T.R.N.C

Near EAST UNIVERSITY
INSTITUTE OF HEALTH SCIENCES

Resistance to Trastuzumab in Clinical Her2+ve Breast Cancer: Possible Relation to Cancer Stem Cells

A THESIS SUBMITTED TO THE GRADUATE INSTITUTE OF HEALTH SCIENCES NEAR EAST UNIVERSITY

BY:

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In Partial Fulfillment of the Requirements For

The Degree OF

Master of Science in Clinical Pharmacy

NICOSIA 2016

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NICOSIA 2016

To My Family

ACKNOWLEGGEMENTS

I would like to express my sincere gratitude and deep appreciation to **Prof Dr Bilgen Basgut**, Associate Professor of Pharmacy, Faculty of Pharmacy, Near East University for her patience, faithful supervision and revision of every item of this work. It was an honor to be under her supervision.

I would like to express my sincere gratitude and deep appreciation to **Prof Dr Sahar**, **Abdelbary ElBassiony**, Professor of Pathology, Faculty of Medicine, Cairo University, for revising the thesis and helping me in accomplishment of the work.

I wish to express my grateful thanks and deep appreciation to **Dr Shaimaa Lasheen,** Lecturer of Medical Oncology, Faculty of Medicine, Cairo University for revising the thesis and helping me in collection of the patients' data.

I wish to express my deep appreciation to **Prof Dr Laila Rashed**, Professor of Biochemistry, Faculty of Medicine, Cairo University, for helping me in performing the quantitative polymerase chain reaction.

I would like to express my sincere gratitude and deep appreciation to **Prof Dr Maha Baligh Zickri**, Professor of Histology, Faculty of Medicine, Cairo University for helping me performing the immunohistochemistry, photographing, image analysis, statistical analysis and revising the results.

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Abstract

The study aimed at investigating the proposal of involvement of epithelial mesenchyme, molecular subtype and CSCs Biology transition in the development of trastuzumab resistance in HER2 +ve BC, recommending the use of Ab combinations/modifications that are able to suppress CSCs and overcome trastuzumab resistance.

This retrospective study (2012-2015) was conducted in the Departments of Medical Oncology, Pathology, Biochemistry, Histology and Cell Biology, Faculty of Medicine, Cairo, Egypt in collaboration with Faculty of Pharmacy, Near East University North Cyprus. The study was approved by the institutional review board. It was conducted on 40 BC patients age range 27-63 years, 20 served as control group and the other 20 as metastatic (predicted resistance) group. All BC patients gave informed consent according to the institutional review board approved protocol. Clinicopathological analysis of data, including the age at onset, menopausal status, weight, height, family history and diabetes mellitus (DM) were recorded. Date of diagnosis, breast imaging, surgical procedures, tumor stage according to TNM classification, grade and proliferation index were collected. HER2 status, ER status, PR status, baseline ejection fraction (EF), chemotherapy regimen and number of herceptin cycles were gathered from hospital records and pathology reports. All tumor paraffin embedded tumor specimens that had a 3+ stain intensity by immunohistochemistry (IHC) were considered HER2+. The predicted resistance to treatment was further investigated by IHC for: **molecular subtypes markers:** HER2 and cytokeratin (CK) 5/6 immnostaining**, cancer stem cell marker:** CD44. **A morphometric study** was performed for area% of HER2, CK 5/6 +ve immunoexpression (IE) and CD44 +ve cells. Evaluation of formalin-fixed paraffin-embedded (FFPE) BC specimens by **real-time quantitative polymerase chain reaction (qPCR)** and **statistical analysis** was performed.

As regards the clinicopathological results, a significant increase in the mean proliferative index (PI) and the mean of ER +vety was detected in the metastatic group compared to the control group. No nodal involvement was found in the control group. In the metastatic group, 2 cases only were N0, but had distant metastasis. PR was +ve in 15 cases in the metastatic group. In the control group, **HER2 immunostained sections** demonstrated obvious +ve IE, while in the metastatic group, less obvious IE appeared in specimens of cases with low PI, low ER and PR +vety. Minimal IE was detected in the specimens of patients with high PI, ER and PR +vety. In the control group, **CK immunostained sections** demonstrated -ve IE among the malignant cells. While in the metastatic group, cases with low PI, ER and PR +vety revealed +ve CK IE in some malignant cells forming solid masses or lining ducts. Obvious IE was detected in multiple malignant cells lining ducts or forming solid masses in the specimens of patients with high PI, ER and PR +vety. In the control group, **CD44 immunostained sections** demonstrated few +ve spindle cells among the malignant cells of duct carcinoma. While in the metastatic group, some of the cases with low PI, ER and PR +vety revealed some +ve spindle cells among the malignant cells of duct carcinoma, in the surrounding CT and among malignant cells forming solid masses**.** Specimens of patients with high PI, ER and PR +vity recruited multiple +ve spindle cells in the CT surrounding malignant cells lining ducts**,** in the CT between groups of malignant cells. In addition, multiple +ve spindle cells were found inside and near blood vessels. The mean area% of HER2 +ve IE indicated a significant decrease in the metastatic group.

The mean area% of CK IE was -ve in the control group. In the metastatic group a significant increase was found in cases with high PI, ER and PR +vety compared to cases with low PI, ER and PR +vety. The mean area% of CD44 IE expressed a significant increase in the metastatic cases with low PI, ER and PR +vety compared to the control group, also in cases with high PI, ER and PR +vety compared to low PI, ER and PR +vety. In addition, the mean values of PCR indicated a significant decrease in the metastatic group compared to the control group**.**

It can be concluded that the development of resistance to trastuzumab in the metastatic group of HER2+ve BC is related to the development of the basal like subtype and the associated overexpression of CD44 +ve CSCs. This nicititates performing IHC assessment of the molecular subtype and the type of CSCs during the follow-up of course of treatment to allow the choice of the most appropriate treatment regimen or modifying it giving the best expected prognosis.

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LIST OF ABBREVIATIONS

Abbreviations	Words
BC	Breast cancer
IDCs	Invasive ductal carcinomas
NOS	Not otherwise specified
ILC	Invasive lobular carcinoma
HR	Hormone receptors
HER2	Human epidermal growth factor receptor
RR	Response rate
PFS	Progression-free survival
OS	Overall survival
IV	Intravenous
SC	Subcutaneous
NAT	Neoadjuvant therapy
CSCs	Cancer stem cells
CT	Connective tissue
WHO	World Health Organization
ER	Estrogen receptor
PgR	Progesterone receptor
FFPE	Formalin-fixed and paraffin-embedded
qRT-PCR	Quantitative real-time reverse transcription-polymerase chain reaction
EGFRs	Epidermal growth factor receptors
CICs	Cancer initiating cells
cHer2+	Clinical HER2+
IHC	Immunohistochemistry
EGF	Epidermal growth factor
MRI	Magnetic resonance imaging
US	Ultrasound
EC	Extracellular
MMP	Metalloproteases
MBC	Metastatic BC

eBC	Early BC
PI3K	Phosphatidylinositol 3-kinase
PTEN	Phosphatase and tensin homolog
SCs	Stem cells
ESCs	Embryonic stem cells
iPSCs	Induced pluripotent stem cells
ASCs	Adult stem cells
BM	Bone marrow
HSCs	Hematopoietic stem cells
MSCs	Mesenchymal stem cells
UCB	Umbilical cord blood
AT	Adipose tissue
MYOs	Myoepithelial cells
MaSCs	Mammary stem cells
CD	Cluster of differentiation
UCMSCS	Umbilical cord MSCs
WJMSCs	Wharton's jelly MSCs
PLMSCs	Placental MSCs
BMMSCs	Bone marrow MSCs
DM	Diabetes mellitus
EF	Ejection fraction
Mam	Mammography
MRM	Modified radical mastectomy
Cth	Chemotherapy
AC-T+Hercept	Adriamycin Cyclophosphamide-Taxol+Herceptin
Carbo	Carboplatin
FEC	FluoroUracil Epidoxorubicin Cyclophosphamide
FAC	FluoroUracil Adriamycin Cyclophosphamide
CK	Cytokeratin
SPSS	Statistical Package for the Social Sciences
Fc	Fraction crystallizable
kd	kilodalton
DAB	Diaminobenzidine

ABC	Avidin-Biotin Complex
HIER	Heat-Induced Epitope Retrieval
Ab	Antibody
PBS	Phosphate buffer saline
Rt	Room temperature
cDNA	Cyclic DNA
RT	Reverse transcriptase
SDS	Standard deviation score
+vity	Positivity
SD	Standard Deviation
IE	Immunoexpression
PI	Proliferation index

1. Introduction

Breast cancer (BC) is a highly heterogeneous disease, different types of this neoplasm exhibit variable histopathological and biological features, different clinical outcome and different response to systemic interventions. BC cannot be viewed as a single clinico-pathological entity. Histopathological classification includes 20 major types and 18 minor subtypes with minimal prognostic & predictive implications. 70%–80% of the all BCs belong to either 1 of the 2 major histo-pathological classes invasive ductal carcinomas (IDCs) not otherwise specified (NOS) or invasive lobular carcinoma (ILC) **(Li et al, 2015)**.

Biological classification includes expression of hormone receptors (HR) or human epidermal growth factor receptor (HER2) status in BC. 3 main classes of HR+ve BC include, highly endocrine responsive (treated by endocrine therapy and adding chemo-therapy according to tumor size, grade and proliferative fraction). Not endocrine responsive BC and endocrine responsiveness uncertain (treated by cytotoxic drugs) and (treated by trastuzumab in HER2 overexpression). Molecular classification represents clustering analysis of gene expression profiling. Luminal HER2-enriched and basal-like classes were proved by molecular classification and were found to have distinctive biological and clinical features. Prognostic value is confirmed **(Prat et al, 2015)**.

The HER2 gene is located on the long arm of chromosome 17 and encodes a 185-kDa transmembrane protein. HER2 gene is amplified in 25–30% of BCs and this amplification causes overexpression of the encoded protein in 95% of cases. HER2+ve BC is a more aggressive phenotype and adverse disease prognosis was recorded. 1st-line treatment of HER2 +ve BC achieved a response rate (RR) of 30%–35%, a median progression-free survival (PFS) of 5–6 months and a median overall survival (OS) of 20–23 months. Addition of trastuzumab, a humanised monoclonal antibody with specificity for the HER2 protein, resulted in RR of 50%–72%, median PFS of 11-12 months and median OS of 25–36 months. It is administered as intravenous (IV)

infusion, in part with chemotherapy or subcutaneous (SC) which is well tolerated with fewer adverse events **(Huszno and Nowara, 2015)**.

Adjuvant trastuzumab has become foundation of care for HER2 +ve early BC. Neoadjuvant therapy (NAT) is a successful approach to convert patients inoperable at diagnosis to operable/ make conserving surgery possible instead of mastectomy. Although trastuzumab is an effective treatment in early stage HER2+ BC, the majority of advanced HER2+ BCs develop trastuzumab resistance. Continued use of trastuzumab may increase the frequency of cancer stem cells (CSCs) and metastasis potential **(Burnett et al, 2015)**.

Based on the spectrum of molecular BC subtypes and the CSC-determined sensitivity to trastuzumab, a better delineation of the predictive value of cHER2+ BC can be provided, by incorporating CSCs-driven intra-tumor heterogeneity into clinical decisions **(Martin-Castillo et al, 2015b)**.

Aim of Work:

Consequently, the study aimed at investigating the proposal of involvement of epithelial mesenchyme, molecular subtype and CSCs Biology transition in the development of trastuzumab resistance in HER2 +ve BC, recommending the use of Ab combinations/modifications that are able to suppress CSCs and overcome trastuzumab resistance.

2. LITERATURE REVIEW

2. 1. Breast Cancer

2.1.1.Anatomy and Histology of Mammary Gland

Each mammary gland consists of 15–25 **lobes** of the compound tubuloalveolar type whose function is to secrete milk to nourish newborns. Each lobe, separated from the others by dense connective tissue (CT) and much adipose tissue, has its own **excretory lactiferous duct**. These ducts, 2–4.5 cm long, emerge independently in the **nipple,** which has 15–25 openings, each about 0.5 mm in diameter. The histological structure of the mammary glands varies according to sex, age, and physiological status **(Junqueira and Carneiro, 2005)**.

Before puberty, the mammary glands are composed of **lactiferous sinuses** and several branches, the **lactiferous ducts**. During puberty the breasts increase in size and develop a prominent nipple, due to accumulation of adipose tissue and CT, with increased growth and branching of lactiferous ducts due to increased estrogens. In boys, the breasts remain flattened. The **lobe** in the adult woman is developed at the tips of the smallest ducts and consists of several ducts that empty into one terminal duct. Each lobe is embedded in loose CT. A denser, less cellular CT separates the lobes **(Fig1) (Mesher, 2010)**.

The **nipple** has a conical shape and may be pink, light brown, or dark brown. Externally, it is covered by keratinized stratified squamous epithelium continuous with that of the adjacent skin. The skin around the nipple constitutes the **areola.** The color of the areola darkens during pregnancy, as a result of the local accumulation of melanin. After delivery, the areola may become lighter in color but rarely returns to its original shade. The epithelium of the nipple rests on a layer of CT rich in smooth muscle fibers. The nipple is abundantly supplied with sensory nerve endings **(Mesher, 2013)**.

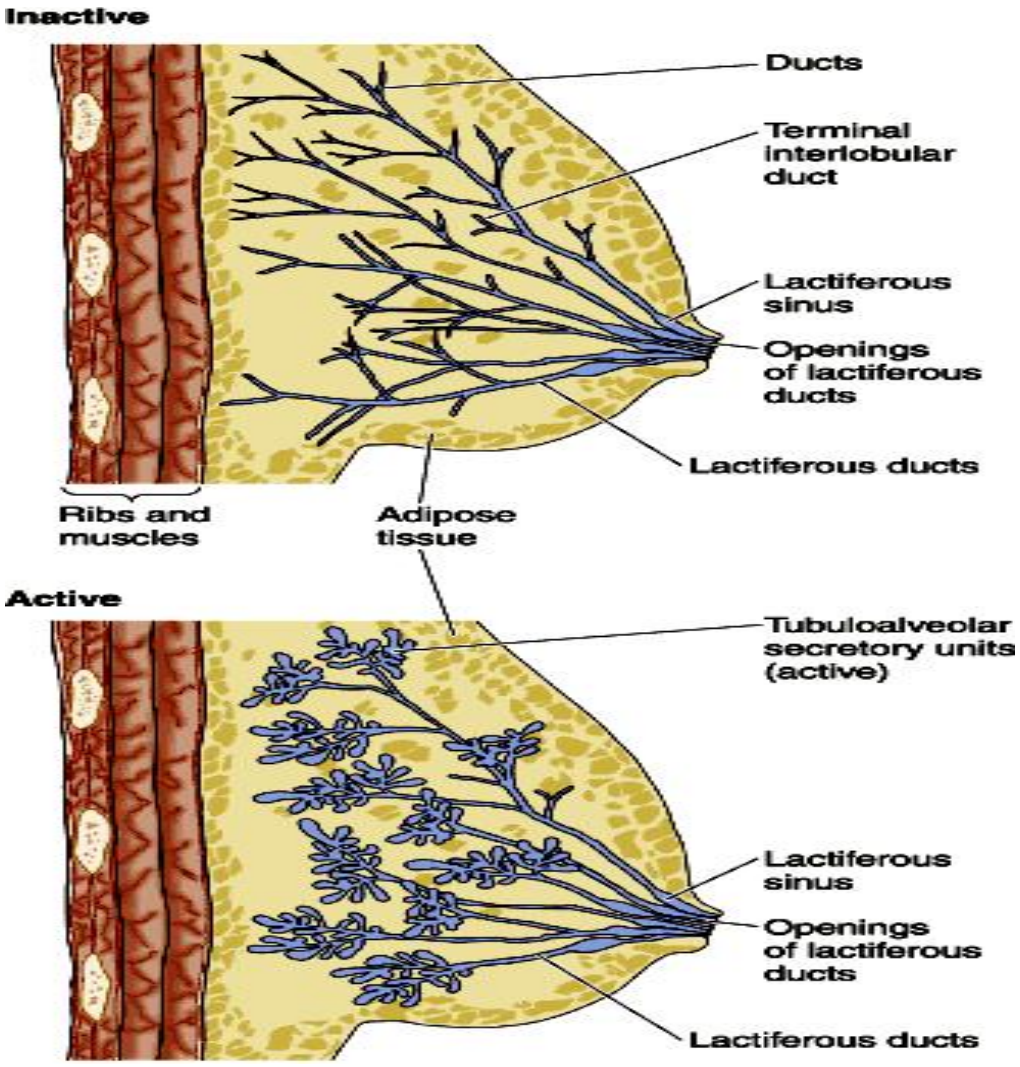


Fig (1): Schematic drawing of the female breast showing inactive and active mammary glands **(Junqueira and Carneiro, 2005)**.

Resting, or nonsecreting, mammary glands of nonpregnant women have the same basic architecture as the lactating (active) mammary gland, except that they are smaller and without developed alveoli, which occur only during pregnancy. Near the opening at the nipple, lactiferous ducts are lined by stratified squamous (keratinized) epithelium. The lactiferous sinus and the lactiferous duct are lined by stratified cuboidal epithelium, whereas the smaller ducts are lined by a simple columnar epithelium. Stellate myoepithelial cells located between the epithelium and the basal lamina also wrap around the developing alveoli and become functional during pregnancy **(Fig 2) (Gartner et al, 2011)**.

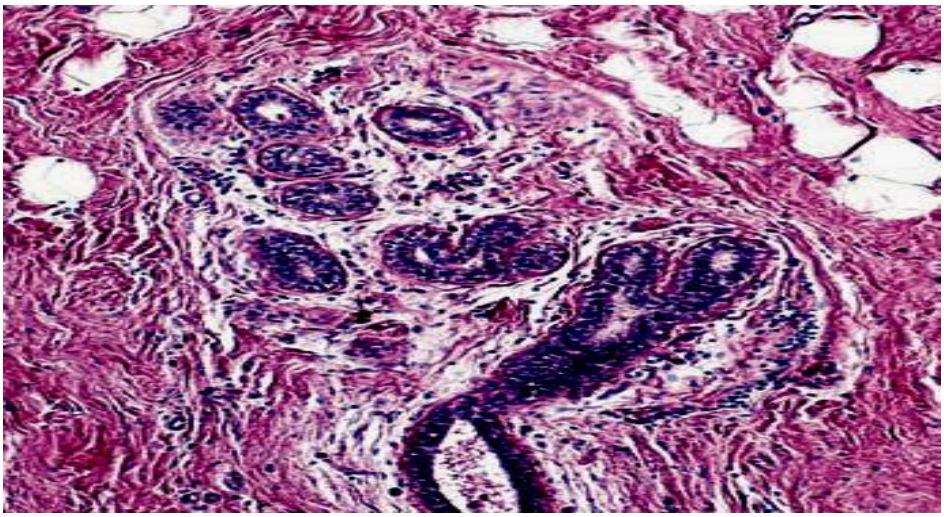


Fig (2): Resting mammary gland (Gartner et al, 2011).

