

6. ይቅም ይታወቀና በማንኛውም የከተሚመረ ይህን በማዳረግ ይህ ከውሣኔ ያስው ይህ ለለና በምክንያቱ ያክርክርን ያስገዴድትም፡፡ ይህን በማንኛውም ያአደርጉ ያስለማች ይህን በታች ያከተበረት ይህን በሚስጥር ያችሊለ፡፡

7. ይስጥር ያስተቀር የሚገኝን ከተለሽ ከገኝ ከተጠቅም መረጃ የሚገኝን ከተለሽ ከገኝ ከተጠቅም ያስተቀር የሚገኝን ከተለሽ ከገኝ ከተጠቅም ያስተቀር የሚገኝን ከተለሽ ከገኝ ከተጠቅም ያስተቀር የሚገኝን ከተለሽ ከገኝ ከተጠቅም ያስተቀር የሚገኝን ከተለሽ ከገኝ ከተጠቅም ያስተቀር የሚገኝን ከተለሽ ከገኝ ከተጠቅም ያስተቀር የሚገኝን ከተለሽ ከገኝ ከተጠቅም ያስተቀር የሚገኝን ከተለሽ ከገኝ ከተጠቅም ያስተቀር የሚገኝን ከተለሽ ከገኝ ከተጠቅም ያስተቀር የሚገኝን ከተለሽ ከገኝ ከተጠቅም ያስተቀር የሚገኝን ከተለሽ ከገኝ ከተጠቅም ያስተቀር የሚገኝን ከተለሽ ከገኝ ከተጠቅም ያስተቀር የሚገኝን ከተለሽ ከገኝ ከተጠቅም ያስተቀር የሚገኝን ከተለሽ ከገኝ ከተጠቅም ያስተቀር የሚገኝን ከተለሽ ከገኝ ከተጠቅም ያስተቀር የሚገኝን ከተለሽ ከገኝ ከተጠቅም ያስተቀር የሚገኝን ከተለሽ ከገኝ ከተጠቅም ያስተቀር የሚገኝን ከተለሽ ከገኝ ከተጠቅም ያስተቀር የሚገኝን ከተለሽ ከገኝ ከተጠቅም ያስተቀር የሚገኝን ከተለሽ ከገኝ ከተጠቅም ያስተቀር የሚገኝን ከተለሽ ከገኝ ከተጠቅም ያስተአን ያስተቀር የሚገኝን ከተለሽ ከገኝ ከተጠቅም ያስተቀር የሚገኝን ከተለሽ ከገኝ ከተጠቅም ያስተቀር የሚገኝን ከተለሽ ከገኝ ከተጠቅም ያስተአን ያስተለቷ ይህን ለለና በምክንያቱ ይህን በሚስጥር ያችሊለ፡፡ ይህን በማንኛውም ያአደርጉ ያስለማች ይህን በታች ያከተበረት ይህን በሚስጥር ያችሊለ፡፡

1. እቶ እንዲሁ በወን ከ.ቁ 0911-61-28-17/
2. ዓ/ር ወንዳ ከፋ / ከ.ቁ 0911-23-27-54/
3. ዓ/ር ወንዳመሆን ከፋ / ከ.ቁ 0911-89-73-56/
4. ዓ/ር ውስ ከፋ ከፋ / ከ.ቁ 0911-40-58-99/
5. ዓ/ር በእ ከፋ / ከ.ቁ 093-89-20-35/
6. እ.ተክ.ት.ሪ.ስ ወ/ር. የር / ከ/ቁ 015538743/