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College of Science

Department of Biology

Importance of Regulatory T cells in Breast Cancer and its Association with Tumor Markers in Women of Kerbala Governorate , Iraq

A Thesis

Submitted to the Council of the College of Science, University of Kerbala in Partial Fulfillment of the Requirements for the Degree of Doctorate of Philosophy in Science of Biology

By

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بسم الله الرحمن الرحيم

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Dedication

- *I thank Allah Almighty for what He has guided me to*
- *I also dedicate this little effort to the position of the Prophet and his projensy Peace be upon them.*
- *To my father soul , whom I miss, may Allah have mercy on him and grant him paradise....*
- *To my mother who carries the true meaning of love, kindness and tenderness and who has never been without her prayers and support for me.....*
- *To all my dear brothers and sisters,*
- *To my lovely husband (Qusay) who encouraged and supported me all that time and still is, I also thank his love, care, and patience.*
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Summary

Regulatory T cells (T reg) are considered one of the most important types of cells in suppressing immunity, and their percentage is low in the blood. They are one of the types of T cells that mediate immune tolerance and prevent autoimmunity by inhibiting multiplying T cells and cytokines. Cancer cells contribute to the production of Treg cells, as they are found in several types of cancers because Cancer cells produce TGF-beta to stimulate myeloid cells, which plays an important role in the development of organisms by converting original T cells into T reg.

The aims of this study were to evaluate the role of T reg, CD28 and (cytotoxic T lymphocyte antigen 4 (CTLA-4), which were calculated by using the flow cytometry technique, in breast cancer, and to evaluate some of the existing tumor markers estrogen receptor (ER), progesterone receptor (PR), human epithelial receptor 2 \neu, B cell lymphocyte 2 and ki67 . In breast tissue using the immunehistochemical technique, 75 women were subjected to the current study. 25 women were normal, which was the control group. 25 women had malignant breast cancer, and the remaining 25 were women with benign breast cancer.

Samples (blood and biopsy) were collected as an initial diagnosis of the type of breast cancer, whether benign or malignant was achieved. In Kerbala Governorate, female patients were classified according to age group into young patients, whose ages range from 29 to 49, and the second older category, which ranges from 50 to 70 years.

The results showed that there was a clear and significantly increased in Treg cells in (benign and malignant) breast cancer compared to the control group, (45.61 and 43.42)% respectively . As for the molecules CD28 and their expression on the T reg cells, it was found that their expression decreased in (benign and

malignant) breast cancer compared with control (87.37 and 90.39)% respectively, and the significant differences. As for the mean fluorescence intensity (MFI), the group of patients showed an increased comparative with the control group, especially the benign breast cancer group (41.20)%. As for the expression of the CTLA4 molecule, there was a decrease in the percentage of these molecules compared to the control group, (56.14% in benign) and (49.53% in malignant). There was a significant and noticeable decrease, as the $p \leq 0.0001$ compared to the control group.

The effect of age on the percentage of breast tissue expression of tumor markers for the malignant breast cancer group was studied. The results were showed that KI67 increased its expression in the breast tissue of the age group (29-49) years, with clear significant differences. The $p \leq 0.01$, but for the rest of the markers (ER, PR, HER2\neu and BCL2), no significant differences appeared. Clearly significant, its expression in breast tissue and the age of patients is evident.

As for the effect of age on Treg cells, the results showed that there was a clear increased in these cells in the two patient groups (benign and malignant), compared to the control group for both age groups. In addition, the control group with an age group of more than 50 years showed significant increased compared with the younger age group less than 50 years in the same group, with a significant difference, the $p \leq 0.01$. While the group has the highest percentage of Treg cells, it was the benign cancer group, under 50 years of age, with a clear significant difference, $p < 0.0001$, while in the malignant breast cancer group, there was an increase in Treg cells with the same significant difference. The same can be said with the age group over 50 years compared to the control group.

The relationship of Treg cells with histological tumor markers of breast cancer was also studied. It was observed that there was non-significant an increase in the

percentage of these cells in women with positive expression of these tumor markers. As for the tumor marker Ki 67, there was a positive relationship between it and Treg cells.

As for the patients' blood group, its effect on the increased or decreased of T reg cells was studied. The results showed that the highest percentage of these cells appeared in the B blood group (51.98%) in the group of patients with benign breast cancer, followed by the blood group O(51.47%), then blood group A(43.32%), and finally blood group AB(29.32%). While the highest percentage of Treg cells was in blood group O(47.55%), then blood group A(45.02%), and the least in blood group B (32.46%) in the malignant breast cancer group.

As for the correlation of blood group with breast cancer, it was investigated, and the results showed that in benign breast cancer the highest percentage was blood type A(40%), blood group O (36%) it was the most closely related to blood group A. As for the malignant breast cancer group, the majority of women with breast cancer were of blood group A (44%), followed by blood group O (36%), and finally blood group B.

In the histological study, the results showed, The percentage of breast tumors expressing estrogen receptor reached 86%, and the percentage of progesterone in tumor tissues was 64%, while her2-neu had a positive expression rate of only 7%, while 93% of patients did not express this marker in their tissues. As for BCL2, the expression percentage was approximately 64% positive expression.

Ki 67, in the tissues of the patients was differed in the percentage of expression of this tumor marker. The percentages ranged from 12 to 80% and they were divided into two categories. The first category was the lower expresseion (35%) of this marker and the other category was the higher expression of 35%. The results

showed that the percentage of women who had their breast tissue expressed the second category, which is more than 35%, and them reached 75%.

Also studied the percentage of the relationship between hormone receptors and breast cancer. The largest percentage was when the tumor was double positive for receptors for the hormones estrogen and progesterone, and the percentage of women was 57%, while the relationship of age with double receptors. Hormones: The highest percentage of women with double positive receptors for the hormones progesterone and estrogen were among women who were younger than 50 years, while the relationship of double receptors with the tumor marker Her2-neu was the percentage of positive expression of this marker in women with positive expression of estrogen and negative expression of progesterone. As for BCL2, it was the highest expression rate was in women whose tissues showed double positive expression of the hormones progesterone and estrogen.

Finally, the conclossion about this study , T regulatory cell might be stopped immune activity normal reactivity to cancers, which could explain why immune system-based therapies for breast and other malignancies do not work. Every essential medicine stop growth of T regulatory cell in carcinoma may work in conjunction with regular or boosted immunity to help fight tumors.

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List of Abbreviations

Abbreviations	Abbreviations Meaning
ADCC	Antibody-dependent cellular cytotoxicity
APC	Antigen-Presenting Cell
BCC	breast cancer cells
Bcl2	*B-cell lymphoma 2*
BMI	Body Mass Index
BRCA2	BReast CAncer gene 2
CCR4	C- Chemokine Receptor Type 4
CD28	Cluster of Differentiation 28
CD4+	Cluster of Differentiation 4 positive
CD8+	Cluster of Differentiation 8 positive
CSCs	cancer stem cells
CTLA4	Cytotoxic T-lymphocyte associated protein 4
DAMP	damage-associated molecular patterns
DC	dendritic cells
DCIS	ductal carcinoma in situ
ECM	Extra cellular matrix
EGFR\ ERBB	Epidermal growth factor receptor
ER	Estrogen receptor
Fas	CD95
FcR	Fc receptor
Foxp3+	forkhead box P3

G0	resting phase
GHs	growth hormones
GPER	G protein-coupled estrogen receptor 1
GPER/GPR30	G-protein-coupled estrogen receptor 1 (*GPER*), also known as G protein-coupled receptor 30 (*GPR30*)
GPR30	G protein-coupled receptor
Her2/neu	Human Epidermal growth factor Receptor 2
HLA	Human leukocyte antigen
HMGB1	high-mobility group box-1 protein
ICAM-3	Intercellular adhesion molecule 3
IHC	Immunehistochemistry
IGF-1	insulin-like growth factor 1
ILC	Infiltrating lobular carcinoma
JAK\STAT	Janus kinase/signal transducers and activators of transcription
KIR	Killer-cell Immunoglobulin-like Receptor
LAG3	lymphocyte activation gene-3
LCIS	lobular carcinoma in situ
MAPK	mitogen-activated protein kinase
MHC	major histocompatibility complex
MIB-1	Molecular Immunology Borstel -1
MICA/B	MHC class I chain related-proteins A and B
MRI	Magnetic resonance
mTOR	mechanistic target of rapamycin
NFκB	Nuclear factor kappa B
NK	natural killer

NKG2D	natural killer group 2 member D
NOD	nucleotide-binding oligomerization domain
PI3K	phosphatidylinositol-3 kinase
Piwi	*P-element-induced wimpy testis*
PIWIL1	*Piwi-like protein 1*
PR	Progesterone receptor
PS/Gas6	Protein S (*PS*) and growth arrest-specific gene 6 (*Gas6*)
Raf	Rapidly Accelerated Fibrosarcoma
RANKL	receptor activator of nuclear factor kappa beta(NFkB ligand)
Ras	A family of genes that make proteins involved in cell signaling pathways that control cell growth and cell death
RIG	retinoic acid-inducible gene-I
TAA	tumor associated antigen
TAM	tumour-associated macrophage
TCR	T Cell Receptor
Th1	T-helper type 1cell
Th2	T-helper type 2
TIM	T cell (or transmembrane) immunoglobulin and mucin domain protein
TME	tumor microenvironment
TNF	tumor necrosis factor.
TRAIL	TNF-related apoptosis-inducing ligand
Treg	T regulatory cell

Chapter One

Introduction

Chapter One

Introduction

1. Introduction

According to Siegel *et al.* (2019), The main reason of cancer-related death in women global and the most frequently detected cancer in women is breast cancer. Breast cancer is a varied illness both etiologically and clinically. Numerous risk factors, mostly hormonal in nature, have been found, and the relationships with breast cancer subtypes can differ. With the development of screening mammography and improved treatments over the past few decades, survival has grown. However, not all races or ethnicities have shown similar advancement, nor have all breast cancer subtypes (such as triple negative).

There are several types of breast cancer. The type is determined by the sort of breast cells that are affected. Carcinomas account for the bulk of breast cancer cases. Most prevalent type of breast tumors is "adenocarcinomas" which refers to cancers that start in the gland cells of the milk ducts. Examples of these tumors include invasive carcinoma and ductal carcinoma in situ (DCIS). Two more cancers that can arise in the breast are angiosarcoma and sarcoma, although they are not the same as breast cancer since they start in different breast cells (Elżbieta Senkus-Konefka, *et al.* 2018).

Histological proof of malignancy and assignment of histopathological phenotype has been a principal diagnostic method . It is supplemented by analysis of specific tumor cells products or markers to determine a molecular subtype of BC. Common biomarkers currently include estrogen (ER) and progesterone (PR) receptors, human epidermal growth factor type 2 receptor (HER2). The BC samples obtained

by biopsy and/or from post-surgery specimen can be currently processed by various methods (Litton *et al,* 2019).

Novel molecular markers, such as Ki-67and BCL-2 are thus emerging as tools for classifying BCs, guiding therapy, and predicting treatment response and prognosis. Tumor markers have shown to be prominent tools for determining prognosis and informing treatment plans. The current study therefore aimed at investigating the relationship between Ki-67 and BCL-2 expression with clinical and histopathological factors in patients with BC (Zaha, 2014)

In the past, breast cancer was thought to be one of the malignancies that elicited the least amount of immune responses (Gatti-Mays *et al*. 2019). There has been a rise in the literature for studies on the immunological impacts on breast cancer during the past 20 years. This has led to a deeper comprehension of the immune system's interactions with carcinoma. The immunological environment of carcinoma is composed of a variety of cells and cytokines, some of which have anti-tumorigenic and others pro-tumorigenic or immunosuppressive roles. Through persistent inflammation, these cells and cytokines can also accelerate the development of breast cancer. Designing treatments that make use of the immune system and the microenvironment to treat breast cancer benefits from research into how the immune system behaves and influences the disease. Additionally, it can make it possible to develop more predictive models for a better comprehension of the biology of breast cancer, as well as more precise prognoses and better options for treating breast cancer patients. Scientists are developing immunotherapies to carry out a variety of activities, such as immunizing against breast cancer, using the immune response to breast cancer to their benefit, enhancing the immune system's

ability to combat breast cancer or reducing breast cancer mortality when combined with chemotherapy (Jarnicki *et al* 2006).

The cytokines that produce from Tcells are essential for the immune system response to be effective. Most scientists agree that T-helper type 1 (Th1)-induced inflammation slows tumor growth and hard tumors are linked to a pathogenic change in the pattern of T-helper type 2 (Th2) cytokine release.

A particular subgroup of T cells famous as T reg cells has the capacity to inhibit both humeral (Th2) and cell-mediated (Th1) reactions (Schmidt 2006).

Regulatory T cell is a specific group of T cells that have the ability to suppress both humeral (Th2) and cell-mediated (Th1) reactions. These T regulatory cells contain the transcription factor Foxp3 and are CD4+ and CD25hi (Shevach 2002). The maintenance of self-tolerance depends on these T reg cells, according to studies conducted on mice and people. Foxp3 mutations in scurfy mice result in decreased T reg populations and the severe autoimmune illness (Foussat *et al* 2003).

There is confirmation that tumors can excite T regulatory cells in cancer, which then prevent the immune system from responding to antigens in tumors. It has been demonstrated by Akbar and colleagues that T reg cells can be produced by humans in the peripheral blood, suggesting that T reg cells may be susceptible to apoptosis (Akbar *et al*. 2003). T regulatory cells might require ongoing for survival, immunological activation. T reg cells may prevent the normal clearance of tumor cells since they appear to be created by malignancies by the immune system. Jarnicki and colleagues demonstrated T regulatory cell (CD4+Foxp3+), (TGF), and IL-10 inhibited T cells from a developing tumor in mice (Jarnicki *et al* 2006). These results imply that development of cancer boosts T regulatory cells, and that

the T regulatory cells possess the capacity to inhibit natural immunological response towards tumor. T regulatory cell has been found to be elevated with periphery blood supply and significantly elevated in the tumor-specific environment of the breast, according to research by Liyanage and colleagues (Liyanage *et al*., 2002). In a mouse breast cancer model, Knutson and associates expanded on this discovery and demonstrated a T regulatory cell is enhanced in a naturally animal form of carcinoma (Knutson *et al* 2006). They went on to demonstrate that targeted reduction of T regulatory cells significantly reduced development of cancer and preserved a potent and long-lasting anticancer immunological reaction. The reaction is a particular T regulatory cell adoption into animals lacking T regulatory cells totally stopped the immunological reaction.

Lymphocytes, like T cells, T regulatory cells, and NK cells, additionally the patterns of cytokines generated by these cells, are responsible for the initial prevention of breast cancer as well as the subsequent prevention (i.e., relapse or recurrence). The way the immune system works may have an impact on how well a cancer patient may fare. Investigational immune-therapies that regulate NK and T-cell activity are still being developed (Bozward, *et al*, 2021).

1.2. Aims of Study and Objectives :

1. Investigating the correlation between regulatory T cell with the breast cancer in women

2. Assessment of Carcinoma with its relation of histopathologic markers (ER,PR,HER2\neu , BCL2 and KI67).

3. To find out the relationship between the studied immunological markers and T regs with the histopathologic markers of breast cancer tissues.

Chapter Two

Literature Review

Literature Review

2.1 Breast

There are three key ways that the breast differs from other organs. First, its primary purpose is to ensure the sustenance and endurance through nutrition of a different person, the child. It also experiences dynamic structural changes over the course of a person's lifetime, including the development of the lobular system during menarche, periodic remodeling in maturity, particularly both throughout and following pregnancy, then regression and involution in the end. At last, unlike other organs, breasts have special significance in society, culture, and the individual as emblems of femininity. The cause, manifestation, and management of breast illnesses are all impacted by these characteristics. Knowing the breast's natural structure and cellular make-up is necessary to comprehend illnesses of the breast. (Kumar *et al* ., 2017)

2.1.1 Breast Anatomy

The two primary tissue types that comprise the breast are the glandular tissues and the stromal tissues. The lobules and ducts that produce milk were found in the glandular tissues, while the stromal tissues are made up of the breast's fatty and fibrous connective tissues (Jagsi *et al.* , 2019) . In addition, the breast has two varieties of stroma (intralobular and interlobular), two types of epithelial cells (myoepithelial and luminal), lymphatic tissue, and immune system tissue that eliminates cellular waste. These elements can all result in benign and cancerous tumors. During the reproductive years, the female breast undergoes the most significant and dramatic changes. Hormones like estrogen, progesterone, and prolactin influence the further branching of ducts and the creation of lobules. The

breast changes with each menstrual cycle, much like the endometrium does. The lobules are comparatively dormant throughout the menstrual cycle's initial half (Sung *et al*., 2021). The number of acini per lobule rises after ovulation due to increased cell proliferation caused by estrogen and rising progesterone levels. The hormone levels drop after menstruation, causing the lobules to retreat. The breast doesnot fully develop and function until a woman is pregnant. The quantity and size of lobules gradually grow. Towards when a pregnancy comes to an end at term, the breast consists almost completely of lobules separated by little stroma. The lobules create colostrum, a high-protein substance, after parturition before moving on to milk, which has a higher fat and calorie content. (Kumar *et al.*, 2017)

2.1.2. Breast cancer

Breast cancer has emerged as the most prevalent malignancy and is one of the principal causes of cancer-related fatalities in recent years. According to the World Health Organization, it is estimated that by 2022, one out of every eight women will suffer from breast cancer (Sung *et al*., 2021). The DNA and RNA of cancer cells are remarkably similar to the cells of the body from which they developed, yet they are not identical. Because of this, the immune system seldom recognizes them, particularly if it is already weakened. (Sharma *et al*., 2010).

2.1.3. Types of Breast Cancer

Numerous tumor forms can show up in different breast areas. Most breast tumors are the consequence of benign alterations that are not malignant. Fibrocystic alteration is one instance of a non-cancerous illness in women who suffer areas of thickening, breast soreness, discomfort, or lumpiness; it also causes

cysts, which are stored packets of fluid; and fibrosis, which is the growth of connective tissue that resembles scars (Blows *et al.*, 2010). The cells that line the ducts are where most breast cancers, also known as ductal tumors. While a tiny portion (lobular malignancies) start in other organs, others originate in the lobule-lining cells. (Jagsi *et al.* , 2019)

2.1.3.1 Breast Cancer According to Hormonal Receptors

Breast cancer is separated into four biological subcategories according to the condition of hormone receptors and the expression human epidermal growth factor receptor (HER2). The luminal types A and B, HER2-enriched and basal-like (with no ER expression) subtypes are some of these subtypes (Perou *et al.*, 2000). The most prevalent breast cancer subtype, Luminal A, is distinguished by ER+ and/or PR+/HER2 status, low-grade tumor, and favorable prognosis (Perou *et al.*, 2011; Blows *et al.*, 2010 ; Carey *et al.*, 2007). According to histological and genetic features, clinical practice currently employs a surrogate classification of five subgroups (Harbeck *et al.* ,2019)

2.1.3.2. Breast Cancer According To Site

2.1.3.2.1. Breast Cancer Without Invasion

Restricted cells within the ducts donot infect the fatty and connective tissues that surround the breast tissues.

- Ductal carcinoma in situ is the primary etiology of 90% of cases of non-invasive breast cancer (DCIS). DCIS has the potential to progress into an

aggressive form of breast cancer even though it is not currently malignant. Although they are not yet in the healthy breast tissue, the cancer cells in this particular form of breast cancer are found in ducts. (Elżbieta Senkus-Konefka *et al*., 2018).

- Localized lobular carcinoma, or LCIS Changes in the cells lining the lobules are less common and they are referred to as lobular neoplasia (previously called lobular carcinoma in situ). This condition is believed to be a marker for an elevated danger of breast cancer (Harbeck *et al.* ,2019). Lobular neoplasia is not the same as breast cancer, but most women with it do not go on to develop breast cancer and will instead receive routine checks (Sharma *et al,* 2010)

2.1.3.2.2. Breast Cancer That is Invasive

Cells that pass through ducts and lobular walls of breast to infect the fatty and connective tissues around it. Cancer can be invasive without metastasizing, or spreading, to the lymph nodes or other organs, according to Sharma *et al.* (2010). Breast cancers that invade the ducts and the lobules are two types of breast cancer that have expanded outside of the ducts are referred to as invasive breast cancer. By examining their histology, these can be further divided; for instance. Papillary, medullary, tubular, and mucinous breast tumors are less prevalent forms of breast cancer (Elżbieta Senkus-Konefka *et al*., 2018).

2.1.3.3. Frequently Occurring Breast Cancer

- **ILC(Infiltrating lobular carcinoma)**

- ILC is sometimes mentioned to as invasive lobular cancer (Fayed , 2009). The breast's milk glands, or lobules, are where ILC typically begins, even though it frequently spreads (metastasizes) to other bodies. Between 10% and 15% of breast cancers are ILC (Fayed , 2009).

- Infiltrating ductal carcinoma (IDC): Invasive ductal carcinoma is another term for IDC. IDC that begins in the breast's milk ducts, penetrates the duct wall, and infects the breast's fatty tissue as well as possibly other body areas. IDC cases account for 80% of all breast cancer diagnoses, making them the most common type (Sharma *et al*, 2010; Fayed , 2009).

2.1.3.4. Breast Cancer That Occurs Less Frequently

- **Medullary carcinoma**: A type of invasive breast cancer in which the tumor and surrounding healthy tissue are clearly separated. Less than 5% of cases of breast cancer are due to medullary carcinoma (Stephan, 2010)**.**

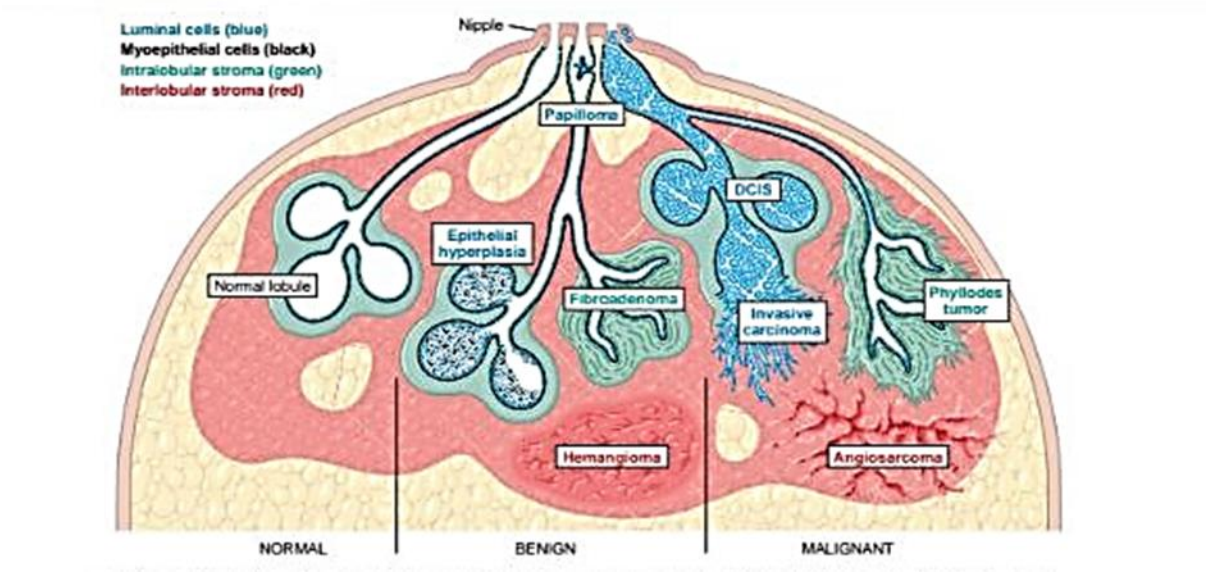
- **Mutinous carcinoma**: Mutinous carcinoma, also known as colloid carcinoma, is a rare form of breast cancer created by malignancies that release mucus. Women who have mutinous carcinoma usually have a better prognosis than those who have more common invasive carcinoma forms (Stephan, 2010)

- **Cancer of the tubules** A unique variety of penetrating (invasive) breast carcinomas are tubular carcinomas. Women with tubular carcinoma usually have a better prognosis than those with more common invasive carcinoma types. Tubular carcinomas account for slightly more than 2% of all diagnosis of breast cancer (Fayed , 2009)

- **Inflammatory breast cancer**: Cancerous breasts with thick ridges, dimples, and/or red, hot breasts result from the obstruction of lymphatic veins or channels in the skin covering the breast. Although inflammatory breast cancer is rare roughly 1% of all breast cancers it spreads swiftly(Stephan , 2010).

- **The nipple-related Paget disease:** Only around 1% of instances of Paget's disease of the nipple is breast cancer. an uncommon kind of the illness that starts in the milk ducts and moves to the areola and nipple skin(Stephan , 2010).

- **Tumor of Phylloides**: It is also possible to describe either benign (not cancerous) or malignant (cancerous) phylloides tumors by the term "phyllodes". If phylloides tumors develop in the connective tissues of the breast, they may be surgically removed. Every year, less than ten American women graduate away from Phylloides tumors, an extremely rare form of breast cancer (Stephan , 2010).



(Fig 2-1) Anatomy of Breast Cancer (Normal , Benign and Malignant) (Kumar, *et al* 2017)

2.1.4. Signs and Symptoms

The following are the most typical or severe indications and symptoms that warrant clinical evaluation of breast lesions:

- Pain is a typical symptom that can either be noncyclic or cyclic with menstruation (mastalgia or mastodynia).
- Inflammation causes erythema and edema that affect all or part of the breast.
- Nipple discharge that is tiny in size and bilateral may be typical. Milky discharge (galactorrhea) may also be experienced by people oral contraceptives methyldopa, phenothiazines, tricyclic antidepressants. Hypothyroidism, endocrine and ovulatory abnormalities, and elevated prolactin levels (resulting, for instance, from a pituitary adenoma) are

associated with it. A spontaneous, unilateral, and bloody abortion is most frequently the result of cancer..

- Breast lumpiness, also known as diffuse nodularity, is typically a sign of healthy glandular tissue. When severe, imaging tests can be required to rule out the existence of a distinct mass.
- Palpable masses are often discovered when they are 2 to 3 cm in size and can result from stromal cell or epithelial cell proliferations. The majority (about 95%) are benign; they frequently have a round or oval shape, are flexible and movable, and have defined borders.
- A density. breast lesions when adipose tissue is replaced with radiodense tissue produce mammographic densities. Most frequently, rounded densities are not malignant tumors like fibroadenomas or cysts, whereas aggressive carcinomas typically take the shape of irregular masses.
- Calcium deposits. Calcifications commonly accompany benign lesions including hyalinized fibroadenomas, sclerosing adenosis, and apocrine cysts. They can develop on secretions, stroma hyalinized, or necrotic detritus. Malignant calcifications typically have tiny, uneven, frequent, and grouped calcifications (Kumar *et al* ,2017)

2.1.5. Identification of Breast Cancer

Alterations in the breasts, such as a lump, alterations to the nipple, drainage from the nipple, or changes in the breast skin, are the most typical signs of breast cancer. A physical examination, mammogram, and ultrasound scan are the first steps in the initial inquiry for breast cancer. Magnetic resonance imaging (MRI) of the breast may also be done in specific circumstances. Before deciding on a course

of therapy if a tumor is discovered, a biopsy will be performed to determine the cancer (Elżbieta Senkus-Konefka *et al.*, 2018).

2.1.6 Risk Factors of Breast Cancer

According to Zendehdel *et al*. (2018), breast cancer is a complex disease that is influenced by a numeral of different features. Regardless of the disease's global prevalence, significant geographical variations exist in the incidence, mortality, and survival rates of the disease. This can be attributed to a multitude of factors such as environment, genetics, lifestyle, and population structure (Hortobagyi *et al.* , 2019)

2.1.6.1. Non Hormonal (Demographic) Risk Factors

2.1.6.1.1 Gender

Less than 1% breast cancer, which often affects mainly women, accounts for all instances of cancer in men. The danger of breast carcinoma is higher in older adults. Men who have experienced hormone imbalance, radiation exposure, and family history, and among males, the most prevalent indicator for this illness is BRCA2 gene mutation (Giordano *et al*, 2002 ; Abdelwahab Yousef , 2017)

2.1.6.1.2. Age

Prior to the age of thirty, the occurrence of breast cancer is incredibly low (occurrence of 25 cases per 100,000). From that point on, the incidence climbs linearly until the age of eighty, when it reaches a plateau of slightly less than 500 cases per 100,000. Among the most well-established danger features for breast cancer (in addition to many other malignancies) is this one. According to Ries *et*

al. (1999), the frequency of breast cancer increases noticeably with age, peaks at menopausal ages, and then gradually decreases or stays constant.(Kim *et al*. 2015) However, In a case-control study, there was a correlation between age beyond 50 and an increased risk of breast cancer. Nonetheless, breast tumors in younger women are typically greater, positivity in lymph nodes in later stages, and have a poorer prognosis (Assi *et al,* 2013)

2.1.6.1.3. Blood Groups

According to Meo *et al.* (2017), females with blood type A who test positive for rhesus are more likely to acquire breast cancer than those with blood group AB who are Rhesus negative. Despite the fact that these findings were supported by a research in 2015 (Saxena *et al*.2015) Numerous studies have revealed no connection between blood type and breast cancer (Gates *et al*., 2012).

2.1.6.1. 4. Drinking Alcohol

Numerous approaches have been used to correlate alcohol consumption to an elevated danger of carcinoma. According to Kavanagh *et al*. (1998), the suggested processes range from the somewhat specialized (increasing the metabolism of carcinogens like acetaldehyde) to the more universal (decreasing DNA repair effectiveness or decreasing intake of preventive nutrients). Despite this, it seems as though alcohol use has a negligible impact on the incidence of breast cancer. According to numerous studies, having one drink per day or less does not significantly affect the incidence of breast cancer. (Harvey *et al*., 1987; Manisto *et al.*, 2000). This was supported by a meta-analysis of pertinent studies published

between 1966 and 1999 by Ellison *et al*. (Ellison *et al.,* 2001). According to (Royo *et al*, 1997), people with a Body Mass Index (BMI) higher than the median were at an elevated relative risk of drinking alcohol.