Mammary glands undergo dramatic proliferation and development during pregnancy **(Young et al, 2014)**.

• **First trimester** is characterized by elongation and branching of the terminal ductules. The lining epithelial and myoepithelial cells proliferate and differentiate from breast progenitor cells found in epithelium of terminal ductules.

• **Second trimester** is characterized by differentiation of alveoli from the growing ends of the terminal ductules. The development of the glandular tissue is not uniform, even within a single lobule. The cells vary in shape from flattened to low columnar.

• **Third trimester** commences maturation of the alveoli. The epithelial glandular cells become cuboidal. They develop extensive secretory vesicles and lipid droplets that appear in the cytoplasm. Subsequent enlargement of the breast occurs through hypertrophy of the secretory cells and accumulation of secretory product in the alveoli **(Ross and Pawlina, 2011) (Fig 3)**.

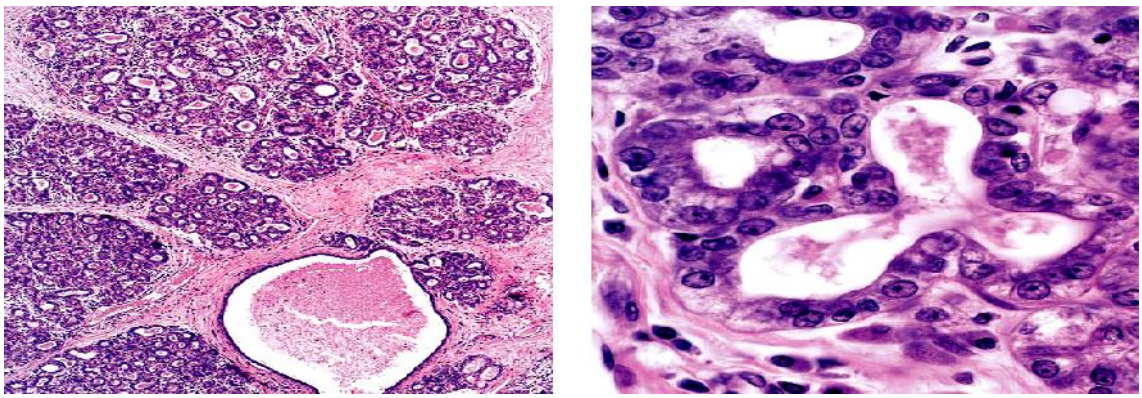


Fig (3): Lactating mammary gland **(Ross and Pawlina, 2011).**

2.1.2. Classification

Breast cancer needs a suitable classification that 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 BC still has not been written. More recent classification schemes, based on the immunohistochemical characterization of BC for the assessment of HR status, HER2 gene over-expression or amplification and proliferative fraction or gene expression profiles, correlate much better with the clinical outcome and may be used to inform the choice of the systemic therapy **(Viale, 2012).**

The histopathological classification of BC is based on the diversity of the morphological features of the tumors. By the world health organization (WHO) in 2003 it includes 20 major tumor types and 18 minor subtypes. A major drawback of this classification is that 70%– 80% of the all BC cases will eventually belong to either one of the two major histopathological classes, namely IDCs or ILC. As a result, the histopathological classification has minimal prognostic and predictive implications, and its clinical utility is quite modest **(Tavassoli and Devilee, 2003)**.

In a large study of the International Breast Cancer Study Group on 9374 patients, ILC was associated with older age; larger, better differentiated estrogen receptor (ER) +ve tumors; and less peritumoral vascular invasion. ILC was associated with increased incidence of bone events but a decrease in regional and pulmonary metastases **(Pestalozzi et al, 2008)**.

In a recent study, ILC of the 'classic' type was compared with an equal number of cases of IDCs. There was no substantial difference in disease-free or OS, locoregional relapse or time to distant metastasis among the lobular and ductal groups, although the lobular group showed a trend to earlier appearance of contralateral BC **(Viale et al, 2009)**.

Minor tumor subtypes, the tubular and cribriform carcinomas, pursue an almost indolent clinical course with an extremely good OS **(Colleoni et al, 2012)**.

The metaplastic carcinomas, may have a substantially worse clinical outcome than the IDCs NOS **(Leibl et al, 2005)**. Pure ILC OS (57% with the 'classic' type; 19% with the alveolar type; 11% with the solid type; and the remaining 13% characterized by pleomorphic features). Classic histotype of lobular carcinoma showed lower risk of axillary lymph node metastases and lower tumor grade **(Orvieto et al, 2008)**.

Biological classification is relying on the expected responsiveness of the tumors to different therapeutic approaches. The systemic therapy of early BC is mainly informed by expression of HR and HER2 status Guidelines and recommendations described the optimal performance of the immunohistochemical assay for assessing the HER2 status **(Wolff et al, 2007)** and **(Goldhirsch et al, 2009)**.

It included three main classes, tumors considered to be highly endocrine responsive, not endocrine responsive and endocrine responsiveness remain uncertain. Estrogen receptor +ve and progesterone receptor (PgR) +ve tumors were defined by showing 1% or more immunoreactive cells. The higher the number of +ve cells, the larger is the expected benefit of endocrine therapies. In addition, it is recommended to report on the average intensity of the staining **(Hammond et al, 2010).**

Ki67 immunolabeling is a prognostic and predictive marker, extensively investigated in both the neo-adjuvant and adjuvant settings **(de Azambuja et al, 2007).** However, mainly due to the lack of standardization in the performance of the assay and in the interpretation and scoring of the results, the measurement of Ki67 has not been considered a useful prognostic marker in the updated recommendations for the use of tumor markers **(Jonat and Arnold, 2011).**

New approaches have been considered to unveil the molecular basis for heterogeneity of BC. By using a clustering analysis of gene expression profiling, it was able to identify molecularly defined classes of BC (luminal, HER2-enriched, basal-like and normal-like) with distinctive biological and clinical features. **This molecular classification** has been shown to have prognostic value and to be predictive of the response to chemotherapy in the setting **(Rouzier et al, 2005).**

The original molecular classification has been derived from investigations on fresh frozen tissue, and it is not applicable to formalin-fixed and paraffin-embedded (FFPE) material. More recently, however, a gene expression assay using 50 genes (PAM50) has been developed for use on FFPE tissue. The assay, based on quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR), accurately identifies the major molecular subtypes of BC and generates risk-of-relapse scores **(Parker et al, 2009).** Its prognostic value has been confirmed in several retrospective investigations using tumor samples of patients with long-term follow-up data **(Nielsen et al, 2010)** and of patients enrolled in randomized, clinical trials **(Ellis et al, 2011).**

2.1.3.Human Epidermal Growth Factor Receptor BC

Human epidermal growth factor receptor overexpression is present in approximately 20-30% of BC tumors. HER2 overexpression is associated with a more aggressive disease, higher recurrence rate, and shortened survival. HER2 is part of the epidermal growth factor family, along with 3 other receptors: epidermal growth factor receptors (EGFRs) HER1, HER2, HER3 and HER4. The HER2 gene is located on the long arm of chromosome 17 and encodes a 185-kDa transmembrane protein. The HER2 receptor extracellular domain has no identifiable ligand, unlike the other EGF family receptors. It is present in an active conformation and can undergo ligand-independent dimerization with other EGF receptors. The most active and tumor promoting combination is thought to be the HER2/HER3 dimer **(Mitri et al, 2012) (Fig 4)**.

Overexpression of HER2 was correlated with the age of the patient and tumor size. Increased expression is associated with reduced survival. Poorer survival was found when BC patients have both ER and HER2 positivity. It was also commented that HER2 and EGFR3 are linked with tumor proliferation. HER2 normally has to heterodimerize with another EGFR family member for activity, however, if the HER2 gene is amplified, HER2 activity is induced and the tumor growth is dependent on HER2 activity**.** Recently mutations in HER2 have been detected in BC patient samples which lack HER2 gene amplification. HER2 is expressed in the cancer initiating cells (CICs) population and modulated by the tumor microenvironment. Targeting of HER2 in these CICs may be an appropriate therapeutic approach **(Davis et al, 2014)**.

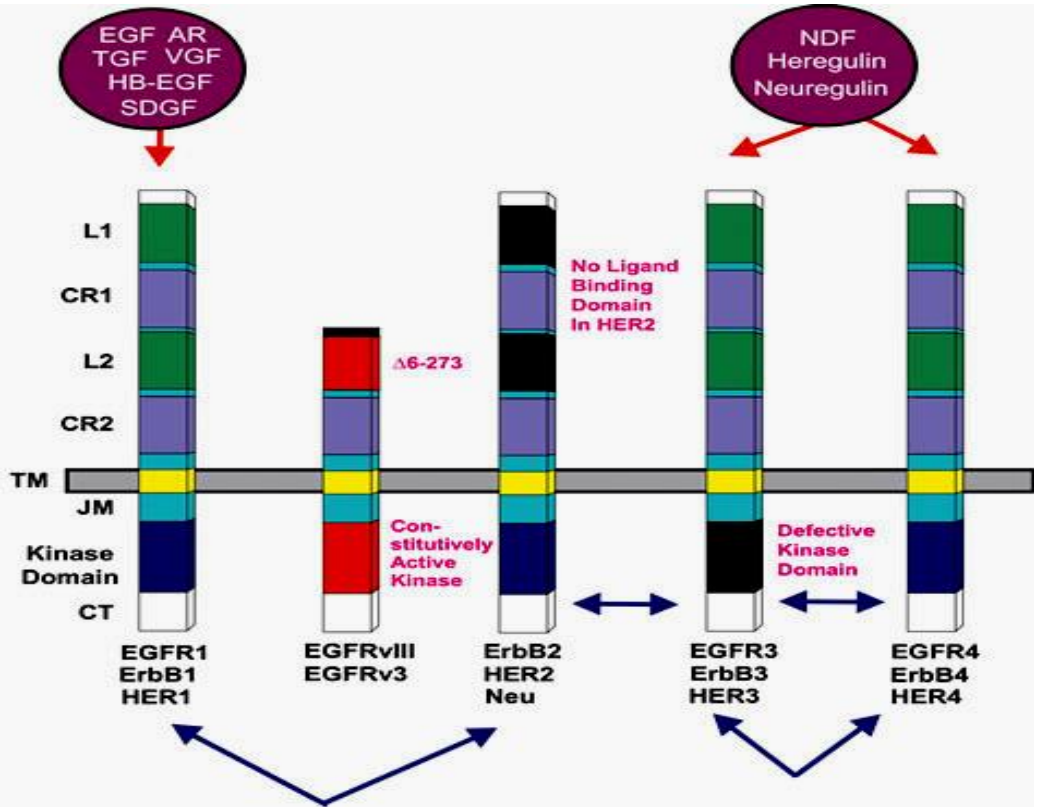


Fig (4): Epidermal Growth Factor Receptor Family**.** Arrows between receptors indicate possible heterodimer formation **(Davis et al, 2014)**

Clinical HER2+ (cHER2+) BC, as exclusively determined by immunohistochemistry (IHC) of HER2 protein overexpression and/or fluorescence in situ hybridization of HER2 gene amplification, has been largely considered a single

disease entity. Presumably, this is due to the apparent dominant role of the HER2 receptor itself on the biology and clinical behavior of HER2+ cells. In other words, the distinct and intrinsic molecular subtypes appear to retain their biological function and more importantly, their clinical outcome, regardless of the cHER2+ status. **(Martin-Castillo et al, 2015b) (Fig 5)**.

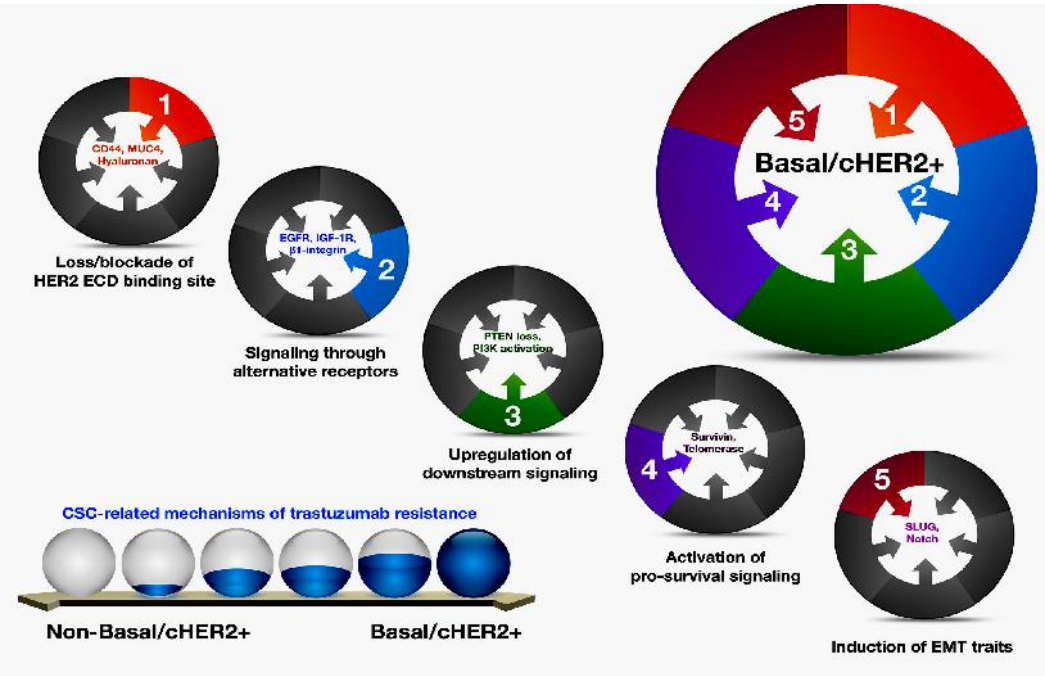


Fig (5): Basal-HER2+ BC and trastuzumab resistanc, key mediators are closely linked to CSCs **(Martin-Castillo et al, 2015b)**.

Established risk factors for BC include reproductive factors (early menarche, nulliparity, age at first pregnancy over 30 years, use of high-dose hormonal contraceptives, late menopause and hormone replacement therapy), increasing age, obesity, high breast tissue density and family history of cancer, especially BC. Additional factors that modulate BC risk include nutritional factors, physical activity, history and duration of breast feeding, obesity in post-menopause, smoking, alcohol consumption, exposure to ionizing radiation and socio-economic level **(Singletary, 2003)**.

Family history, having benign breast disease, high-dose radiation to chest, never breastfed a child, tobacco smoke, recent and long-term use of hormone replacement therapy. In addition, consumption of spicy foods, stressful life, occupational risk factors and environmental pollution. Deficiency of physical activity and recent oral contraceptive use are also considered risk factors **(Che et al, 2014)** and **(Abu Samah et al, 2015)**.

Developing countries are observing alarming rates of BC, particularly among young women **(Ahmadian et al, 2014)**. A recent study carried out in 24 countries across Asia, Africa and America revealed that 35.4% of the women were not aware of any risk factors influencing BC **(Peltzer and Pengpid, 2014)**. BC is a multi-factorial disease in which genetic and environmental factors contribute to its occurrence **(MacDonald, 2011)**. Educational knowledge and clinical role in the prevention and early detection of BC, especially hereditary BC are important **(Prolla et al, 2015)**.

Insulin-like growth factor and epidermal growth factor polypeptide growth factors are closely correlated to malignant transformation and all the steps of the BC metastatic cascade **(Vodouri et al, 2015)**.

The HER-2 status in primary versus metastatic breast cancer testing approaches include testingHER-2 status in circulating tumor cells **(Ross et al, 2009)**. Risk factors for disease progression in HER2 +ve BC were the menopausal status (central nervous system metastases), history of cancer in the family (lung metastases) and history of cigarette smoking (liver metastases) **(Huszno and Nowara, 2015)**.

2.2. Management of BC

2.2.1. Diagnosis

Key to the developing use of **magnetic resonance imaging (MRI)** has been the development of MRI-guided biopsy techniques **(Heywang-Kobrunner et al, 2009)**. Sensitivity over 90% and specificity 97 % has been established **(Sardanelli et al, 2011)**. The diagnostic accuracy of MRI in detecting additional lesions was proved to find a positive predictive value of 67% **(Plana et al, 2012)**.

The best program of screening within a multidisciplinary high-risk management program recommends starting at 25–30 years or 10 years before the youngest relative developed BC **(Lowry et al, 2012)**. **Ozanne et al, (2013)** documented that current provisions only recommend MRI in women under 50, but for some this age limit may not be applicable. Some clinics suggest alternate 6 monthly MRI with mammogram **(Cott Chubiz et al, 2013)**. Current guidelines recommend breast MRI beginning 8–10 years after chest irradiation, combined with mammography, which has a sensitivity of 94% **(Ng etal, 2013)**.

Early detection of BC through screening has contributed significantly to the decline in cancer-related mortality. For women at “high risk,” the American Cancer Society has recommended MRI of the breast as an adjunct to annual screening mammography **(Lin et al, 2015)**. Interest in nuclear breast imaging is increasing because of technical improvements in dedicated devices that allow the use of relatively low doses of radiotracers with high sensitivity for even small BCs. Cancer imaging technology, standardized interpretive criteria, direct biopsy guidance, and acceptable cost-effectiveness are under study **(Berg, 2016)**.

Mammographic screening is the only proven modality to reduce mortality of BC, with the rates ranging from 10 to 30% **(Kalager et al, 2010)**. Screening **breast ultrasound (US)** is known to have a substantial role in the detection of early BC in high risk women or women with dense breasts. It was recommended that all mammography reports inform patients of the availability of screening breast US and MRI to women

with dense breasts. Subsequently, many facilities in Western countries have experienced a marked increase in the number of screening breast US **(Parris et al, 2013) (Fig 6)**.

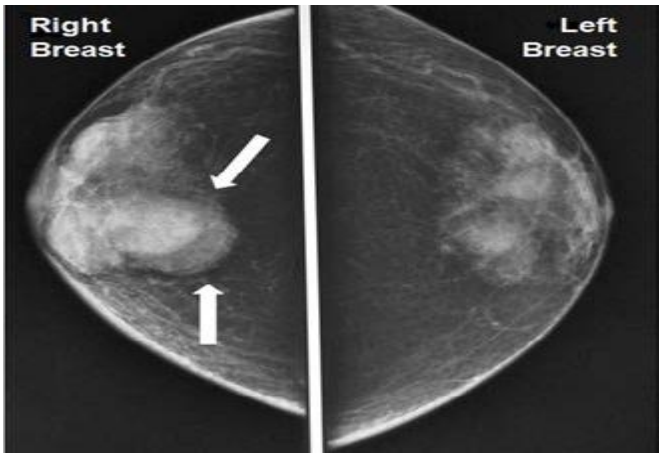


Fig (6): Craniocaudal mammographic view shows well circumscribed lesions mainly in the right breast **(Mandanas et al, 2015).**

The American College of Radiology Imaging Network multicenter trial reported that combined screening using both mammography and US could detect 4.2 cancers per 1000 women **(Berg etal, 2008) (Fig 7)**.