2.1.6.1.5. Ionizing Radiation

When compared to age-matched control participants, young women who had mantle radiation for Hodgkin's lymphoma have a significantly greater chance of getting breast cancer (Goss and Sierra , 1998). In addition, females who survived the World War II nuclear bombings of Japan have a relatively high incidence of breast cancer (Land , 1995), most likely as a result of somatic mutations brought on by radiation exposure. In both situations, it is hypothesized that radiation exposure during adolescence, when breast growth is active, amplifies its effects (Goss and Sierra , 1998).

2.1.6.2. Hormone Receptor Susceptibility Factors

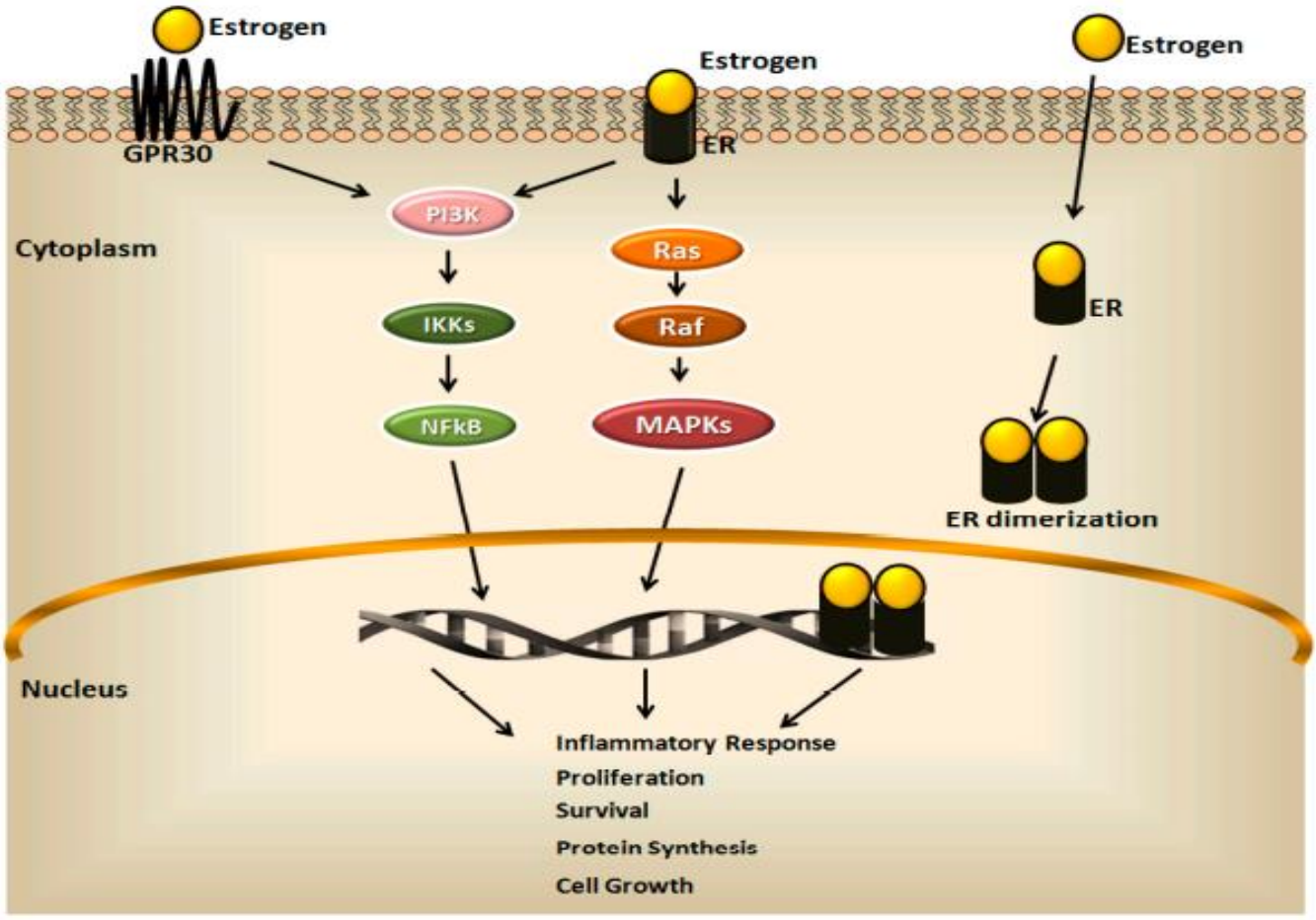
The genesis and growth of steroid hormones have a significant impact on breast, ovarian, and other types of cancer (Mahfouz *et al.,* 2016). They might be as easy as separated into corticosteroids, which are categorized into glucocorticoids and mineralocorticoids, and Sexual hormones, comprising androgen (a male hormone) and estrogen and progesterone (a female hormone). The five types of nuclear steroid receptors are as follows: glucocorticoid receptors, mineralocorticoid receptors, androgen receptors, progesterone receptors (PRs), and estrogen receptors (ERs), are generally activated by these hormones to regulate cell function (Mahfouz *et al.,* 2016). Human epidermal growth factor receptor 2

(HER2, encoded by ERBB2), progesterone and estrogen receptors, and/or BRCA mutations in addition to hormone receptors are known to be involved in the molecular pathways that cause breast cancer to be a hormone-dependent illness (Harbeck *et al*, 2019). Through Progesterone and estrogen receptors, in turn, govern the formation and growth of human tissues, such as the reproductive system and breast tissue. These receptors are also essential in establishing the medical diagnosis of cancers of the reproductive system, including breast cancer.

2.1.6.2.1 Estrogen Receptors in Breast Cancer

Estrogen works biologically by attaching to ERs, which are typically made up of nucleus ERs (ER and ER) and membrane ERs (mainly G protein-coupled receptors). Through these receptors, estrogen predominantly controls cancer stem cells (CSCs) (Kumar *et al*., 1987). The prognosis of patients with ER-positive cancers is better, and as these tumors are thought to be hormone-dependent, they can be treated with tamoxifen or other alternatives that interfere with the activity of estrogen. Three distinct ERs mediate the effects of estrogen: (1) The nearly 75% of BCs that are propelled by the nuclear receptor ER (Siersbæk *et al.*, 2018) ;(2) nuclear estrogen receptor; 3) cytoplasmic G protein-coupled estrogen receptor 1 (GPER) (Huang *et al*., 2015). Five distinct domains, referred to as A/B, C, D, E, and F, each with a comparable mechanism of action, share common structural properties with ER and ER (Gérard, *et al.*, 2018). Typically, estrogens attach to ERs in the cytoplasm, diffuse through the cellular membrane to move the cell passively, and then are delivered to the nucleus (Chen *et al*., 2020). The receptor-ligand contact leads to a conformational change in receptors, which allows the ERs to create dimers, bind to DNA, and initiate transcription of genes. via nuclear

translocation and attachment to specific response components, ERs control transcriptional processes in this example, affecting the control of gene expression. The primary component of estrogen signaling is the stimulation of the intracellular estrogen receptor (ER), which moves to the nucleus after dimerization and ligand interaction and directly binds target genes involved in protein synthesis, cell survival, and proliferation. Conversely, estrogens induce non-genomic effects and initiate intracellular signaling by binding to the G protein-coupled receptor (GPR30) on the plasma membrane and ER variants. This binding triggers (NFκB) and mitogen-activated protein kinase (MAPK), which control the expression of the estrogen target gene, as well as the (PI3K), (Ras), and rapidly accelerated fibrosarcoma (Raf) gene. (Saczko, *et al*, 2017)



(Fig 2-2). The Signaling Mechanism For Estrogens

primary component of estrogen motioning is the stimulation of the intracellular estrogen receptor (ER), which translocates to the nucleus after dimerization and ligand binding. There, it binds directly to the target genes' receptive regions that are Participated in protein synthesis, cell development, inflammation, and survival. On the other hand, estrogens bind to G protein-coupled receptors (GPR30), ER variations, and plasma membrane receptors to mediate non-genomic actions and initiate intracellular signaling. This binding causes the transcription factors (NFκB) and (MAPK) to activate rapidly, as well as the protein kinases (PI3K), (Ras), and (Raf). These factors control expression of genes that are targetted by estrogen.(Saczko *et al*, 2017)

In endometrial cancer cells, the promoter region of PIWIL1, a critical gene for stem cell self-renewal, is bound by ER in response to estrogen., causing PIWIL1 to

be overexpressed and promoting the growth of cancer cells (Chen *et al*., 2020; Morimoto *et al.*, 2009). Estrogen can activate the GPER/GPR30 estrogen membrane receptor, which is involved in rapid non-genomic signaling. Transmembrane receptor GPER/GPR30, which is expressed by both ER+ and ER breast cancer cells, contributes to the growth of breast cancer. (Wei *et al* ., 2014)

2.1.6.2.2 Progesterone Receptors in Breast Cancer

The main step of young growth in the breast is driven by estrogens, although estradiol together with progesterone are accountable for the proliferation of cells in the memory gland, according vivo investigations of hormonal replacement therapy after ovariectomy for hormonal ablation (Brisken, 2013). Progesterone participates in the pathways that control CSC activity by:

1. Binding to cell membrane PRs (mPRs) as well as nuclear PRs genes. It takes little time for this technique to activate the transcription of target genes and their translation into proteins. (Vares *et al.*, 2015)

2. The relationship between CSC activity and various isoforms and posttranscriptional alteration of PRs (Truong *et al*., 2019)

3. Progesterone controls CSC activity by PR+ and PR cells' paracrine interactions. Progesterone alters the tumor microenvironment via acting on PR+ cells, which in turn influences neighboring PR cells. (RANKL) receptor activator and WNT4 are paracrine markers of progesterone-induced CSC growth. (Finlay-Schultz and Sartorius , 2015)

4. According to certain research, progesterone can control the miRNA expression necessary for CSC development and proliferation. (Zhang *et al*., 2010).

5. CSC activity is also impacted by the intricate interactions linking progesterone to other hormones like growth hormones and prolactin (GHs). (Sato *et al.*, 2014)

   PRs are essential to the growth of CSCs and lead to poor prognoses. PR-A and PR-B are the two isoforms of nuclear PRs. These have varied transcriptional and functional activity as a result of being produced from the same gene via two separate promoters. (Kastner *et al.,* 1990)

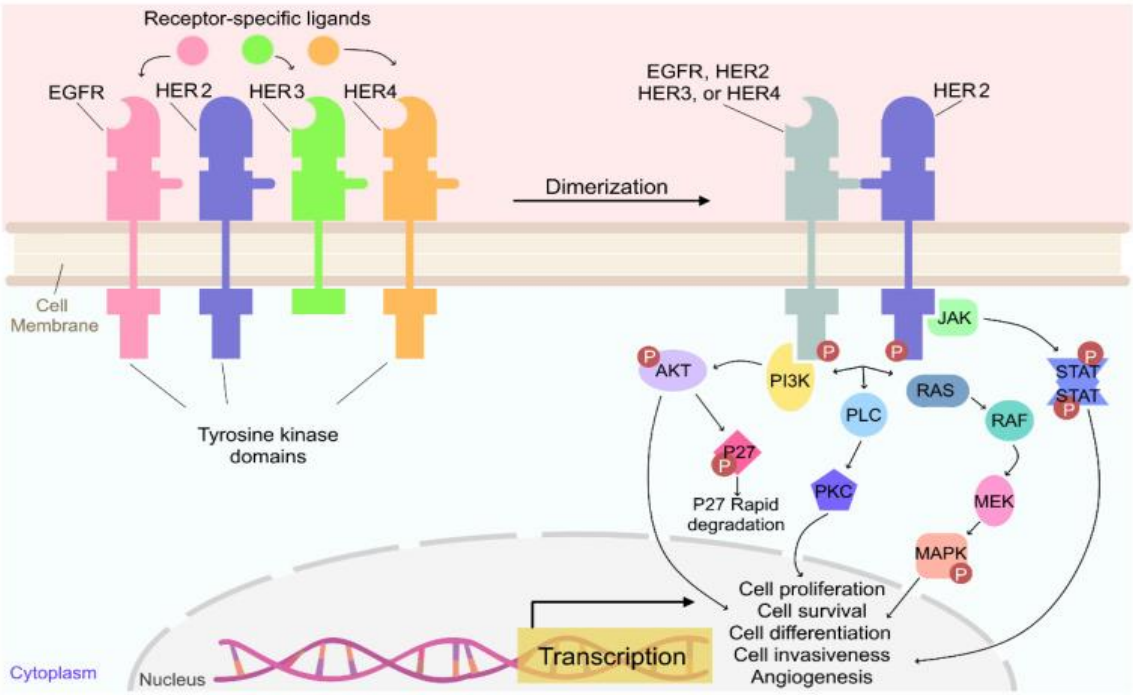
2.1.6.2.3 HER2/neu Receptors in Breast Cancer

Because it resembles HER1, in structure, HER2 received its name. Neu is called after a rodent glioblastoma cell line, a particular kind of cerebral tumor. (Coussens *et al*, 1985). The (HER/EGFR/ERBB) family includes HER2. It has a significant impact on the onset and course of some aggressive forms of breast cancer. For about 30% of patients with breast cancer, the protein has recently emerged as a key biomarker and a therapeutic target (Mitri *et al.*, 2012). Latest years, a significant increase in attention role of HER2/neu in cancer, both as a prognostic marker and as a predictor of therapeutic response. Evaluation of HER2/neu status in patients with metastatic breast cancer has grown in importance as a clinical factor since the introduction of the medication Herceptin. (Paik ,*et al*, 2000). According to earlier research, between 25 and 30 percent of breast cancers

with invasive ductal carcinomas exhibit HER2/neu overexpression or amplification. In contrast, according to Slamon *et al.* (1987), the prevalence of HER2/neu excess expression or amplification in situ ductal carcinoma is about 60%. When inactive, Membranes on the surface of cells harbor monomers of the EGFR family of receptors, which are mainly composed of three domains: an intracellular tyrosine kinase domain, a lipophilic transmembrane segment, and an external cysteine-rich domain.Except for HER2, which does not bind to any ligands, the extracellular domain of the EGFR family of receptors is largely activated by ligand interaction. This facilitates the intracellular tyrosine kinase domains of the receptors' dimerization, autophosphorylation, and transphosphorylation. (Figure 2-3). When HER2 forms homodimers with another HER2 monomer or heterodimers with EGFR, HER3, or HER4 without ligand-induced dimerization or activation, it is said to be active. Downstream signaling pathways including PI3K/AKT, RAS/MEK/MAPK, JAK/STAT, and PKC are also activated when HER2 is active. (Citri *et al.*, 2006).

Several studies have shown that the most active and powerful tumor-enhancing effect is caused by the HER2/HER3 dimer, which mainly acts through the downstream activation of PI3K/AKT, MAPK/ERK, and JAK/STAT pathways and is accountable for treatment failure and enhanced resistance to medicines in breast cancer patients. Along with EGFR family members, HER2 can form dimers with other membrane-bound receptors like insulin-like growth factor 1 (IGF-1), which increases HER2's phosphorylation and thus activates downstream signaling pathways that support tumor growth. HER2 is not connected to every known ligands, in contrast to the other EGFR family receptors. Instead, heterodimerization

with other activated EGFR family of receptors or heterodimerization with activated HER2 receptors activates HER2. Tyrosine residues are phosphorylated by receptor dimerization, which triggers signal transmission. The most prevalent signaling pathways that activate a number of downstream cascades and promote a variety of outcomes, including cell proliferation, survival, differentiation, angiogenesis, and invasion are PI3K/AKT, RAS/MEK/MAPK, JAK/STAT, and PKC. Additionally, active PI3K/AKT also causes the cell-cycle inhibitor p27Kip1 to be degraded, promoting the continuation of the cell cycle. (Nahta *et al*, 2005)



(Fig 2-3) An Overview of the Signaling Mechanism for HER2 (Nahta . *et al*, 2005)

Early breast carcinogenesis events, including HER2 amplification and mutations that activate HER2 are present in about 50% of in situ carcinomas and are maintained in 20% of instances as the illness progresses to the invasive type

(Gutierrez and Schiff , 2011). Additionally, HER2-amplified breast tumors have higher susceptibility to some chemotherapy drugs, like doxorubicin, increased resistance to some hormonal treatments, such as tamoxifen, and increased propensity to metastasis to the liver, lungs, and brain (Gabos, *et al*., 2006). These results demonstrate the importance of the prognostic marker HER2 and the predictive value of HER2 in breast cancer. (Slamon *et al*, 1987)

2.1.6.2.4 KI-67 in Breast Cancer

According to Kloppel and La Rosa (2018), the name of this biomarker is derived from its place of origin, Kiel, and its placement inside a 96-well plate. Ki-67 is a proliferation marker that is found in all proliferating cells and is of great interest (Nishimura *et al*, 2010). It distinguishes malignancies of luminal A and B and is a nuclear marker of cell proliferation. Breast cancers with low Ki67 and PgR positivity and a low chance of recurrence are known as luminal A-like tumor risk Among HER2-negative/ER-positive tumors according to multigene expression test. In the meantime, luminal B-like tumors are described as tumors with a negative or low positive reaction for PgR, high Ki67 (20%) index, and high recurrence risk (Goldhirsch *et al*, 2013). The expression of the Ki-67 in all cell stages other than G0 has been demonstrated. The immunohistochemical examination of the Ki-67 antigen utilizing the monoclonal antibody MIB-1 against human Ki-67 and reporting the proportion of cancer cells with a positive stain is most often used analysis approach (Inwald *et al* ,2013). Throughout the cell cycle, Ki67 expression changes, peaking during mitosis. Although Ki67's significance in cell division and ribosomal RNA production has not yet been fully understood,

there is evidence of this (Duchrow *et al*, 1996). According to numerous studies (Urruticoechea *et al*, 2005), the proliferation marker Ki-67 is a reliable independent predictor and prognostic factor in early breast cancer. Chemotherapy is more effective in treating breast cancer with elevated Ki-67 expression (Jones *et al*, 2010). The Ki-67 antigen is only found in cell nuclei during interphase, however during mitosis, the majority of the protein is moved to the surface of cellular chromosomes. (Cuylen, *et al* , 2016)

2.1.6.2.5 BCL2 in Breast Cancer

The BCL family of proteins, which controls apoptosis, includes the BCL2 protein. The bcl-2 gene produces the bcl-2 protein. Animal models have shown it to have tumor-causing potential (McDonnell and Korsmeyer, 1991). It also plays an anti-apoptotic effect, suppresses cell death, and prolongs cell life (Vaux *et al.*, 1988). Estrogen is known to increase the expression of Bcl-2, which is commonly found in both breast cancer cells and normal breast epithelial cells (Leek *et al*., 1994). According to studies, Bcl-2 expression is favorably correlated with differentiated markers or good prognostic indicators in breast cancer, including ER/PR expression, HER2 negative, slow proliferation, small tumor size, and others. To choose the most effective anti-cancer medications, it is crucial to identify specific therapeutic targets in cancer tissues. Determining the expression of the (ER), (PR), and (HER2) in breast cancer has long been standard procedure. Trastuzumab is administered to individuals with HER2-positive cancers while hormone receptor-positive (ER-positive and/or PR-positive) tumors are candidates for hormonal therapy. Chemotherapy is the only treatment option for patients with so-called triple-negative malignancies, which are ER-negative/PR-negative/HER2-

negative. Patients with breast cancer require more therapy options, especially when their tumors lack established therapeutic targets. Bcl-2 is overexpressed in many malignancies and helps in tumor development, growth, and treatment resistance. (Ohmori *et al* , 1993 ; Lee *et al* ,2007). Bcl-2 targeted therapy may be an effective treatment for many malignancies, according to mounting evidence. The majority of investigations have come to the conclusion that Bcl-2 expression forecasts a successful clinical result. Bcl-2 has been demonstrated TO separate predictor of clinical outcome for patients receiving endocrine therapy, but not for those receiving only local or regional treatment, considering the therapeutic regimen into account. Given Bcl-2's anti-apoptotic properties, a positive clinical outcome in Bcl-2-positive patients is unexpected; nevertheless, the association of Bcl-2 with differentiated markers appears to be at least partially responsible for these outcomes. Endocrine therapy is routinely administered to individuals who are positive for hormone receptors malignancies, regarding the relationship between ER/PR expression and Bcl-2 expression may mask the independent function of Bcl-2. (Daidone *et al*, 1999).

2. 2. Immune Response

Reactions to some non-infectious substances, such as safe tumors, environmental chemicals, and even intact host components are regarded as forms of immunity (autoimmunity, tumor immunity, and allergies). In a biologic setting, the word "immunity" has historically been used to describe disease resistance. The network of organs, tissues, and cells that makes up the immune system functions as a defense the body against "foreign" invaders. The majority of them are microorganisms (germs), which include bacteria, viruses, parasites, and fungus. An

immune response is these molecules' and cells' coordinated response to other chemicals and diseases. The human body is a suitable habitat for many germs, so they try to get inside. The immune system's objective is to either destroy them or, in the event that it is unable to keep them out. Although the immune system's functions in tumor growth and treatment have long been hypothesized, they have only recently come to light and been the subject of mechanistic research. (Hanahan and Weinberg, 2011 ; Janeway *et al* , 2001). The immune system consists of two parts: the innate immune system and the adaptive immune system.One of the features of cancer is the development of an immunosuppressive tumor microenvironment, which is facilitated by T and B lymphocytes from the adaptive immune system and macrophages, neutrophils, mast cells, myeloid cells, dendritic cells, and natural killer (NK) cells from the innate immune system. (Gasser *et al* , 2005)

2.2.1. Innate Immune

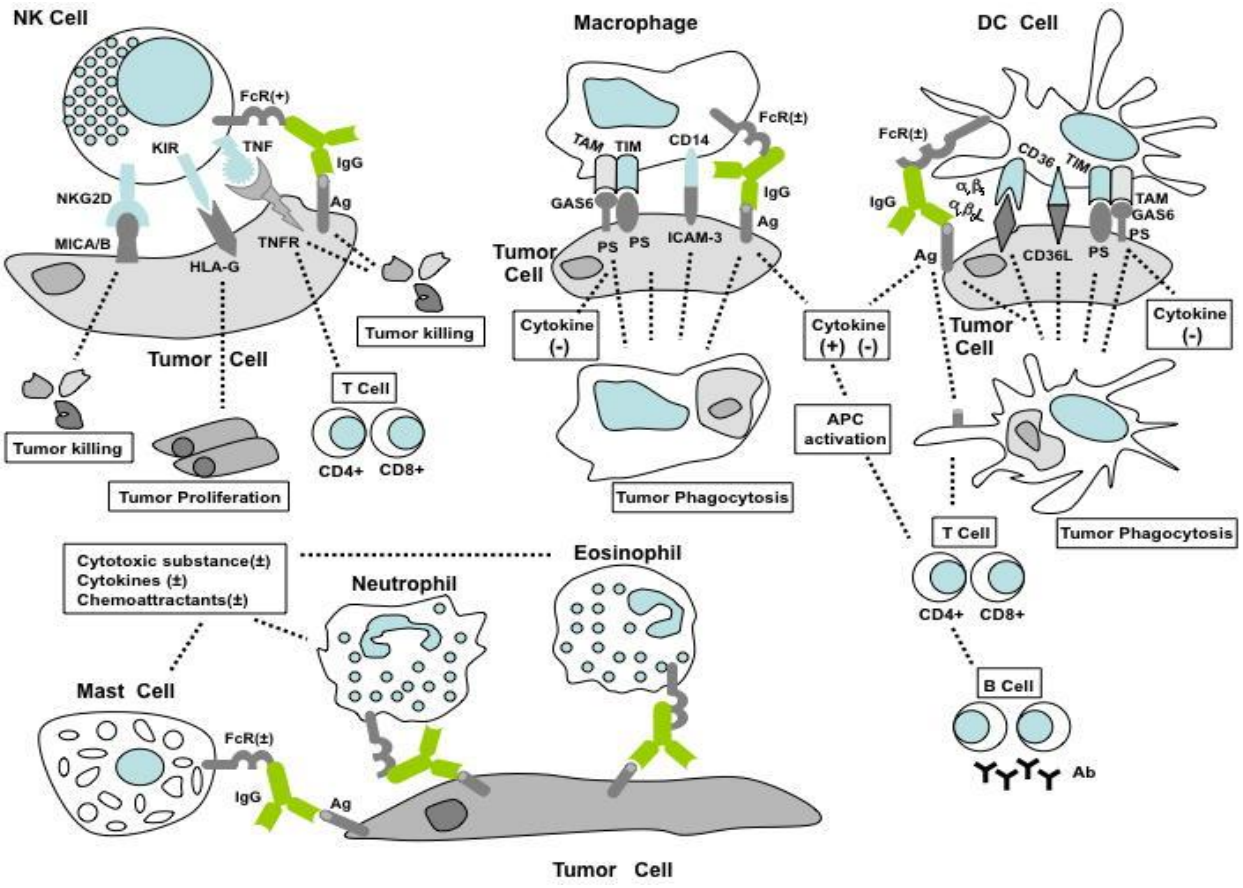
One of the two primary immunity mechanisms in vertebrates, together with the adaptive immune system, is the innate, or nonspecific, immune system. Plants, fungi, insects, and early multicellular organisms all have an immune system that is predominately innate, which is a different type of defense mechanism (Gasser *et al* , 2005)

serve as a second-line physical or chemical barrier, such as the blood-brain barrier, which, in the event of a contusion or other injury that breaches the first-line physical barrier, releases clotting factors in blood and acts as a physical and

chemical barrier to infectious agents. This protects the nervous system from pathogens that have already entered the host (Gasser *et al* , 2005)

2.2.1.1. The Direct Interaction Between the Innate Immune System and Cancer

The primary immune system effector cells that attack cancerous cells specifically are (NK), (DC), macrophages, PMN, which includes (neutrophils, eosinophils, and basophils), mast cells, and cytotoxic T lymphocytes. NK cells, DC, PMN, mast cells, and macrophages are the first-line effectors to eradicate cells, including cancer cells. NKT and T cells serve as elements of the innate and adaptive immune systems through close interaction with cells of the latter, including memory-preserving CD4+ and CD8+ T lymphocytes. (Armean *et al*, 2008). The following direct molecular interactions between malignancies and innate immune effector cells have lately brought attention to how crucial the innate immunity system is in preventing cancer growth. (figure 2-4)



(Fig 2-4) Cancer recognition by the innate immune system (Liu and Zeng , 2012).

direct identification of cancer by the immune system at large. Innate immune system cells include neutrophils, eosinophils, DC, mast cells, NK, and macrophages. MICA/B on malignancies is preferentially recognized by NKG2D, a stimulatory receptor on NK cells, which promotes cell death. To stop NK cell cytotoxicity, inhibitory receptors like KIR identify the non-classical MHC class I molecule HLA-G on tumors. TNF family ligands on NK cells bind to TNF family receptors on tumor cells, causing cancer apoptosis that produces CD4+ and CD8+ T lymphocytes in the process. NK cell activity is also mediated by FcR/CD16 activation via ADCC. Macrophages use the CD14, TIM, TAM, and FcR receptors to bind to ICAM-3, PS, PS/Gas6, and immune complex, respectively, to phagocytose apoptotic cancer cells. The "bridge" that connects PS and TAM receptors is Gas6. Macrophages have both activating and

inhibitory Fc receptors (FcR+, activating; FcR−, inhibitory), which are linked to the synthesis of superoxide compounds and cytokines. The release of cytokines by DCs and macrophages stimulates antigen-presenting cells, which triggers an adaptive immune response that is both humoral and cellular. FcR on DC is activated to aid in the presentation of tumor cell antigen. Neutrophils, eosinophils, and mast cells also express activating and inhibitory FcR. These cells use their direct recognition of antibody-coated tumors to either stimulate or suppress the release of cytokines and chemokines from innate immune cells. (Gasser *et al* , 2005)

The native defense mechanism is composed of its cellular constituents, macrophages, NK, DC, neutrophils, eosinophils, and mast cells elements. A NK cell's stimulatory NKG2D receptor particularly detects MICA/B on malignancies to promote cell death. To stop NK cell cytotoxicity, inhibitory receptors like KIR find the non-classical MHC class I molecule HLA-G on tumors. CD4+ and CD8+ T cells are produced as a result of cancer apoptosis, which is triggered by the binding of TNF family ligands on NK cells to TNF family receptors on tumor cells. Activating FcR/CD16 via ADCC is another way that NK cells function. ICAM-3, PS, PS/Gas6, and immune complex, respectively, interact with CD14, TIM, TAM, and FcR receptors on macrophages to cause them to phagocytose apoptotic cancer cells. The "bridge" between PS and TAM receptors is played by Gas6. On macrophages, there are both activating and inhibitory Fc receptors (FcR+, activating; FcR, inhibitory), and they work together to produce cytokines and superoxide compounds. Activation of antigen-presenting cells is aided by the production of cytokines by DC and macrophages, which triggers a cellular and humoral adaptive immune response. FcR activation on DC aids in the presentation of tumor cell antigen. Additionally, neutrophils, eosinophils, and mast cells express activating and inhibitory FcR, which are used to either enhance or prevent the

release of cytokines and chemokines from innate immune cells in response to antibody-coated malignancies (Liu and Zeng , 2012).

2.2.1.2. Direct Interactions Between Cancer and NK Cell

There are now a few NK-cell-based cancer treatments that are being evaluated in clinical trials. The majority of them make use of the NK cell's direct cytotoxic effect against cancer, which involves blocking surface inhibitory receptors or stimulating NK cell-surface stimulatory receptors. Considering preclinical research revealing that genetic overexpression of NKG2D causes tumor regression, a number of medicines that specifically activate NKG2D ligands on malignant cells have been developed to supplement treatments involving chemotherapy, such as 5-FU and cisplatin, which destroy DNA (Terme *et al*, 2008).

Hepatocellular carcinoma and breast cancer in humans have also been treated with low-dose bortezomib, a proteasome inhibitor. (Terme *et al*, 2008 ; Sheridan , 2006), to boost NK activating ligands, which will lead to tumor lysis. Even after chemotherapy, which results in cancer cells being resistant to the intrinsic apoptotic pathway, TNF- related apoptosis – inducing ligand (TRAIL) on NK cells may effectively cause cancer cell death. As a result, a novel method for combining chemotherapy with NK cell-based treatment is to modulate the TRAIL pathway on NK cells (Robak and Robak , 2011 ; Weiner *et al*, 2010). Individuals suffering from sudden myeloid leukemia and multiple myeloma have undergone clinical studies with medicinal monoclonals, such the monoclonal antibody against KIR, that disrupt inhibitory signaling in NK cells in addition to activating NK-surface stimulatory receptors. (Kawai and Akira , 2010).

Rituximab, Tositumomab, and Veltuzumab, which are B-lymphocyte antigen CD20-targeted humanized monoclonal antibodies, are some of the therapeutically relevant monoclonal antibodies that have been authorized for lymphoma and leukemia. These antibodies work partially through ADCC. (Takeuchi and Akira , 2010)

2.2.1.3. Cancer and the Innate Immune System Interactions via DAMP and Their Partner Receptors

A multitude of chemicals generated due to the demise of cancer cells may operate as damage – associated molecular patterns (DAMP) and act together with innate immunity cells in addition to the direct interactions between cancer and the native immune system. These extracellular matrix (ECM) and intracellular chemicals that are released by necrotic and apoptotic tumor cells are examples of cancer-derived DAMP. (HSPuric acid, mitochondrial DNA, mitochondrial formyl peptides, adenosine triphosphate (ATP), high-mobility group box-1 protein (HMGB1), and are examples of intracellular compounds that can act as DAMP. According to Takeuchi and Akira (2010), TLRs on innate immunity cells are the main pattern recognition receptors that detect danger signals connected to DAMP. Other receptors that are important in reacting to DAMP produced from malignancies include cytoplasm nucleotide – binding oligomerization domain NOD-like receptors and retinoic acid – inducible gene-1 RIG-I-like receptors (Medzhitov and Janeway, 2000).

2.2.2. Adaptive Immune System

The components of the adaptive immune system, T and B cells, display unique antigen-specific receptors that are specific to particular antigens and were created by somatic DNA recombination. Due to its large number of receptors, the adaptive immune system is very selective because only a tiny percentage of these cells will be able to identify and multiply in response to a particular antigen (Bonilla and Oettgen 2010). Identification of specific "non-self" antigens and their distinction from "self" antigens, the creation of pathogen-specific immunologic effector pathways that eliminate specific pathogens or pathogen-infected cells, and the establishment of an immunologic memory that can swiftly eradicate a particular pathogen in the event of The primary functions of the adaptive immune response are to combat subsequent infections (Murphy *et al* 2007). Adaptive immune responses are necessary for effective immunization against infectious diseases. Two cell types make up the adaptive immune system: antigen-specific T cells that proliferate when they are activated by APCs, and B cells that mature into plasma cells and produce antibodies. (Murphy *et al* 2007)

2. 2.2. 1. Antigen-Presenting Cells (APCs) and T lymphocyte Cells

T cells mature in thymus after migrating from bone marrow's hematopoietic stem cells. These cells express the (TCR), a distinct class of antigen-binding receptors, on their membrane. A single type of TCR is expressed by each T cell, and if the right signals are given to it, it can divide and multiply quickly. As previously indicated, in order for T cells to identify a particular antigen, APCs

typically dendritic cells, but also macrophages, B cells, fibroblasts, and epithelial cells—must act. (Schroeder and Cavacini 2010).

A group of proteins called Major Histocompatibility Complex (MHC) are expressed on the surfaces of antigen-presenting cells (APCs). MHC is categorized into two groups: class I, commonly referred to as HLA, or human leukocyte antigen. A, B, and C, which are present on every nucleated cell, as well as class II, or HLA (M, O, P, Q, and R), which are present only on specific immune system cells such as B cells, dendritic cells, and macrophages. T cells are presented with class I MHC molecules exhibit endogenous (intracellular) peptides, while class II molecules are found on APCs when a cell phagocytoses foreign proteins or organisms or becomes infected with an intracellular pathogen, like a virus, the MHC protein displays fragments of antigens (peptides). Numerous distinct TCRs on T cells allow them to attach to particular external peptides. During the immune system's development, T cells that would respond to antigens typically present in the body are mainly removed. T cells become activated when they come into touch with an APC that has degraded an antigen and is displaying the proper antigen fragments (peptides) linked to its MHC molecules (Schroeder and Cavacini 2010).

The lymphatic and circulatory systems, which transport T cells throughout the body, as well as the lymph nodes where APCs are found, increase the likelihood that the proper T cells will come into contact with an APC that is carrying the right peptide MHC complex. The MHC-antigen complex activates the TCR, which prompts the T cell to release cytokines to further control the immune response. T lymphocytes are activated by these antigen presentation mechanism to develop

mostly into T-helper (Th) cells (CD4+ cells) or cytotoxic T cells (CD8+ cells) (Schroeder and Cavacini 2010).

The primary functions of CD8+ cytotoxic T lymphocytes are the elimination of foreign agent-infected cells, such as viruses, and the destruction of tumor cells that express the proper antigens. Their TCR interacts with peptide attached to MHC class I molecules to activate them. Cytotoxic T cells that undergo clonal growth give rise to effector cells, which secrete chemicals that cause target cells to undergo apoptosis. When the infection resolves, the majority of effector cells perish and are eliminated by phagocytes. When the same antigen is encountered again, Certain cells are retained as memory cells, which have the ability to quickly differentiate into effector cells (Schroeder and Cavacini 2010).

Helper T cells that are CD4+, are crucial for initiating and optimizing the immune response. These cells are unable to eliminate infections or kill infected cells directly because they lack cytotoxic or phagocytic function. But by instructing other cells to carry out these functions and controlling the kind of immunological response that arises, they "mediate" the immune response. When class II MHC molecules bind to antigen, TCR recognition of those molecules activates Th cells. When Th cells become activated, they elicit cytokines that affect the function of various cell types, including the APCs that initially stimulated them. The immune response is also influenced by the regulatory T cell (T reg), a subgroup of CD4+ T cells. T reg cells may regulate atypical reactions to self-antigens and the onset of autoimmune illness by limiting and suppressing immune responses. T reg cells can aid in the resolution of typical immune responses by

removing infections or antigens. According to Schroeder and Cavacini (2010), these cells are also essential for the establishment of "immune tolerance" to specific foreign antigens, such as those present in food.

2.2.2.2. B cells

After developing from bone marrow-derived hematopoietic stem cells, B lymphocytes express a distinct (APC) on their membrane and exit the marrow. B cells, in contrast to T cells, have specific antibodies produced on their cell surface that allow them to directly identify antigens without the aid of APCs. B cells' primary job is to produce antibodies in response to foreign antigens, which necessitates further differentiation of the cells (Murphy *et al*., 2007; Schroeder and Cavacini, 2010). B cells can function as APCs in specific situations. When foreign antigens that they have the proper antigen-specific receptors on them stimulate B cells, they proliferate and become antibody-secreting memory B cells or plasma cells. Being "long-lived" survivors of prior infections, memory B cells still express antigen-binding receptors. These cells can be relied upon to respond quickly to a reintroduction of an antigen by producing antibodies and eliminating it. On the other hand, when the immune response's trigger is eliminated, plasma cells usually undergo apoptosis and have a brief lifespan. Conversely, however, these cells generate a lot of antibodies that travel throughout the body and coat tissues, effectively warding off infections (Murphy *et al*., 2007).

B cells are important in the immunological reaction mediated by antibodies because of their role in producing antibodies; this is in contrast to the

immunological response mediated by cells, mostly regulated by T cells (Li, *et al* 2015)

2.3. Role of the Immunity in The Breast Cancer

The immune systems, either innate or adaptive, contain the cells that are involved in immune response. Innate immune system cells have a function in the initial reaction to a pathogenic challenge. These cells employ a variety of strategies to combat infections, including phagocytosis, complement cascade, immune cell recruitment, and the release of toxins. Solid tumors, such as breast tumors, are extremely complicated assemblages of immune and stromal fibroblast cells as well as neoplastic cells. The cells in this community have different effects on one another. Tumor cells can use immunological system to their benefit, suppressing the reactions of the immune system that fight cancer, while immune cells can inhibit or promote tumor growth (Dunn *et al* , 2004). Early on in the tumorigenesis process, Chemokines, including vascular endothelial growth factor and proteins of the C-X-C motif chemokine family, are expressed by rapidly proliferating neoplastic cells to stimulate angiogenesis after they exhaust the resources at the site of origin. This is when the autoimmune reaction to a solid tumor starts. Stress causes the innate immune system's typically immature myeloid cells to be drawn to the location, which causes a tissue healing response akin to what happens in a sterile wound (Grivennikov *et al* 2010). Because the tumor microenvironment lacks an immunogenic stimulation, these myeloid cells are usually immunosuppressive. On the other hand, in response to this stimulus, these myeloid cells begin removing and breaking down tumor proteins in order to send them to T cells within the lymph node of the adaptive immune system.

Two types of antigenic proteins that the immune system's adaptive response can identify are expressed by tumors. (TAA) are typical proteins that are expressed in atypical tissues or at uncommon stages of development. These are the first type of antigens. Tissue differentiation antigens, cancer/testis antigens, and secondly, common proteins that are overexpressed in cancer are examples of TAAs (Criscitiello 2012). Oncogenic tumor virus antigens are also occasionally regarded as TAAs. Apart from TAAs, oncogenic cell somatic mutations can also change some proteins to create neoantigens, which are distinct immunogenic peptides that the immune system has never encountered before (Schumacher and Schreiber 2015).

Merely a small percentage of these unique peptides can trigger a potent anti-tumor immune response by T cells and are effectively digested and provided by cells that present antigens. Dendritic cells absorb the neoantigens if there is enough immunogenic stimulation present in the tumor, and they then go to the lymph node. These cells expose their tumor-driven antigens to CD4+ T cells and cytotoxic T lymphocytes (CTLs) at the lymph node, which encourages them to proliferate into clonal populations that are devoted to specific antigens and travel to find the sources of their corresponding antigens (Gajewski *et al* 2013).

These T cells carry out effector tasks after being identified in an effort to eradicate the tumor. If they are effective, this leads to a decrease in the tumor burden and the elimination of the antigen-positive tumor subpopulation. Nonetheless, signals to dendritic cells inside the microenvironment of the tumor significant influence on T lymphocytes' presentation of antigens in lymph nodes,

and some of these signals even obstruct this process. Instead, through cell signaling and the production myeloid cells, including immature dendritic cells, may release immunosuppressive cytokines." respond to these signals by establishing a tolerogenic environment. These cytokines prevent the presentation of antigens and attract or produce (Treg) cells, which react to antigens within the tumor but block other immune cells' ability to proliferate, so shielding the tumor from an immunological onslaught (Mahnke *et al* , 2007; Palucka *et al* , 2013). Consequently, the immune-suppressive tumor microenvironment inhibits the growth and activation of helper CD4+ T cells and CD8+ CTLs, which would otherwise aid in the neoplasm's removal.

The interaction between both the immune systems—innate and adaptive in breast cancer is starting to be described, but there is still more to learn. Since the initial papers from the 1980s, using immunohistology, that detailed cancer as having varying degrees of T cell infiltration, many studies have reported the presence of adaptive immune infiltrate in breast tumors (Bhan and DesMarais, 1983 ; Rowe and Beverley , 1984 ; Hurlimann and Saraga , 1985). (HER2), a normal protein has been demonstrated to exhibit cancer-specific overexpression, is one of the TAAs specific to breast cancer that have been found (Lee *et al* , 1985). Furthermore, neo-antigens have been linked to stimulating the adaptive immune response, even though the frequency of mutations in breast cancer is lower than in other types of tumors. There may be a connection between the prevalence of neo-antigens, the anti-tumor immune response, and patient survival because the existence of these neo-antigens has been linked to higher T cell density in these tumors and better patient survival. (Gentles *et al* , 2015).

2.4. The Cellular Immune Response in the Development of Breast Cancer

Numerous types of adaptive immune cells infiltrate breast cancers, each of which has a distinct influence on the growth and development of the tumor. T lymphocytes are the most well-characterized of these cell types. T cells can be found inside breast cancers at different percentages; an early study (Baxevanis *et al*., 1994) estimated that they comprised 1% to 45% of the cellular tumor mass. In 1997, Stewart and Heppner calculated that these cells make up, on average, 26% of the Leucocytes connected with tumors present in breast cancers, based on an in silico approach. These cells are capable of broadly classified into three forms: immunosuppressive CD4+ CD25+FOXP3+ Treg cells, CD4+ T helper cells, and CTLs. Fascinatingly, several findings have asserted that certain T-cell subsets are pre-eminent. According to certain analyses (Gajewski *et al*., 2013; Zhang and Bevan, 2011), CD4+ T cells dominate CD8+ T cells, while other reports (Baxevanis *et al*., 1994) found the opposite. These disparities might result from variations in stromal composition between patient biopsies or subtype heterogeneity between breast cancer samples. Gaining insight into the interactions between different T cell subsets inside the breast tumor microenvironment will help us comprehend the immune system's adaptation to breast cancer and can also provide important predictive data (Gajewski *et al*., 2013).

2.4.1. Cytotoxic T cells

The CTLs have been described as cells that eradicate tumors in a change of cancer forms. They are T cell receptor-positive cells on them that can identify TAAs and neoantigens unique to malignancy. These cells expand clonally upon identification of an antigen prior to moving through the body in circulation pursuit of the antigenic source cell population. Once this population has been located, antigen-expressing tumor cells are killed by these cells acting through the granzyme and interferon perforin pathways (Liu *et al* , 2011). The bulk of effector CTL cells die if the antigen source is eradicated, but a tiny number of CD8+ memory T cells remain that can quickly trigger a cytotoxic response when their committed antigen is encountered again. Numerous studies have attempted to define the function of CTLs in the development of breast cancer. Basal phenotype and higher histological grade are linked to these cells' infiltrates, which are inversely correlated with the expression of the progesterone and estrogen receptors (ER and ER, respectively) (Varn *et al*, 2016). According to recent data, tumor-resident CD8+ memory T-cells Elevated levels of immature effector populations could be a sign of insufficient immune response, while subpopulations may be the main factor influencing a favorable patient prognosis. Collectively, these findings imply that CD8+ CTLs play a significant role in immune responses against tumours, and that these responses can extend patient survival. In the future, it will be crucial to measure the number of tired and anergic T cells in the breast microenvironment as well as to further characterize CTL infiltration in terms of its memory capacity. Subsets of these cells most likely have a significant impact on how well the adaptive immune response functions. (Beyer and Schultze , 2006)

2.4.2. Regulatory T Lymphocytes

CD4+ CD25+FOXP3+ Among T-cell subtypes, Treg cells are distinct in that they are always immunosuppressive, and studies have indicated that they help cancer's immune system evade the body (Watanabe *et al* , 2010; Liyanage *et al* , 2002). Normally, Treg cells use immunosuppressive mechanisms to keep the immune system tolerant to self-antigens and to stop the overindulgence and multiplication of effector T cells (Watanabe *et al* , 2010).

These cells are more prevalent in peripheral blood samples from breast cancer patients than they are in normal samples (Bates *et al* , 2006). Treg counts are also greater in invasive ductal cancer samples compared to breast tissue in normal condition, with samples of ductal carcinoma in situ showing the greatest concentration of Treg cells (Ghebeh *et al* , 2008). In invasive ductal carcinoma, elevated Treg cell levels are linked to poorer overall and relapse-free survival as well as adverse prognostic markers such high tumor grade, presence of lymph nodes, and ER negative. (Gobert *et al* , 2009). The ability of Treg cell activity to determine which patients are likely to experience a relapse after five years. sets it apart from other prognostic markers, which is interesting (Ghebeh *et al* , 2008). Mechanistic investigations have supported the idea that Treg cells have immunosuppressive properties, which is supported by these connections. According to one study, CCR4 release attracted Treg cells to breast tumors, and tumor-associated antigen-presenting dendritic cells stimulated them. In the tumor infiltration, Treg cell was discovered to be near CTLs, indicating immunosuppression (Liu *et al* , 2011). Despite the immunosuppressive nature of Treg cells, a high ratio of CTLs to Treg cells in the surrounding tumor tissue is

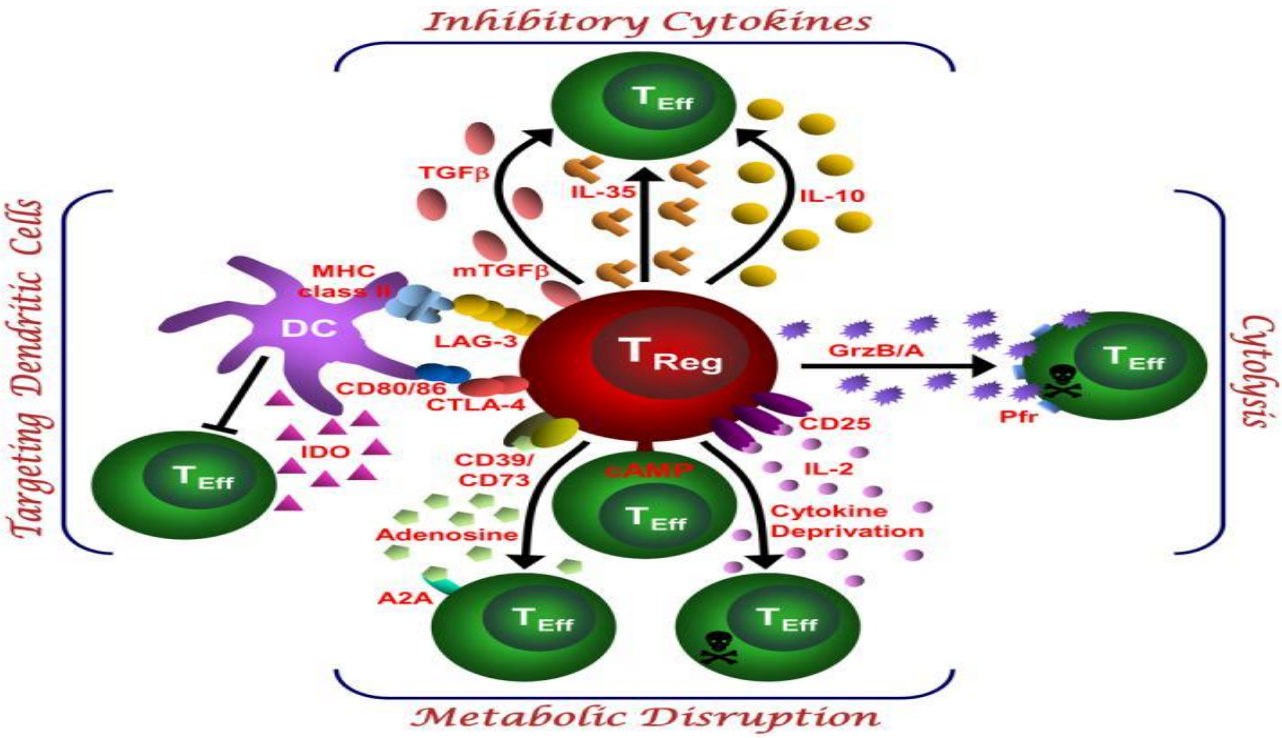
associated with improved overall survival, even though this association does not apply at the tumor bed (Ladoire *et al*, 2008). In a similar vein, patients with breast cancer had independent predictive value for PCR when their levels of CTLs and Treg infiltration were high (Albert *et al* , 2006). All of these findings point to Treg cells' immunosuppressive function in the breast tumor microenvironment. Patients with breast cancer may benefit therapeutically if these cells are eliminated or inhibited.

The naturally occurring CD4+CD25+ Tregs (nTregs) are among the various varieties of regulatory T cells (Tregs) that have been well studied and show great promise as immunotherapy for the induction of tolerance in autoimmunity or transplantation (Albert *et al*, 2006). The thymus's requirement for the generation of nTregs depends on Foxp3, a transcription factor with a winged helix-forkhead DNA-binding domain (Fontenot and Rudensky ,2005). While it is generally acknowledged that CD4+CD25+Foxp3+ Tregs originated in the thymus, mounting data indicates that, in specific circumstances, CD4+ non-Treg progenitors can create identically phenotypic Tregs in the periphery. For instance, all CD4+ cells that are derived from RAG−/− TCR transgenic (Tg) mice are CD25−; nevertheless, a minor fraction of these cells undergo phenotypic conversion to CD25+ Treg upon adoptive transfer into Ag-bearing animals or mice that receive a dose of peptide Ag that is tolerable. In thymectomized animals, de novo production of CD4+CD25+ Tregs from CD4+CD25− cells also happens, and B7 costimulation is necessary for this conversion. The name "induced Tregs" (iTregs) was also used to describe these Tregs that were induced in the peripheral (Apostolou and von Boehmer 2004; Kretschmer *et al* , 2005; Liang *et al* , 2005). There is proof that the members of the

CD28 family are essential nTreg regulators in addition to being important costimulatory molecules. When it was discovered that blocking CD28 ligation with CTLA4-Ig made autoimmune illness worse in NOD mice, it provided the first indication of the crucial role the CD28 family plays in nTreg activity. Mice lacking either CD28 or its ligands, CD80 and CD86, exhibit significantly fewer nTregs (Salomon *et al* , 2000).

2.4.2.1. Mechanisms of Suppression of Treg Cells

Laboratory investigations have demonstrated that Treg cells' suppressive activity is not dependent on a single mechanism. Consequently, the following mechanisms are proposed: A) Secretion of cytokines. The inhibitory cytokines TGF-β and IL-10 are secreted by Treg cells *in vivo* (Asseman *et al,* 1999). It has been demonstrated that Treg cell-mediated inhibition of effector CD4+ T cells requires TGF-β. B) Treg cells may employ cell-to-cell interaction as one method of suppression. Research by Nakamura *et al*. (2001) has demonstrated that TGF-β bound to the membrane plays a role in cell-cell contact-mediated suppression. Additionally, suppression has been linked to the cell surface molecules CTLA-4, Granzyme B, Fas, and LAG3 (Read *et al,* 2000; Huang *et al,* 2004; Cao *et al,* 2007). c) Rivalry for growth factors such as IL-2 may be a factor in Treg cells' ability to suppress. (De la Rosa *et a.,* 2004)(figure 2-5)



(Fig 2-5) Mechanisms of Treg Cell Suppression **(**Vignali, *et al.,* 2008)

2.4.2.2. Role of CD28 in Breast Cancer

The majority of T-cells express the glycoprotein CD28. Research indicates that CD28 polymorphism raises the possibility of developing cervical and breast cancer (Shen *et al.,* 2018). In peripheral blood from humans, the CD28 surface receptor is generally expressed on 95% of CD4+ T-cells and roughly 50% of CD8+ T-cells (June *et al* , 1990).

Consequently, in comparison to CD28 wild type (WT) mice, NOD mice on CD28 knockout (KO) backgrounds have a more severe and fast onset of autoimmune diabetes. Some research unequivocally shows that CD28 is necessary for nTreg survival and homeostasis in the periphery as well as for nTreg formation in the thymus (Tai *et al* ,2005). Therefore, these animals lack both nTregs, the

most potent mediators of self-tolerance, and robust costimulation (CD28) for T effector cells, resulting in a balanced deficit that preserves Ag-mediated activation (Riley and June , 2005). It is unknown if CD28 plays a significant part in the development of iTreg. Empirical data indicates that, as opposed to nTreg formation, CTLA4 or ICOS may be crucial for effector function (Akbari *et al*, 2002). Through its interactions with CD4 and CD8, CD28 regulates tumor immunity (Shen *et al.*, 2018). The CD28 is essential for CD4+ T-cell growth and survival. Activated T-cells express more CD28 (Turka *et al.*, 1990).

2.4.2.3. Breast Cancer and the Function of CTLA-4

The primary effector cells which are responsible for identifying tumor antigens and facilitating immune responses against tumors are CTLs. The interaction between co-stimulatory pathways and checkpoints governs CTL activity. Following the presentation of tumor antigen, antigen-presenting cells (APCs) surface protein B7 (CD80/CD86) is bound by the CTL-associated CD28 receptor, activating CTLs. Then, after being translocated to the CTL membrane, (CTLA-4) connected to B7 more strongly than CD28 and competes for its attention, blocking the pathways that had previously been engaged (Qureshi *et al* 2011). About 50% of breast carcinomas express CTLA-4, whereas normal breast tissues do not (Kassardjian *et al.*, 2018).

Among the simplest immunosuppressive substances, CTLA-4, strong negative modulator of the T cell reaction. According to Holmgaard *et al.* (2013), CTLA-4, which is expressed on the surface of active T cells and some Tregs, has the potential to raise the threshold for T cell activation early in carcinogenesis process,

which would reduce the antitumor response and increase the vulnerability of the tumor to growth. (Yan *et al* ,2013). There is proof that the tumor microenvironment and blood circulation contain higher Treg levels in breast cancer patients. Constitutive expression of CTLA-4 on Tregs inhibits the interaction between the CD80/86 receptor on DCs and the CD28 ligand on T cells. This results in a reduction in DC activation, suppression of CD8+ cytotoxic T lymphocytes (CTLs) proliferation, T cell cycle arrest, and inhibition of IL-12 production (Verma *et al*, 2013). Moreover, CTLA-4 suppresses the production of an efficient antitumor response, downregulates peripheral tolerance and T-cell response, and ultimately induces tumor immune tolerance. Furthermore, it would be anticipated that CTLA-4 which is constitutively expressed by natural Tregs, would engage leftover B7 molecules more effectively than responder T cells, so encouraging suppression as opposed to T-cell growth. (Bolton *et al* , 2015)

Recent research has demonstrated that in addition to activated T cells and Tregs, CTLA-4 is expressed on nonlymphoid cells in liver, skeletal muscle, placental fibroblasts, monocytes, leukemia cells, and certain solid tumor cells. By binding with the recombinant version of the CTLA-4 ligands, CD80/CD86, Contardi *et al*. 2005 discovered that CTLA-4 which is expressed on cancer cells might cause apoptosis linked to the sequential activation of both caspase-8 and caspase-3. Consequently, CTLA-4 expressed in tumor cells might be useful. Moreover, a higher clinical stage and evident axillary lymph node metastases were linked to increased expression of CTLA-4 in breast cancer tissues. In the current investigation, we postulated that CTLA-4, which is expressed by breast cancer cells (BCCs), may potentially impede human DC maturation and function in the

tumor milieu, just as it did for Tregs. In addition, study examind the impact of the CTLA-4 antibody on the maturation and functions of DCs that have been recovered, as well as the potential signal transduction route that contributes to the maturation of conditioned DCs. Investigations were also conducted on the direct impact of the CTLA-4 antibody on breast cancer cells' biological activities (Mao *et al*., 2010).

Chapter Three

Materials and Methods

Chapter Three

Materials and Methods

3.1 Materials and Methods

This is a case-control study (also known as a "case-referent study"), which is a type of observational study (Tenny et al., 2022). In this type of study, two groups of patients and one group of control were compared.

The patients newly diagnosed with breast cancer attended the Breast Cancer Early Detection Center at Al- Hospital. The first group, women who we expected to be healthy (apparently healthy), is called the control subject group, healthy controls had no history of autoimmune diseases or malignant diseases. The second group includes women who have been diagnosed with benign breast cancer. The third group includes women with malignant breast cancer. We have taken into consideration that all women must be free of any other disease, whether infectious or chronic, and non-smokers. Samples were collected from July to December, 2022. The consultant diagnosed the patients based on clinical examination, ultrasound, mammography, immunological tests, and supervision of the medical staff at the center. Questionnaires were designed to obtain the information of control subjects and case groups . The patients were then categorized into different groups.

3.1.1.Control Subject

The study was executed on 25 control subjects ,25 patients in each group, All groups were divided into two age groups, the first category included ages 29-49 years, and the other category included ages 50-70 years.