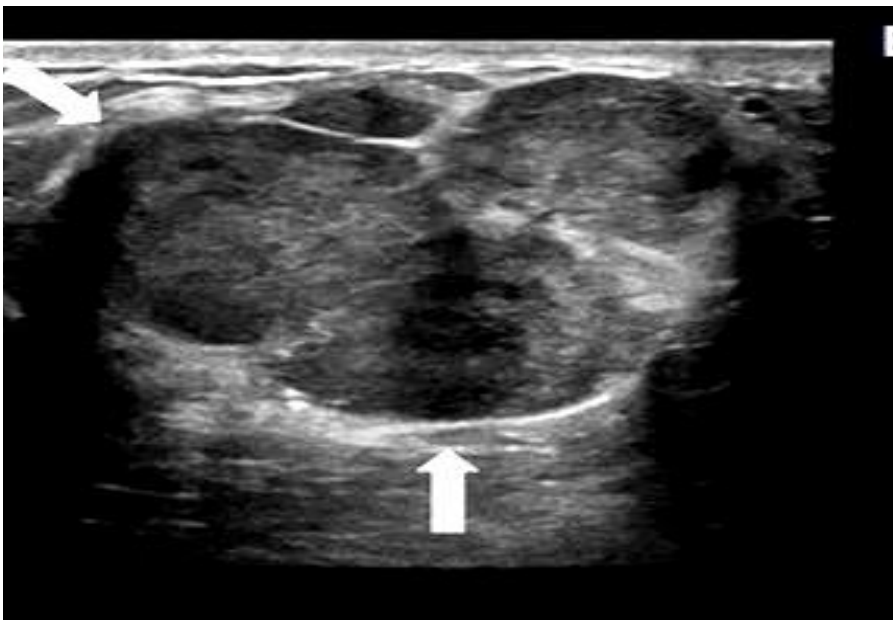


Fig (7): US showing a hypoechoic mass with lobular appearance and hyperechoic diaphragms **(Mandanas et al, 2015).**

Screening procedure US includes bilateral whole breasts and both axillary areas. Mammography can be performed before US. During US examinations, any abnormality including cysts, solid nodules, distortion-like abnormalities, or focal heterogeneity was stored as representative images. If no abnormalities were seen, the representative normal parenchymal pattern of any plane was imaged quadrant-by-quadrant. Each US examination can take approximately 10 minutes. In addition, US can detect hypoechoic ill defined nodules **(Hwang et al, 2015)**.

The results of IHC assay of HER2 can be presented as 0-3+. Negative HER2 was defined as 0 or 1+, while positive HER2 was defined as 3+. The samples with results of 2+ and 1+ for HER2 were then tested with a fluorescent assay **(Hu et al, 2015) (Fig 8)**. The expression of Ki-67 (biomarker of cellular proliferation) can be assessed and samples were considered +ve for Ki-67 in cases that scored ≥ 20% **(Li et al, 2015)**.

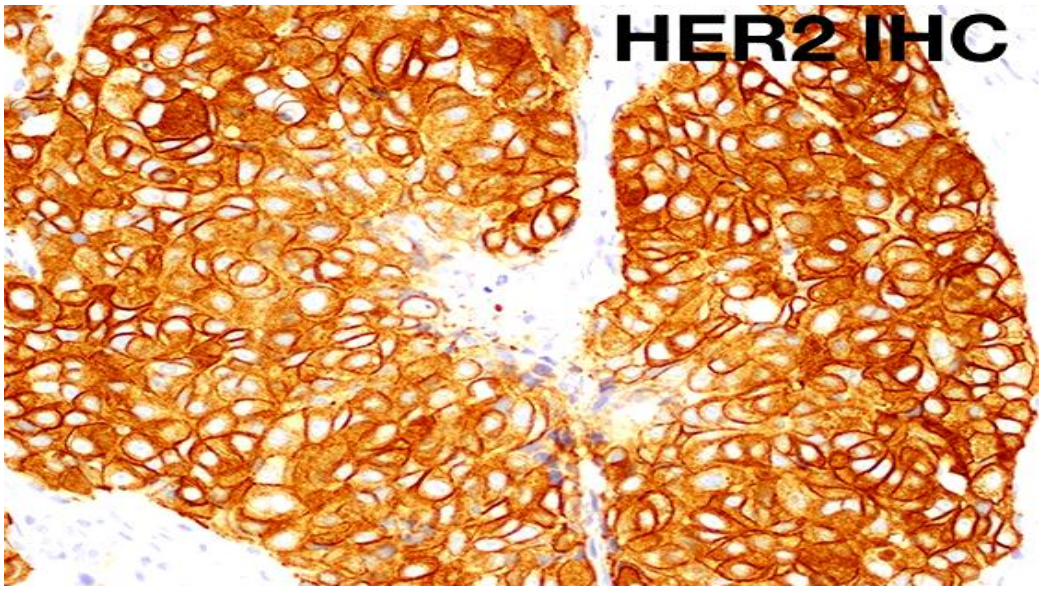


Fig (8): HER2 +ve overexpression in BC **(Martin-Castillo et al, 2015b)**

Alternative, expression of basal/myoepithelial cell proteins identified by immunohistochemical staining of basal cytokeratins is accepted **(Bertucci et al, 2008)** in basal like HER-2 molecular subclass **(Rakha et al, 2015)**.

2.2.2. Trastuzumab Treatment

It is a recombinant Ig G1 Kappa humanized monoclonal antibody. It consists of 2 light and 2 heavy chains with chemical formula C6470H112N1726O2013S42. Molecular weight 145531.5 Da. Selectively binds with high affinity in a cell based assay to the extracellular domain of HER2 protein produced in CHO culture. It is removed by opsonization via reticuloendothelial system **(Block and Jr, 2011).**

Three important pockets, two electrostatic and one hydrophobic, exist in the interaction region between trastuzumab and HER2. Hydrogen bonding and hydrophobic interaction in the binding pocket of HER2-herceptin were described **(Fig 9)**.

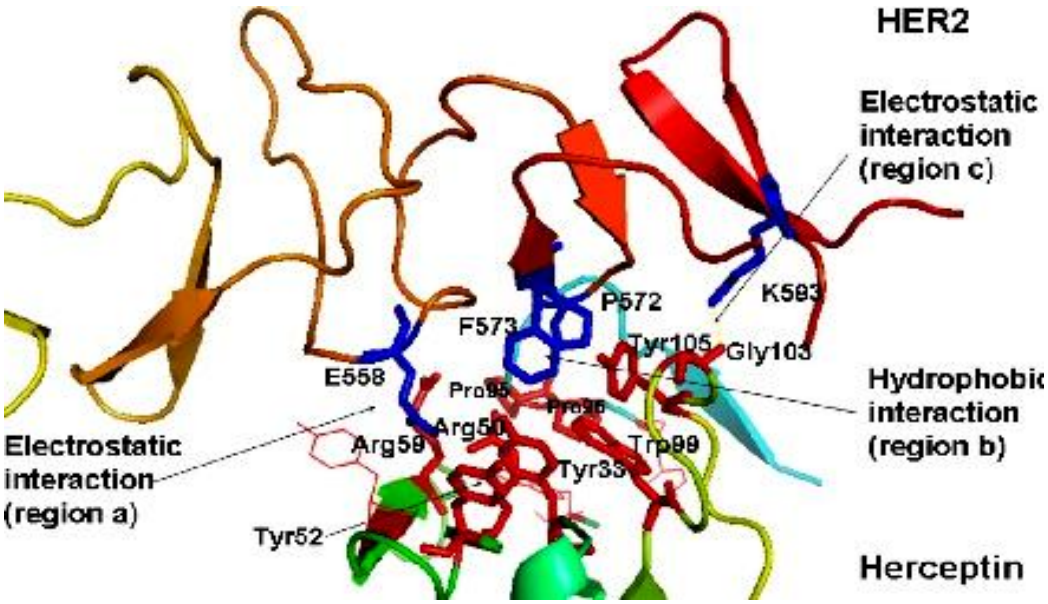


Fig (9): Trastuzumab interacting with crystal structure of part of domain IV of HER2. Residues from herceptin are shown as red sticks and residues from HER2 protein domain IV are shown in blue **(Satyanarayanajois, 2009).**

In normal cells, activation of this EGFR tyrosine kinase family triggers signaling pathways that control normal cell growth, differentiation, and motility. Binding of extracellular (EC) ligands, such as EGF to the EC ligand binding domain of EGFR, results in receptor homo-heterodimerization, activation of tyrosine kinase activity, and autophosphorylation of the receptors. Consequently, initiating a mitogenic signaling

cascade. Blockade of HER2-mediated multimerization, results in inhibition of phosphorylation, ultimately leading to control of cell growth. Monoclonal antibodies specifically directed against the EC domain of HER2 have been shown to be selective inhibitors of the growth of HER2-overexpressing cancer cells. The extracellular region of HER2 consists of four domains (I-IV). Domain II of HER2 is known to participate in dimerization with other HER receptors. Domain IV has an important cleavage site for matrix metalloproteases (MMP). Herceptin, has specificity for the HER2 protein; binds to the EC domain IV of HER2 and inhibits the cleavage site of MMPs. This leads to indirect inhibition of dimerization, phosphorylation and signal transduction **(Satyanarayanajois et al, 2009).**

The mechanism of action of Trastuzumab is perceived to be through both innate and adaptive immunities. Innate mechanisms lead to cell cycle arrest, with a noted decrease in cyclin D1 and cyclin-dependent kinase 2 activity. Trastuzumab alone does not seem to promote a significant level of apoptosis, but is synergistic with most chemotherapeutics in preclinical models. This synergism is felt in part to be explained by inhibition of the signaling pathway, which normally promotes cell survival. Nevertheless, the innate response alone does not fully explain the effect of Herceptin on tumor regression. Adaptive mechanisms are also present and involve antibody dependent cell-mediated cytotoxicity. This is likely to be T-cell mediated, through activation of the receptor, leading to increased cell death **(Park et al, 2010) (Fig10)**.

In preclinical studies trastuzumab provided evidence that it is safe and clinically active in patients with HER2 overexpressing metastatic BC (MBC), with a response rate of 12–40% **(Vogel et al, 2002)**. Clinically, trastuzumab resulted in more favourable RR, PFS and OS **(Andersson et al, 2011)**.

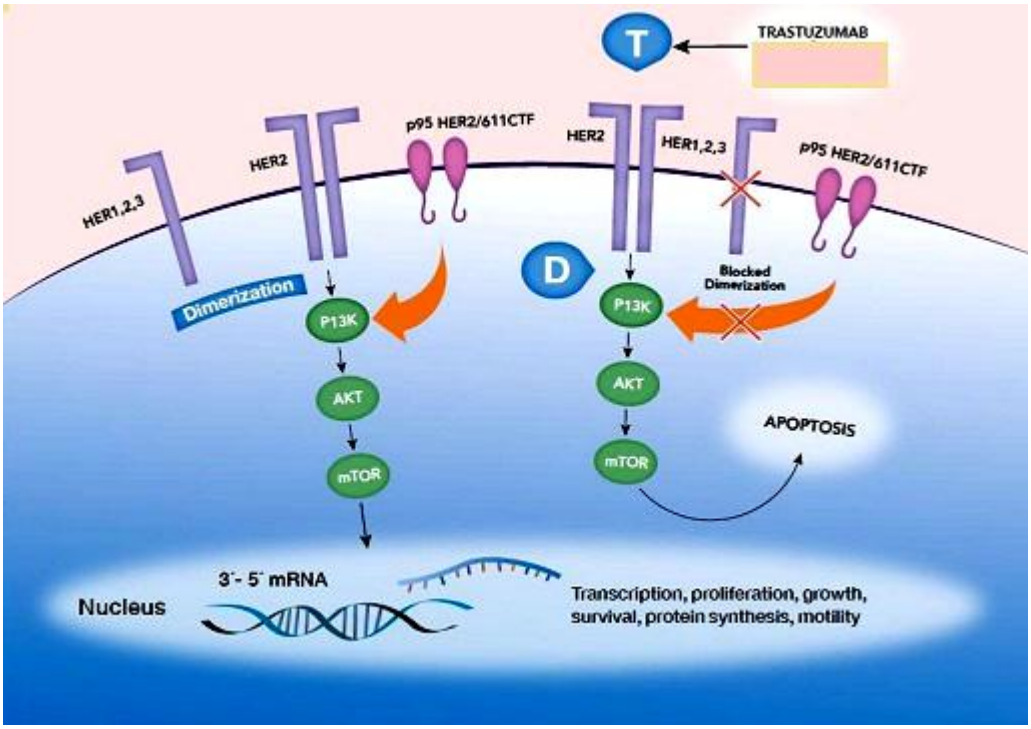


Fig (10): Mechanism of action of trastuzumab in HER2+ve BC **(Gagliato et al, 2016)**

Significant clinical benefits of trastuzumab in the treatment of early BC (eBC) have been observed. Four large trials (and several smaller trials) evaluating adjuvant trastuzumab demonstrated significant improvements in PFS and OS, irrespective of tumor size, nodal status, HR status, or age **(Slamon et al, 2011)**. Trastuzumab provides significant clinical benefit as monotherapy and in combination with chemotherapy as either first- or second-line therapy. On the basis of data from several trials, adjuvant trastuzumab has become the foundation of care for HER2 +ve BC **(Perez et al, 2011)**. A pathologic complete response after NAT is an excellent outcome of HER2 +ve BC **(Cortazar et al, 2014)**.

Trastuzumab is standard treatment for HER2 +ve eBC and MBC, being administered as IV infusion, in part with chemotherapy, as an every-3-weeks regimen for 12 months in eBC (17 cycles), or until disease progression in MBC. Trastuzumab IV needs to be reconstituted into solution for infusion, with the dose calculated on the basis of patient bodyweight. IV trastuzumab treatment starts with a loading dose 8mg/Kg infused over 90 minutes, followed by subsequent maintenance doses 6mg/Kg infused over 30 minutes **(Roche, 2015)**.

A SC formulation of trastuzumab was developed to allow drug administration over a shorter time period approximately 5 minutes, with the goal of improving convenience and compliance, particularly during long-term therapy. This is facilitated by the fact that trastuzumab has a wide therapeutic window. In addition, trastuzumab SC is given as a fixed dose that does not require adjustment based on bodyweight **(Launay-Vacher, 2013)**. SC administration of volumes more than 1 mL has been limited by the structure and physiology of the SC layer, which contains a matrix of hyaluronan and collagen fibers. To overcome this issue, the trastuzumab SC formulation includes recombinant human hyaluronidase as a novel excipient, the effects are temporary and reversible within 24 hours **(Leveque, 2014)**.

Pharmacokinetic modeling, determined that a 600 mg fixed dose of trastuzumab SC **(Ismael et al, 2012)** was noninferior to trastuzumab IV with respect to efficacy and had an equivalent tolerability profile **(Wynne et al, 2013)**. The recent development of a SC formulation of trastuzumab is an important step towards improved patients' care. This results in substantial time savings for patients, pharmacists, physicians and nursing staff, with a healthcare professionals' and patients' preference largely going to SC. In addition, the possibility to treat patients with poor venous access or to spare patients' venous capital when necessary may also be of interest **(Launay-Vacher, 2013)**. A recent study provided an indication that switching from IV to SC administration of trastuzumab has beneficial effects on resource use, including consumables needed to administer treatment. These reductions contribute to health care cost efficiencies that have the potential to influence how and where HER2-+ve BC treatment is delivered in the future **(North et al, 2015)**.

Weekly trastuzumab and paclitaxel was proved to be safe and effective inHER2 MBC **(Gori et al, 2004)** and a median PFS of 8.5 months **(Kaufman et al, 2009)**. The results of carboplatin in HER2 MBC were controversial **(von Minckwitz et al, 2014)**. Anthracycline combination improved cure rate **(Huang et al, 2015)**. The targeting of HER2 with more than one agent is better than use of a single agent in a first/second-line setting. A recent study supported the treatment with trastuzumab emtansine as well as pertuzumab in combination with trastuzumab and docetaxel **(Yu et al, 2015)**. The best strategy for the treatment of HER2 MBC is trastuzumab and lapatinib **(Qin et al, 2015)**.

Pertuzumab is a HER2-targeted monoclonal Ab that provides a more complete blockade of the HER pathway in combination with trastuzumab (dual blockade), resulting in augmented anticancer activity in patients with HER2 +ve BC. It is also recommended in patients with HER2 MBC who might not be good candidates for docetaxel **(Moya-Horno and Cortés, 2015).**

The efficacy of trastuzumab in HER2 eBC **(Perez et al, 2011)** is at the cost of significant cardiotoxicity, which manifests usually as either asymptomatic left ventricular dysfunction or as symptomatic heart failure. The incidence of cardiotoxicity was highest in patients receiving trastuzumab and anthracyclines (27%) with lower risk in patients receiving trastuzumab plus paclitaxel (13%) or trastuzumab alone (3-7%) in MBC. In significant cardiotoxicity trastuzumab was terminated early. Although cardiac complications of trastuzumab therapy are frequent, they seem not to be harmful. Most of them are transient, asymptomatic and reversible and do not affect long-term outcome **(Piotrowski et al, 2012)**. Trastuzumab cardiotoxicity presents most frequently in the form of asymptomatic decrease in left ventricular ejection fraction **(Cao et al, 2016).**

Risk factors for the development of chemotherapy-related congestive heart failure include cumulative doses and increasing patient age. In trastuzumab therapy, prospective cardiac function monitoring was performed. A reduced incidence of heart failure was seen in adjuvant (post-surgery) trials, compared with metastatic setting (**Xie et al, 2015)**.

Despite the clinical benefit of HER2-targeted therapies, a substantial percentage of patients die of disease progression due to drug resistance. Multiple mechanisms of resistance have been proposed, including the deregulation of the phosphatidylinositol 3-kinase (PI3K) pathway, which is caused by activating mutations or a loss of phosphatase and tensin homolog (PTEN). The reduced receptor-antibody binding affinity and the increased signaling via alternative HER and non-HER family receptor tyrosine kinases were also suggested **(De Mattos-Arruda et al, 2015)**.