3.1.2.Exclusion criteria

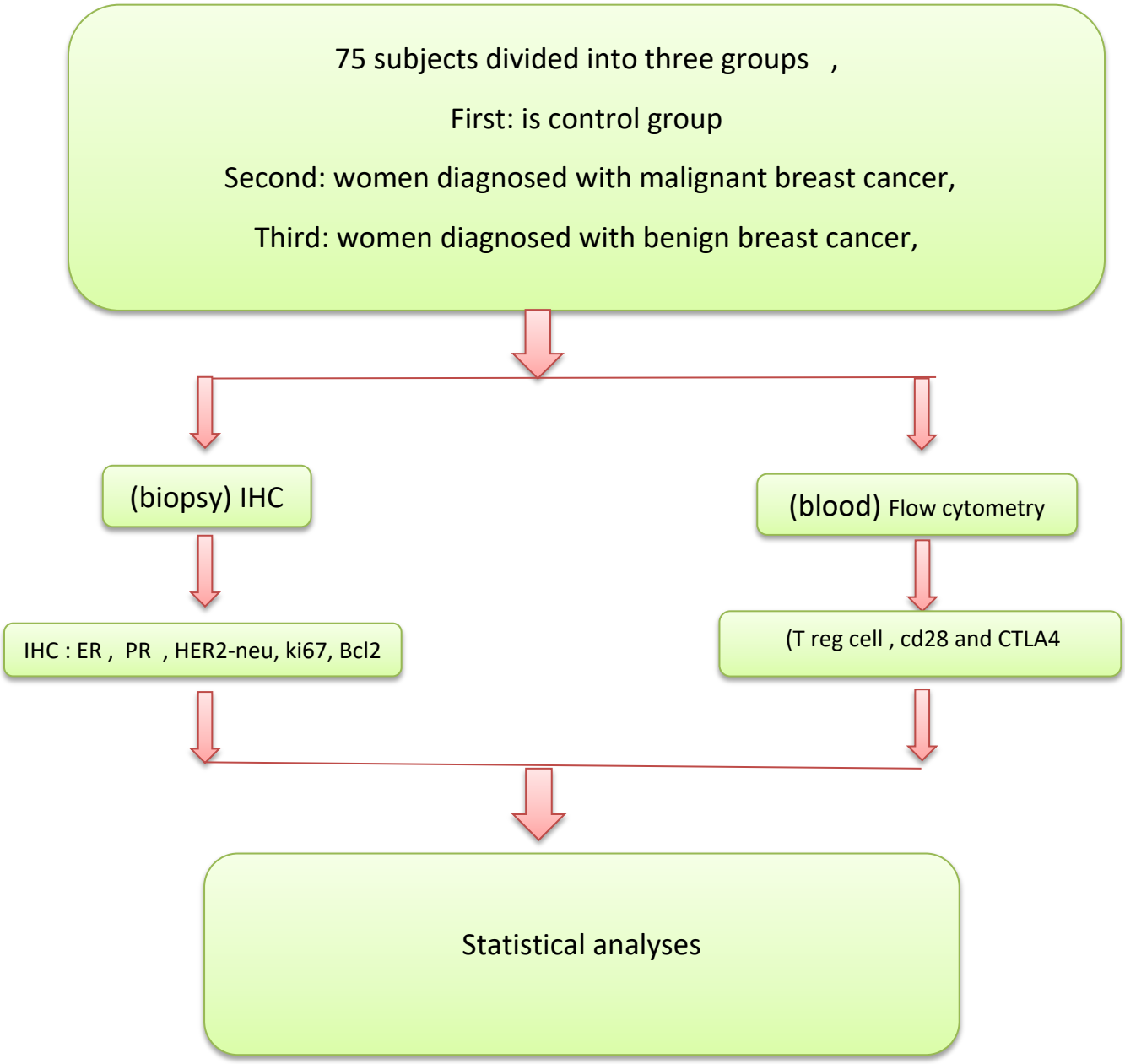
There were a number of patients' conditions considered exclusion criteria for this study:

- patients with any other autoimmune disease.
- male patients.
- patients with chronic disease
- all subjects ages down 29 and up to 70 years of age .
- Take antibiotics

3.1.3.Ethical Approval

As a mandatory step for taking samples from patients, approval for this study is obtained by the ethical committees, which include: Research Committee / Training and Human Development Center (No. 126 dated 5/16/2022), Kerbela Health Department / Holy Kerbela Governorate - Iraq.

3.1.4. Steps of study



(Fig. 3-1) Steps of study

3.2 Materials

3.2.1 Laboratory Equipment (Medical Tools and Instruments)

The medical tools and instruments utilized in the present study along with their comments in the table. (3.1).

Table. (3.1). Tools and Instruments

	Tools and apparatus	Company that manufactures	Origin
1.	BD polystyrene round bottom tube	BD bioscience	USA
2.	Calibrated adjustable micropipettes	humapette	Germany
3.	Centrifuge with rotor for 5ml tube	Hettich Rotofix	Germany
4.	Chargeable slid or positively charged slid	pathnsitu	USA
5.	Cool box	VB	China
6.	Digital section flotation bath	Thermo scientific	USA
7.	Disposable lab. Gloves		China
8.	Disposable Pasteur pipette3 ml		China
9.	EDTA Tubes 3 ml	Qeak lab.	China
10.	Eppendorf Tube 0.5 ml		
11.	Flow Cytometry	BD bioscience	USA
12.	Gemini AS slide stainer	Thermo scientific	USA

13.	Micropipette tips		China
14.	Microtome	Leica	Germany
15.	Nexty Micro pipette 100 µL to 10 µL	Watson bio lab.	Japan
16.	Nexty Micro pipette 1000 µL to 100 µL	Watson bio lab.	Japan
17.	Oven	Memmoret	Germany
18.	Plain tube without additives 10 ml	AFCO	Jordan
19.	PT module antigen retrieval	Thermo scientific	USA
20.	Refrigerator		
21.	Syringe 5 ml	Q Ject	Qatar
22.	Vortex	HumaTwist	Germany

3.2.2 Kits

Table (3-2): Solutions and Kits

	Kit	Manufacturing Company	Origin
1.	Bcl2	Dako	Denmark
2.	BD Human FOXP3 buffer A	BD bioscience	USA
3.	BD Human FOXP3 buffer B	BD bioscience	USA
4.	BD mouse anti human CD25	BD bioscience	USA
5.	BD mouse anti human CD28	BD bioscience	USA
6.	BD mouse anti human CD4	BD bioscience	USA
7.	BD mouse anti human CTLA-4	BD bioscience	USA

8.	BD mouse anti human FOXP3	BD bioscience	USA
9.	Cell wash	BD bioscience	USA
10.	Detection kit	Pathnsitu	Denmark
11.	ER	Dako	Denmark
12.	Ethanol solution	Cristalco"	France”
13.	Her2-neu	Dako	Denmark
14.	Ki67	Dako	Denmark
15.	Lysing solution	BD bioscience	USA
16.	PR	Dako	Denmark
17.	retrieval solution	Dako	Denmark
18.	Ultrapure water	Brawn	
19.	Xylene	SRL	India

3.3. Methods

3.3.1. collection of Samples

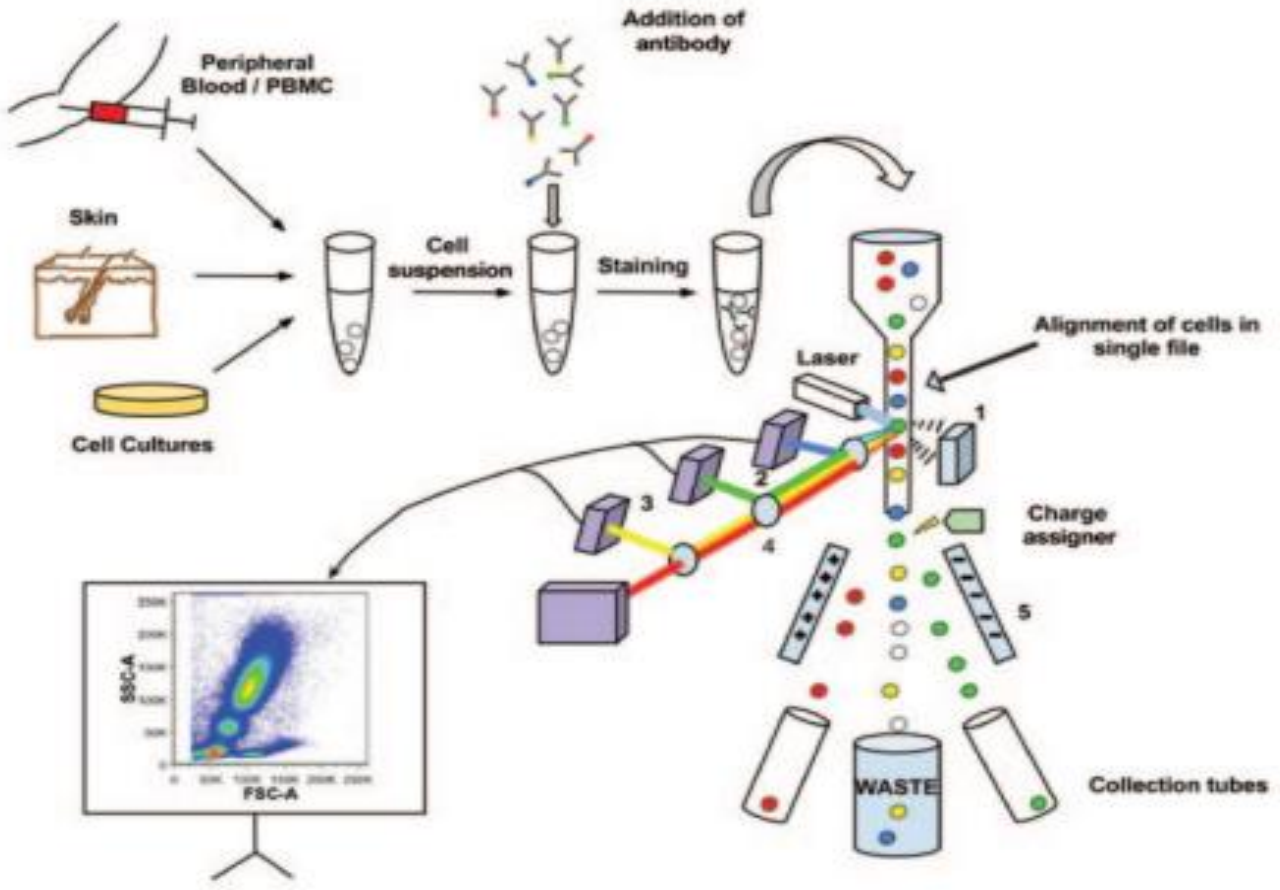
3.3. 2. The Measurements of Regulatory T Cells

By using flow cytometry, could be counted the number of human regulatory T cells.

3.3.2.1. Primary Assessment

The work of flow cytometry technique is based on the fact that a designated antibody adheres to the specific antigen in the sample followed by a series of washing**,** buffering, and staining procedures. This produces a cell suspension that is

ready for flow cytometric analysis. A liquid sheath place on a particular hydrostatic pressure on the cell or particle suspension as it entered the flow cytometer apparatus, creating a single stream of cells or particles. When a target cell or particle that has been identified with an antibody and crosses a laser focused at this stream, it will be counted or isolated in accordance with the method or the intended use **(**Figure 3-2).



(Figure 3-2) Diagrammatic Depiction of a Flow Cytometer (Richard R. Jahan-Tigh *et al*., 2012)

3.3.2.2. Assay Procedure (Flow Cytometry)

Kit materials were allowed to acclimate for 15 minutes at room temperature. The operation was then completed by following the kit's instructions:

3.3.2.3. Preparation of Buffer Solution :

Prior to usage (for each experimental set, fresh functioning solutions for human Foxp3 buffers A and B must be prepared)

1. One X Foxp3 buffer **A** preparation : Foxp3 buffer A was dilute (10 X concentration) 1: 10 with room temperature deionized water (300µl +2700 µl)
2. working solutions of buffer C preparation : dilute Foxp3 buffer B(50 x) into 1X Foxp3 buffer A at a ratio of 1: 50 (buffer B : buffer A). (16 µl +784 µl).

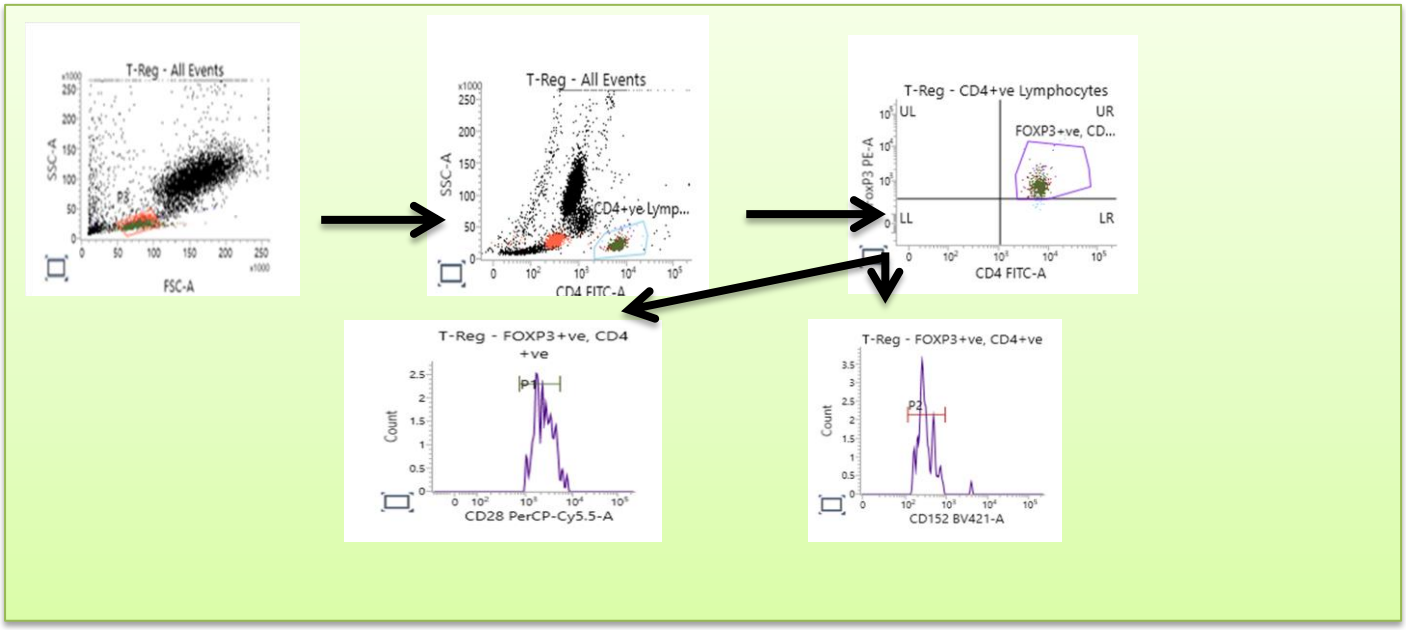
3.3.2.4.Procedure for Surface and Cytoplasmic Markers in One Tube Modified for Foxp3

1. In a BD tube add 100 µl of sample.
2. Add the surface markers to the tube : 5 µl CD 152 , 20 µl CD 25 , 20 µl CD28 , 20 µl CD4, Vortex 3-5 sec, and leave in darkness 20 min.
3. Add 2ml of lysis solution , mix by inverting them several times.
4. Incubate in darkness 10 min.
5. Centrifuge at 3000 rpm /800g for five minutes.
6. Carefully eliment the supernatant, and wash once with 2ml cell wash
7. Centrifuging at 2100 rpm for 5 minutes. Carefully Eliminate the excess fluid.
8. To fix cells re-suspend the pellet with 2ml of working solution A, Vortex 3-5 sec, and leave in darkness for 10 min.

9. Centrifuge at 2100 rpm for 5 min and remove the supernatant.
10. Wash the cells by resuspend the pellet with 2ml of cell wash .
11. Centrifuge at 2100 rpm for 5 min and remove the supernatant.
12. Add 500 ul of working solution C(A+B), and Incubate for 30 min.
13. Wash cells 2 times with cell wash then centrifuge at 2100 rpm for 5 min.
14. Add 20 ul of Foxp3 with gentle shake or vortex.
15. Incubate in the dark for 30 min, Wash twice.
16. Resuspend the pellet with 500ul cell wash and read by flow cytometry.

3.3.2.5. Calculation of Results

The outcomes determined by the cube 6 Partec device's specialized computer application.as the figure (3-3).



(Figure 3-3) Diagrammatic Depiction of a Step of Flow Cytometry

3.4. Immunohistochemistry Assay

3.4.1. Principle Assay

Immunohistochemistry (IHC) is a method for locating antigens in cells of a tissue segment that is based on the premise that antibodies attach specifically to antigens in biological tissues. Antibody-antigen binding can be observed by using a variety of techniques. Enzymes like horseradish peroxidase (HRP) or alkaline phosphatase (AP) are routinely used to catalyze a color-producing process.

IHC is widely used in many research and clinical laboratories because it permits the imaging of the distribution and localization of specific cellular components both within cells and in the appropriate tissue context. Different IHC techniques can be used to locate an antigen. The method should take specimen types and test sensitivity into account. (fig 3-4).

3.4.2. Kit Contents

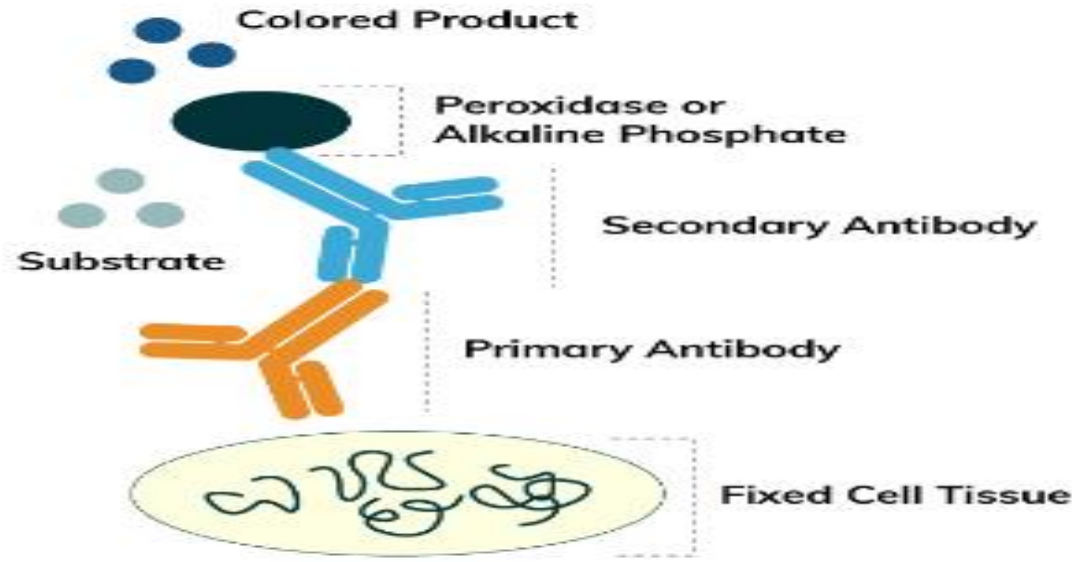
PolyExcel Peroxidase Quencher (H2O2)

PolyExcel Target Binder

PolyExcel PolyHRP

PolyExcel Stunn DAB Substrate Buffer

PolyExcel Stunn DAB Substrate Chromogen



(Figure 3-4) Immunohistochemistry (IHC) Assay. (Magaki, *et al.,* 2019).

3.4.3. Tissue Specimen

Typically, tissue specimens are that collected from many origins, involve surgery and biopsy.

- After gathering, fixing, and splitting the samples, use caution. It is worth noting that the samples were taken from the histopathological laboratory at Al-Husseini Hospital in the form of blocks ready for cutting

- Use razor-sharp scissors and knives to prevent extrusion damage.
- The cutter should be flat, thin, and small (the standard size is 3-5 cm).
- Immediaely after sectioning, Prepare tissue that has been frozen or paraffin-embedded, or keep the tissues in a liquid nitrogen container in the freezer at -70°C.

- The blocks are cut by a microtome with a size of 4 mm, and the knife must be sharp so as not to cause damage to the tissue and give a blurred image.
- The cut slices are taken and placed in a water bath with a constant temperature, after which the slices are taken and placed on a charged slide

3.4.3.1. Staining procedure

Preparation workable solutions: DAB (DAB is a possible carcinogen; use reasonable caution): Add one drop of Stunn DAB chromogen to one milliliter of Stunn DAB buffer. The preparation should be thoroughly mixed before being stored in darkness. When kept between 2 and 8 oC, this solution stays stable for one week. For clear and crisp results, always prepare fresh.

3.4.3.2. De-paraffinization:

1. Deparaffinize tissue samples in three xylene changed.
2. Hydrate by adding progressively stronger alcohols to water. Pretreatment Solution/Protocol following each incubation, washed tissue sections with antibody wash buffer according to the staining technique.
3. H2O2, a peroxidase inhibitor Peroxide quencher should be applied to the tissue segment for five to ten minutes.
4. Primary Antibody: For information on incubation time and temperature, please see the relevant primary antibody datasheet.
5. PolyExcel Target Binder: Apply PolyExcel Target Binder to the tissue pieces and let them sit at room temperature for 10 minutes.
6. PolyExcel PolyHRP: Apply PolyExcel PolyHRP to the tissue pieces and let them sit at room temperature for 10 minutes.

7. PolyExcel StunnDAB: Cover the tissue sections with the StunnDAB working solution (please refer to the preparation section for instructions on preparing the working solution), and then incubate them for 5-7 minutes at room temperature.
8. Hematoxylin: Apply Hematoxylin to the tissue sections and allow to sit at room temperature for the necessary amount of time.
9. Graded alcohols and xylenes are used to dehydrate slides, and the slip is then covered with the proper mounting media.

3.5. Statistical Analysis

Data of studied specimens were entered and analyzed using the statistical package for social sciences (S.P.S.S.) version 25. The results were given as mean ± Standard Error (Mean ± S.E.). Statistical analysis for the significance of differences of the quantitative data was conducted by using independent-sample T test. The probability levels were indicated as follows (one sign $P < 0.05$, two signs $P < 0.01$, three signs $P < 0.001$ and four signs $P < 0.0001$) (Newman *et al.*,1997)

Chapter Four
Results and Discussion

Chapter Four: Results and Discussion

4.1. Characteristics of Patients and Healthy Groups

4.1.1. Age of Patients

The patients were divided according to severity indicator to benign and malignant breast cancer, to examine the type of cancer that effect on development the immunity, and their age of the patients, to examine whether age had an effect on the development and type of the disease. The group of women with benign breast cancer was divided into two age groups. Each group of the patients divided into two subgroup, 29-49 and 50-70 years with mean 38 years and 55 respectively in benign group, and 41 years and 58 years in malignant group.

4.2. Immunological Studies

4.2.1. Frequency of Regulatory T cells

The results of the current study showed that the percentage of regulatory T cells (CD4+Foxp3 +) increased in patients groups compared with control group. However, the highest percentage was in the benign group 45.61%., following by malignant 43.42% . The differences between patients groups and control have reached to the significant levels p value $\leq$ 0.0001as shown in fig 4-1.

One of the most interesting immunosuppressive subgroups of CD4+ (CD25+) T cells are regulatory T cells, which make up approximately 5% of the overall CD4+ T cell population under normal circumstances. They are mostly represented by master transcription factor 3 (FoxP3) in the periphery (Mougiakakos *et al*,2010). It is a particular subset of T cells that play a major role in mediating immunologic tolerance. Tregs typically stop autoimmunity by preventing T cell growth and

cytokine production. After being exposed to inflammatory circumstances in the tumor microenvironment (TME), they develop a robust immune-suppressive function phenotype (Kondelkova *et al*., 2010). Cancer cells control their distinctive metabolic reprogramming within the TME in order to adjust to the physicochemical properties of the surrounding milieu. By encouraging the development and proliferation of tumor-infiltrating Tregs (TI-Tregs) and releasing immunosuppressive mediators, this metabolic reprogramming influences the biologic features of TI-Tregs (Wang *et al*, 2018). TI-Tregs directly assist in immune evasion and encourage a protumorigenic TME.

They also have a diverse phenotypic and functional profile, with upregulated markers linked to increased stimulation and repression (Chaudhary and Elkord, 2016). Moreover, mTOR activity is suppressed upon the stimulation of AMP-activating protein kinase signaling, resulting in the production of Tregs. Through enhancing nicotinamide adenine dinucleotide oxidation, increasing oxidative phosphorylation, and suppressing glycolysis and Myc expression, the transcription factor forkhead box P3 (FoxP3) reprogrammes triple metabolisms. The suppression of T-cell proliferation and activities caused by lactate is avoided, and Tregs are better able to adjust to environments that are high in lactate and low in glucose (Angelin *et al* ,2017). Tregs help the TME avoid immunological destruction by promoting peripheral immune tolerance. By triggering immune-inhibitory and protumor signals, the concentration of Tregs in the TME reduces the impact of radiation and chemotherapy on the response to treatment (Oweida *et al*., 2019). In order to promote the spread of tumor cells, tregs activate genes that mediate advanced-stage differentiation and suppressive action. A wide variety of membrane-bound chemokine receptors are expressed by Tregs, and these receptors

play a dynamic role in Tregs' intratumoral migration. TME-invading Tregs have distinct characteristic transcripts that span the course and stages of tumors and encode useful targets for cancer immunotherapy (Hensler *et al* ,2020 ; Zhang *et al* ,2020). T cells are the primary immune response in tumor growth; however, upon prolonged activation and interactions with tumor cells, they transform into suppressive CD4+ and CD8+Treg cells, which promote rather than inhibit development and progression of cancer (Mokhtar *et al*, 2007).

The findings of the current study concurred with those of previous research projects, including those of (Ola Sayed *et al.* 2017; Zahran *et al.* 2021).

T-reg cells may provide fresh perspective on how to enhance cancer treatments. Combining immune modulatory drugs with chemotherapeutic treatments which have been demonstrated to impact T-reg can significantly slow the growth of tumors and increase the length of time that patients survive (Xia *et al.*, 2017 ; Hekim *et al.*, 2017).

The discovery that increased numbers of Treg cells have been identified in many types of cancer can be clarified by the observation that tumor cells frequently contribute to the creation of Treg cells. By transforming naïve T cells into Treg cells, TGF-β—which is released by the tumor or tumor-stimulated myeloid cells—appears to play a key role in tumor-mediated Treg cell formation (Perez *et al*, 2007). We speculate that the increase in Treg cells may be due to TGF- β released from cancer cells.

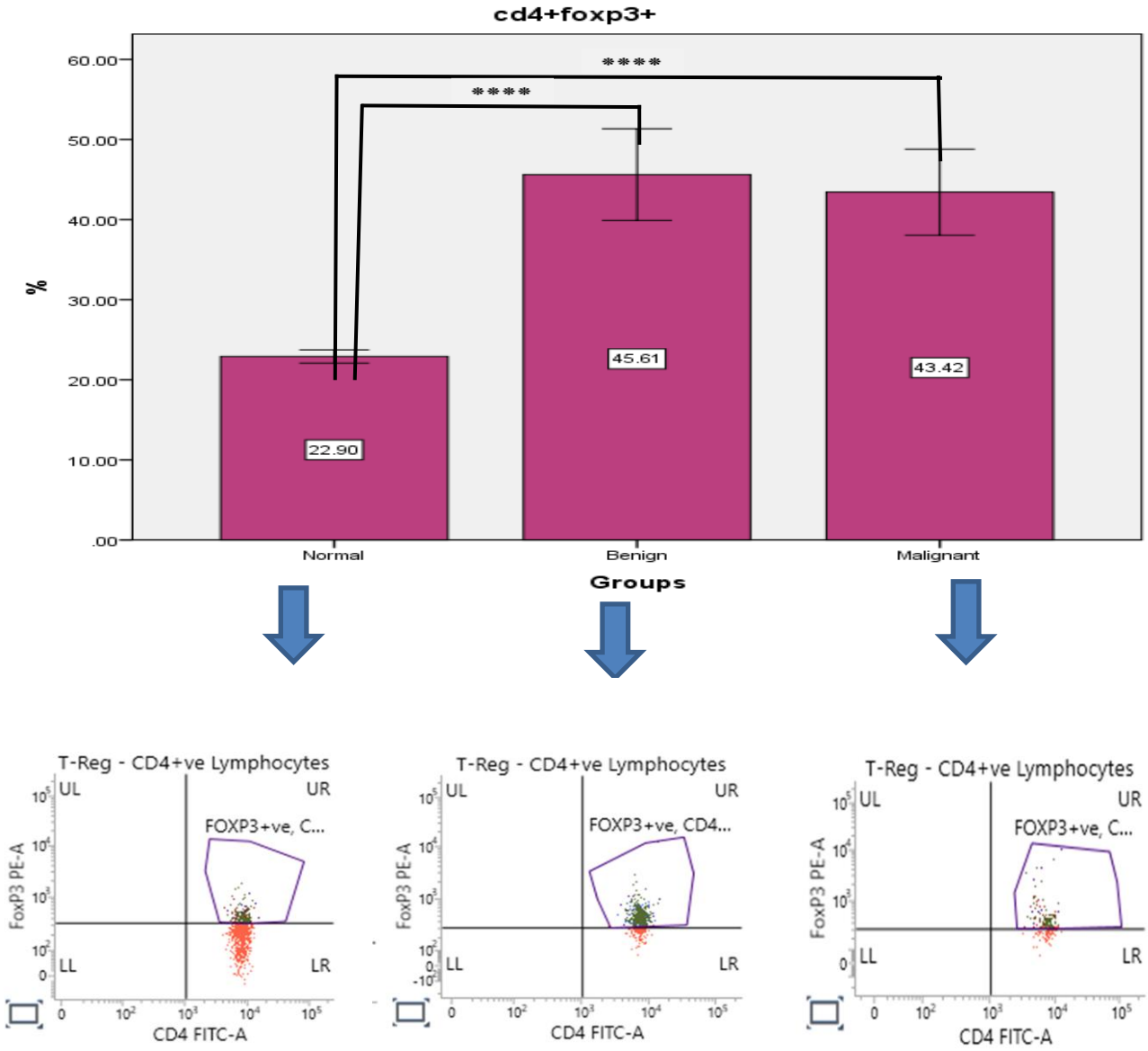


Figure 4-1: The Percentage of T reg Cells According to the Group's Classification.

4.2.2. Frequency of The Expression of CD28 Molecule by Regulatory T Cell

In the current study the percentage and expression of CD28 have been investigated. The results showed that there was a decrease in the expression rate of

this molecule on the regulatory T cells in each of two groups of women with benign and malignant breast cancer, its (87.37 and 90.39) % respectively , in contrast to the group under control. The differences reached to the significant level for both patients groups compared to control as shown in the figure (4-2). The results showed also that there was a clear increase the mean in the group of women with benign breast cancer, as reached 41.2% while it decreased in the malignant group, as the results were 3.334%. And would also like to point out that there were clear significant differences between the benign group from the control group, so the p value was < 0.031, while the significant differences were also clear among the control group and malignant group, as it reached a value of ($p < 0.001$). As for the significant differences that appeared between the group of malignant and benign group, the $p < 0.034$, as shown in Figure (4-3).

Compared to control group patients' peripheral blood CD28+ T cell percentages were noticeably reduced. This result was agreed with (Melichar *et al*, 2001 , and Gruber *et al* ,2008). Jana *et al*,2004 indicated that CD4+ T-lymphocytes in the paracortex and germinal centers had the lowest level of CD28 expression. The down-regulation of CD28 in peripheral T-cells of breast cancer patients may lead to the hypothesis of systemic immunosuppression, which is currently being investigated and could pave the way for tumor cell dissemination via the blood stream.

CD28 molecules expressed by T-cells attach themselves to antigen-presenting cells (APCs) via B7 ligands (B7-1/CD80 and B7-2/CD86). T-cell survival, differentiation, and proliferation are stimulated by B7–CD28 interactions (Chen, 2004). Conversely, there hasn't been much research done on cancer patients' blood

levels of sCD28. In certain auto-immune conditions, like Sjogren's syndrome, systemic sclerosis, and systemic lupus erythematosus, serum levels of circulating CD28 rise and may be related to the severity of these conditions (Hebbar *et al*, 2004). The CD28 co-stimulatory pathway is a crucial channel that might initiate the naïve.

T cells may be involved in immunization against cancer (Esensten, *et al*, 2016). T cell persistence, production, and increased IL-2 production are all enhanced when CD28 molecules are ligated (Boise *et al*, 1995). lacking in CD28 or any of its ligands (B7, CD80, or CD86) have much less nTregs (Tai *et al* 2005). The decreased CD28 expression might be the result of continuous antigenic stimulation, aiming at reconstituting the non-responsiveness of T-cells. Thus, the functional significance of decreased CD28 expression remains complex to interpret.

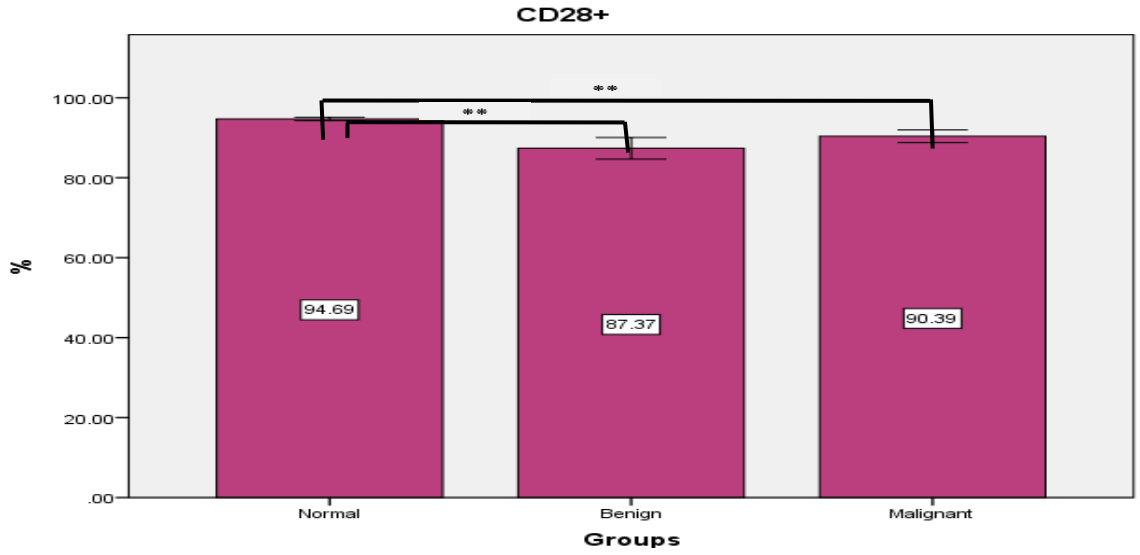


Figure 4-2: The Percentage of CD28+ T reg cell in Three Groups.

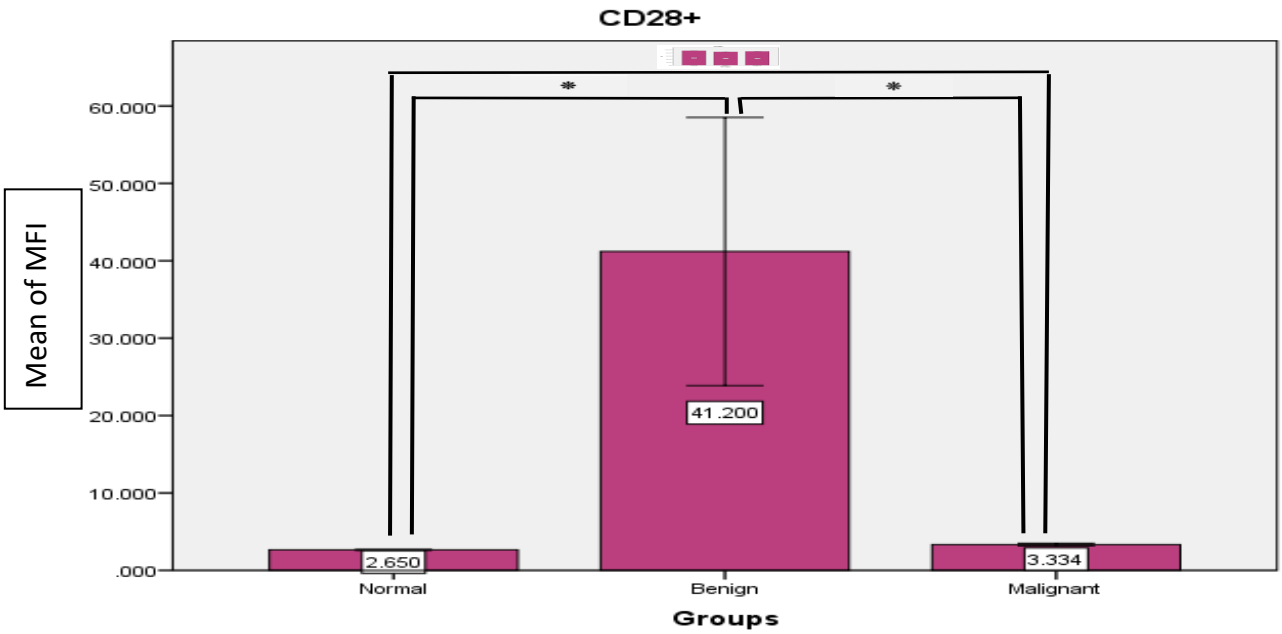


Figure 4-3: The expression of CD28 Mean Fluorescent Intensity (MFI) in three groups .

4.2.3. Frequency the Expression of CTLA-4 Molecule on Regulatory T Cells

The expression rate of CTLA4 was measured on the regulatory T cells, and the results showed a clear decrease in the expression rate in the both patients groups compared to the control group, as the percentage was higher in the control group, where it was 61.47, while the percentage for patients groups, malignant and benign were 49.53 and 56.14, respectively. Significant differences of malignant group compared with the control group, the p value was ($p \leq 0.01$), as shown in Figure (4-4)

On the surface of regulatory T cells (Tregs) and naive effector T cells, CTLA-4 is typically expressed at low levels. Following stimulation of a naive T cell via the

T-cell receptor, CD8+ T cells, CD4+ T cells, and Tregs up-regulate membrane CTLA-4 and generate soluble CTLA-4 (Ward *et al*., 2013). Different CTLA-4 isoforms restrict T-cell activation as a negative feedback to maintain immunological self-tolerance and homeostasis through internal or extrinsic regulation of T-cell activity. After CD28 attaches to B7 receptors on antigen-presenting cells and mediates activating signals in T cells, the full-length CTLA-4 binds B7 and starts inhibitory signals via its intracellular signal-transducing domain, including cell-cycle arrest and decreased cytokine production cells Intracellular calcium levels rise in response to T-cell activation, and secretary granules holding pre-synthesized The translocation of soluble CTLA-4 to the immunological synapse's central super a molecular activation cluster (cSMAC) in order to produce the dissolve CTLA-4. Through its interaction with B7, soluble CTLA-4 keeps CD28 out of the cSMAC. According to Ward *et al*. (2013), soluble CTLA-4 was found to impede human T-cell responses to antigen in *ex vivo* tests. Conversely, inhibiting soluble CTLA-4 greatly increased (PBMC) reactions triggered by antigens. Antagonism of B7-CD28-mediated co-stimulatory signals is one of CTLA4's modes of action. This happens because CTLA-4 has a far greater affinities for B7 than CD28 does. 500–2500 times more CTLA-4 binds to CD80/86 than to CD28 (Edgardo *et al*, 2015).

Nevertheless, earlier research also showed that CTLA-4 was associated with a bad outcome in cases of breast and esophageal cancer (Yu *et al*. ,2015). Thus, postulated that the prognosis of individuals with luminal B HER2-negative breast cancer would be related to CTLA-4 expression. Moreover, Yu *et al*. (2015) found that tumor CTLA-4+ was a separate risk factor for breast cancer patients' prognosis. The findings also indicated a negative correlation between survival rate and CTLA-4 expression and T cell activation, with low T cell activation score being associated

with high CTLA-4 expression(Edgardo *et al*, 2015). Results from a clinical trial using tremelimumab, a CTLA-4 blocker, for the treatment of breast cancer revealed that immune system in peripheral blood activity improved in most of the patients (Vonderheide *et al*,2010). Thus, for the therapy of luminal B HER2-negative breast cancer, CTLA-4 may be a potential target. Vonderheide *et al*.'s investigation, however, showed that there was no connection between clinical outcomes and peripheral blood immunological function. When it comes to anti-tumor immunotherapy, the tumor microenvironment might be more important than the peripheral blood's immune state. When CTLA-4 is used as an immunotherapy target for breast cancer, the tumor microenvironment may be taken into consideration (Vonderheide *et al*,2010)

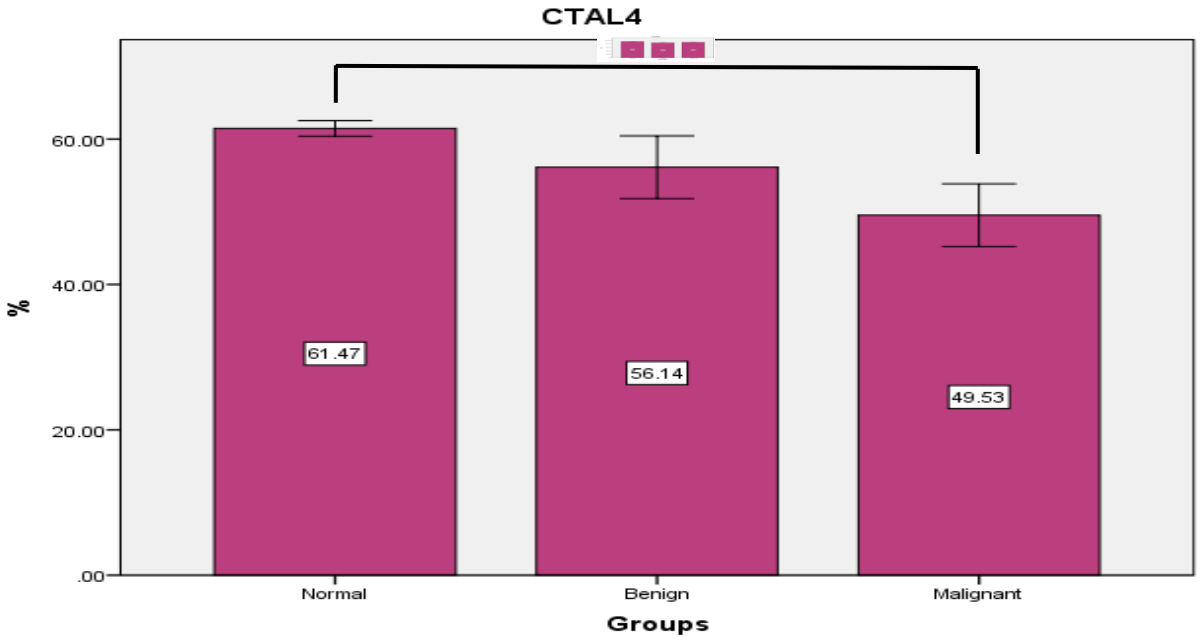


Figure 4-4 The Percentage of CTLA-4 Expression on T Regulatory Cells.

The results of current study showed that there was a significant decrease in the ratio of MFI of the CTLA-4 molecule when comparing the both groups of women with breast cancer, compared to control group, as the ratio in the control group was 17.37, while the ratio of MFI was in the groups of malignant and benign breast cancer were242.07 and 224.67 respectively. As shown in the figure below (Figure 4-5), there were very clear significant differences between the control group and the group of women with malignant breast cancer, as the value of $p \leq 0.0001$, and the same value between the group of women with benign breast cancer and the control group.

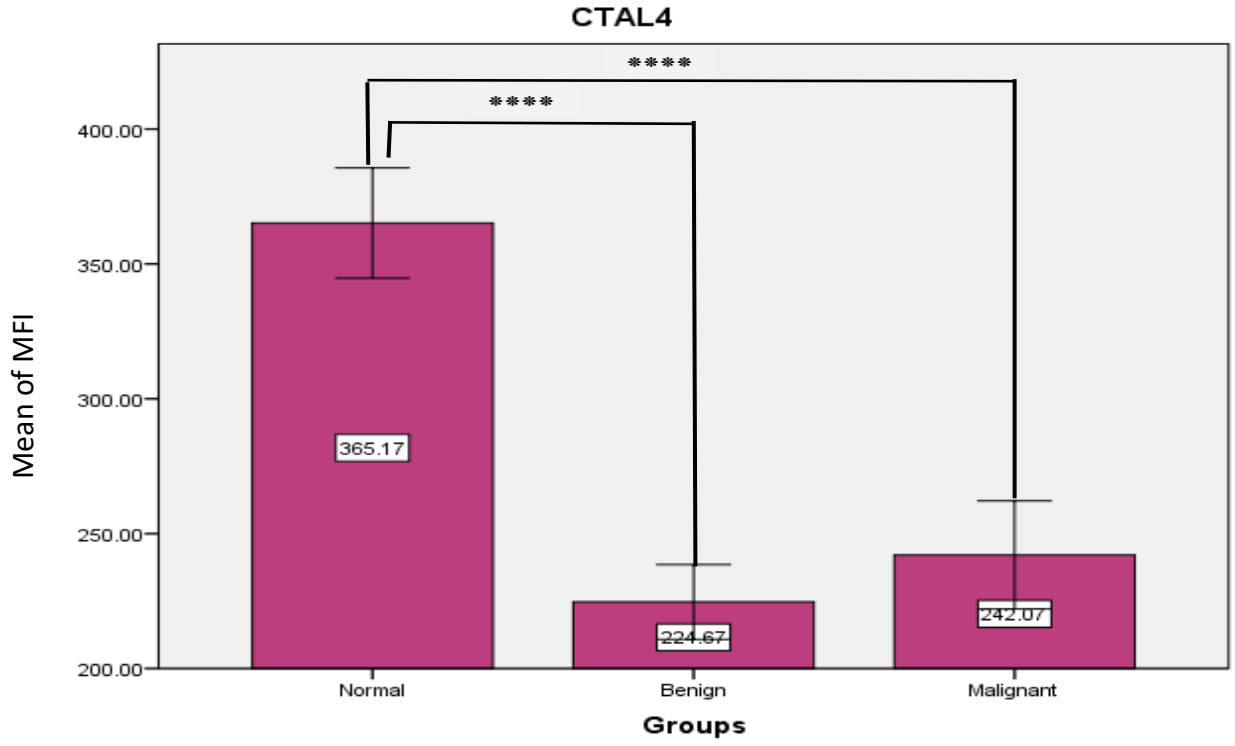


Figure 4-5 : The Expression of CTLA-4 Molecule by Treg Mean Fluorescent Intensity (MFI) in Three Groups .

This result agreed with the results of others such as (Prasanna Kumar Vaddi *et al , 2023)* In comparison to healthy donors, they discovered that the Treg cell fractions of individuals suffering from metastatic melanoma had 70% less CTLA4 expression. These findings reveal a significant down-regulation or near-complete elimination of CTLA4 expression levels in Treg cells from patients with metastatic melanoma, suggesting that CTLA4 mRNA induction in human Treg cells is actively suppressed either through transcriptional or post-transcriptional mechanisms. According to these findings, CTLA-4 adversely controls the growth of peripheral Treg cells, and down-regulated CTLA-4 may promote the growth of circulating Treg cells without lessening their ability to suppress. Mice shielded from autoimmunity by adult Treg cells' conditional ablation of CTLA-4 (Paterson *et al.* 2015). T regs have been shown to inhibit CTL and Th1 cell activity, thereby accelerating the growth of tumors in breast cancer. (Gupta *et al* ,2007).

4.2.4. Frequency The Relationship of Patients' Age to Tumor Markers

In the current study of breast cancer, the ages of patients were taken into consideration with malignant breast cancer and determined whether there was a relationship between age and the expression of these tumor markers. The malignant group was divided into two categories. The first category included ages from (29 years old to 49 years old), and this group is considered the youngest among women. As for the second category, ages fell under it (50 years to 70 years of age). Below was an explanation of whether there was a relationship between each of the tumor markers, which include ER, PR, Her2-neu, KI67, and Bcl2l, with these two categories.

4.2.4.1. ER With The Age of The Patients

The results showed that the mean of nuclear expression of the estrogen receptor in the age group (29-49) years is 1.071, and this group has less expression than the second age group, which included ages over 50 years, as the mean of expression for this receptor increased to 1.27, as shown in the figure (4- 6).

Steroid hormones called estrogens control growth, differentiation, and function in a variety of target tissues throughout the human body. Estrogen receptors (ER) α and β, which belong to a broad nuclear receptor superfamily, mediate the biological actions of estrogens. These receptors function as transcription factors that are ligand-activated. Estrogen binds to receptors in the nucleus in the traditional method of ER action. After that, the receptors dimerize and attach to certain response elements, known as estrogen response elements (EREs), which are present in the target gene promoters. Additionally, the ligand binding region of the receptors undergoes a conformational shift brought on by hormone interaction, which makes it possible to recruit co-activator proteins. (Nilsson *et al*, 2001). It has been demonstrated in numerous investigations that ERs can control transcription without physically attaching to DNA. In these situations, the receptors are attached to a transcription factor complex that makes contact with the DNA via protein-protein interactions. Through this method, ERs control the expression of several genes that respond to estrogen but lack EREs. Members of the nuclear receptor superfamily frequently employ this method, which is also known as transcriptional cross talk (Gottlicher *et al.*, 1998).

This result was agreed with (AlZaman, *et al* , 2016) they found Estrogen receptor (ER) positivity was more common in elderly women. Numerous

investigations have revealed that a poor prognosis for breast cancer is independent of age (Lobbezoo *et al 2013).* ER positivity increased with rising age (Azizun-Nisa *et al* , 2008).

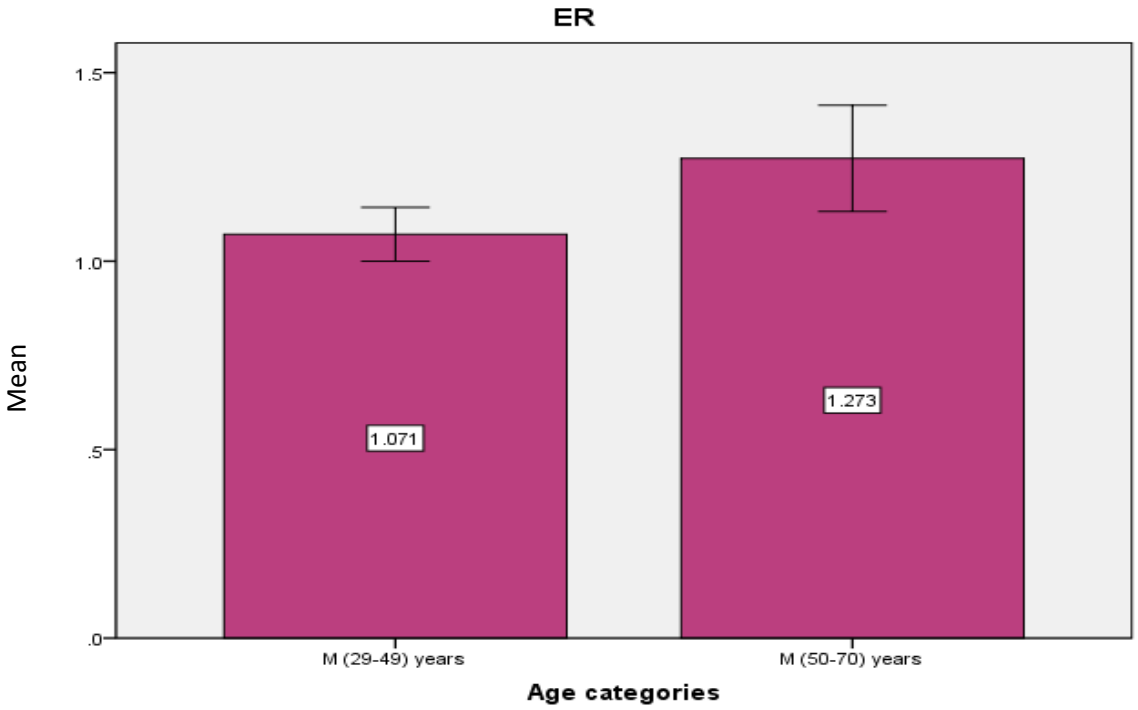


Figure 4-6: Relationship of Estrogen receptor (ER) with the age in the patients with malignant breast cancer.

4.2.4.2. PR with the Age of the Patients

The results of the current study showed that the expression of progesterone receptor protein in the tumor tissue in the first age group, mean reached to 1.286, while in the second age group, which included ages over 50 years, the expression of the progesterone receptor in the tumor tissue increased until it reached 1.455, as shown in Figure (4-7).

Progesterone is the primary proliferative steroid hormones that indicated the development of the mammary gland in the mammary epithelium. During early puberty, ductal elongation requires both estrogen and epithelial ERα signaling; progesterone/PR is not required at this time. Progesterone-mediated PR signaling in the epithelial compartment is necessary for side branching and ductal elongation in response to elevated estrogen levels (Brisken *et al.,* 1998). About 15–30% of luminal epithelial cells were positive for both estrogen receptor (ER) and progesterone receptor (PR), but not in other breast tissues. By using dual-label immunofluorescence techniques, it was revealed that every cell that expressed PR also had ERα. 96% of steroid receptor-positive cells are able to manufacture both ER and PR, according to a study by Robert B. Clarke *et al*. that used twofold labeling with antibodies to ER and subsequently to PR (Clarke *et al* ,1997*).*

Estrogen and ER are needed for the synthesis of PR, an estrogen-regulated gene, in both healthy and malignant cells. There is a significant degree of cyclical fluctuation and heterogeneity in nuclear hormone receptor activity between lobules. Peak ER values were recorded in the proliferative phase (days 3–8) of the menstrual cycle, and the second peak ER was recorded in the luteal phase (days 25 and 26). Days 13–14 mark the first PR peak, while days 21–23 mark the second peak, which happened during the luteal phase. Certain breast cancers do not experience the monthly cycle of ER and PR, which happens in the healthy mammary gland. Because of this research's specificity, the majority of what we know about the functions of ER and PR in the breast comes from a mouse model, which provides insight into the biology of breast development in humans. PR is necessary for the formation of lobulo-alveolar organs in mice because ER is needed early to cause ductal elongation. Only a small portion of the basal epithelium of the mature mouse

mammary gland expresses PR, and progesterone can act through a paracrine mechanism on a subset of mammary epithelial cells to promote alveolar expansion. In PR-positive breast luminal cells, progesterone binds to the receptor of RANKL to upregulate its expression. Through a paracrine mechanism, PR mediated connections between epithelial and myoepithelial cells. By increasing the number of mammary stem cells, progesterone can stimulate the growth of the mammary gland, and PR can control this process (Alferez *et al.*, 2018). In the absence of PR, alveolar formation during pregnancy is nonexistent.

Higher levels of PR expression were typically observed in cancers that have a better prognosis at baseline (i.e., luminal A) as opposed to tumors that have a worse prognosis at baseline (i.e., luminal B). PR is a significant predictive indicator for breast cancer. Hydrolysis is the suggested description of luminal A cancers based on immunohistochemistry (IHC). PR more than 20% (Prat *et al*, 2013*).*

In the normal growth of the mammary gland as well as the origin and progression of breast cancer, PR was a crucial steroid hormone receptor. Another biomarker that was frequently used at diagnosis to describe breast cancer was PR. PR had an important the treatment choices and took part in the molecular subtyping process. Its complex roles in many contexts, however, require clarification. A higher endocrine response is shown in the clinic with PR-positive breast cancer as opposed to PR-negative breast cancer. It is not particularly helpful as an indicator of how endocrine therapy will work. (Li *et al* ,2022).

The results of the current study agreed with other studies such as Shah *et al*., (2022). They found statistically significant relationship between PR and age. Relationship between PR status and age (41–50 years) was significant. Between

young people (ages 27–39) and older people (ages 40–80), another study found significant differences.

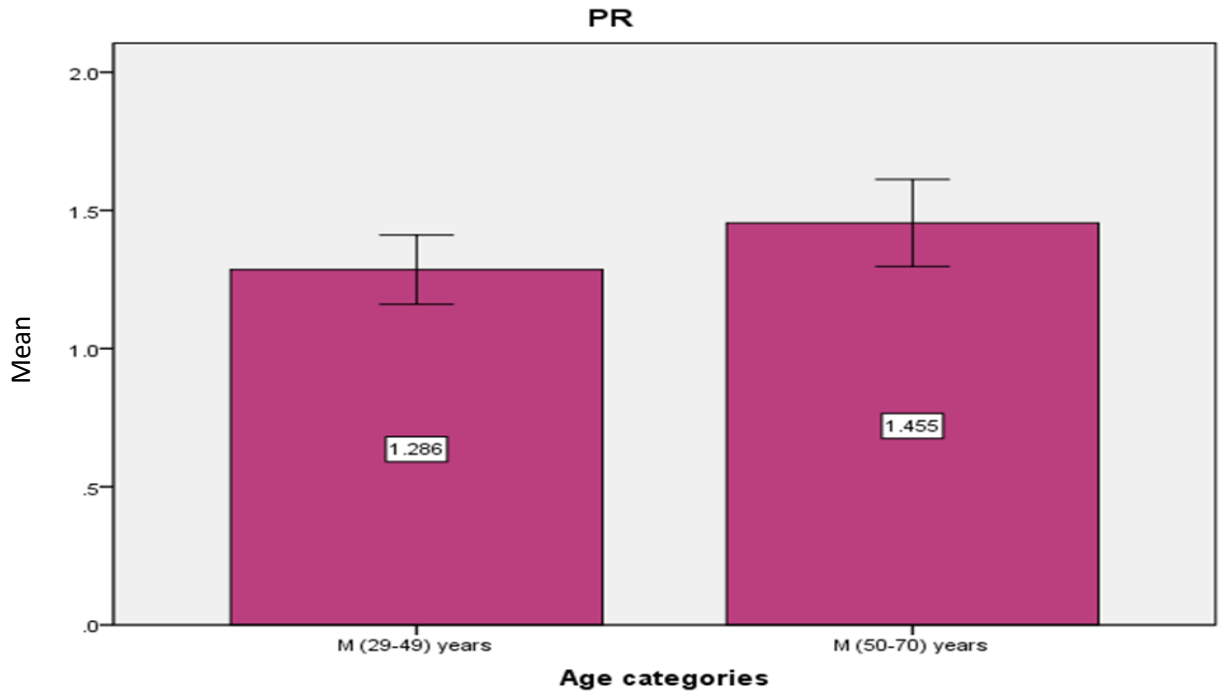


Figure 4-7: Correlation of progesterone receptor (PR) with the age in the patients with malignant breast cancer.

4.2.4.3. Her2- neu With The Age of The Patients

The tumor marker her2-neu and its relationship to the age of patients with malignant breast cancer have been investigated. The results showed that the mean of expression of this tumor marker in tissue reached 1.929 in the age group under 50 years, while in the other age group over 50 years, the mean of expression decreased slightly and reached 1.909, as shown in Figure (4-8).

In the majority of breast cancer cases (85%), HER2/neu signaling pathways were the primary drivers of cell proliferation and survival. In terms of systems biology, the HER2 pathway was a complex biological network made up of three layers: an output layer that regulates genes that impact different cellular functions, a core system processing layer that consists of protein kinases that transmit the signal to the nucleus, and an input layer made up of membrane receptors and their ligands that trigger the signal coming from outside the cell. The predominant TK receptor in breast cancer, HER2, was increased in 20% of patients. HER proteins experience dimerization and intracellular domain transphosphorylation upon ligand attachment to their external domains. Since it lacks a ligand, HER2 must either homodimerize with itself when produced at very high levels or heterodimerize with another member of the family in order to be activated. Numerous genes involved in cell proliferation, survival, differentiation, angiogenesis, invasion, and metastasis are regulated by transcription factors that are activated by the route. According to Citri *et al*. (2006), HER2 exhibits the highest catalytic kinase activity and heterodimers including HER2 have the highest signaling activity.

Complexing with other membrane receptors, such as insulin-like growth factor receptor I, can activate HER2 (Nahta *et al*., 2005).

The complement of HER2 membrane protein was modest in normal tissues. Twenty percent of breast cancers, as well as some ovarian and gastric tumors, overexpress HER2, which worsens the biological behavior and clinical aggressiveness of breast cancer. According to Kallioniemi *et al*. (1992), breast tumors have the potential to produce up to 25–50 copies of the HER2 gene and 40–100 fold more HER2 protein, which results in 2 million receptors expressed on the

surface of the tumor. One factor that makes HER2 a good target for treatment is the difference in HER2 expression between normal tissues and malignancies.

As the first HER2 targeting medication, trastuzumab, is relatively specific to cancer cells overexpressing HER2, it is well tolerated in patients with little toxicity.

Breast cancer that developed in youth typically has a worse prognosis than breast cancer that developed in old age, according to several studies. Accordingly, age affects both the prognosis and treatment of breast cancer (Abubakar *et al.*, 2018).

Nothing noteworthy in relation to HER2/neu status. The findings of the present study were accepted with (Shah *et al* , 2022).