Resistance to trastuzumab suggests that CSCs may be at the root of resistance of HER-2 targeted therapy. A variety of novel HER-2 targeted approaches have demonstrated promising preliminary clinical activity. Future clinical trials should involve the assessment of novel HER-2 targeted therapies on HER-2 positive CSCs **(Bedard et al, 2009)**. In MBC, it was reported that to initiate metastases, disseminated tumor cells with characteristics of CSCs must acquire the ability to intravasate into and survive in the peripheral circulation **(Giordano et al, 2012).** Cytokeratins and EGFR expression were proposed to be predictive of worse prognosis in HER2 +ve BC patients treated with trastuzamab **(Chung et al, 2015) (Fig 11)**.

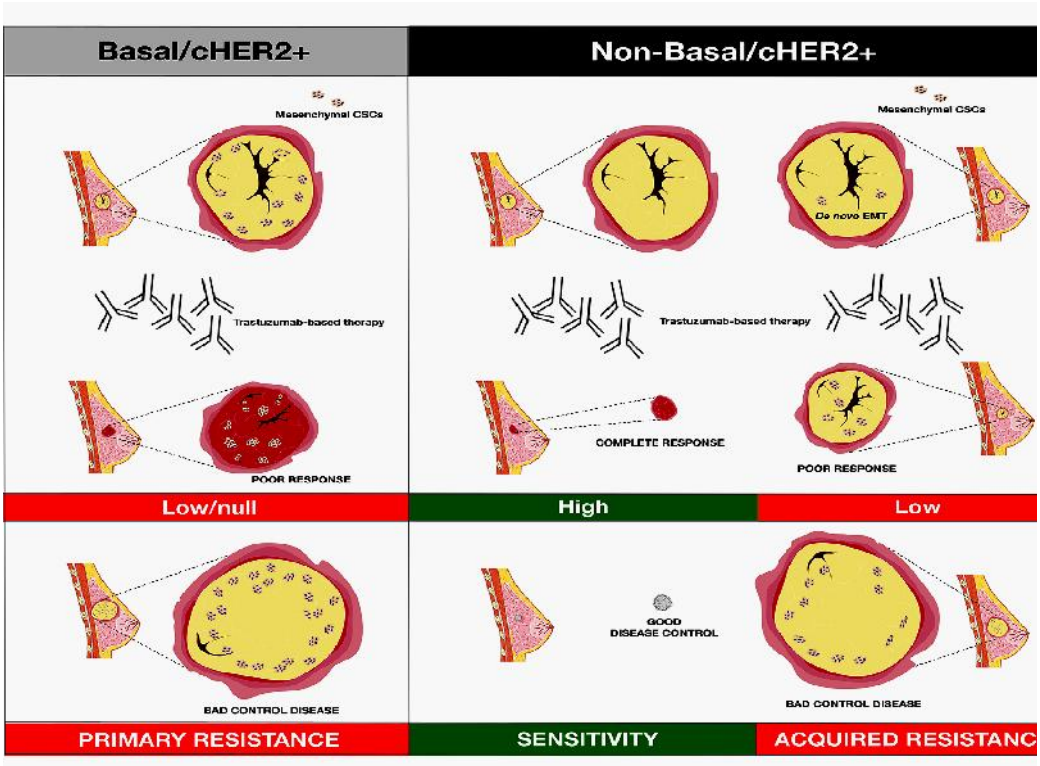


Fig (11): CSC mixed phenotypes and evolutionary response of cHER2+ BC to Trastuzumab **(Martin-Castillo et al, 2015b)**.

2.3.Stem Cells

2.3.1.Introduction

Stem cells (SCs) treatments are a type of genetic medicine that has the potential to change the face of human disease and alleviate suffering. (**Weissman, 2000).** They are slow cycling but highly clonogenic with a great potential for error-free division. They are a self-renewing population of cells that can proliferate indefinitely (**Fuchs and Segre, 2000).** Adult stem cells, residing in multiple tissues are activated depending on cellular signals **(Tsai and McKay, 2000).**

The classical definition of SCs should fulfill three criteria. The first is self-renewal, undergoing symmetrical or asymmetrical cell division. The second is potency, SCs must be capable of multilineage differentiation. The third is in-vivo functional reconstitution of given tissue **(Ulloa-Montoya et al**, **2005)**.

Medical researchers anticipate one day being able to use technologies derived from adult and embryonic SCs research to treat cancer **(Singec et al, 2007).** More research is needed concerning SCs behavior **(Gurtner et al, 2007)**.

Types of SCs include: **Totipotent cells** produced from fusion of an ovum and sperm and cells produced by first few divisions. They have potential to differentiate into derivatives of all three embryonic germ layers. **Pluripotent cells** are the descendants of totipotent cells and can differentiate into nearly all cells derived from the three germ layers except trophoblast cells **(Tandon, 2009)**. **Multipotent (progenitor) cells** can give rise to other types of cells but they are limited in their ability to differentiate. **Oligopotent cells** can differentiate into only a few cells, such as lymphoid or myeloid SCs. **Unipotent cells** can produce only one type of cells that is only of their own. They

have the property of self- renewal which distinguishes them from non-stem cells, e.g. muscle SCs **(Fig 12) (Mitalipov and Wolf, 2009)** and **(Skurikhin et al, 2014).**

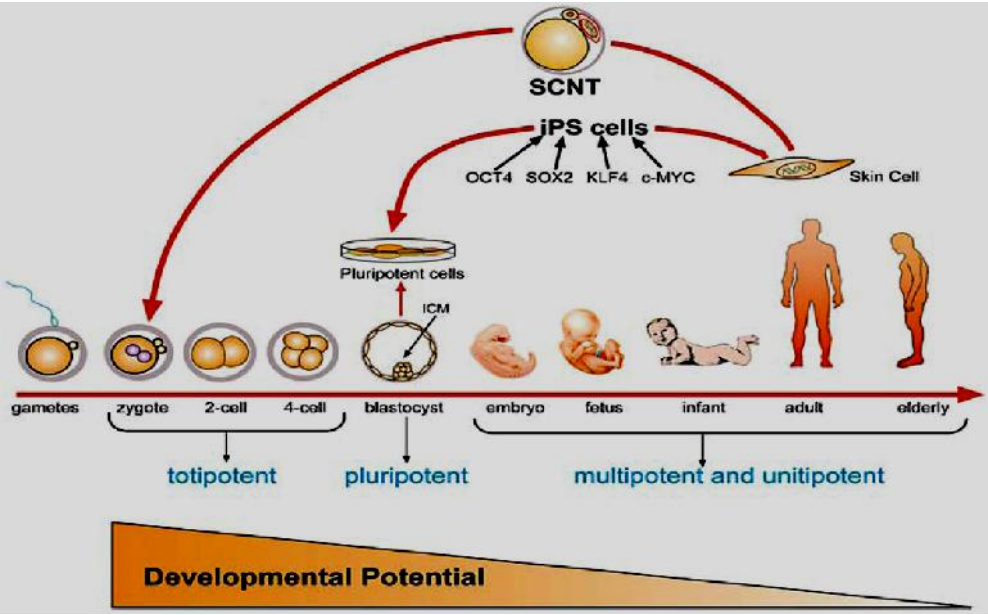


Fig (12): Totipotent, pluripotent, multipotent, oligopotent, unipotent stem cells and terminally differentiated somatic cells that develop into whole organism **(Mitalipov and Wolf, 2009).**

There are predominantly three classes of stem cells: embryonic stem cells **(ESCs)**, induced pluripotent stem cells **(iPSCs)** and adult stem cells **(ASCs)**. The ESCs are thought to be the most naïve SCs because they are extracted in the early developmental stages of the embryo. Nevertheless, ESCs have number of limitations. A single embryo produces a limited amount of SCs, spontaneous uncontrolled differentiation and developmental abnormalities and increased immunogenicity. In addition to lacking a fully developed G1 check point, a common characteristic of cancer cells, which increases the chances of acquiring mutations and transformation into tumor cells. Moreover, the isolation of embryonic stem cells involves the destruction of the embryo, which created some serious ethical and religious problems **(Fig 13) (Werbowetski-Ogilvie et al, 2009)** and **(Christopherson and Nesti, 2011)**.

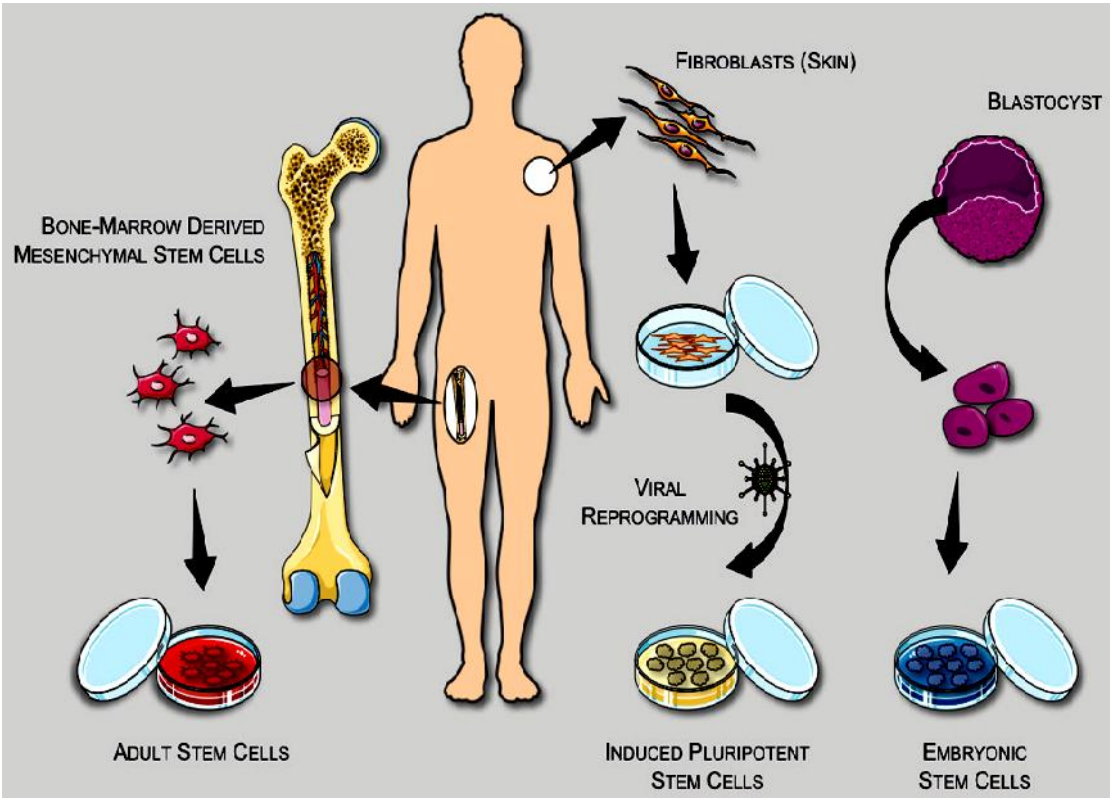


Fig (13): Classes of SCs: Embryonic, induced pluripotent and adult SCs **(Christopherson and Nesti, 2011).**

An iPSC is a SC that has been created from an adult cell - such as skin or blood - through the introduction of genes that reprogram it into a cell with the characteristics of an ESC. The iPSCs have the advantage of not having to be derived from human embryos, a major ethical consideration. Another critical advantage of iPSC technology is by passing issues of immune rejection **(Christopherson and Nesti, 2011)**.

2.3.2. Somatic or Adult Stem Cells (ASCs):

Adult Stem Cells are undifferentiated cells occurring in tissues and organs of adults that may be converted to differentiated cells of the tissue where they are present. The potential ASCs plasticity is termed transdifferentiation **(Poulsom et al, 2002).** SC plasticity for transdifferentiation are more pronounced in embryonic SCs compared to ASCs **(Bishop et al, 2002)**.

In most cases, SCs from adult tissues are able to differentiate into cell lineages characteristic of niche where they are located. For example, the SCs of the central nervous system have the ability to generate neurons, glial supportive cells **(Alison and Islam, 2009)**.

Adult stem cells use has no ethical conflicts nor involves immune rejection problems in cases of autologous implantation**.** They offer alternative possibilities for tissue repair and originate from easier sources, such as fat, skin, olfactory cells and peripheral blood **(Placzek et al, 2009)**.

Adult stem cells have been isolated and characterized in various body tissues, such as bone marrow (BM), umbilical cord, epithelium, dental pulp and more recently, adipose tissue **(Yarak and Okamoto, 2010)**. The adult SCs could be haematopoietic stem cells (HSCs) giving rise to all blood cells and adherent stromal/mesenchymal stem cells (MSCs) **(Abdulrazzak et al, 2010).**

2.3.3. Mesenchymal Stem Cells (MSCs):

They are also known as marrow stromal cells or mesenchymal progenitor cells. MSCs are adult multipotent SCs found mainly in the BM, but the BM harbors weak points. These include an invasive procedure, a high degree of viral exposure and a significant decrease in cell numbers, proliferation and differentiation capacity with advancing age **(Kern et al, 2006).**

An alternative source for MSCs is umbilical cord blood (UCB). It can be obtained by a less invasive method, without harm for the mother or the infant. It is well accepted that UCB is considered a source of human MSCs and transplantation of cord blood is a part of clinical practice **(Lee et al, 2004)**. The second common source from which MSCs are derived is adipose tissue (AT) **(Schaffler and Buchler, 2007).**

Mesenchymal stromal cells, as defined by the International Society for Cellular Therapy, are plastic-adherent cells with a specific surface phenotype **(Fig 14) (Uccelli et al, 2008)** and **(Stanko et al, 2014)**.

Umbilical cord-MSCs, adipose-MSCs and bone marrow-MSCs exhibit similar morphology, while the immunophenotypes of these MSCs coincide with identification criterion for MSCs, The difference-associated mechanisms need to further study **(Chi et al, 2014)**.

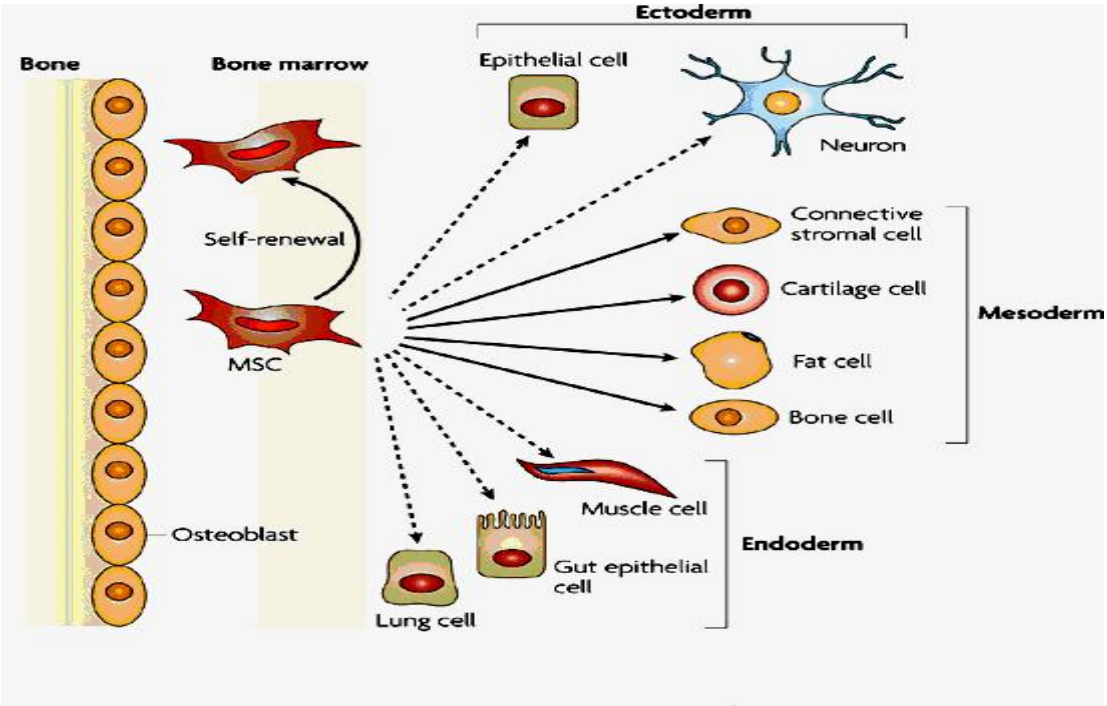


Fig (14): Differentiation and trans differentiation of MSCs **(Uccelli et al, 2008)**.

2.3.4. Cancer Stem Cells (CSCs):

The requirement of the breast to undergo the high level of proliferation observed during puberty and pregnancy suggests the presence of a stem cell population within the breast. There is evidence that a population of cells within BC display stem cell properties. In addition, mammosphere culture has been used to assess both normal and CSCs activity. CXCR4 is the most common chemokine receptor expressed on tumour cells and has been detected in 23 different types of cancer. CXCR4 signalling has been linked with aggressiveness and the promotion of metastasis **(Zlotnik, 2006)**, with cells

expressing the receptor homing to tissues secreting stromal cell factor-1 (**Ablett et al, 2013)**.

Strong evidence is emerging supporting the hypothesis that CSCs with similar features to normal tissue SCs are resistant to standard chemotherapy and drive tumor regrowth after therapy finishes. We hypothesised that biological properties of normal SCs are reactivated in tumour cells to facilitate metastasis. Genes expressed in SCs of the normal mammary gland might therefore carry prognostic information for relapse and metastasis in BC **(Smalley et al, 2013**).

The mammary epithelium consists of two main layers, the luminal and basal layers. The luminal layer consists of ER-ve cells (mainly proliferative progenitors) and ER+ve cells (mainly non-proliferative differentiated cells). The basal layer consists of myoepithelial cells (MYOs) and mammary stem cells (MaSCs), the latter are characterized by their robust growth in transplantation. Only one previous study has purified basal SCs and compared their gene expression profile to MYOs; however, that study identified only four genes expressed >2-fold more highly in SCs compared to MYOs (**Soady et al, 2015)**.

The tumor microenvironment plays an important role in tumor evolution. There is growing evidence that the stromal microenvironment around cancer cells influences the growth, invasiveness, and metastatic behavior of cancer cells, and may be a factor in therapeutic response. Tumor associated stroma contains various cell types and molecules secreted into the EC matrix. There is emerging evidence that fibroblasts may actively function in the induction of cancers, and that tumor invasion is influenced by external signals from the tumor associated stroma **(Quail and Joyce, 2013)**. Inhibition of these microenvironment signals represents a new therapeutic strategy against cancer metastasis. Targeting of stromal cells with less genetic plasticity than cancer cells opens new avenues for investigation of novel therapeutic agents **(Xu et al, 2016)**.

It was demonstrated that genetic disruption of Ki-67 in human epithelial breast cancer cells depletes the CSC niche. Ki-67 -ve cells had a proliferative disadvantage and displayed increased sensitivity to various chemotherapies. Ki-67 -ve cancer cells showed decreased tumor formation, which was associated with a reduction in CSCs markers. The maintenance of Ki-67 expression is associated with metastatic potential and maintenance of CSCs niche, which has potential diagnostic and therapeutic importance for human malignancies **(Cidado et al, 2016)**.

2.3.5. Stem Cell Markers:

Specific monoclonal antibodies, that recognize the surface markers, have been used for prospective identification of self-renewing and multipotent neural SCs from human fetal brain **(Uchida et al, 2000).** Expressed mesenchymal SC markers, cluster of differentiation (CD) 105, CD166 on their cell surface **(Nagatomo et al, 2006).** Additional mesenchymal SC markers CD29, CD73, CD44, and CD90 were reported. Hematopoietic SC markers CD34, CD45, CD117, and CD133 were recorded **(Fig 15) (Watt et al, 2010)** and **(Nartprayut et al, 2013).**

Recently**,** immunohistochemical staining technology was used to detect the expression of the SC marker CD133. The latter is considered one of the markers for cancer SCs in human leukemia, brain tumors, prostate cancer and laryngeal tumors **(Zhou et al, 2007)**. Teratoma cells derived from human embryonic SCs were proved to express high levels of CD56 **(Su et al, 2011).**

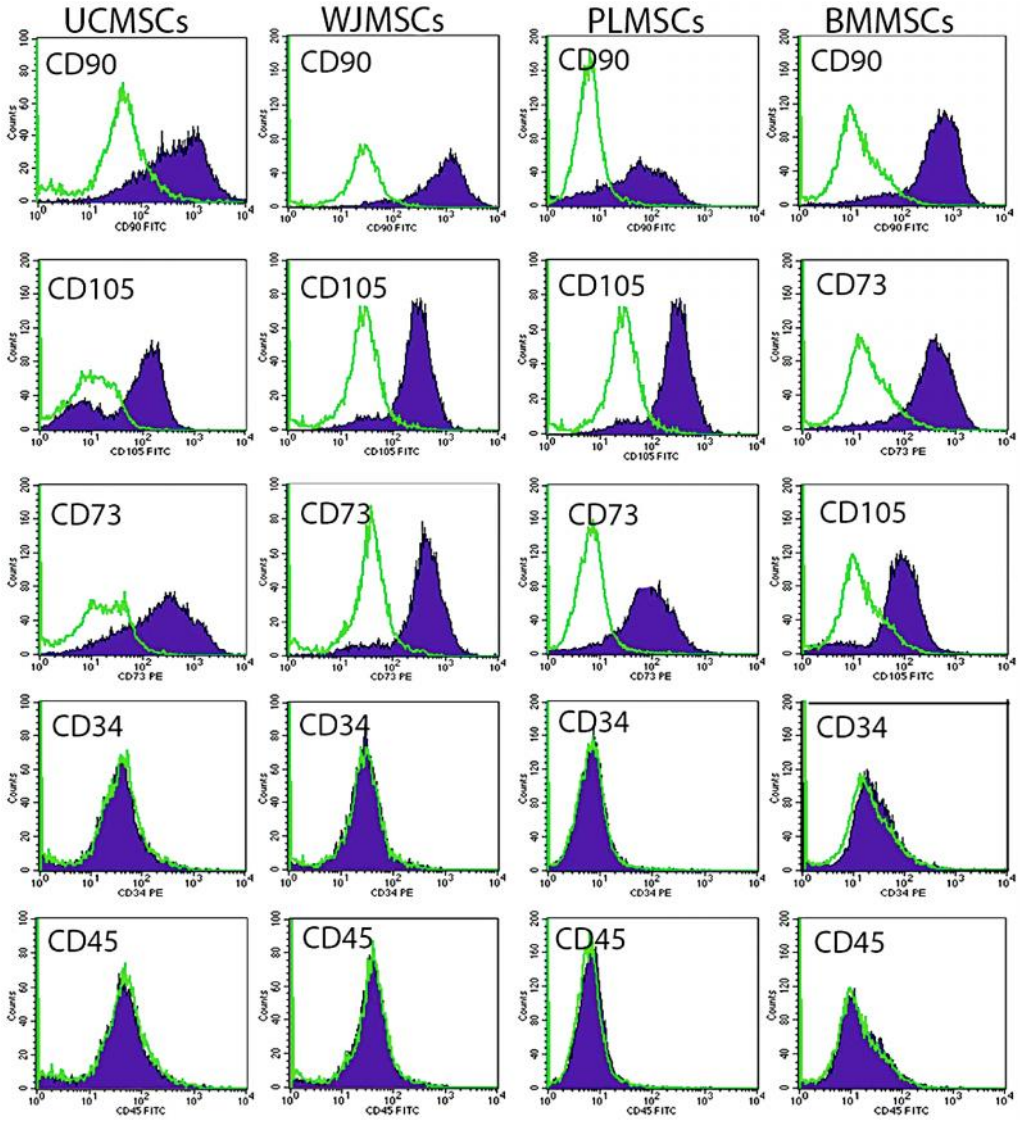


Fig (15): MSC positive and negative markers **(Nartprayut et al, 2013).**

Umbilical cord MSCs (UCMSCS), Wharton's jelly MSCs (WJMSCs), placental MSCs (PLMSCs) and bone marrow MSCs (BMMSCs)

3. MATERIALS AND METHODS

Studied Subjects:

This retrospective study (2012-2015) was conducted in the **Departments of Medical Oncology, Pathology, Biochemistry, Medical Histology and Cell Biology, Faculty of Medicine, Cairo, Egypt** in collaboration with **Faculty of Pharmacy, Near East University North Cyprus.** The study was approved by the institutional review board.

The study was conducted on 40 BC patients age range 27-63 years (average 45 years), 20 served as **control group** and the other 20 as **metastatic (predicted resistance) group**. All patients had BC confirmed with a microscopic examination with proven IHC analysis of HER2+ disease, in postoperative specimens or samples obtained by biopsy. All BC patients gave informed consent according to the institutional review board approved protocol.

3.1. Clinicopathological Analysis:

The study group comprised inclusion criteria for trastuzumab therapy and medical history without serious comorbid conditions such as unstable ischemic heart disease, valvular heart disease, chronic hypertension with cardiovascular problems or uncontrolled diabetes (uncontrolled diabetes is classified as a random blood glucose level ≥ 11.0 mmol/l in diabetic patients). Subsequent inclusion criteria were the peripheral blood count, renal and liver function within normal values. The data, including the age at onset, menopausal status, weight, height, the history of neoplasms in the first degree family and diabetes mellitus (DM) were recorded **(Tables Ia and Ib)**. Date of diagnosis, breast imaging, surgical procedures, tumor stage according to TNM classification, grade and proliferation index were collected **(Tables IIa and IIb)**. HER2 status, ER status, PR status, baseline ejection fraction (EF), chemotherapy regimen and number of herceptin cycles **(Tables IIIa and IIIb)** were gathered from hospital records and pathology reports.

Table Ia.Control Cases Data

Age	Weight	Height	Meno-pause	Family History	D M
37	**57**	**158**	**pre**	**-**	**-**
48	**73**	**158**	**pre**	**-**	**-**
48	**72**	**153**	**pre**	**-**	**-**
27	**59**	**160**	**pre**	irrelevant	**-**
62	**83**	**160**	**post**	**-**	**-**
48	**95**	**160**	**pre**	**-**	**+**
49	**85**	**155**	**pre**	**-**	**+**
49	**72**	**160**	**pre**	**-**	**-**
45	**74**	**151**	**pre**	**-**	**-**
60	**77**	**160**	**post**	**-**	**-**
33	**80**	**162**	**pre**	**-**	**-**
45	**75**	**159**	**pre**	**-**	**-**
34	**74**	**155**	**pre**	**-**	**-**
53	**68**	**157**	**post**	**-**	**-**
40	**82**	**167**	**pre**	**-**	**-**
37	**75**	**171**	**pre**	**-**	**-**
61	**90**	**156**	**post**	**-**	**+**
41	**90**	**160**	**pre**	**-**	**+**
30	**78**	**165**	**pre**	**-**	**-**
60	**65**	**155**	**post**	**-**	**-**

DM=Diabetes mellitus

Table Ib.Predicted Resistant Cases Data:

Age	Weight	Height	Meno-pause	Family History	D M
32	**63**	**150**	**pre**	irrelevant	**-**
60	**65**	**150**	**post**	irrelevant	**-**
32	**63**	**150**	**pre**	**-**	**-**
39	**57**	**165**	**pre**	**-**	**-**
39	**62**	**150**	**pre**	**-**	**-**
44	**70**	**155**	**pre**	irrelevant	**-**
40	**78**	**155**	**pre**	**-**	**-**
63	**96**	**150**	**post**	**-**	**-**
60	**103**	**161**	**post**	**-**	**-**
50	**70**	**145**	**pre**	**-**	**-**
55	**149**	**153**	**post**	irrelevant	**-**
35	**96**	**168**	**pre**	**-**	**+**
45	**84**	**160**	**pre**	**+**	**-**
41	**80**	**155**	**pre**	**-**	**-**
39	**85**	**163**	**pre**	**-**	**-**
60	**91**	**155**	**post**	**-**	**+**
34	**65**	**159**	**pre**	**-**	**-**
33	**125**	**185**	**pre**	**-**	**-**
50	**75**	**155**	**pre**	**-**	**-**
28	**63**	**166**	**pre**	**-**	**-**

Table IIa.Diagnostic Criteria of Control Cases:

Date of diagnosis	Breast Imaging	BC surgery	TNM stage	Grade	Ki-67
2014-8	Mam	Mastectomy	T2N0M0	II	10%
2014-12	Mam+US	Biopsy	T3N0M0	II	10%
2015-11	Mam+US	Biopsy	T2N0M0	II	20%
2014-5	Mam	Biopsy	T2N0M0	III	20%
2014-3	Mam	Mastectomy	T2N0M0	II	20%
2015-4	Mam+US	Biopsy	T3N0M0	II	20%
2014-8	Mam	Biopsy	T1N0M0	II	20%
2014-5	Mam	Biopsy	T2N0M0	II	12%
2013-10	Mam	Biopsy	T2N0M0	II	10%
2015-3	Mam	Biopsy	T3N0M0	II	20%
2014-6	Mam	Biopsy	T1N0M0	II	20%
2013-2	Mam	MRM	T2N0M0	II	20%
2015-6	Mam+US	Biopsy	T4N0M0	II	20%
2015-6	Mam+US	Mastectomy	T1N0M0	II	18%
2015-3	Mam	MRM	T2N0M0	II	18%
2013-11	Mam	Biopsy	T2N0M0	II	14%
2014-6	Mam	MRM	T2N0M0	II	25%
2015-7	Mam+US	Biopsy	T2N0M0	II	20%
2015-3	Mam	Mastectomy	T1N0M0	II	20%
2014-2	Mam	Biopsy	T2N0M0	II	14%

Mam=mammography MRM=modified radical mastectomy

US=Ultra sound Ki67= Proliferation index

Table IIb.Diagnostic Criteria of Predicted Resistant Cases:

Date of diagnosis	Breast Imaging	BC surgery	TNM stage	Grade	Ki-67
2015-1	Mam	Biopsy	T3N1M0	II	50%
2015-2	Mam	Biopsy	T2N1M0	II	20%
2014-12	Mam+US	Biopsy	T3N1M0	II	50%
2014-10	Mam	Biopsy	T2N3M0	II	14%
2013-10	Mam	Mastectomy	T3N1M0	II	40%
2014-9	Mam	Biopsy	T3N1M0	II	50%
2014-2	Mam+US	Mastectomy	T2N1M1	III	14%
2014-1	Mam+US	Biopsy	T1N2M0	II	20%
2014-10	Mam	Biopsy	T2N0M1	II	40%
2012-9	Mam	Mastectomy	T2N0M1	II	40%
2014-12	Mam	MRM	T2N1M0	II	10%
2015-1	Mam	Mastectomy	T3N3M0	II	40%
2015-1	Mam	MRM	T2N3M0	II	70%
2014-1	Mam+US	MRM	T4N3M0	II	40%
2014-3	Mam+US	Biopsy	T3N2M0	II	60%
2015-3	Mam+US	Biopsy	T2N1M0	II	40%
2015-5	Mam	MRM	T3N1M0	II	25%
2014-12	Mam+US	Mastectomy	T2N2M0	II	80%
2014-2	Mam+US	Biopsy	T1N1M1	II	30%
2015-2	Mam	Biopsy	T2N2M0	II	20%

Table IIIa.Diagnostic Data and Chemotherapy (Cth) Regimen of Control Cases:

ER	PR	HER2	Baseline EF	CthRegimen	No of cycles
0/8	0/8	3+	70%	AC-T+Hercept	16
0/8	0/8	3+	61%	AC-T+Hercept	12
0/8	0/8	3+	72%	AC-Taxotere+Hercept	3
0/8	0/8	3+	71%	AC-T+Hercept	15
0/8	0/8	3+	67%	AC-T+Hercept	17
0/8	0/8	3+	60%	AC-T+Hercept	12
0/8	0/8	3+	70%	FEC-Taxotere-Hercept	11
0/8	0/8	3+	70%	FEC -T-Hercept	10
0/8	0/8	3+	70%	AC- T+Hercept	12
0/8	0/8	3+	70%	AC-T+Hercept	12
3/8	0/8	3+	70%	AC-Taxotere+Hercept	14
3/8	0/8	3+	67%	FEC -T-Hercept	16
0/8	0/8	3+	70%	FAC -T-Hercept	9
3/8	0/8	3+	70%	AC-Taxotere+Hercept	9
0/8	0/8	3+	57%	FEC -Taxotere-Hercept	17
0/8	0/8	3+	61%	AC-T+Hercept	14
3/8	0/8	3+	70%	FEC -Taxotere-Hercept	17
0/8	0/8	3+	62%	FEC -Taxotere-Hercept	10
0/8	0/8	3+	65%	AC-T+Hercept	12
0/8	0/8	3+	65%	FEC -Hercept	14

ER=Estrogen receptor EF=Ejection fraction

PR=Progesterone receptor

Table IIIb .Diagnostic Data and Cth Regimen of Predicted Resistant Cases:

ER	PR	HER2	Baseline EF	CTRegimen	No of cycles
3/8	0/8	3+	65%	AC-T+Hercept	15
4/8	4/8	3+	70%	T- Carbo+Hercept	14
6/8	0/8	3+	65%	AC-T+Hercept	15
3/8	4/8	3+	70%	AC-T+Hercept	17
3/8	3/8	3+	70%	AC-T+Hercept	13
4/8	8/8	3+	70%	AC-T+Hercept	17
0/8	0/8	3+	65%	T- Carbo+Hercept	12
3/8	3/8	3+	74%	AC-T+Hercept	15
3/8	3/8	3+	75%	FEC -Taxotere-Hercept	17
0/8	0/8	3+	70%	AC+Hercept+Tykerb Xeloda	11
7/8	8/8	3+	61%	FEC -Taxotere-Hercept	12
8/8	5/8	3+	70%	AC-Taxol+Hercept	14
7/8	7/8	3+	65%	AC-Taxol+Hercept	15
6/8	6/8	3+	70%	AC-Taxol+Hercept	10
8/8	3/8	3+	68%	AC-Taxol+Hercept	16
7/8	6/8	3+	70%	FEC -Taxotere-Hercept	14
3/8	3/8	3+	65%	AC-Taxol+Hercept	12
0/8	4/8	3+	63%	AC-Taxol+Hercept	17
0/8	0/8	3+	70%	AC-Taxol+Hercept	17
3/8	3/8	3+	70%	AC-Taxol+Hercept	15

AC-T+ Hercept=Adriamycin Cyclophosphamide-Taxol+Herceptin

Carbo=Carboplatin FEC =FluoroUracil Epidoxorubicin Cyclophosphamide

FAC= FluoroUracil Adriamycin Cyclophosphamide

All tumor paraffin embedded specimens were assigned a study identification number that is distinct from the patient's medical record number. The histological type and grade of invasive disease were coded according to the TNM classification system. Tumor specimens were analyzed based on evidence of IHC staining. Specimens that had a 3+ stain intensity on **IHC analysis** were considered HER2+.

Disease status was evaluated every 6-12 weeks using the same imaging techniques and the treatment schedule until end of follow-up or relapse (development of resistance).

The 40 HER2 +ve BC patients received trastuzumab treatment and the predicted resistance to treatment was further investigated for by:

3.2. Histological Analysis:

Performed in Medical Histology and Cell Biology Department.

3.2.1. Molecular Subtypes Markers:

HER2 **(Rouzier et al, 2005)** and cytokeratin (CK) 5/6 immnostaining **(Chung et al, 2015)**

3.2.2. Cancer Stem Cell Marker:

A mesenchymal subset of CSCs was defined as cells with a CD44+ immunostaining **(Fillmore and Kuperwasser, 2008) (Chen et al, 2015)**.

3.2.3. Morphometric Study:

Measurements of:

a. Area% of HER2 +ve immunoexpression

b. Area% of CK 5/6 +ve immunoexpression

c. Area% of CD44 +ve cells

3.3. Quantitative real time reverse transcription polymerase chain reaction (qRT-PCR)

Evaluation of FFPE BC specimens by qPCR was performed **(Pu et al, 2015)** by real time reverse transcription **(Wang and Seed, 2003)** in Biochemistry Department.

3.4. Statistical Analysis:

A P value of <0.05 was considered statistically significant. All statistical analyses were conducted using unpaired student t-test in **Statistical Package for the Social Sciences** (SPSS) 16 software **(Emsely et al, 2010)**.

Methods

3.1. Clinicopathological Analysis:

Analysis was performed using the patient's files at the Archieve Unit of the Medical Oncology Department.

3.2. Histological Analysis:

3.2.1. Molecular Subtype Markers HER2 and CK5/6

3.2.2. Cancer Stem Cells Marker CD4 (Isabel, 1990) after (Zickri et al, 2015)

Immunostaining provides a way of identifying substances in tissues using antigen-antibody reactions which can be made microscopically visible through the incorporation of a suitable label. Using the avidin-biotin immunoperoxidase polyclonal kit provided by Lab. Vision Corporation.

• Biotin:

This non-enzymatic label is finding increasing use in immunocytochemistry, in combination with avidin. Many mole molecules of biotin can be conjugated to the fraction crystallizable (Fc) portion of one immunoglobulin molecule and, since biotin combines very tightly with avidin, avidin labelled with enzyme can be used to reveal the site of antigen-antibody reaction.