Figure 4-8: Effect the age in the patients with malignant breast cancer on expression of Her2-neu

4.2.4.4. KI67 with the Age of the Patients

In this study, histological sections were examined and the percentage of tumor cellular expression of the tumor marker Ki67 was examined in each of the two age groups. It was found that the percentage of Ki67 in the first age group, which includes ages under 49 years, reached 42.5 percentage, while in the second age group, which included ages older than the first, the percentage decreased until it reached 26.55 percentage. The results also showed that there were clear significant differences between the two age groups, with the p value $\leq$ 0.014, as shown in Figure 4-9.

The results of this study were in consistent with the study that was conducted by (Nishimura,*et al,* 2010 ; Madani *et al,* 2016). It demonstrated that the rate of increased expression of the tumor marker Ki67 in the tumorous breast tissue of women under 50 years old is greater than in women who are older than this age.

Younger age was substantially connected with a higher Ki-67 index (Nishimura *et al.,* 2010). According to a study, age and the proportion of cells that are positive for Ki-67 in breast cancer have been a significantly association with age (Sahin *et al,* 1991).

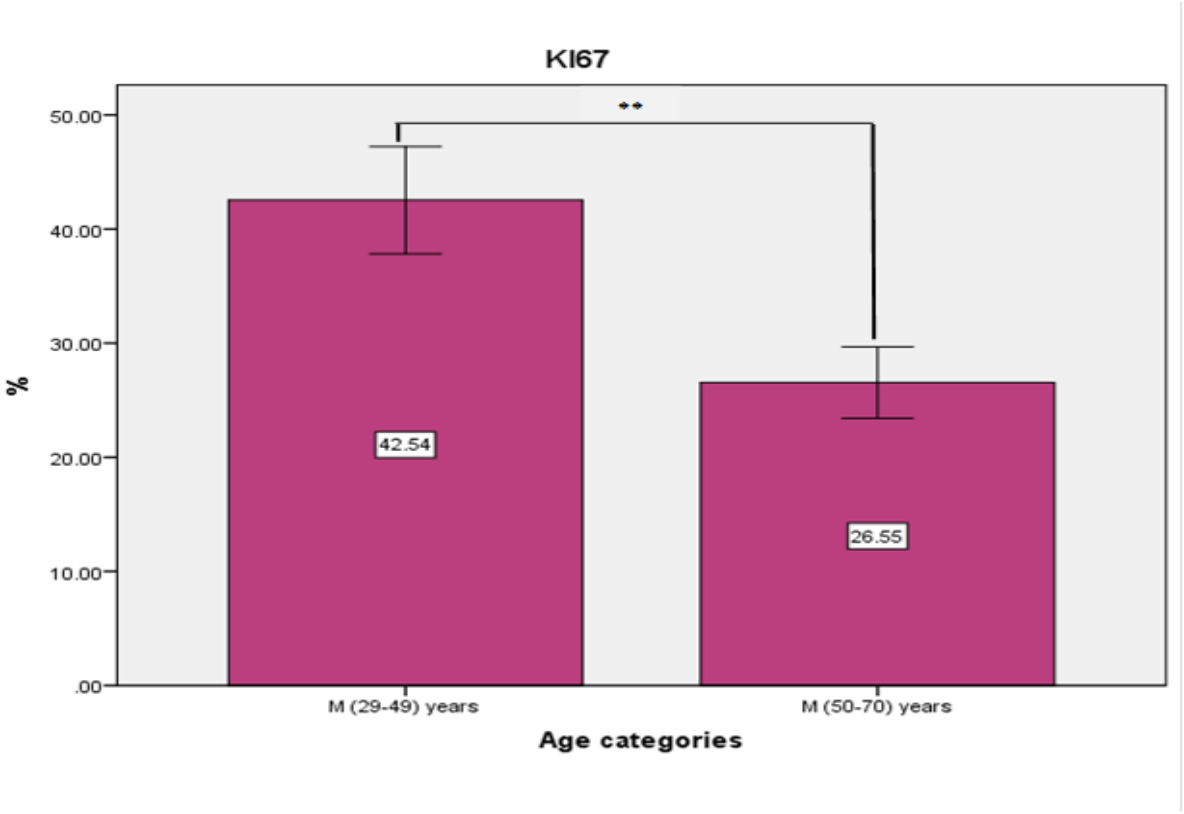


Figure 4-9: Association of Ki67 with the age in the patients with malignant breast cancer.

4.2.4.5. Bcl2 with the Age of the Patients

The tumor marker BCL2 was considered the last tumor marker whose relationship to the age of patients with malignant breast cancer has been studied. The results of this study showed that the mean of tumor tissue expression of this marker in the first age group reached 1.357, and this value was very close to the expression rate of this marker in the second age group (50-70 years), as it decreased very slightly, reaching 1.364, as shown. In Figure (4-10).

Bcl-2 is known to be increased by estrogen and is commonly expressed in both breast cancer and normal breast epithelial cells (Honma, *et al*, 2015). Rather than

speeding up cell division, BCL-2 prevents cell death, which results in an increase in the overall number of cells. Since cancer developed from these cells' inability to die, it made sense to conclude that BCL-2 prevents a type of intentional cell death. The word "apoptosis" (Greek: "falling off, like a tree leaf") was originally used to describe the form of cell death that BCL-2 blocks a few years prior (Hockenbery *et al.*, 1991).This result agreed with other studies such as (Heba *et al*, 2020 ; Holmqvist *et al* , 1999).

It was yet unknown how Bcl-2 contributes to the onset and spread of breast cancer. It has been reported that ductal carcinomas exhibit both overexpression and absence of Bcl-2 expression (Heba *et al*, 2020)

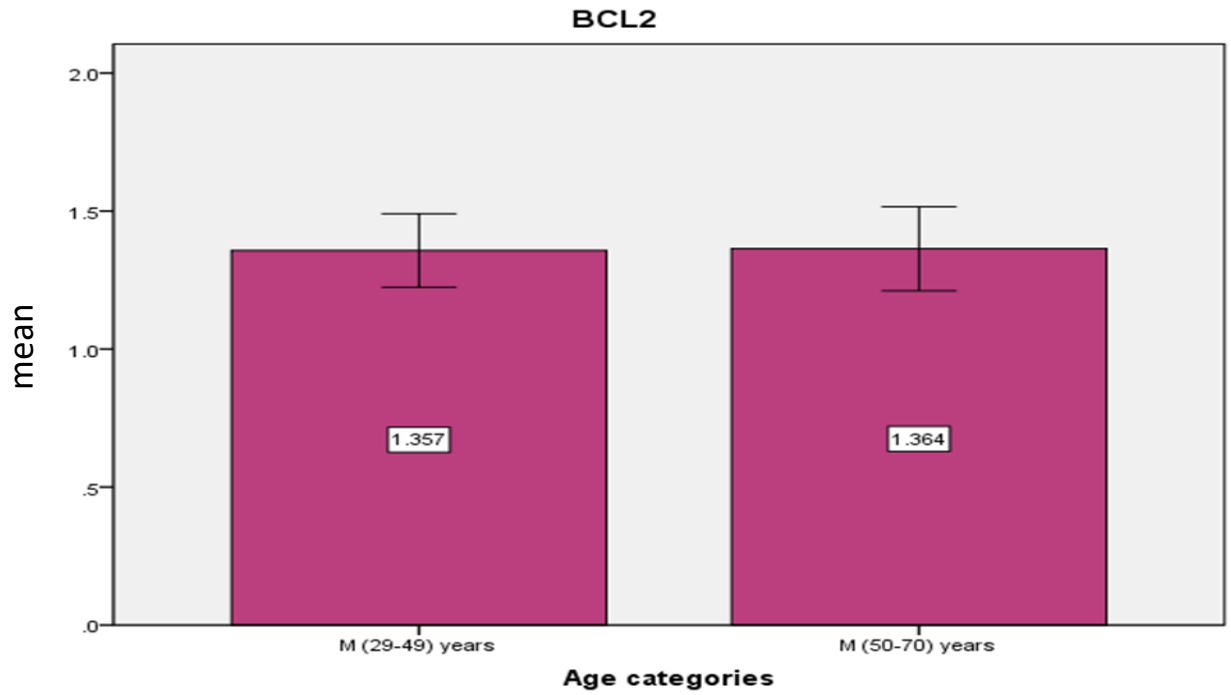


Figure 4-10: Relationship of Bcl2 with the age in the patients with malignant breast cancer.

4.2.5. The Relationship The Age With Regulatory T lymphocytes Cell

In the current study, was examined whether there was a relationship between women's age and the rate of production of regulatory T lymphocytes from the immune system.

The results showed that the group of women with the highest production of regulatory T lymphocytes was in the younger age group (29-49) years among women with benign breast cancer, where the expression rate reached 48.58%, and a clear significant difference appeared compared to the control group, as it was p value $\leq$ 0.0001. While the second age group, which was the oldest (50-70) years in the same group (the group of women with benign breast cancer), the expression rate of the cells reached 36.22%.

While the results showed exactly the opposite in the group of women with malignant breast cancer, where the highest percentage of Treg cells was in the second age group (50-70) years, when the percentage of the cells reached 45.26%. Also, significant differences appeared when compared with the control group, where p $\leq$ 0.0001. While in the first age group of the same group, women produced less Treg cells, as the percentage of cells decreased to 41.97%. Also, significant differences appeared and became clear when compared with the control group, and the p value was $\leq$ 0.003.

As for the control group, the percentage of Treg cells in the first age group, which was the youngest, the percentage of cell production was 22.22%, while in women in the oldest age group, which is (50-70) years, the percentage of Treg cells

production increased to 27. 89%, and a significant difference appeared when comparing the two age groups with each other for the same control group, $p \leq 0.022$. As shown in the figure (4 – 11).

The CD4+ regulatory T cell (Treg) is a major orchestrator of immunosuppression and has been the focus of cancer immunology research since its discovery (Plitas and Rudensky 2020; Yano *et al.* 2019). In both original breast cancers and their metastases, T regs can be widely distributed (Syed Khaja *et al*. 2017). Nevertheless, because immune cell cross-talk and metastatic illness are complex processes, pinpointing their precise role and significance in the advancement of breast cancer has been difficult. The biology of Tregs in breast cancer has recently been revealed through intriguing new insights derived from fundamental and preclinical research. This was significant since immune checkpoint drugs have shown mixed effectiveness in the early stages of breast cancer. (Planes-Laine *et al.* 2019).

Measuring CD4+ Treg counts may be useful for monitoring the course of BC and as a key target for treatment. According to reports, patients with invasive high grade BC had considerably more CD4+ Tregs, which may be associated with a shorter time to both a relapse-free and overall survival (Banin *et al*, 2018). These results imply that a significant factor in the prognosis of BC patients may be the higher frequency of CD4+ Tregs (Matsumoto *et al*, 2016). Nevertheless, in other investigations, there was no direct correlation found between the frequency of CD4+ Tregs and the clinical stage of BC (Perez *et al*., 2007).The immunosuppressive action of CD4+ Tregs may facilitated the growth and progression of tumors.

Even while the original T cells became fewer in number and the thymus gland shrinks with age, they survive longer and proliferate less. This was the case for healthy individuals, but researchers have shown that in cancer patients, older individuals have higher T reg cell counts than do healthy individuals (Hou *et al*, 2017).

These results were similar to the previous results that was accomplished by (Zahran *et al*, 2021). They discover that compared to healthy individuals and malignant tissue, the mean percentages of CD4+CD25+highT cells and Tregs were higher in peripheral blood from TNBC patients. Furthermore, the frequencies of Tregs and CD4+T cells that penetrate tumors were higher in the peripheral blood of cancer patients. Patients with local recurrences had considerably greater levels of Tregs than those without; only these Tregs that infiltrate tumors have increased in amount as tumor size has increased. Tregs also shown a strong inverse relationship with DFS and a direct relationship with the level of peripheral Tregs.

According to Chen *et al.* (2003), tumor cells' produced TGF-β and IL-10 factors have the potential to either directly or indirectly stimulate Treg proliferation in the surrounding tumor environment and peripheral circulation. Growing numbers of Treg cells promote the growth of tumors by further suppressing antitumor immunity. Treg cell elevation in clinical patients may be monitored on a regular basis to assist determine their prognosis, and suitable therapies to limit CD4+CD25+FoxP3+ Treg elevation may help prevent tumor growth.

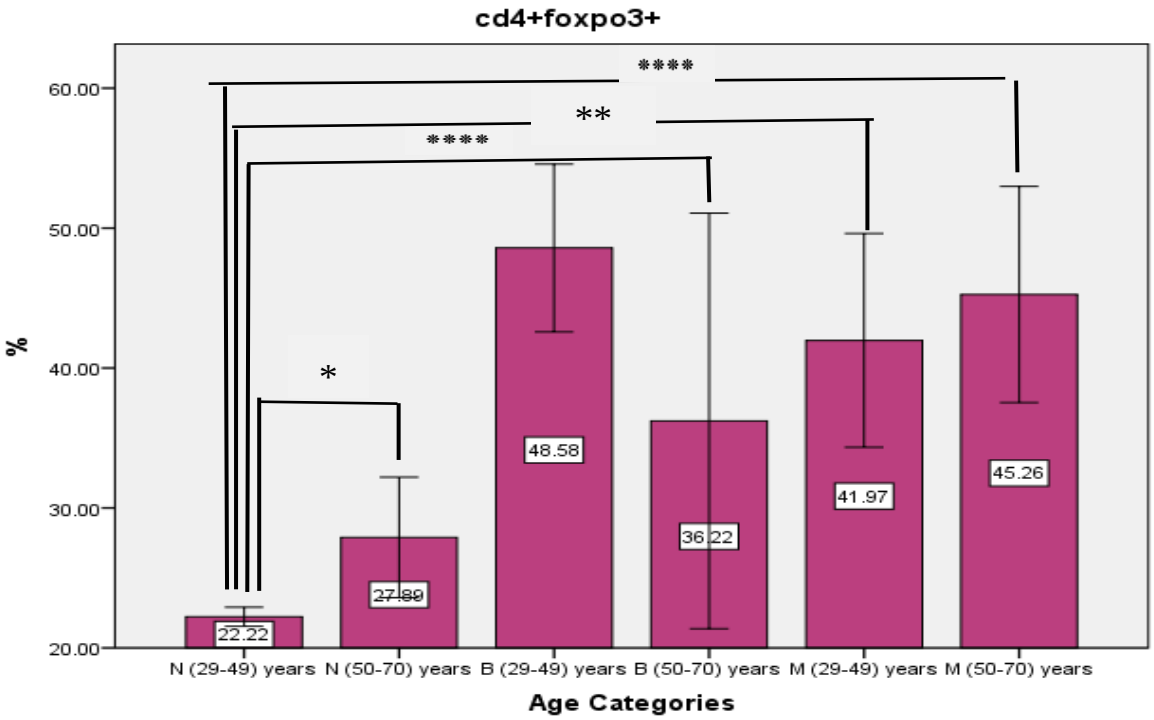


Figure (4-11) The effect of age of women on expression of regulatory T lymphocytes. N=normal , B= Benign , M=malignant.*p≤0.05 ** p≤ 0.01 *** p ≤0.001 **** p=0.0001

4.2.6. The Relationship of Regulatory T lymphocytes With The Tissue Expression of Tumor Markers in The Tumor

4.2.6.1. The Relationship of T reg With ER

Estrogen modifies the T cell development of lymphocytes in tumor tissue that have invaded the tumor.

The percentage of malignant group expressed the estrogen receptor has been studied and known. The percentage of Treg cells was 44.98%, while for women

whose tumor tissues did not express the estrogen receptor (negative expression), the percentage of Treg was 35.25% shows that in Figure (4-12)

T regulatory cells (Treg) (CD4+ FoxP3+) significantly increased in ER mutant breast cancer, while CTLs had no difference, indicating the correlation between ER and Treg differentiation (Williams *et al*,2021). In addition, the study also showed that high levels of estrogen were the only conditions necessary for estrogen-induced Treg activation. But in low estrogen levels, the outcome completely changed (Tulchiner *et al*., 2021).

Similar findings also demonstrated that low estrogen levels enhanced Th1 cell differentiation, which in turn promoted Th1-induced CTL activation and up-regulated anti-tumor immunity. Conversely, high estrogen levels increased the proportion of Th2 phenotype, which in turn caused immunity inhibition (Salem *et al.,* 2004). In this context, estrogen suggested a bidirectional role in Treg differentiation, which was dose-dependently regulated differently in cancer immunity.

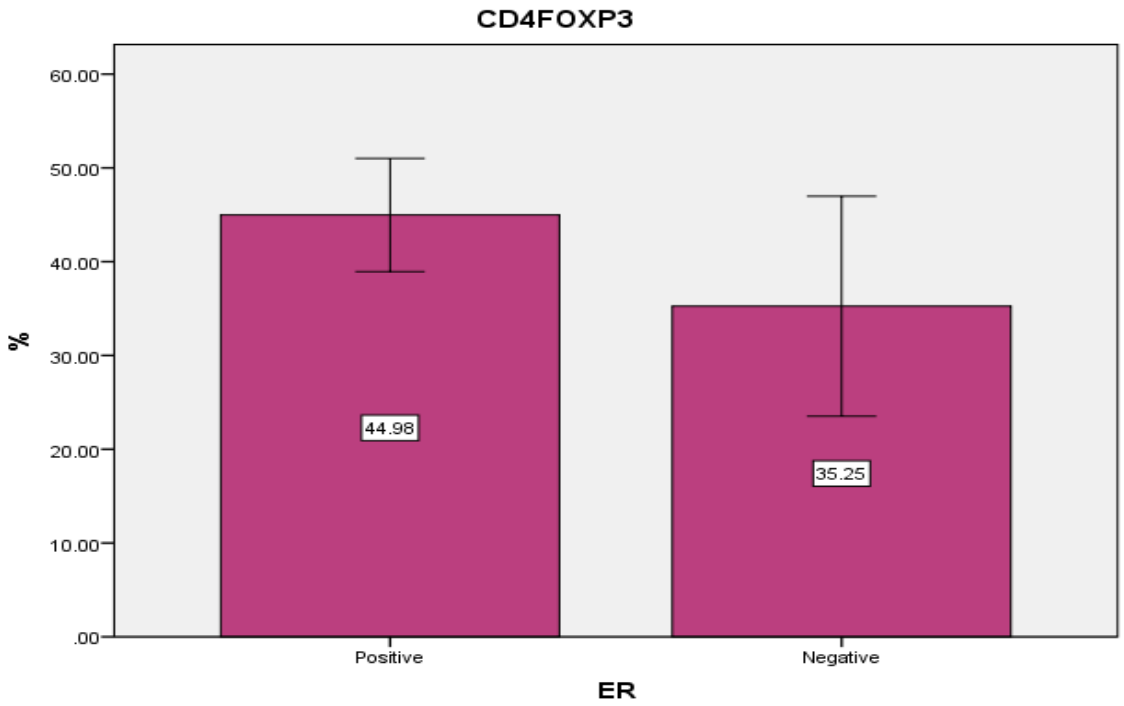


Figure 4-12: Correlation between Treg cell with the estrogen receptor marker in the patients with malignant breast cancer

4.2.6.2. The Relationship of T reg With PR

The results of current study showed that in women whose tumor tissue expresses the progesterone receptor (PR positive) the percentage of regulatory T lymphocytes reached 49.56%, while women whose immune tests using the immunohistochemical staining technique showed negative in the tumor tissue of the breast also had a decrease in the percentage of regulatory T lymphocytes. It reached 32.51%, as shown in Figure (4-13).

Progesterone should attach to the PR in order to have its effect because it can encourage the down-regulation of pro-inflammatory cytokines and chemokines

(Hall *et al*, 2017). PGR polymorphisms and mutations are linked to carcinogenesis and cancer risks, including endometrial, breast, and ovarian malignancies (Lee *et al*, 2010). By modifying Th1 and Treg activity, PR can modify the immune response (Hughes *et al*, 2013).Additionally, research has revealed a connection between the PR and the infiltration of a subset of Tregs, which suggests a worse prognosis for breast cancer (Dziobek *et al*, 2018).

These results were similar to the study that is carried out by (Amany *et al*, 2020) observed higher frequency of CD4+ Tregs in the peripheral blood of Egyptian females with BC. with PR positive than PR negative, but the association did not reach statistical significance

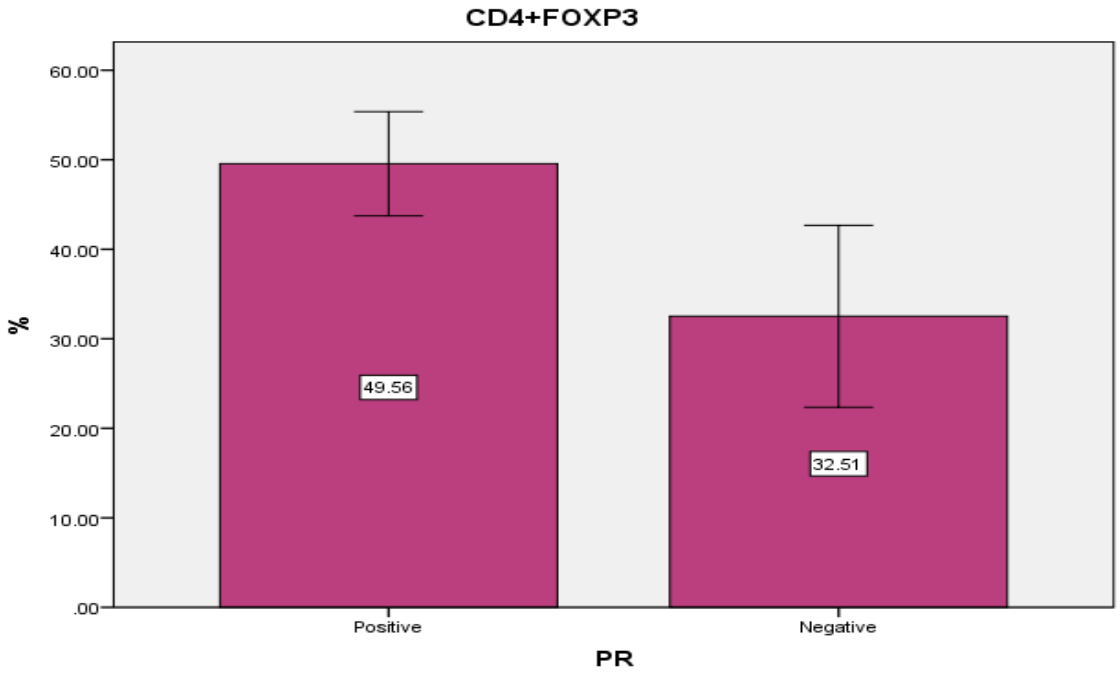


Figure 4-13: Relationship Treg cell with the progesterone receptor marker in the patients with malignant breast cancer

4.2.6.3. The Relationship of T reg With Her2-neu

The relationship between Treg and the expression of the tumor marker HER2-neu have been studied. The results showed that in women who showed expression of the tumor marker HER2-neu through IHC technology, the percentage of regulatory T lymphocytes reached 69.70%. As for women whose IHC tests showed tumorous breast tissue (her2-neu negative), their percentage of Treg cells decreased to 41.14%, as shown in Figure (4-14).

The complement of HER2 membrane protein was modest in normal tissues. Twenty percent of breast cancers, as well as some ovarian and gastric tumors, overexpress HER2, which worsens the biological behavior and clinical aggressiveness of breast cancer. One factor that makes HER2 a good target for treatment was the difference in HER2 expression between normal tissues and malignancies.

In order to inhibit the onset of innate and adaptive immunity, effector function, and memory response—all of which could create an environment that was conducive to the development of cancer—tumors aggressively seek out and activated regulatory T cells. A transcription factor belonging to the forkhead/winged-helix family, forkhead box protein 3 (FoxP3) controls the growth and operation of the immune system. According to Coffer and Burgering (2004), this gene is crucial for the production of immunosuppressive CD4 + CD25 + regulatory T cells (Tregs), which promote immunological tolerance to antigens.

These results were agreed with several studies in the last few years which have seen the publication of meta-analyses that demonstrate the correlation between high levels of FoxP3 in hormone receptor positive breast tumors and poor survival, high

grade, and involvement of lymph nodes (Jiang *et al.* 2015, Wang *et al*. 2016). The results of these analyses also demonstrated a significant relationship between high levels of FoxP3+ and high histological grade, as well as HER2 positive.

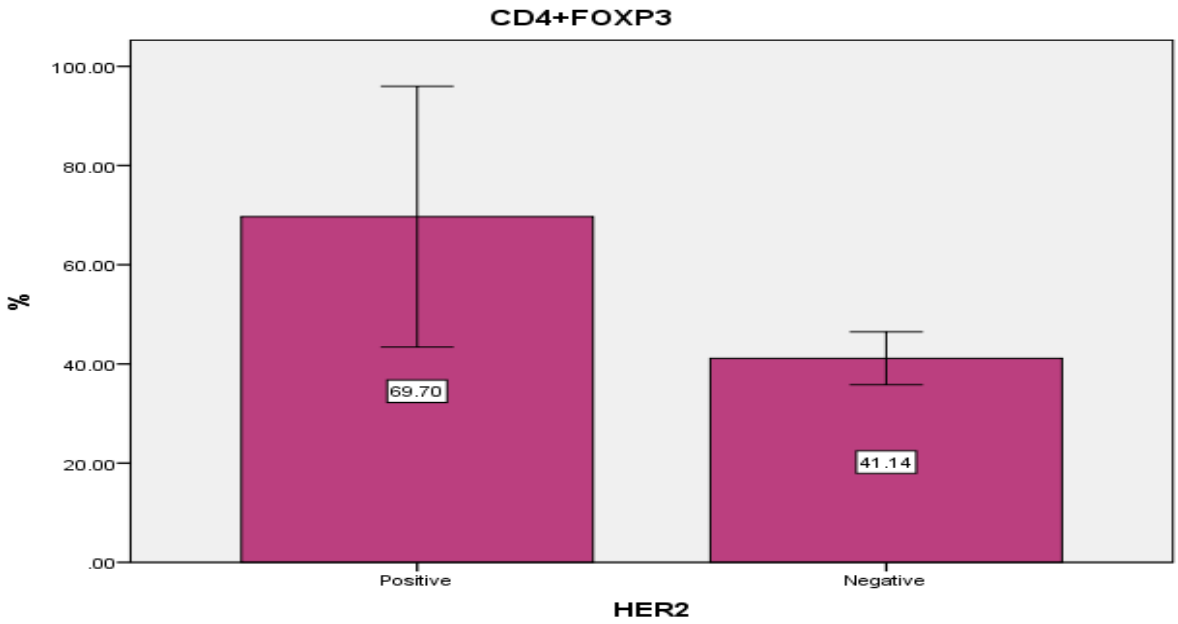


Figure 4-14: Correlation of Treg cell with the Her2-neu marker in the patients with malignant breast cancer

4.2.6.4. The relationship of T reg with Bcl2

The relationship of regulatory T lymphocytes with tumor tissue expression of the marker bcl2 was also examined. The results showed that for patients with malignant breast cancer whose test result was (bcl2 positive) the percentage of regulatory T lymphocytes was reached 50%, while for women whose test result was (bcl2 negative) the percentage of cells had decreased to 31.73%. As shown in Figure (4-15).

An essential clinical breast cancer prognostic sign is the anti-apoptosis protein B-cell lymphoma 2 (Bcl2). Because the activity of Bcl2 depends on the state of the estrogen receptor (ER), different molecular subtypes may have different effects. Depending on the source, Bcl-2 or Mcl-1 can induce lymphadenopathy, aid in the development of cancer (Campbell *et al*., 2010), and induce autoimmunity in mice (Egle *et al*., 2004).

This results indicated an increase in lymphocyte regulatory cells in women who showed positive expression of BCL2. This indicated that the immunosuppressive action represented by Treg cells contributed to the development of the tumor, especially with the increased of the anti-apoptotic marker represented by BCL2 and vice versa, as a decrease in the percentage of Treg cells may leads to a reduction in the proportion of BCL2, and this was what was pointed out by researchers.

These results were similar to the result of (liu *et al* 2022).They found the increased of Treg cell with increased the Bcl2 in tumor cell. One of the causes of immunological escape is elevated Tregs in the TME (Yang *et al* 2009). According to De Matteis *et al.* (2018), Tregs release TGF-β and IL-10, which depress the immune system. High Treg levels were predictor of when cancer patients may start therapy, according to research by (Weiss *et al.*, 2011).