• Avidin:

Avidin is present in the egg white and is composed of four subunits, which form a tertiary structure processing four biotin-binding hydrophobic pockets. The oligo-saccharide residues

present in egg white avidin and its charge properties appear to give it some affinity for tissue compartments, particularly lectin-1ike proteins, which results. in non-specific binding. A similar molecule, streptavidin, molecular weight 60 kilodalton (kd) is extracted from the culture broth of the bacterium streptomyces avidinii. The lack of oligosaccharide residues and its neutral isoelectric point give streptavidin some advantages, and it is becoming widely used. If streptavidin is not available, using a pH 9 buffer will help to prevent non-specific attachment and will not affect the immuno-reaction.

• Peroxidase (Enzyme Label):

The most useful reaction product is derived from the oxidative polymerization of 3, 3'-diaminobenzidine (DAB) tetra-hydrochloride. The action of antibody-bound peroxidase on hydrogen peroxide produces oxidation of the DAB and the reactions product is localized on the site of the antigen-antibody reaction as a dark brown and insoluble deposit giving good contrast with the unreactive parts of the section. If there is active peroxidase present in the tissue to be incubated, it is blocked before the peroxidase labelled antibody is applied in order to confirm whether the end product is endogenous or antibody-associated enzyme. For paraffin sections the blocking agent is usually hydrogen peroxide-methanol, which must be fresh.

• Avidin-Biotin Methods:

Three methods have been described using avidin-biotin that use biotin-labeling of the primary (direct) or secondary (indirect) antibody. In the labelled avidin method the tracer is attached directly to the avidin molecule **(Fig. 1)**. In the Avidin-Biotin Complex (ABC) method, a complex of avidin and biotinylated tracer, which contains free avidin-binding sites, is applied to the biotinylated antibody **(Fig. 2)**. In these techniques as most biotin molecules can be attracted to a single antibody, a high tracer to antibody ratio may be achieved. This gives high sensitivity, allowing a high dilution of the primary antiserum to be used. The avidin-biotin technique has every indication of becoming the standard laboratory immunocytochemical method employing peroxidase.

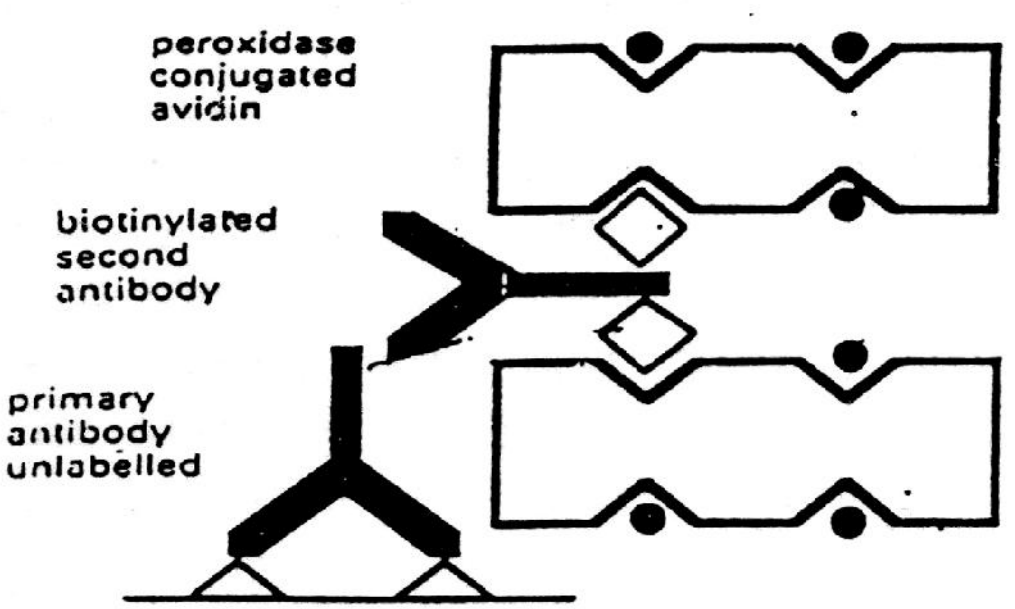


Fig (16): Labeled Avidin method (Noorden, 1990)

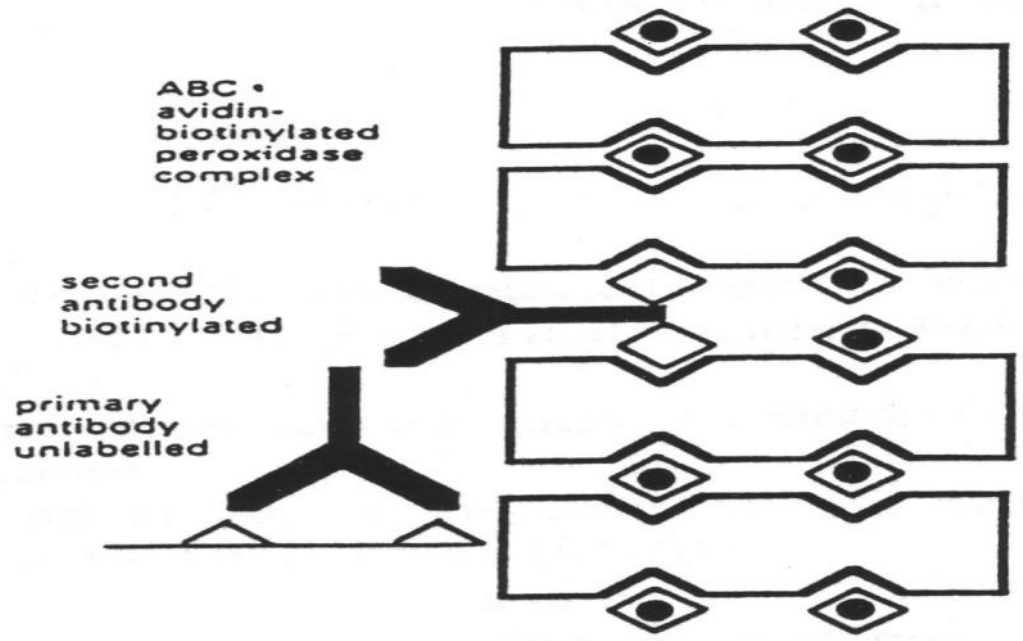


Fig. (17): Avidin-Biotin Complex (ABC) method (Noorden, 1990).

Materials Required:

* 60 ml or 125 ml of ready to use. Hydrogen peroxide block.

* 50 ml (10X) concentrated sodium citrate buffer 10 mM. pH 6.0 for: Heat-Induced Epitope Retrieval (HIER).

* 60 ml ready to use ultra V block (serum blocking solution).

* a) 0.2 ml diluted primary antibody (Ab) **(c-erbB-2 oncoprotein)** (A048529) (Dako).

* b) 0.1 ml prediluted primary Ab **(CK5/6)** (M7237) (Dako).

* c) 0.1 ml diluted primary Ab **(CD44)** (IW-PA1021) (IHW, Ellicott City, USA).

* 125 mL ultra Ab diluent.

* 60 ml ready to use biotinylated goat anti-polyvalent (secondary Ab).

* 60 ml ready to use streptavidin peroxidase conjugate (enzyme conjugate).

* 125 ml of concentrated phosphate buffered saline (PBS) pH 7.6.

* 2 ml of concentrated peroxidase-compatible chromogen (DAB).

* 60 ml DAB substrate.

* 60 ml ready to use haematoxylin solution.

* 15 ml ready to use histomount for DAB.

* Xylene and ethanol.

* Distilled or deionized water.

Staining:

* 5 um thick serial sections were cut and taken on to poly-lysine coated slides.

* Sections are deparaffinized with xylene and rehydrated in graded series of ethanol. Avoid any dryness of the section.

* Boiling of tissue sections in 10 millimoles citrate buffer pH 6.0. for 10-20 minutes and then leave to cool at room temperature (Rt) for 20 minutes.

* Incubate slide in hydrogen peroxide block for 10-15 minutes at 37 °C.

* Wash 2 times in PBS.

* Apply ultra V block and incubate for 5 minutes at Rt and don't exceed 10 minutes [incubate in moist chamber].

* Apply

a) 0.2 ml diluted polyclonal rabbit antihuman **HER2 Ab** and incubate at Rt in moist chamber for 60 minutes.

b) 0.1 ready to use mouse monoclonal **CK5/6 Ab** and incubate at Rt in moist chamber for 60 minutes.

c) 0.1 ml prediluted primary rabbit polyclonal **CD44 Ab** and incubate at Rt in moist chamber for 60 minutes.

* Wash 4 times in **PBS**.

* Apply biotinylated goat antipolyvalent and incubate for 10 minutes at Rt in moist chamber.

* Wash 4 times in **PBS**.

* Apply streptavidin peroxidase and incubate for 10 min. at Rt in moist chamber.

* Wash 4 times in **PBS**.

* Incubate with peroxidase-compatible chromogen (DAB) mixture for 5-15 minutes at RT. *N.B.:* This mixture is prepared by mixing 1-2 drops of DAB chromogen with each 1 ml of DAB substrate.

* Wash slides in distilled water.

* Counterstain the slides with Mayer-Haematoxylin for 1-3 minutes.

* Wash slides in tap water and put into PBS for 30 seconds. Wash in distilled water.

* Dehydrate slides with graded series of alcohol, clear in xylene.

* Add histomount to the slide and mount with coverslip.

Results:

HER2 reaction is membranous/ cytoplasmic. Human tonsil is used as positive control sections for CK 5/6 and CD44 Ab. Cellular localization is cytoplasmic for CK 5/6 and membranous for CD44 **(Fig. 3)**. On the other hand, three of the BC sections were used as a negative control by omitting the step of applying the primary antibodies **.**

The standard CD44 isoform is found in most cells, whereas the variant isoforms are expressed on tumor cells and promote tumor progression and metastatic potential **(Misra et al, 2015).**

3.2.3. Morphometric Study

Using Leica Qwin 500 LTD (Cambridge UK) computer assisted image analysis, assessment of area% of CD44 +ve cells, that of HER2 and that of CK5/6 +ve immunoexpression in the BC immunostained sections were performed. The measurements were done in 10 high power fields in control and resistant sections using binary mode.

3.3. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) (Wang and Seed (2003)

Tissue sampling

We collected paraffin-embedded tumor tissues from 40 BC patients who underwent breast surgery at Kasr ALAini Hospital. Samples where tumor cells comprised ≥ 70% of the sample were selected. Specimens with a small sample size were excluded.

Time required

Cyclic (c) DNA synthesis: 2 hours.

real-time PCR: 2 hours.

Dissociation curve analysis: 0.5 hour.

Reagents and Equipments

- Oligonucleotide Primers. Gene specific primers were retrieved from Primer Bank **(www.harvard.edu/primerbank)**. All the primers (5'ATGAGCTACCTGGAGGATGT 3' 5'CCAGCCCGAAGTCTGTAATTT 3') were desalted and both ultraviolet absorbance and capillary electrophoresis were used to assess the quality of primer synthesis.
- Human total BC RNA (Stratagene).
- Human total RNA master panel (BD Biosciences / Clontech).
- SYBR Green PCR master mix, 200 reactions (Applied Biosystems).
- Optical tube and cap strips (Applied Biosystems).
- SuperScript First-Strand Synthesis System for reverse transcriptase (RT)-PCR (Invitrogen).
- 25 bp DNA ladder (Invitrogen).
- ABI Prism 7000 Sequence Detection System (Applied Biosystems).

- ABI Prism 7000 standard deviation score (SDS) software (Applied Biosystems).
- 3% Ready Agarose Precast Gel (Bio-Rad).
- Agarose gel electrophoresis apparatus (Bio-Rad).

Detailed procedure

Reverse Transcription

Reverse Transcription is carried out with the SuperScript First-Strand Synthesis System for RT-PCR. The following procedure is based on Invitrogen's protocol.

1. Prepare the following RNA/primer mixture in each tube:

5 µg	Total RNA
3 µl	random hexamers (50 ng/µl)
1 µl	10 mM deoxynucleotide Triphosphate mix
to 10 µl	Diethyl Pyrocarbonate H_2O

2. Incubate the samples at 65°C for 5 min and then on ice for at least 1 min.

3. Prepare reaction master mixture. For each reaction:

2 µl	` buffer
4 µl	$MgCl_2$
	Dithiothreitol
2 µl	

4. Add the reaction mixture to the RNA/primer mixture, mix briefly, and then place at room temperature for 2 min.

5. Add 1 µl (50 units) of SuperScript II RT to each tube, mix and incubate at 25°C for 10 min.

6. Incubate the tubes at 42°C for 50 min, heat inactivate at 70°C for 15 min, and then chill on ice.

7. Add 1 µl RNase H and incubate at 37°C for 20 min.

8. Store the 1st strand cDNA at -20°C until use for real-time PCR.

Real-time PCR

1. Normalize the primer concentrations and mix gene-specific forward and reverse primer pair. Each primer (forward or reverse) concentration in the mixture is 5 pmol/µl.

2. Set up the experiment and the following PCR program on ABI Prism SDS 7000. Do not click on the dissociation protocol if you want to check the PCR result by agarose gel. Save a copy of the setup file and delete all PCR cycles. Please note the extension steps are slightly different from described in our paper.

1. 50°C 2 min, 1 cycle
2. 95°C 10 min, 1 cycle
3. 95 °C 15 s -> 60 °C 30 s -> 72 °C 30 s, 40 cycles
4. 72°C 10 min, 1 cycle

3. A real-time PCR reaction mixture can be either 50 µl or 25 µl. Prepare the following mixture in each optical tube.

12.5 µl SYBR Green Mix (2x)		25 µl SYBR Green Mix (2x)
0.2 µl cDNA		0.5 µl cDNA
1 µl primer pair mix (5 pmol/µl each primer)	OR	2 µl primer pair mix (5 pmol/µl each primer)
11.3 µl H_2O		22.5 µl H_2O

4. After PCR is finished, remove the tubes from the machine. The PCR specificity is examined by 3% agarose gel using 5 µl from each reaction.

5. Put the tubes back in SDS 7000.

6. Analyze the real-time PCR result with the SDS 7000 software.

3.4. Statistical Analysis

Quantitative data were summarized as means and standard deviations and compared using unpaired student's T test. P-values <0.05 were considered statistically significant. Calculations were made on SPSS software **version 16 (Chicago, USA).**

4. RESULTS

4. 1. Clinicopathological Results:

In the present study, the analysis of 20 control BC cases and 20 metastatic (predicted resistant) BC cases treated with trastuzumab revealed that: The mean age in the control group at diagnosis was (45.35±10.50) and in the metastatic group it was (43.95±10.92), denoting a nonsignificant difference between both groups. Similarly, the mean weight and the mean height in control group at diagnosis was (76.20±9.80) and (159.1±19.18). In the metastatic group the mean was slightly higher (82.00±23.29) and (157.50±13.98), denoting a nonsignificant difference between both groups **(Table IV)**. The premenopausal patients were 15 versus 5 postmenopausal patients in both group. In the control group, family history was -ve in 19 cases and irrelevant in a case, while in the metastatic group it was -ve in 15 cases, irrelevant in 4 cases and +ve in one case. In the control group, DM was +ve in 4 cases and -ve in 16 cases, while in the metastatic group it was +ve in 2 cases and -ve in 18 cases.

On the other hand, the mean proliferative index in control group at diagnosis was (17.55±2.54) and in the metastatic group it was higher (37.65±4.36), denoting a significant difference ($P<0.05$) between both groups. As regards the mean of ER positivity (+vity) in control group at diagnosis it was (0.60±0.17) and in the metastatic group it was much higher (3.90±0.60), denoting a significant difference ($P<0.05$) between both groups. In the control group, 5 cases received up to 10 cycles and 15 more than10 cycles, while in the metastatic group 1 case received up to 10 cycles and 19 more than10 cycles **(Table IV, Fig18)**.

The date of diagnosis of 3 cases was in 2013, 9 cases in 2014 and 8 cases in 2015 in the control group, while in the metastatic group 1 case was in 2012, 1 in 2013, 11 in 2014 and 7 in 2015. Concerning breast imaging 14 cases underwent mammography and 6 cases performed US in addition in the control group, while in the metastatic group 12 cases underwent mammography and 8 cases performed US in addition. In the control group 13 cases underwent biopsy, 3 underwent MRM and 4 mastectomy, while in the metastatic group 11 cases underwent biopsy, 4 underwent

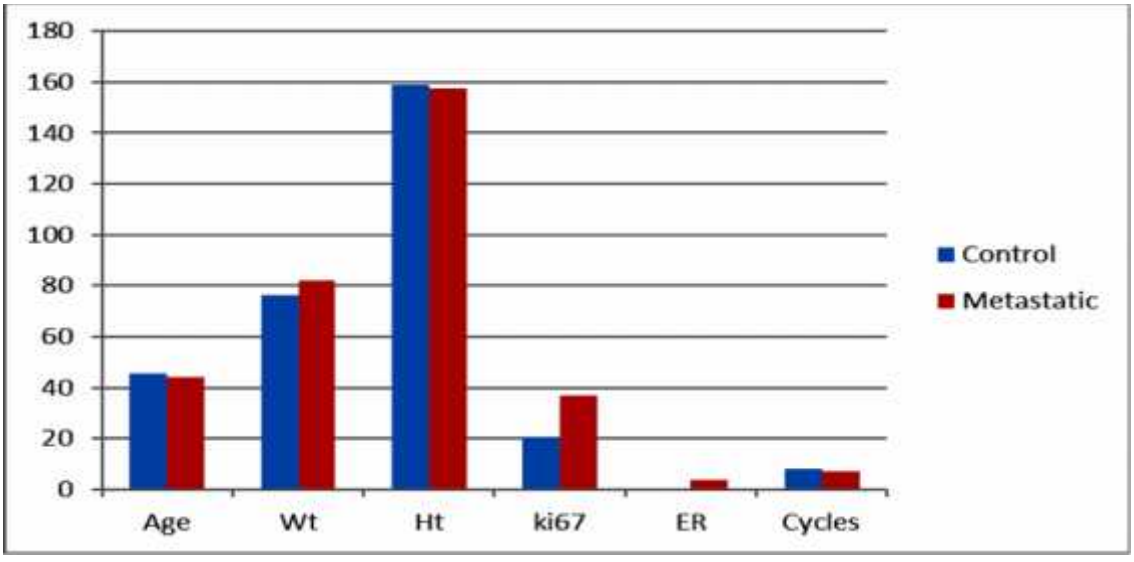
MRM and 5 mastectomy. TNM staging revealed 4 cases T1, 12 cases T2, 3 cases T3 and 1 case T4 and 20 cases N0 in the control group. In the metastatic group, 1 case T1, 10 cases T2, 7 cases T3, 1 case T4, 2 cases N0, 10 cases N1, 4 cases N2, 4 cases N3 and 4 cases M1. PR was +ve in 15 cases in the metastatic group.

Table IV: Mean ± standard deviation (SD) of clinicopathological results

Group	Age	Weight	Height	Ki67	ER+vity	Cycles
Control	45.35± 10.50	76.20± 9.80	159.1± 19.18	17.55± 2.54	0.60± 0.17	12.60± 1.32
Metastatic	43.95± 10.92	82.00± 23.29	157.50± 13.98	37.65± 4.36*	3.90± 0.60*	14.40± 0.96

* significant P<0.05

Fig (18): Mean of clinicopathological results



4.2. Histological Results:

4.2.1. Molecular subtypes markers:

In the control group, **HER2 immunostained sections** demonstrated obvious +ve immunoexpression (IE) among the malignant cells **(Fig 19a).** While in the metastatic group, less obvious IE appeared in the specimens of cases with lower proliferation index (PI) and low ER and PR +vity **(Fig 19b)**. On the other hand, minimal IE was detected in the specimens of patients with high PI, high ER and PR +vity **(Fig 19c).**

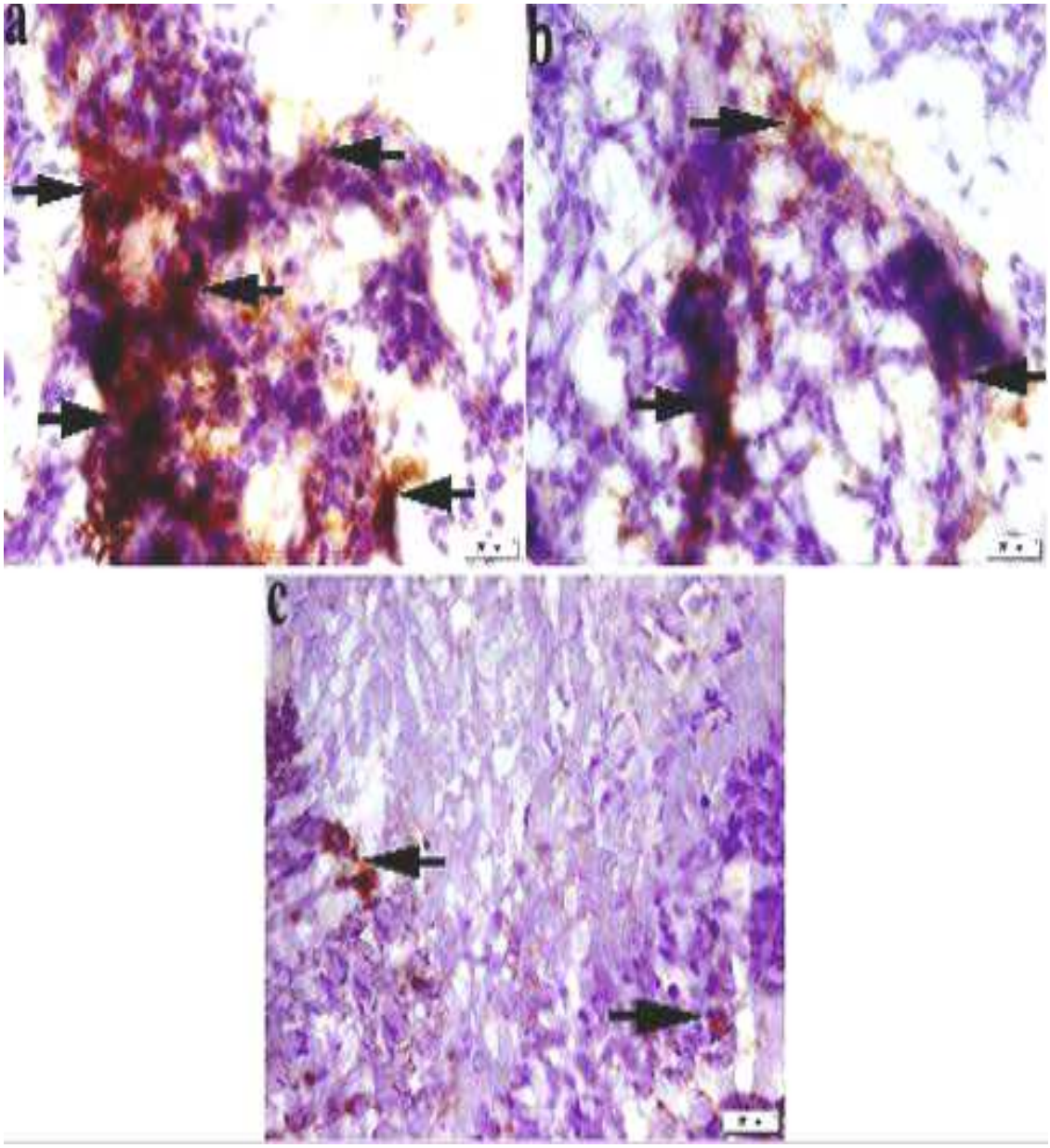


Fig (19): Photomicrographs of BC biopsies showing: **a.** control group with obvious HER2+ve IE (arrows). **b.** metastatic group (low PI, ER and PR +vity) with less obvious HER2+ve IE (arrows). **c.** metastatic group (high PI, ER and PR +vity) with minimal HER2+ve IE (arrows) **(HER2 immunostaining, x 400).**

In the control group, **CK immunostained sections** demonstrated -ve IE among the malignant cells **(Fig 20a).** While in the metastatic group, some of the cases with lower PI and low ER and PR +vity revealed +ve CK IE in some malignant cells lining ducts **(Figs 20b)**. Others with similar clinicopathological features demonstrated +ve CK IE in some malignant cells forming solid masses **(Fig 20c).** On the other hand, obvious IE was detected in multiple malignant cells lining ducts or forming solid masses in the specimens of patients with high PI, high ER and PR +vity **(Figs 20d).**

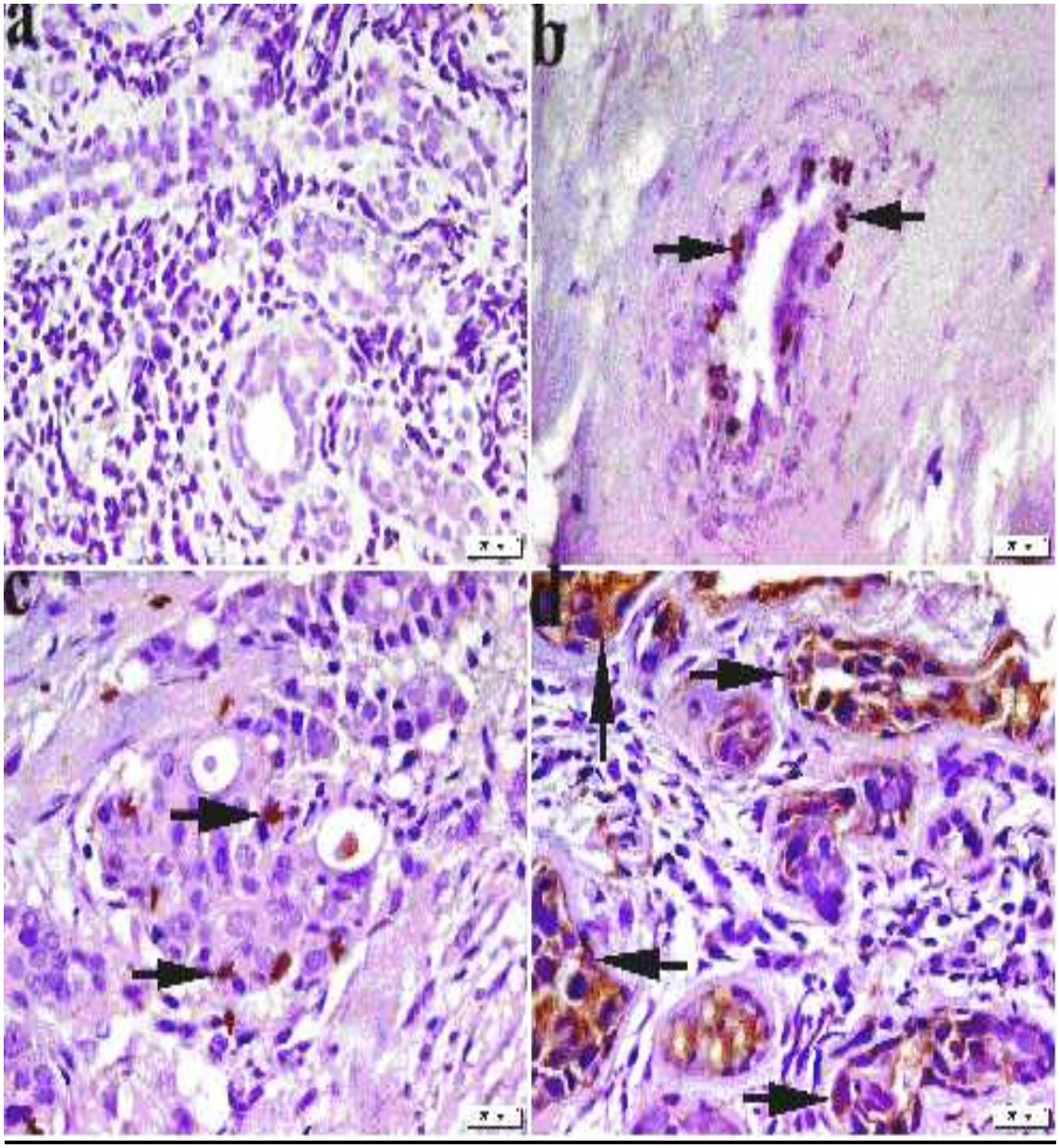


Fig (20): Photomicrographs of BC biopsies showing: **a.** control group with -ve IE. **b.** metastatic group (low PI, ER and PR +vity) with +ve IE in some malignant cells lining a duct (arrows). **c.** metastatic group (low PI, ER and PR +vity) with +ve IE in some malignant cells forming solid masses (arrows). **d.** metastatic group (high PI, ER and PR +vity) with obvious IE in multiple malignant cells lining ducts or forming solid masses (arrows) **(CK immunostaining, x 400).**

4.2.2. Cancer Stem Cell Marker:

In the control group, **CD44 immunostained sections** demonstrated few +ve spindle immunostained cells among the malignant cells of duct carcinoma **(Fig 21a).** While in the metastatic group, some of the cases with lower PI and low intensity of ER and PR +vity revealed some +ve spindle cells among the malignant cells of duct carcinoma and in the surrounding CT **(Fig 21b)**. Others with similar clinicopathological features demonstrated some +ve spindle cells in the CT among malignant cells forming solid masses **(Fig 21c).** On the other hand, specimens of patients with high PI, high ER and PR +vity recruited multiple +ve spindle cells in the CT between groups of malignant cells **(Figs 21d)** and numerous +ve spindle cells in the CT surrounding solid masses of malignant cells of duct carcinoma **(Fig 21e)**. In addition, multiple +ve spindle cells were found inside and near blood vessels in the specimens of the metastatic group **(Fig 21f)**.

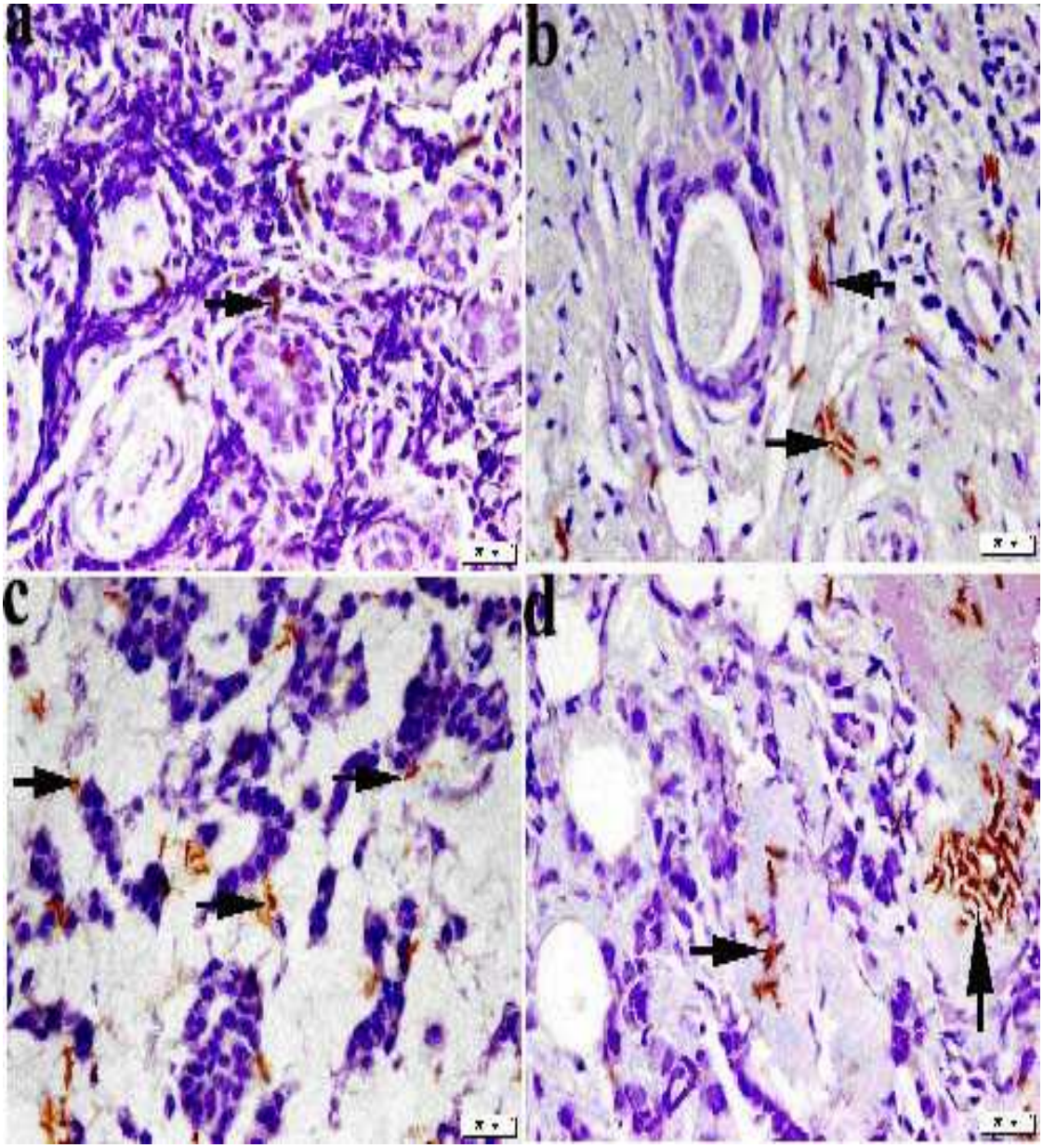
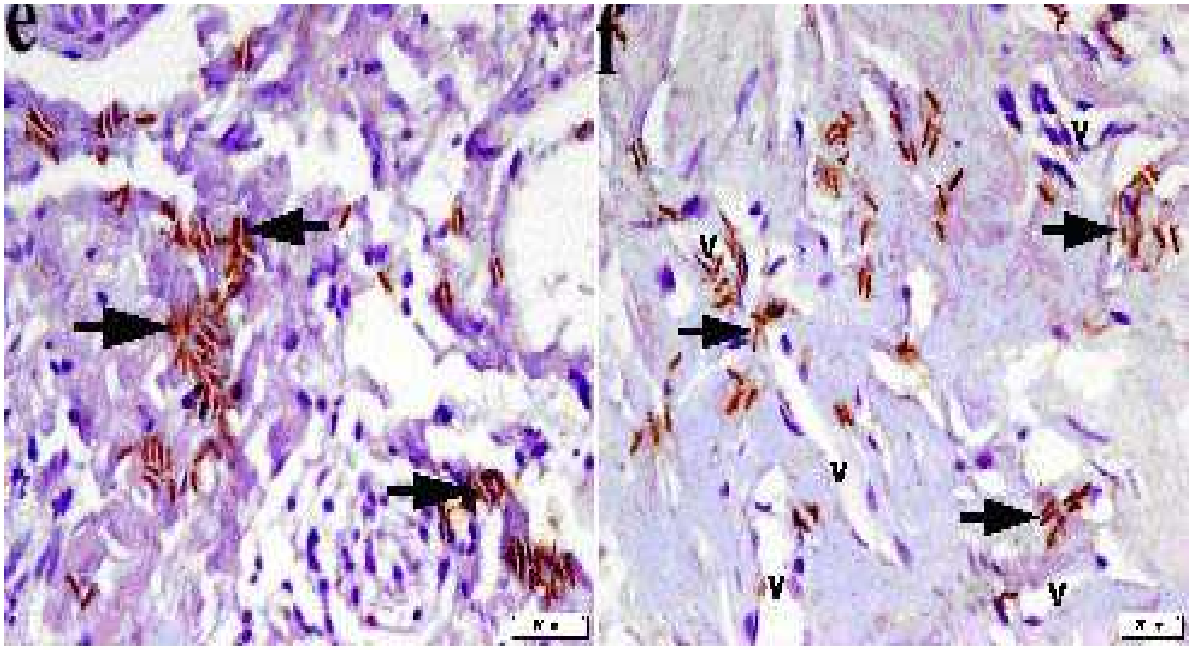


Fig (21): Photomicrographs of BC biopsies showing **a:** control group with few +ve spindle cells (arrow) among the malignant cells of duct carcinoma**. b:** metastatic group (low PI, ER and PR +vity) with some +ve spindle cells (arrows) among the malignant cells of duct carcinoma and in the surrounding CT. **c:** some +ve spindle cells (arrows) among malignant cells forming solid masses**. d:** metastatic group (high PI, ER and PR +vity) with multiple +ve spindle cells (arrows) in the CT between groups of malignant cells **(CD44 immunostaining, x400)**.



e: metastatic group (high PI, ER and PR +vity) with numerous +ve spindle cells (arrows) in the CT surrounding solid masses of malignant cells and **f:** multiple +ve spindle cells (arrows) inside and near blood vessels **(CD44 immunostaining, x400)**.

4.2.3. Morphometric Results:

By the application of paired student's t- test, results of the mean area% of HER2 +ve IE was (**16.01±0.76**) and (**9.11±1.36**) in the control and the metastatic groups respectively, indicating a significant decrease in the mean area% ($P<0.05$) **(Table V, Fig 22)**. On the other hand, the mean area% of CK IE was -ve in the control group. In the metastatic group +ve IE in cases with lower PI and low ER and PR +vity was (**2.22±0.42**) and that in cases with high PI and high ER and PR +vity was (**5.08±0.45**), indicating a significant increase in the mean area% ($P<0.05$). While, the mean area% of CD44 IE was **(0.84±0.21)** in the control group. In the metastatic group +ve IE in cases with lower PI and low ER and PR +vity was (**4.34±0.48**) and that in cases with high PI and high ER and PR +vity was (**8.65±0.89)**, indicating a significant increase in the metastatic group compared to control group and also in cases with high

PI and high ER and PR +vity compared to control and those with lower values (P<0.05) **(Table VI, Fig 23)**.