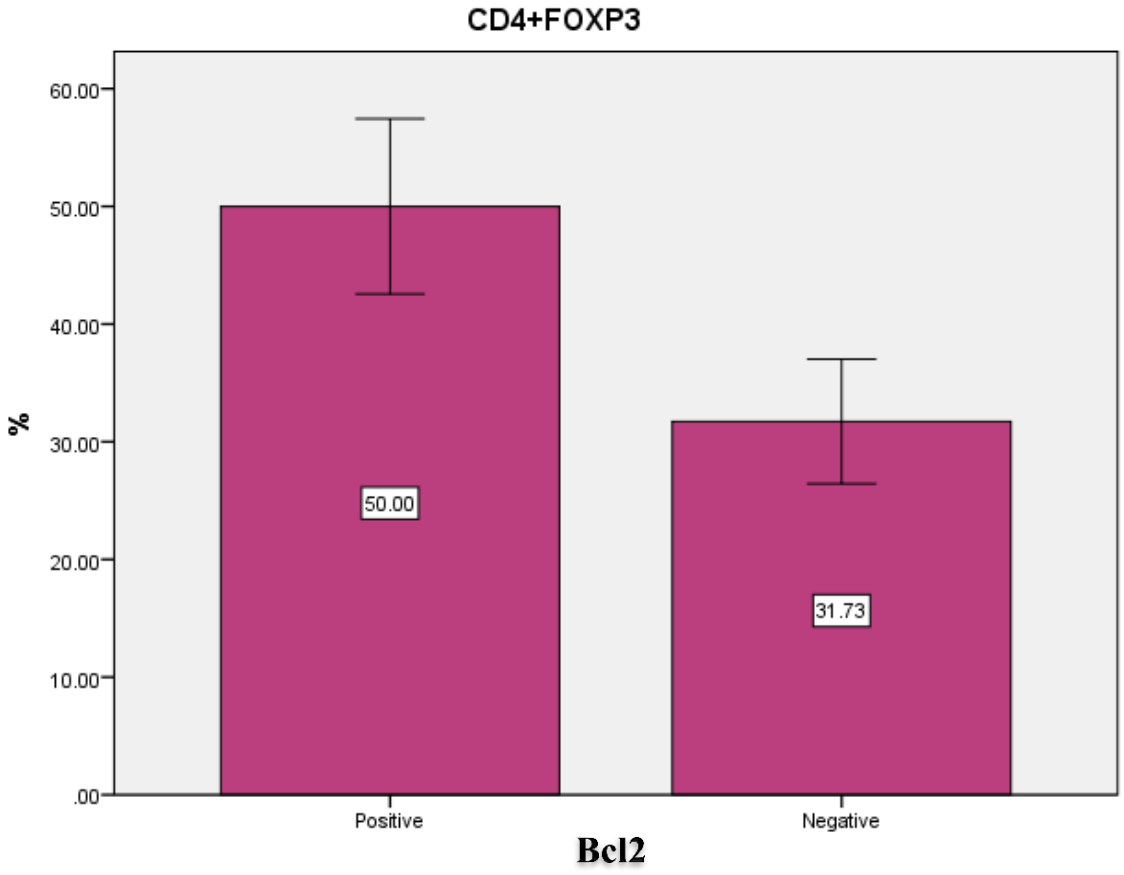


Figure 4-15: Relationship Treg cell with the Bcl2 marker in the patients with malignant breast cancer.

4.2.6.5. The Relationship of T reg With KI67

The relationship of the tumor marker ki67 with regulatory T lymphocytes was investigated. The results showed that this tumor marker has a positive relationship with regulatory T lymphocytes, but in a small percentage, as shown in the figure (4-16).

Nuclear protein Ki-67 was first discovered by (Gerdes *et al* 1983) and is linked to cellular proliferation. The nuclear antigen Ki-67 is expressed during the S, G1,

G2, and M stages of the cell cycle, although it is absent during G0. A significant percentage of tumors from patients with primary breast cancer have Ki-67 detected in them. This implies that even if national standards do not advocate the parameter, it is utilized in clinical routine (Fernández-Cuesta *et al*, 2012). This investigation proved that there was a correlation between Ki-67 and the standard histopathologic markers (Fernández-Cuesta *et al*, 2012). The effect was clearly seen in the association between Ki-67 and grading. This result reinforces the assumption of a similar behavior of these two parameters, both associated with proliferation. Similarly higher tumor stages and higher nodal status were associated with higher Ki-67 quartiles indicating that the more aggressive the tumor is the higher is the percentage of cells positively stained for Ki-67 (Fernández-Cuesta *et al*, 2012).

The results of current study were similar to another study that was conducted by Ko and his colleagues 2017 , they found the tumor marker ki67 was positively correlated with elevated the average proportion of CD4+FoxP3+ Tregs in gastric cancer patients' peripheral blood. Ki-67 was discovered to be expressed at low levels (<3% of cells) in ER-negative cells but not in ER-positive cells in samples of normal breast tissue. This study found that in gastric cancer, there was a positive association between the expression of Ki-67 and the quantity of CD4+FoxP3+Treg cells in peripheral blood. It's possible that cancer cells create the chemokine ki-67, which leads to the buildup of Tregs and immune system escape in cancer (Takkem *et al.,* 2018). One unique tactic to improve the responsiveness of cancer immunotherapy could be to target Ki-67. According to Shirendeb *et al.* (2009), the Ki-67 antigen, which codes for two different protein isoforms, is present in all active cell cycle phases (G1, S, G2, and M), although it is lacking in resting cells (G0). Ki-67 levels drastically drop during the later stages of mitosis, known as

anaphase and telophase. For this reason, it can be utilized as a marker for tumor cells with significant proliferative activity and is thought to be the best target antigen for detecting tumor cell proliferation. Given its potential as a trustworthy marker, Ki-67's prognostic usefulness has been examined in numerous cancer-related studies. It has also been established that gastric cancer has an overexpression of Ki-67, which indicates a higher risk of cancer cell proliferation, metastasis, and recurrence (Takkem *et al.,* 2018). More studies have been conducted recently on Ki-67's function in the tumor microenvironment, particularly in relation to immune modulation (Shirendeb *et al.* , 2009). In breast cancer patients with lymph node metastases, the overexpression of Ki-67 was linked with the proportion of Treg cells infiltrating in tumor tissues (Fernández-Cuesta *et al,* 2012). The concentration of Treg cells was found to be associated with the differentiation and proliferation of tumor cells, as indicated by the degree of Ki-67 expression. In line with earlier research, we discovered that the amount of CD4+FoxP3+ Treg cells in breast cancer patients' peripheral blood was correlated with the protein expression of Ki-67, and that a higher proportion of CD4+FoxP3+ Treg cells was associated with an increase in Ki-67 protein expression. From all of the aforementioned data, it is clear that a tumor's metamorphosis should alter the tissues' natural structure and trigger an immune reaction (Shirendeb *et al.* 2009). Since Ki-67 appears in all active phases of the cell cycle, we believe that tumor cells may create Ki-67 and other chemokines in their microenvironment.

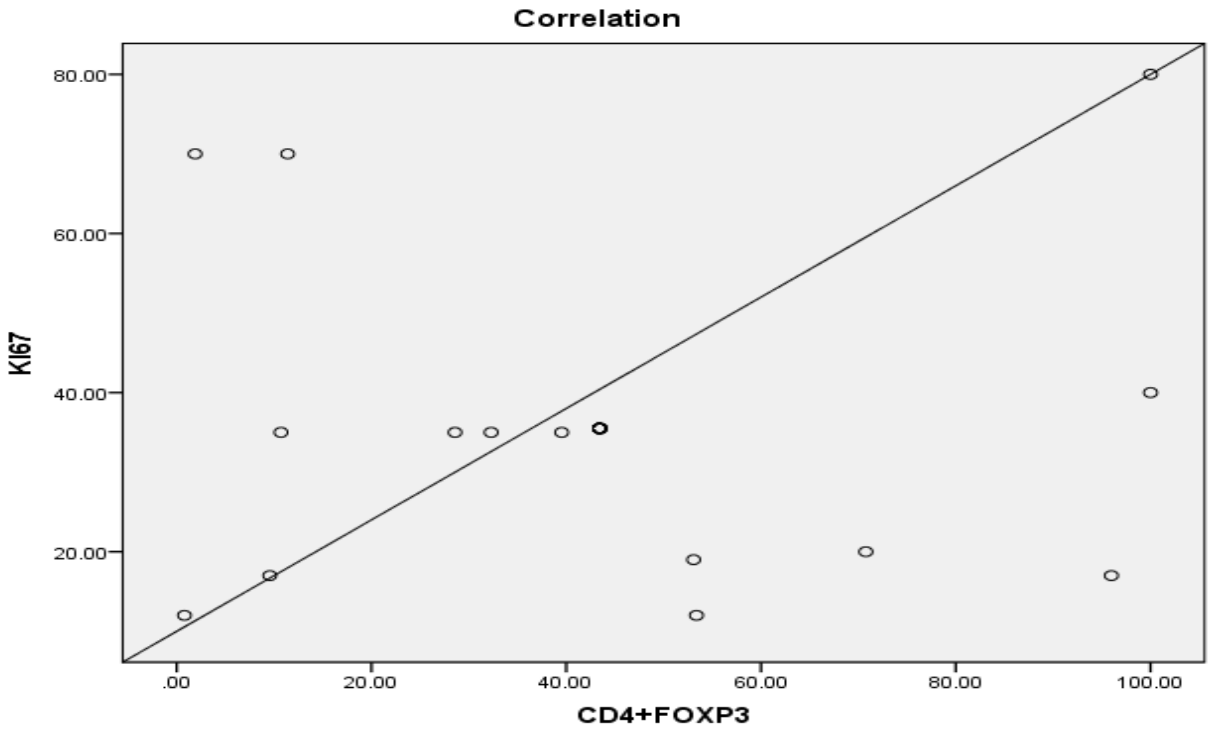


Figure 4-16: Correlation the Treg cell with the ki67 marker in the patients with malignant breast cancer

4.2.7. Effect of Blood Groups

4.2.7.1. The Relationship of Blood Group With The Percentage of Regulatory T lymphocytes

Regulated T lymphocytes and their relationship with blood groups were studied for each of the three research groups (control, benign and malignant). The results showed that in the malignant breast cancer group, the percentage of organized T lymphocytes were the highest in the O blood group, as the percentage of organized T cells in it reached 47.55%, followed by the A blood group, and the lowest percentage of the regulatory T cells were the B blood group. AB It was

worth noting that no women with malignant breast cancer carrying blood type AB were found.

As for the group of women with benign breast cancer, the blood type with the highest expression of regulatory T lymphocytes was blood type B, where the percentage of cells reached 51.98%, followed by blood type O, with a percentage of 51.47%, then blood type A, with a percentage of cells that decreased. To 42.32%, and in last place was the AB blood group from the same group. These results were compared with the blood groups in the control group, as shown in the figure(4 – 17).

This result was agree with (Liu, *et al.*, 2021). They found increased the Treg cells in the patient with blood group B. Though this study did not find the same trend in patients with blood type O, the mechanisms should be further investigated in the future. We hypothesize that the increase in Tregs and FOXP3 expression in these patients with blood type B may be related to the lack of the A antigen on erythrocytes and the increase in anti-A antibodies in the plasma.

Larger trials are still required to confirm these results, but these studies may result in customized blood therapy for patients with various ABO blood types.

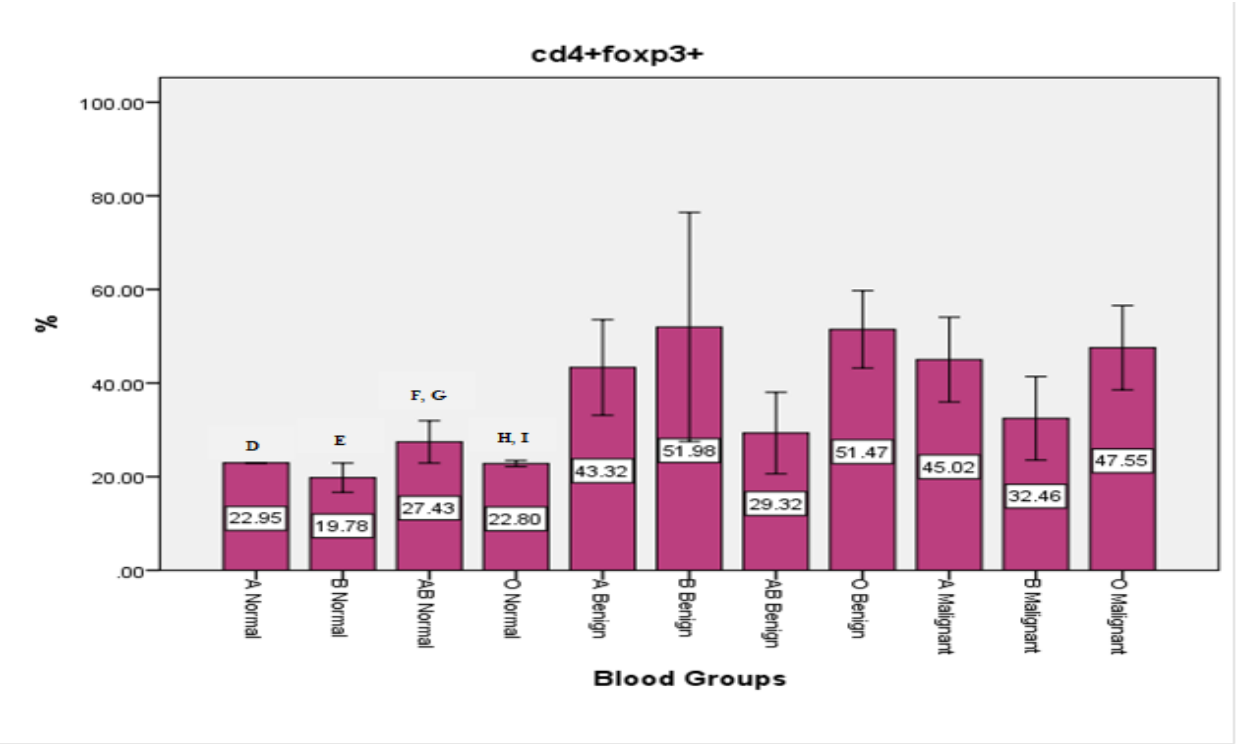


Figure 4-17: The relationship of blood group with the percentage of regulatory T lymphocytes

4.2.7.2. The Relationship of Blood Group With Type of Cancer

In the current study, investigated whether blood type has an effect on the development of breast cancer or not. The results showed that the highest percentage of women with malignant breast cancer were blood type A 44%, followed by blood type O 36%, and finally blood type B 20%.

As for women with benign breast cancer, the highest percentage was among women with blood type A 40%, followed by a small percentage of women with blood type O 36%. As for women with blood types B and AB 12%, their percentage was equal and was the lowest among the benign breast cancer group, as shown in Figure 4-18.

This study was agreed with several studies such as (Sultan Ayoub meo *et al,* 2017).They found Blood groups "A" and "O" had the highest incidences of breast cancer (45.68% and 31.69%, respectively), followed by "B" (16.16%) and "AB" (6.27%). The blood group "AB" had the lowest correlation with breast cancer, while blood group "A" had the greatest.

Similarly , Akin and Altundag (2018) found 43.7 % blood group A, 33.8% blood group O ,14.7%blood group B, and finally blood group AB 7.9%. On the other hand, there were not statistically significant differences determining the relationship of blood types to the risk of breast cancer.

But Zaki *et al* ,(2013) found a significant relationship between the ABO group type and breast cancer, with blood group type (A) having the highest incidence and percentage of patients with breast cancer.

Blood group A individuals may be more susceptible to carcinogenesis or the advancement of cancer due to elevated levels of certain inflammatory mediators, such as soluble intracellular adhesion molecule (ICAM)-1, E-selectin, and P-selectin (Barbalic, *et al.,* 2010).

The AB0 blood type antigens are linked to glycosylation, and further research on the connection between glycosylation and carcinogenesis is a possible avenue of pursuit (Zouine, *et al.,* 2017).

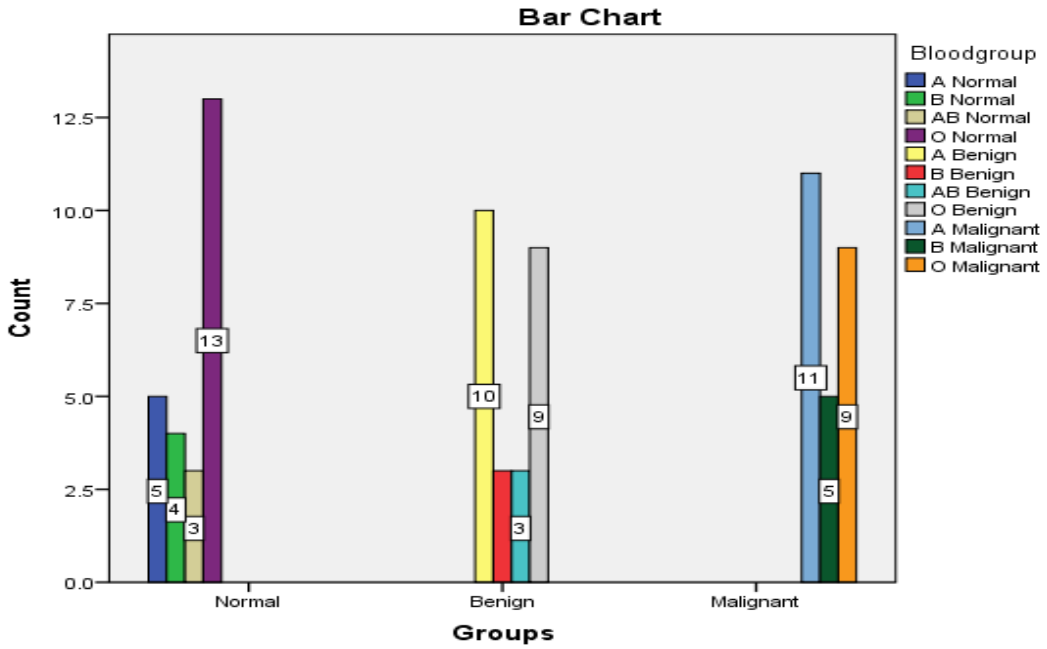


Figure 4-18: The relationship of blood group with type of cancer

4.3. Histological Studies

As mentioned previously, the groups under study were divided into three groups. Among these groups is malignant breast cancer, as we subjected this type of cancer to detect the type and quantities of tumor markers present in the tumors taken from malignant breast cancer patients after confirming that it is a malignant type by conducting some tests such as ultrasound and mammogram of the breast. Current study dealt with the tumor markers (ER, PR, Her2-neu, ki67, and bcl2) in the true cut that take from the breast, as these markers were detected using IHC technology. Below in Figure (4-19) is a picture of normal breast tissue, which shows the milk ducts in the breast, stained with the usual dye (Hematoxylin and eosin stain).

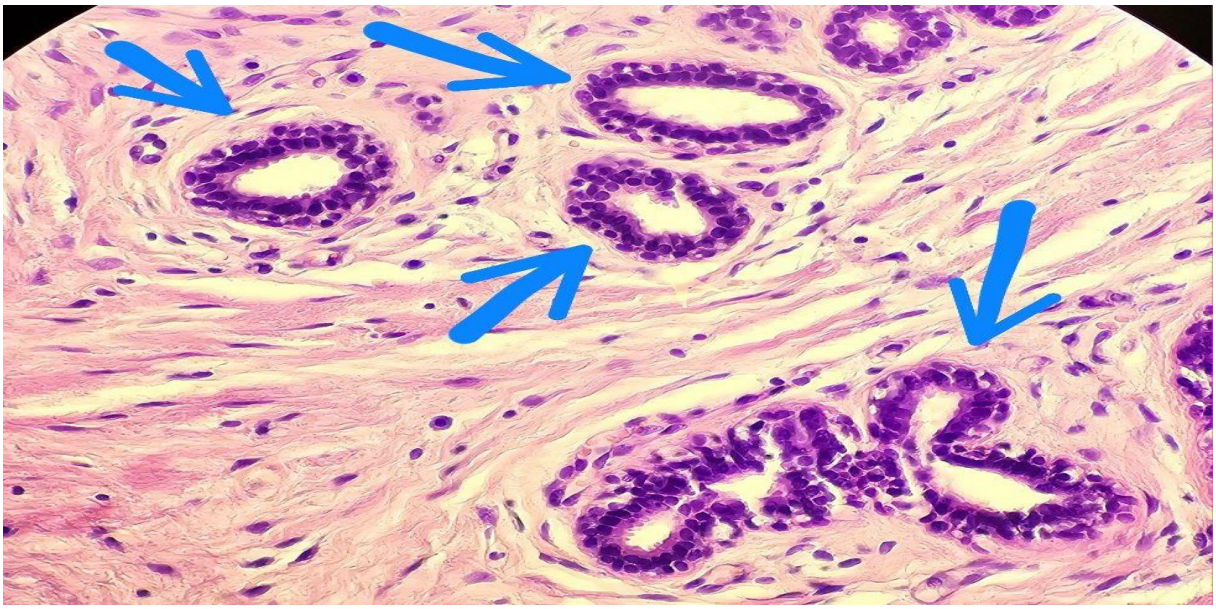


Figure (4-19) : Normal breast ducts ,H&E stain ,x400. The arrow indicates the regularity of the cells that make up the ducts in the natural breast tissue

4.3.1. Determination Tumor Tissue Expression of The Estrogen Receptor:

The results showed that the percentage of women who had been examined and whose tissues expressed the future Estrogen reached 86%, while the remaining women whose tissues did not express the estrogen receptor reached 14%. The histological picture of this marker was shown in figure (4-20)

In order to prepare the breasts for breastfeeding, estrogen plays a key role in influencing breast development throughout puberty and breast maturation during pregnancy (Brisken and Malley, 2010). Estrogen has a major role in the development of the breast, mainly by creating the ductal component of the breast and by promoting the growth of connective tissue and fat deposition (Hilton, *et al.*, 2018). The slides that were made from blocks of tumor tissue taken from women

with malignant breast cancer were stained using the immunohistochemical staining technique so that we could know the percentage of women who showed expression of this marker.

Our result is agree with Dai *et al* (2016). They found ER positive is mostly found at a high level among patients with breast cancer compared to those with ER negative. (Liang *et al* , 2020) discovered that 70% of patients had hormone-dependent breast cancer, which is characterized by luminal A and B tumor cells that express the ER. Estrogens are the main signals in these tumors that are important for the growth and spread of tumor cells (Liang *et al* , 2020). The primary mechanisms via which estrogens operate on cells are through the nucleus ERα, ERβ, and membrane G protein-coupled ER (GPER, also known as GPR30) (Liang *et al* , 2020). The receptor known as ERα is thought to have a major role in the development of breast cancer (Liang *et al* , 2020).

A woman's risk of breast cancer may be ascertained by looking at clinical indications of estrogen exposure, such as bone mineral density, blood estrogen concentrations, and breast density on mammography. In addition to providing a more accurate assessment of risk for individual women, composite risk assessments based on these and other risk factors, such as family and reproductive histories, may contribute to our understanding of the function of estrogen in the pathophysiology of breast cancer (Dai *et al*., 2016).

Breast cancer risk is increased by hormonal factors that impact mammary gland growth, such as early menarche and delayed menopause. Having children lowers the chance of developing breast cancer later on, and breastfeeding further reduces the risk. There is no proof that a woman's usage of oral contraceptives or

menopause hormone therapy affects her chance of developing breast cancer. However, women with a family history of breast cancer in first-degree relatives are more likely to develop the disease (Dai *et al*., 2016). Depending on the particular agonist or antagonist actions on estrogen target tissues, selective estrogen receptor modulators may be helpful in the treatment or prevention of breast cancer (Liang *et al* , 2020)

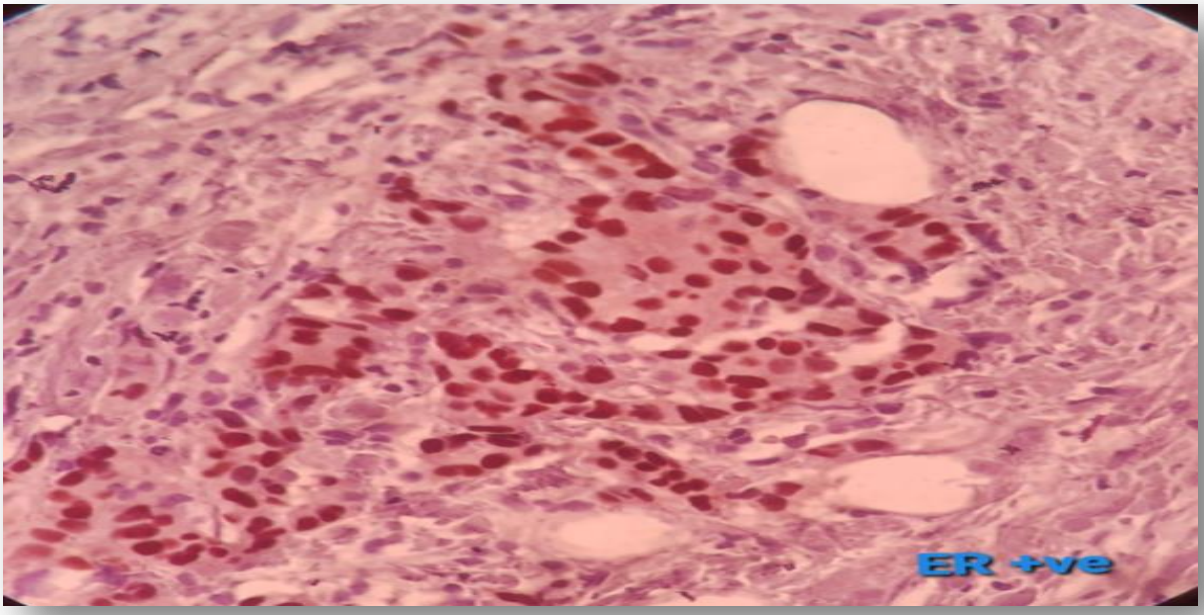


Figure (4-20) : Breast ductal carcinoma in situ, $ER^{+,}$ IHC, nuclear positivity, high power x400.

4.3.2. Determination Tumor Tissue Expression of The Progesterone Receptor:

The one purposes of this study was an analysis of the correlation between cancer patients' prognosis and PR expression. The pathological samples were also

subjected to immunohistochemical staining to determine the expression of the progesterone receptor in the breast tissue by the tumor cells. The percentage of women who showed expression of this marker was 64% PR+, while the percentage of 36% was for women whose tests showed a negative result for this marker PR-. The histological image of the marker is shown in Figure (4-21).

Through the overexpression of target genes, such as those involved in signal transduction pathways (such as EGFR and EGFR ligands, IRS-2, cyclins D and E, and p21), progesterone serves to sensitize breast cancer cells to the activities of growth factors (Faivre and Lange, 2006). Preneoplastic transitions during tumor growth and the control of breast cancer prosurvival are two understudied aspects of progesterone/PR action. Early events in breast cancer cell invasion and metastasis are also included(Faivre and Lange, 2006).

Growth factors that support transcriptional synergy with progestins on PR-target genes include EGF and heregulin (Daniel *et al.*, 2007).

Our result is agree with Shah *et al* , (2022), the progesterone receptor (PR) was found to be positively expressed in 54.6% of cases. The multi-domained PR protein, which is activated by ligand, is responsible for regulating progesterone signaling. The target genes' transcription is either activated or inhibited by the ligand-bound PR protein, which binds DNA, because the PR gene produces at least two isoforms, each of which has a distinct transcriptional activity owing to dimerization status, recruitment of particular coregulators, and the presence of an active inhibitory domain, progesterone signaling is further characterized by its added specificity (Li *et al* . , 2022). Furthermore, independent of DNA binding, the PR protein can bind to SH3 domains to quickly activate signaling pathways. Moreover, progesterone

receptors that traverse membranes might exist and exhibit entirely distinct activities from their nuclear counterparts. Numerous ligand-independent activities of PR in promoting chromatin mobility and repression have been reported in recent investigations. a handful of these numerous PR processes. Lastly, a variety of mechanisms, such as the addition of post-translational modifications and the binding of chaperone proteins inside the cytoplasm, control the function of PR specificity (Li *et al* . , 2022)..

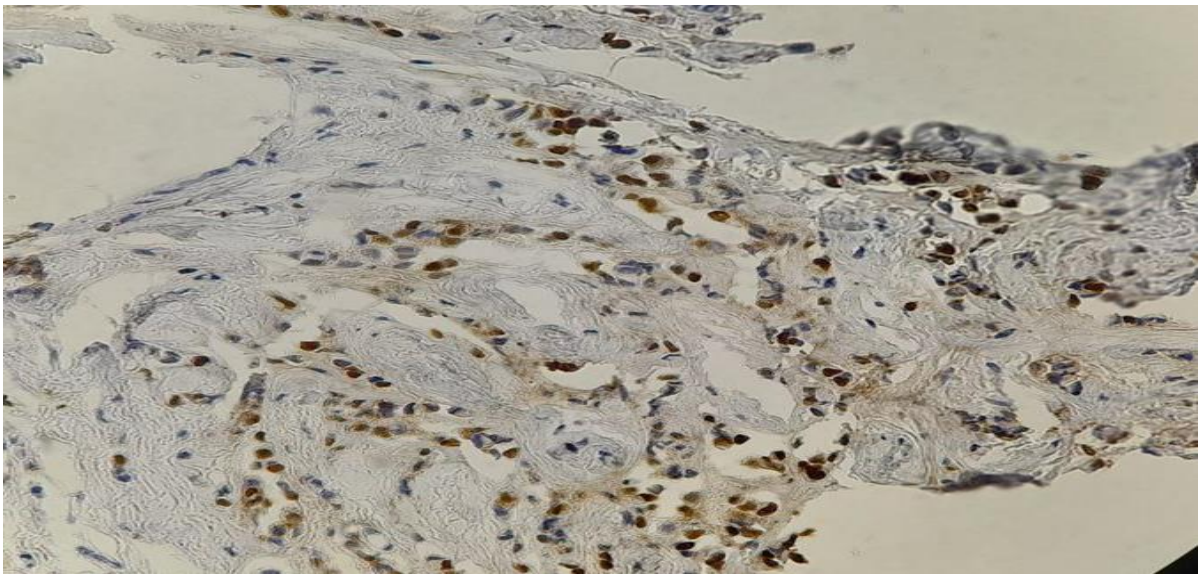


Figure (4-21) Breast ductal carcinoma ,PR IHC ,nuclear positivity ,high power x400.

4.3.3. Determination Tumor Tissue Expression of The Her2\neu

The tumor marker HER2\neu was also investigated, and the results showed that the percentage of this marker in the cell membrane of tissues of women with breast cancer reached 7%, while 93% of women with malignant breast cancer had a negative result in the expression of this marker. The IHC-stained histological image of this marker is shown in (Figure 4-22 a, b).

On chromosome 17, the proto-oncogene HER-2/neu, sometimes referred to as C-erb B2 (HER-2), is found. Fifteen to twenty-five percent of invasive breast cancer cases have it amplified and the protein (HER-2) overexpressed, which is associated with a bad prognosis. According to Hung and Lau (1999), HER-2/neu encodes p185, a transmembrane glycoprotein that is part of the family of epidermal growth factor receptors and has tyrosine kinase activity. While overexpression of HER-2/neu is a strong indicator of trastuzumab (Herceptin) response, it is not a reliable indicator of overall survival or chemotherapy response. For patients with lymph-node-positive breast cancer, HER-2/neu is also an independent negative predictor of time to recurrence and overall survival (Suo *et al.*, 2002).