4.3. PCR Results:

By the application of paired student's t- test, results of the mean values of PCR were **(8.82±1.17)** and **(2.77±1.11)** in the control and the metastatic groups respectively, indicating a significant decrease in the metastatic group (P<0.05) **(Table V, Fig 22)**.

Table V: Mean ± SD of PCR values and HER2 +ve IE

Group	PCR values	HER2 +ve IE
Control	8.82±1.17	16.01±0.76
Metastatic	2.77±1.11*	9.11±1.36*

* significant (P<0.05) decrease compared to the control group

Fig (22): Mean PCR values and HER2 +ve IE

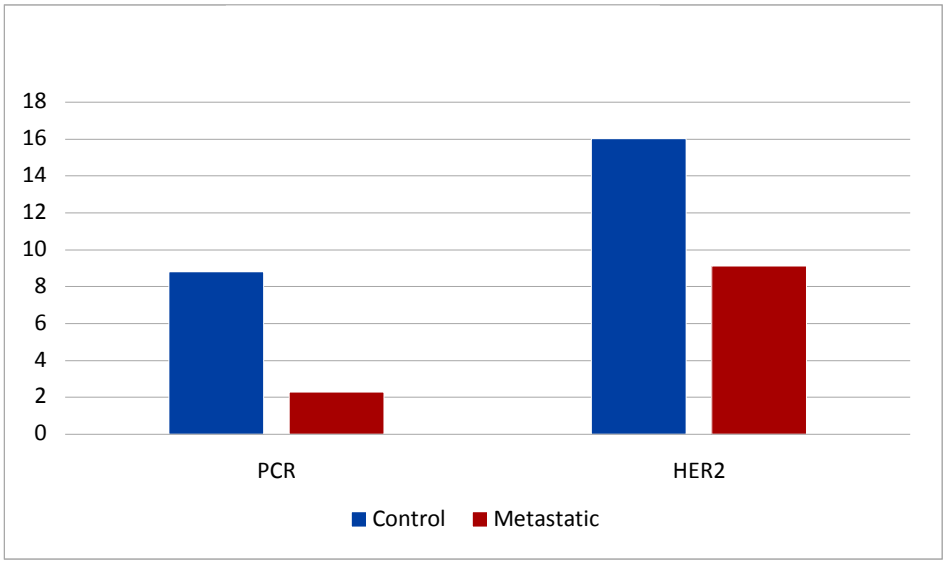
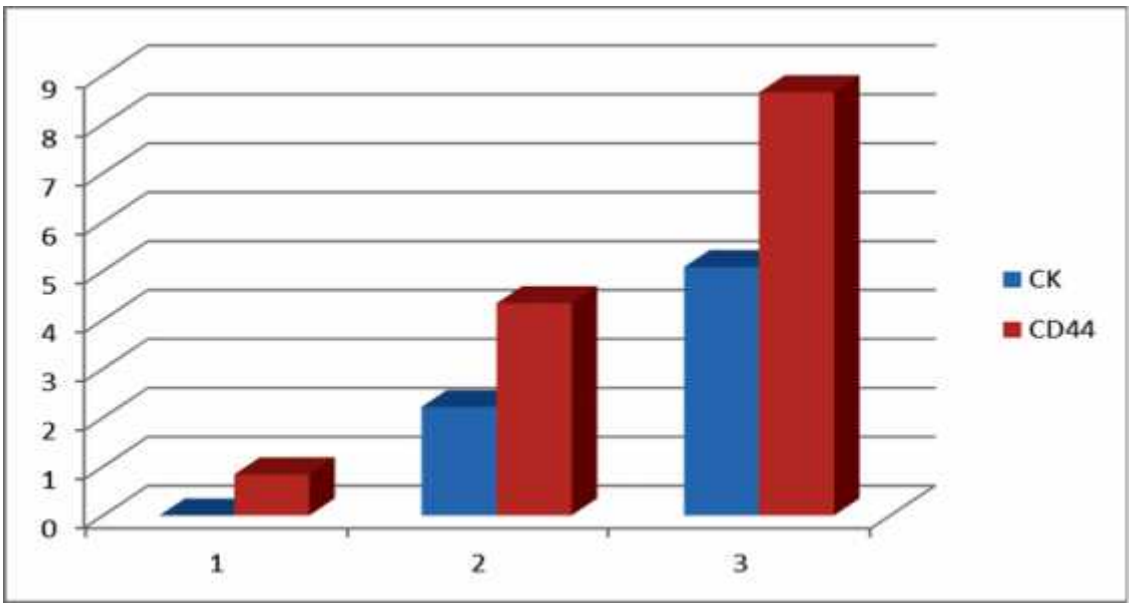


Table VI: Mean ± SD of CK +ve and CD44 +ve IE

Group	CK +ve IE	CD44+ve IE
Control	-	0.84±0.21
Metastatic (low PI, ER and PR)	2.22±0.42	4.34±0.48*
Metastatic (high PI, ER and PR)	5.08±0.45#	8.65±0.89#

- * significant (P<0.05) increase compared to control group
- # significant (P<0.05) increase compared to control and the metastatic cases with low PI, ER and PR +vity

Fig (23): Mean CK +ve and CD44 +ve IE



1:Control

2:Metastatic low PI, ER and PR +vety

3:Metastatic high PI, ER and PR +vety

5. Discussion

In the present study depending on the investigation of 20 control and 20 metastatic BC cases, the clinicopathological data, proved significant differences in the metastatic group. In addition, immunohistochemical and biochemical analysis of the specimens revealed significant changes. The previously obtained results are going to be discussed.

As regards the clinicopathological results, nonsignificant difference was found in the mean age, the mean weight and the mean height between both groups. The number of premenopausal patients, the family history, the incidence of DM and the number of cycles of herceptin did not prove any significant difference between both groups. It can be commented that none of the previous data were proved to be directly related to the possibility of development of trastuzumab resistance in the metastatic group of BC versus the control group. On the other hand, **Huszno and Nowara (2015)** stated that risk factors for disease progression in HER2 +ve BC were the menopausal status (CNS metastases) and history of cancer in the family (lung metastases).

Concerning the TNM staging data, it can be concluded that the tumor stage was not obviously different between both groups. While the nodal staging differed, in the control group no nodal involvement was recorded. In the metastatic group, only 2 cases proved lack of nodal involvement by pathological examination, in fact these 2 cases revealed distant metastasis.

Schramm et al (2015) commented that over the years, multiple HER2-targeting drugs stepped into clinical practice, for the curative as well as the metastatic situation. **Aftimos et al (2016)** added that the recently investigated combination of eribulin and trastuzumab should lead to higher activity in HER2-+ve metastatic BC.

Santa-Maria et al (2016) reported that a better understanding of HER2 biology has led to the development of powerful targeted therapies. Four drugs were approved by

the US Food and Drug Administration for treatment in the metastatic setting (trastuzumab, pertuzumab, lapatinib, and trastuzumab emtansine). However, while the prognosis has improved, metastatic disease is still not curable; newer, better drugs are needed. Novel therapeutics, include small-molecule inhibitors, nanoparticles, immunotherapy, and agents targeting resistance pathways. On the other hand, **Harbeck et al (2016)** recorded that trastuzumab-based therapy remains the treatment of choice for patients with HER2-+ve metastatic BC who had progressed on trastuzumab.

On the other hand, in the present study, a significant increase in the mean PI or Ki67 and the mean of ER +vity was detected in the metastatic group compared to the control group. PR was +ve in 15 cases in the metastatic group and -ve in all cases of the control group.

Balmativola et al (2014) documented the identification of markers of non-response to neoadjuvant chemotherapy. Review of the core biopsies of treated BCs and record of the clinico-pathological findings (histological type and grade; ER, PR, and HER2 status, Ki67, mitotic count) and data regarding the pathological response in corresponding surgical resection specimens was performed. The distribution of the mitotic numbers and the % of Ki67 were high in nonresponders to chemotherapy.

Genrich et al (2016) studied the cell growth and survival by investigating the cell count, Ki67 staining and apoptosis assays in pancreatic ductal adenocarcinoma as parameters of tumor promotion.

Cell viability assays were performed to study the influence of chemotherapy on cell proliferation in hepatocellular carcinoma. In addition, the chemotherapeutic effect was investigated on postoperative recurrence and metastasis in tumor-bearing mice by Ki67 examined by immunohistochemistry **(You et al, 2016)**.

Whisenant et al (2015) investigated trastuzumab response in HER2-overexpressing BC. Trastuzumab-sensitive and resistant cases after two treatments revealed tumor volumes with significantly different Ki67 immunoreactivity (nearly 50%) less in trastuzumab-sensitive xenografts. This can identify nonresponders earlier during the course of therapy and is considered of great interest in order to spare patients a delay in initiation of effective combination therapies.

Chae et al (2016) stated that in addition to survival outcomes, several surrogate markers are also being employed to study the effects of therapy on BC cell population. These include Ki67, which showed significant reduction in staining corresponding to the +ve clinical response. Decrease in tumor cell proliferation inhibits apoptosis of CD8+ T-lymphocytes. In addition, it also increases the effector memory T cell population, thus enhancing the immune response against tumor cells as by check point blockade monoclonal antibodies.

Tamimi et al (2010) analyzed the spectrum of molecular subtypes of BC. ER, PR, HER2, and CK5/6 were used as surrogate markers to classify BC specimens. Correlation of each molecular class with Ki-67 proliferation index was performed. Co-positivity of different markers in varied patterns was seen in 10% of cases. A high Ki-67 PI was seen in basal followed by HER2+ class. The presence of a predominant co-+ve unclassified group also suggested that the currently used molecular analytic spectrum may not completely envolve all molecular classes and there is a need to further refine and develop the existing classification systems. This could, in the future alter and modify the course of therapeutic strategies, that are currently more class specific.

Chen et al (2012) recorded that tumors developing resistance to combined therapy, as well as those acquiring resistance to endocrine therapy alone, may exhibit distinct histological and molecular phenotypes. Prior to the onset of resistance, these tumors showed upregulation and reactivation of HER2 signaling, losing ER protein and the estrogen-regulated gene PR. By the establishment of resistance to endocrine and

trastuzumab therapy, these may shift their molecular phenotype to become more ER +ve, PR +ve and less HER2 +ve.

Advani et al (2015) stated that resistance to trastuzumab may pose a major problem in the effective treatment of HER2+ve BC. Dual HER2 blockade, using agents that work in a complimentary fashion to trastuzumab, has more recently been approved in the metastatic and neoadjuvant treatment of HER2-positive BC. ER +vity may predict which patients will respond best to HER2-targeted therapy. HER2 expression was noted to vary significantly based on the presence of ERs, with only 17% of ER+ tumors exhibiting a HER2-enriched subtype. ER-ve tumors would be more likely to be HER2-enriched and respond to therapy.

Inoue and Fry (2015) documented that ERa36 is a potential regulator for membrane-initiated mitogenic signaling and is a promising diagnostic/prognostic biomarker for therapy-resistant cancer.

Pera et al (2016) assessed the expression level of proteins involved in Ca2+-signalling in an in vitro model of trastuzumab-resistance and to assess the ability of identified targets to reverse resistance and/or act as potential biomarkers for prognosis or therapy outcome. High levels of Ca2+ were associated with poor outcome in patients with ER +vity in BC. In addition, elevated levels of Ca2+ were found in trastuzumab-resistant SKBR3 cell lines. It was concluded that certain protein factors may be possibly proposed to be a driver of trastuzumab-resistance or not. Further studies are now required to elucidate the potential differential roles of these factors in different BC subtypes and its utility as a potential biomarker of prognosis and therapeutic responsiveness in patients with coinciding ER +ve and HER2+ve BCs respectively.

In the control group, **HER2 immunostained sections** demonstrated obvious +ve IE. While in the metastatic group, less obvious IE appeared in the specimens of cases with lower PI and low ER and PR +vity. On the other hand, minimal IE was detected in the specimens of patients with high PI, high ER and PR +vity. This was confirmed by a significant decrease in the mean area% of HER2 +ve IE in the metastatic group

compared to the control group. The previous results may indicate a change in the molecular subtype of the HER2 +ve BC. Going with, **De Mattos-Arruda et al (2015)** documented that a comprehensive molecular understanding of the pathways associated with resistance to trastuzumab and chemotherapy might greatly aid the development of more effective targeted therapies for patients with HER2-amplified BC. **Burnett et al (2015)** added that the transformed resistant cells in HER2 +ve BC exhibited loss of dependence on HER2 family signalling, which increased the frequency of CSCs and metastasis potential.

In the control group, **CK immunostained sections** demonstrated -ve IE among the malignant cells. While in the metastatic group, some of the cases with lower PI and low ER and PR +vity revealed +ve CK IE in some malignant cells lining ducts and those forming solid masses. On the other hand, obvious IE was detected in multiple malignant cells in the specimens of patients with high PI, high ER and PR +vity. This was confirmed by a significant increase in the mean area% of CK IE in the metastatic group cases with higher PI and high ER and PR +vity compared to cases with low PI and low ER and PR +vity. In accordance, **Martin-Castillo et al (2015b)** recorded a basal-HER2+ phenotype established solely on expression of the basal marker CK5/6. **Martin-Castillo et al (2015a)** added building on earlier studies that HER2+ tumors enriched with molecular and morpho-immunohistochemical features classically ascribed to basal-like tumors are highly aggressive and refractory to trastuzumab. The retrospective cohort study of 152 HER2+ primary invasive ductal breast carcinomas first confirmed the existence of a distinct subgroup co-expressing HER2 protein and basal cytokeratin markers CK5/6, the so-called basal- HER2+ phenotype. A CK5/6-based immunohistochemical fingerprint was suggested to provide a simple, rapid, and accurate method to re-classify women HER2+ BC. This is to improve prognosis and therapeutic planning in clinically aggressive basal-HER2+ tumors who are not likely to benefit from trastuzumab-based therapy.

Nagasawa et al (2015) postulated that CK +ve IE is a poor prognostic factor in BC, especially in basal-like BC, a subtype of HER2 +ve BC with aggressive clinical features. This criteria is also observed in samples of triple negative BC. **Wu et al (2015)**

continued that basal-like BC largely overlaps with triple-negative BC and these two groups are generally studied together as a single entity. Differences in the molecular makeup of BCs can result in different treatment strategies and prognoses for patients with different BC subtypes.

Saba et al (2016) described basal-like BC as an aggressive phenotype of breast malignancies that is often associated with poor prognosis. Basal-like cancer cells express genes that are usually seen in basal or myoepithelial cells of normal breast tissue. Triple negative BC, on the other hand, lack HER2 in addition. Although differences between these two types of BC have been demonstrated, they may overlap significantly. These tumors represent a therapeutic challenge because of lack of effective therapy. Although accounting for a small % of BC subtypes, they cause 25% of BC-related deaths due to aggressive and refractory nature.

In the control group, **CD44 immunostained sections** demonstrated few +ve spindle immunostained cells among the malignant cells of duct carcinoma. While in the metastatic group, some of the cases with lower PI and low ER and PR +vity revealed some +ve spindle cells among the malignant cells of duct carcinoma, some +ve cells in the CT and among malignant cells forming solid masses**.** On the other hand, specimens of patients with high PI, high ER and PR +vity recruited multiple or numerous +ve spindle cells among the malignant cells lining ducts and in the surrounding CT**.** In addition, multiple +ve spindle cells were found inside and near blood vessels. This was confirmed by a significant increase in the mean area% of CD44 IE in the metastatic cases with low PI, low ER and PR +vity compared to the control group, also in cases with high PI, high ER and PR +vity compared to low values.

In 2003, with the isolation of CSCs from the first solid tumor, BC and recognition of the tumorigenicity of these cells, this theory suggested that the main reason for therapy failure might be the presence of CSCs. It appears that this small subpopulation of cells, with the capacity for self-renewal and a high proliferation rate, originate from normal stem cells. They are identified by specific markers and

enhance tumor metastasis, invasion and therapy resistance. **(Bozorgi et al, 2015)**. CD44 was proved to influence the P-glycoprotein-mediated multidrug resistance. This is mediated by prevention of accumulation of anticancer drugs within cells by virtue of its active drug efflux capacity **(Pokharel et al, 2016)**.

Gonçalves et al (2016) documented that CSCs have been associated with metastasis and therapeutic resistance and can be generated via epithelial mesenchymal transition (EMT). The EMT is a mechanism to generate CSCs endowed with an invasive and metastatic phenotype. This process is mediated by the activity of growth and transcription factors, resulting in loss of the epithelial cells' typical intercellular junction structure, acquisition of mesenchymal morphology and invasion ability. **Huang et al (2016)** commented on CSCs, that have been invoked in resistance, recurrence and metastasis of cancer. Consequently, curative cancer treatments may be concentrating on CSC selective approaches.

In addition, the mean values of PCR indicated a significant decrease in the HER2 gene expression in the metastatic group compared to the control group. **Tung et al (2016)** stated that real- time PCR analysis indicated the measurement of miR-184 expression level that promotes tumor progression in non-small cell lung cancer**.** Similarly, **Hofmann et al (2016)** reported that RT-qPCR is a precise and cost-effective diagnostic approach for HER2 testing in cancer. The PCR assay is simple, accurate and robust and can easily be implemented and standardized in clinical laboratories.

On the other hand, **Takeshita et al (2016)** demonstrated that droplet digital PCR monitoring of the recurrent estrogen receptor1 mutation in circulating cell free DNA of metastatic BC patients is a feasible and useful method of providing relevant predictive information.

Conclusion:

It can be concluded that the development of resistance to trastuzumab in the metastatic group of HER2+ve BC is related to the development of the basal like subtype and the associated overexpression of CD44 +ve CSCs. This nicititates performing IHC assessment of the molecular subtype and the type of CSCs during the follow-up of the course of treatment to allow the choice of the most appropriate treatment regimen or the modification of treatment giving the best expected prognosis.

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Ethical Approval / Copyright Transfer and Agreement

Thesis Title: Resistance to Trastuzumab in Clinical Her2+ve Breast Cancer: Possible Relation to Cancer Stem Cells

A THESIS SUBMITTE TO THE GRADUATE INSTITUTE OF HEALTH SCIENCES NEAR EAST UNIVERSITY
In Partial Fulfillment of the Requirements For
The Degree OF
Master of Science in Clinical Pharmacy
NICOSIA 2016

Name of Candidate: Ahmed Mahmoud Fouad Reda

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