The result in this study agreed with some studies by Bae *et al* (2015) ; Shah *et al* (2022). They found that the most women patients with breast cancer have the negative expression of her2-neu , and the positive expression to this tumor marker was highest in breast cancer.

Higher histologic grade carcinoma was more closely correlated with HER-2 positive. According to Lal *et al*. (2005), the majority of grade III tumors showed positivity whereas a lower percentage of grade II carcinomas were HER-2 positive. None of the grade I carcinomas were HER-2 positive.

In population, there is a significant push for the clinical significance of these prognostic markers in the management of breast cancer patients in order to improve the poor prognosis and to offer better therapeutic alternatives.

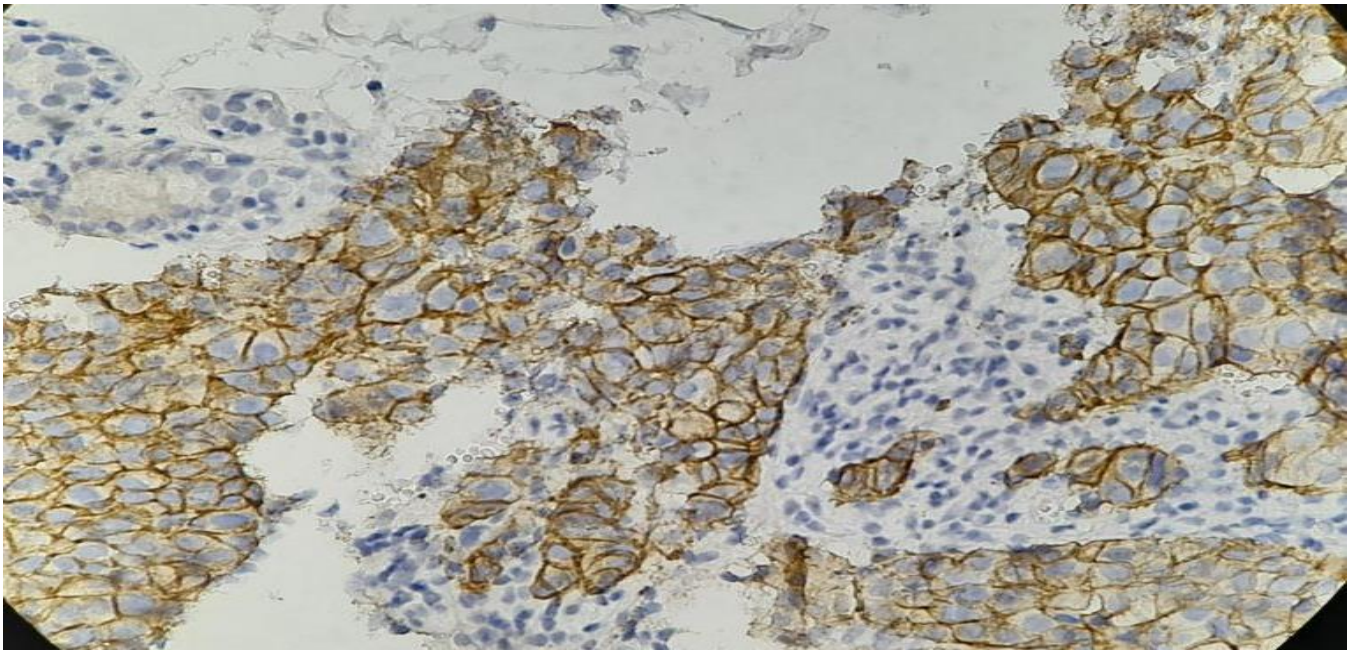
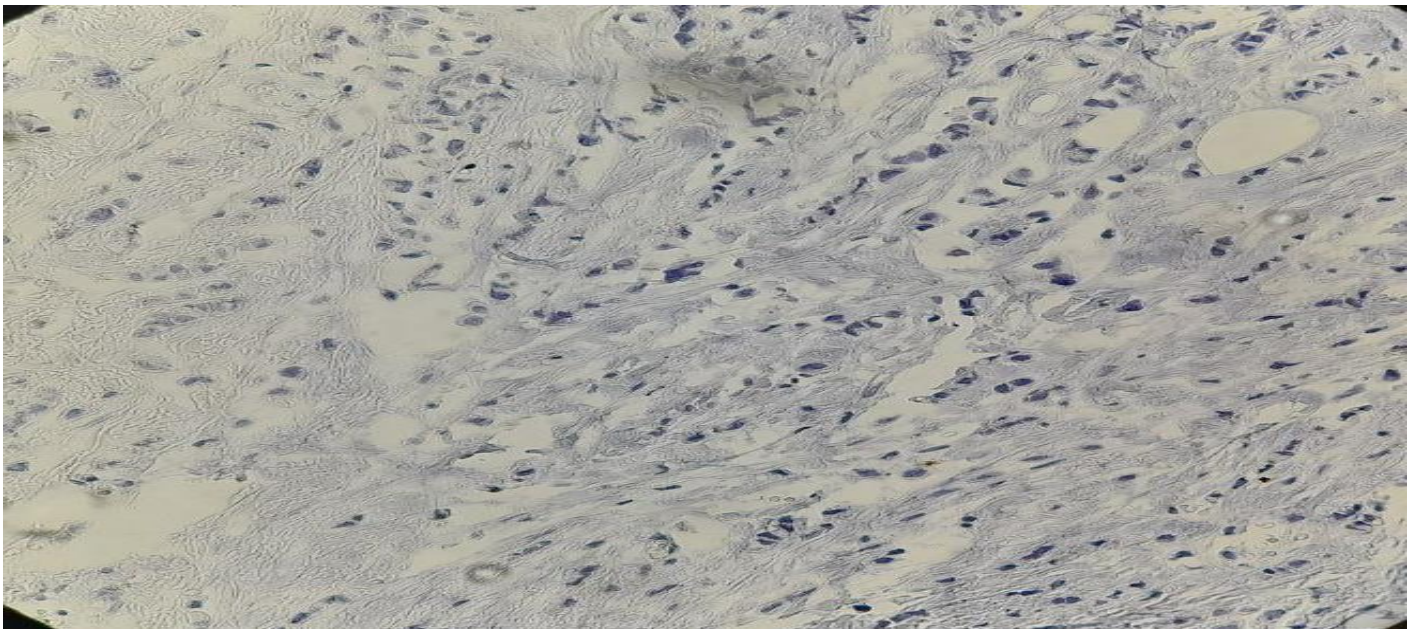


Figure (4-22.a) Breast ductal carcinoma invasive ,Her2\neo, IHC ,cell membrane positivity ,score 3 ,high power x400.



Figure(4-22 ,b) Breast ductal carcinoma , HER2/neu no cell membrane positivity, score (0) ,high power x400.

4.3.4. Determination Tumor Tissue Expression of The BCl2

As for the histological marker BCL2, the results showed that the highest percentage of women with malignant breast cancer had a positive result for this marker, which amounted to 64%, while 34% of women did not have a positive result for this marker. The histological image of this marker is shown in Figure (4-23).

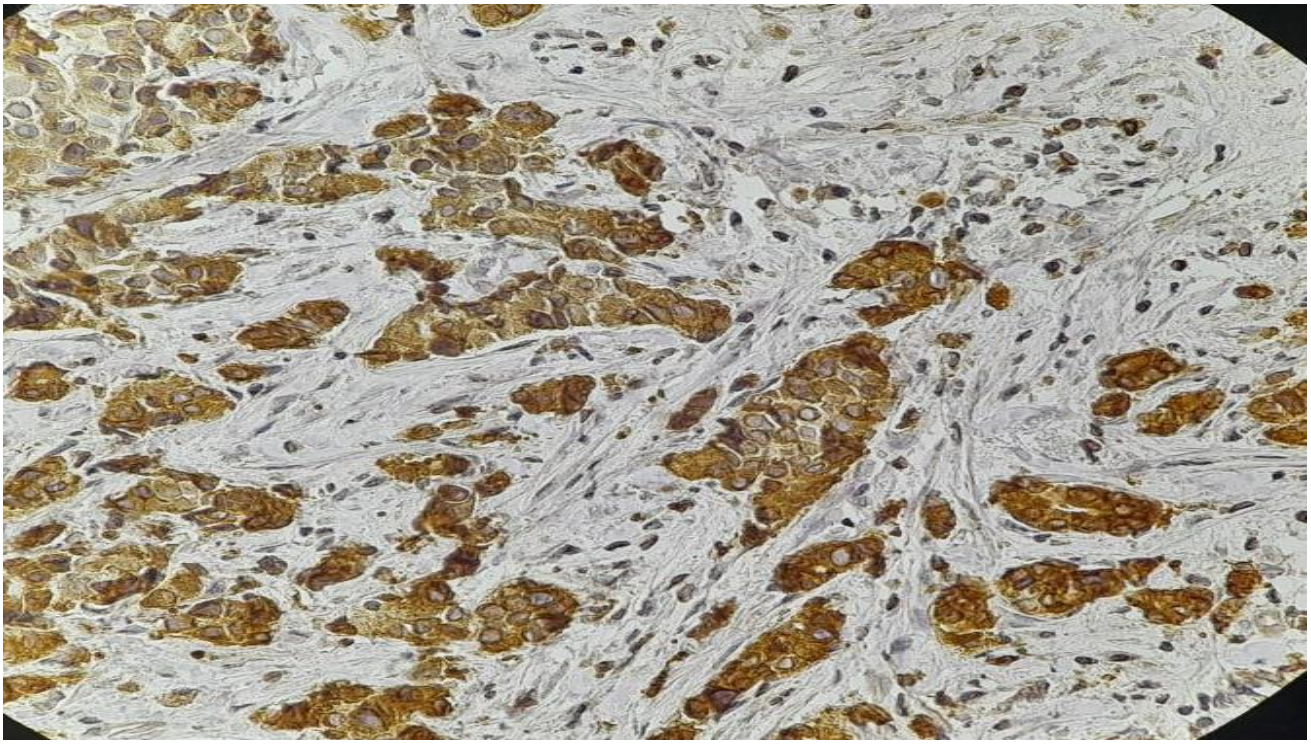
In epithelial cells, like those found in the mammary ducts and lobules, Bcl-2 overexpression occurs in tandem with differentiation. Bcl-2 expression rises with age and supports a function in mammary morphogenesis, from variable immunostaining in the developing breast to specific positivity of terminally differentiated ductal cells in mature breast tissue (Nathan *et al*, 1994).

Although the Bcl-2 effect's molecular cause is still unknown, there is mounting evidence that it is a component of the cell's defense mechanisms against damage which is caused by free radicals (Kane *et al* ,1993).

It is unknown how Bcl-2 contributes to the onset and spread of breast cancer. It has been reported that ductal carcinomas exhibit both overexpression and absence of Bcl-2 expression (Leek *et al* 1994). Expect a relationship between Bcl-2 overexpression and factors for unfavorable clinical outcome based on the theory that Bcl-2 helps and prolongs the survival of transformed cells, thereby providing an opportunity to accumulate further genetic aberrations and promoting malignant progression. Nonetheless, the majority of research conducted up to this point has discovered a highly positive association between Bcl-2 expression and hormone receptor positivity (Leek *et al* 1994)

This study agrees with Binder *et al* (1995) ; Javeed *et al* (2011). They showed the elevated percentage of the expression bcl2 in breast cancer.

It has been demonstrated in numerous *in vitro* and *in vivo* investigations that the protein BCL2 suppresses apoptosis (Vanhaesebroeck *et al* ,1993). Numerous researches have looked into BCL2's prognostic usefulness. Moderate to strong BCL2 expression (abbreviated as BCL2+ tumors) is found to be strongly correlated with a number of favorable prognostic features, including low tumor necrosis and p53 expression, low cathepsin D expression, low mitotic count, and low S-phase fraction size. According to early research, BCL2 expression is linked to low-grade, slowly proliferating ER+ breast cancers (Lipponen *et al.*, 1995), and its association with ER status is thought to be responsible for the increased survival of these tumors (Neri *et al*., 2006). According to a number of studies, BCL2 is a powerful and clinically valid prognostic marker for all forms of early-stage breast cancer, regardless of ER, HER2, or adjuvant therapy received (Dawson *et al*., 2010). Its strong correlation with the hormonal receptor may also be a factor in the higher survival rates seen in BCL2+ breast cancer patients. BCL2 is shown to have predictive value for ER-PR-HER2-breast cancers; patients with ER-PR-HER2-BCL2-tumors were found to benefit from an anthracycline-based regimen (Abdel-Fatah *et al*, 2013).These suggest that while "resisting cell death" is not a decisive element in the aggressiveness of breast cancers, it is a necessary step towards the development of anthracycline resistance in triple negative tumors.



(Figure 4-23) : Breast ductal carcinoma ,BCL-2 IHC , cytoplasmic positivity , x200.

4.3.5. Determination Tumor Tissue Expression of Ki67

When screening for the tumor marker ki67 in women with malignant breast cancer in both age groups. It was found that the percentage of tissue expression of this marker ranged from 12% to 80%. It is noted that the average expression of this marker level higher than 35% was observed in 75% of patients, while in women with breast. The histological images of this tumor marker in malignant breast cancer tissue stained using the IHC technique, shown in (Figure 4-24, a,b), show the nuclear expression of this marker in the tissue cells of women with breast cancer.

Ki67 is the most often utilized proliferation marker in breast cancer and is primarily seen in cycling cells(Jacquemier *et al*., 2009). According to Jacquemier *et al*. (2009), Ki67 has been utilized to forecast the neoadjuvant response or the

result of adjuvant chemotherapy (endocrine therapy for ER positive tumors) in cases of breast cancer. It has also been utilized to provide prognostic and predictive values for breast cancer in conjunction with other indicators. Cheang *et al*. have classified breast tumors using KI67 in addition to ER, PR, and HER2. Based on the expression of Ki67 and HER2, [ER+|PR+] tumors are split into three prognostically different subclasses (Cheang *et al* ,2009). This is consistent with the characteristic of cancer, which is "sustaining proliferative signaling," wherein a patient's clinical result declines with increased propensity for cancer cell multiplication. This classification highlights the significance of using Ki67 in conjunction with ER, PR, and HER2 to distinguish tumors with positive hormone receptors. Specifically, it splits the intrinsic luminal B tumors into two groups: [ER+|PR+]HER2-KI67+ and [ER+|PR+]HER2+KI67+. These groups will be discussed in the next section. Further, the joint use of these four markers has been found to yield as much information as other expensive molecular assays in breast cancer subtyping (Cuzick *et al* ,2009)

This current study result was similar to with previous studies (Soliman and Yussif, 2016 They discovered that 39% of patients experienced recurrence and that 69% of patients had a Ki-67 level more than 15%. This agrees with Yerushalmi *et al* (2010).

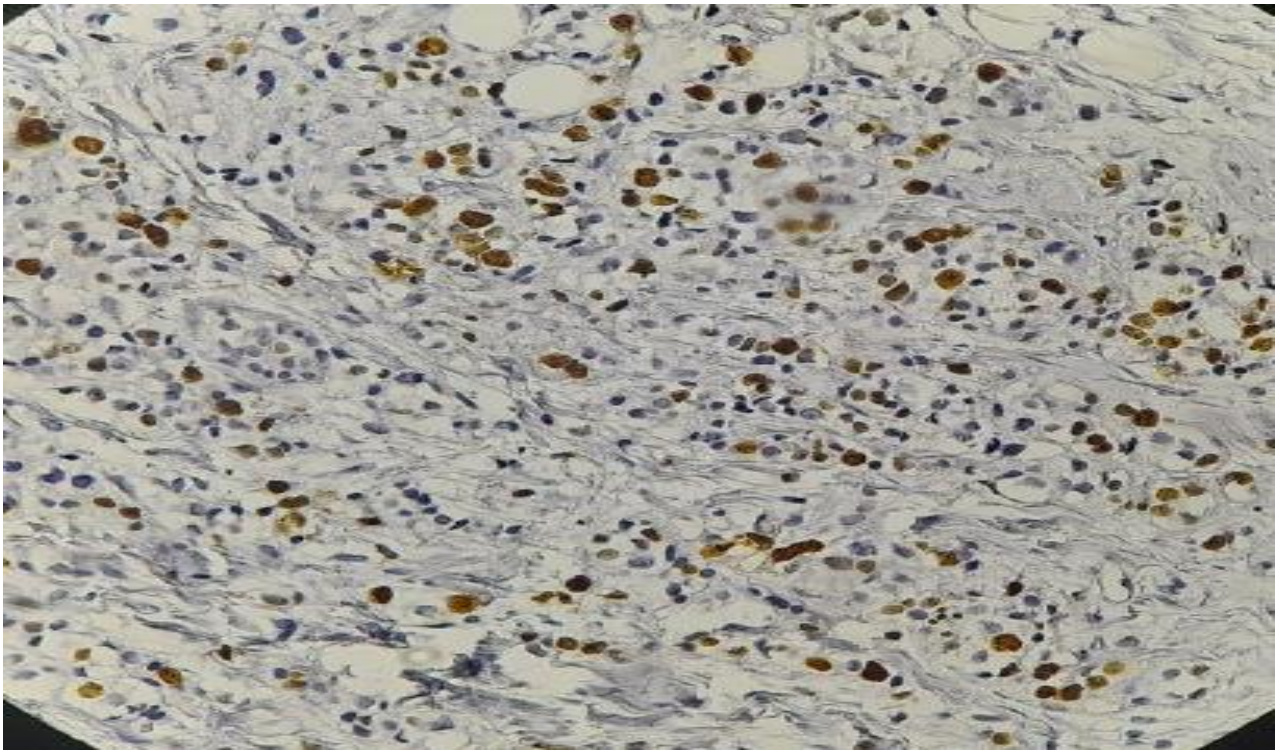


Figure (4-24,a) Breast ductal carcinoma ,ki67 IHC , nuclear positivity , x200.

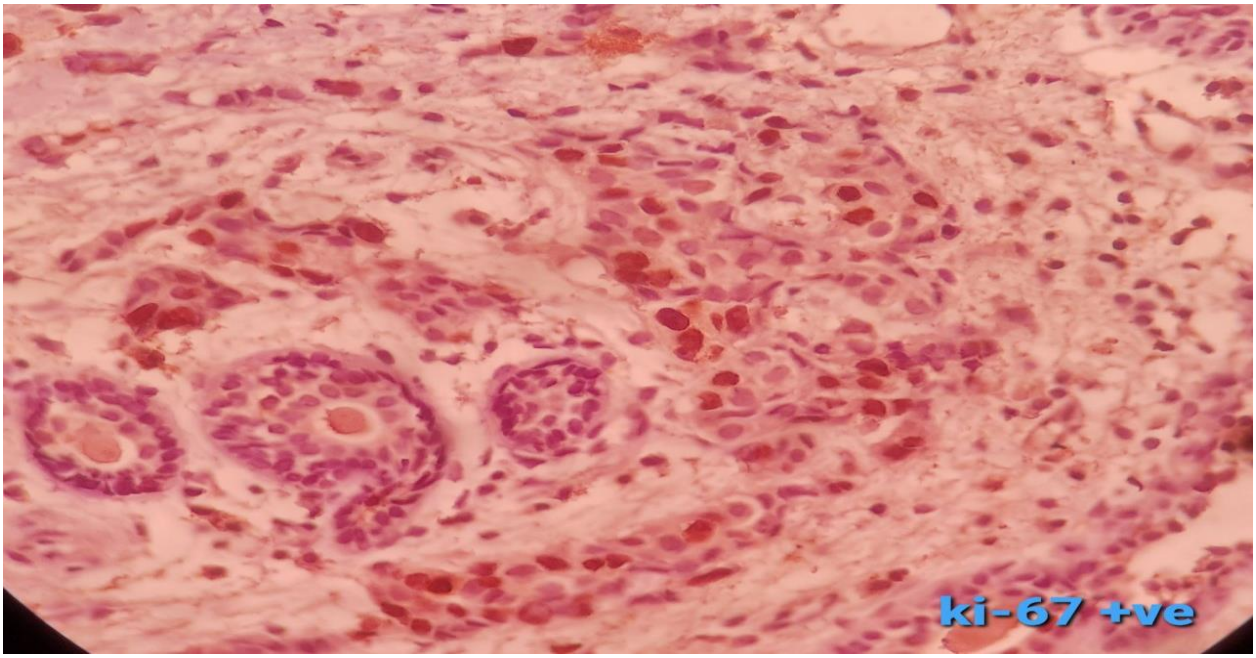


Figure (4-24,b) Breast ductal carcinoma ,ki67 IHC , nuclear positivity , x200.

4.3.6. Patients With Breast Cancer and Their Hormone Receptor Status

Table (4-1) shows that the majority of tumors were ER+/PR+, and their percentage in female patients reached (57%). The least tumors were in (ER-/PR-, ER-/PR+), which the percentage of women with breast cancer decreased until it reached 7%.

This result agreed with Bae, *et al*, (2015). They discovered that in 66.6% of individuals with breast cancer, the combination of progesterone and ER type was positive. In line with Li *et al*. (2020), they discovered that ER upregulates PR expression and that PR-positive/ER-positive breast cancer are frequently encountered. 82.9% of hormone receptor-positive breast cancer cases with PR-positive characteristics were found in a study using SEER data, whereas 15.0% of cases had PR-negative characteristics and 2.0% had both.

Table (4-1) The Status of Hormone Receptors in Patients with Breast Cancer

Hormone receptor	Percentage
ER+ / PR+	57%
ER+ / PR-	29%
ER- / PR+	7%
ER- \ PR-	7%

4.3.7. Hormone Receptor Status in Patients With Breast Cancer Depend on Age

In Table (4-2) below, the dual hormonal receptors and their percentages were investigated in each of the two age groups, where it was noted that the highest percentage of the dual positive receptors ER+/PR+ had the highest percentage of the rest of the dual receptors in both age groups. However, the highest percentage occurred in the age group under 50 years, as the percentage of double positives reached 36%, while it decreased in the age group over 50 years and reached 21%. As for dual hormonal receptors ER+/PR- in both age groups, it reached 14%. On the other hand the percentage of double negative hormonal receptors was highest in the age group that is older than 50 years, but it was the lowest among other hormonal doubles. The ER-/PR+ hormonal double had similar proportions to the negative hormonal receptor double for the same two age groups.

Research has shown that younger people had a lower risk of developing breast cancer (Miller, 2020). Still, the results in younger age groups are not good (Anastasiadi. *et al.,* . 2017). Breast cancer that develops in youth typically has a worse prognosis than breast cancer that develops in old age, according to several studies (Radecka, and Litwiniuk 2016). Therefore, age affects both the prognosis and treatment of breast cancer (Abubakar, *et al.,* 2018) and the other study show no significant association of age with ER and PR (Lee, *et al., 2015*).

This result was agree with (Shah , *et al.*, 2022), and the other study show no significant association of age with ER and PR (Lee, *et al., 2015*).

The disparity could be attributed to various age groups, racial variance, and a lower sample size. Furthermore, the findings of our investigation confirmed that

cancers that grow in each age group differ biologically and call for additional study in this area. We advise determining how age affects the results of treatment tailored to HRS.

Table (4-2) The Status of Hormone Receptors in Patients with Breast Cancer Depend on Age.

Hormonal receptor	Age < 49	Age >50
ER+ / PR+	35.8%	21.4%
ER+ / PR-	14. 3%	14.3%
ER- / PR-	0%	7.1%
ER- / PR+	0%	7.1%

4.3.8. Hormone Receptor Status According to Her-2 Expression

The results that investigated the ratio of the tumor marker her2/neu and its relationship to the four groups of hormonal receptors were shown in Table 4-4. It showed that groups (ER+/PR+, ER -/PR+ and ER -/PR-) had the highest rates of not expressing the tumor marker her2/neu- . The highest percentage among these three groups was double positive for ER+/PR+ receptors that is reached to 57% . On the other hand, the results showed that there was only one group in which Her2/neu+ was present, which is the ER +/PR- group, and the percentage of women in this group was 7%, which is also less than the percentage of women in the same group. However their tumor tissues did not express the tumor marker Her2/neu, as shown, in the table below.

Our result agreed with Bae *et al* (2015).They found that the ER+PR+ was elevated in the Her2\neu negative , reached to 88%, and the lower in the ER-PR- , it

is reached to the 61%. Sheikhpour *et al* (2018) found ER+PR+ was 40 %, ER^{+}/PR^{-} was 12 and ER^{-}/PR^{-} was 13.6

Previous research has shown that PR negative may be associated with cross talk with the EGFR or HER2, which is the epidermal growth factor receptor. High HER2 overexpression was observed in ER + PR-tumors in our investigation. PR negative, however, did not appear to be a significant predictor of outcome in cancers overexpressing HER2. This indicates that in cancers with HER2 overexpression (HER2+), HER2 expression may be linked to the outcome of treatment or may be a more important prognostic indicator than PR loss.

Table (4-3) The Status of Hormone Receptors in Patients with Breast Cancer According to Her-2 Expression

Hormonal receptor	her2/neu-	her2/neu+
ER+ \ PR+	57.3%	0%
ER+ \ PR-	21.4%	7.1%
ER- \ PR+	7.1%	0%
ER- \ PR-	7.1%	0%

4.3.9.Hormone Receptor Status According to BCL2 Expression:

Finally, the expression rate of the four groups of hormonal receptors was investigated, which of these groups expressed the most expression of the tumor marker bcl2 and which expressed the least in women with malignant breast cancer. The results showed that all groups of hormonal receptors had the highest expression of the tumor marker bcl2+, with the exception of the double-negative group of

hormone receptors, where the expression rate was 0%. The group with the highest expression of the hormonal marker was the group of women with double positive expression of the hormonal receptors ER+/PR+, as the percentage reached 36%. Table (4-5) showes that.

Our result was in agreement with Ali, *et al*., (2012). They found elevated the bcl2 with double positive of estrogen and progesterone

Estrogen has shown to induce Bcl-2 expression in ER-positive breast cancer cells (Wang, 1995).

Estrogen is a hormone that plays a key role in the development and growth of breast tissue. In estrogen receptor (ER)-positive breast cancer cells, estrogen can bind to the estrogen receptor on the cell surface, leading to the activation of various downstream signaling pathways. One of the effects of estrogen signaling in ER-positive breast cancer cells is the induction of Bcl-2 expression. Overexpression of Bcl-2 has been associated with increased survival and resistance to cell death in cancer cells, including breast cancer cells. Therefore, when estrogen binds to the estrogen receptor in ER-positive breast cancer cells, it can stimulate the expression of Bcl-2, potentially promoting cell survival and contributing to the growth and progression of the cancer. This mechanism may be one of the reasons why ER-positive breast cancers are often responsive to hormone-based therapies that target estrogen signaling, such as hormonal therapies like tamoxifen or aromatase inhibitors. Understanding the relationship between estrogen signaling and Bcl-2 expression in ER-positive breast cancer cells is important for developing targeted therapies that can effectively disrupt this pathway and improve treatment outcomes for patients with this type of breast cancer.

Table (4-4) The Status of Hormone Receptors in Patients with Breast Cancer According to bcl2 Expression

Hormonal receptor	BCL2+	BCL2-
ER+ \ PR+	35.7%	21.5%
ER+ \ PR-	21.5%	7.1%
ER- \ PR+	7.1%	0%
ER- \ PR-	0%	7.1%

Conclusions

1. High ratio of T cells in pathological groups is recognized .
2. A decrease in CD28 in T cells in breast cancer patients is realized.
3. CD28 is very high in benign breast cancer, and this indicates the possibility of the disease turning into malignant breast cancer
4. CTLA-4 decreases in pathological groups, and this leads to an inverse relationship with T-cell activation
5. It was noted that age has an effect on increasing the production of estrogen hormonal receptors, while the markers her2-neu and bcl2 have no relationship with age, while ki67 has increased expression in cancer cells at ages less than 50 years, and this is very important in giving treatment.
6. There is an increase in T reg cells in patients with malignant breast cancer who were over 50 years old.
7. Blood group was linked to the type of breast cancer, which is that malignant breast cancer is most common in women who have blood types A and O.
8. The binding of the ER and PR hormone receptors increased significantly, and this leads to the development of breast cancer
9. The percentage of triple-negative breast cancer is almost non-existent, and this gives the opportunity to be treated with chemotherapy
10. There is an Increased expression level of BCL2 in women who were bi-positive for the hormones estrogen and progesterone. This indicates the induction of BCL2 production by estrogen.
11. There is an Increased height of T cells with increased expression of tumor histological markers (er+, pr+, her2-neu, bcl2) as well as the direct relationship with ki67. This also gives an important immunological indicator in determining the increase in breast cancer and its development.

Recommendations

1. A study of molecules expressed by T reg cells that have direct effects on inhibition.
2. Utilizing all available markers for T cells to gain a comprehensive understanding of their crucial role in breast cancer and to study it at the molecular level.
3. Conduct a comprehensive investigation into the involvement of cells such as T cells, Ds cells, Th1, Th2, Th17, and B cells in this disease.
4. Extend the number of samples to mitigate bias and extend the duration of the study.
5. Conduct studies that track the same patients prior to and following chemotherapy treatments.
6. Examine the percentage of regulatory T cells in lobular and ductal breast cancer.
7. Check the type of progesterone receptor if it is PR-A or PR-B
8. Detection of Treg cells in tumor tissue
9. Study of the proportion of Ctla4 in tumor tissue.

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الخلاصة :

الخلايا المنظمة تعد احد اهم انواع الخلايا في كبح المناعة وتكون نسبتها قليلة في الدم وهي احد انواع الخلايا التائية التي تتوسط التحمل المناعي والتي تمنع المناعة الذاتية بواسطة تثبيط تكاثر الخلايا التائية وانتاج السيتوكينات. ان الخلايا السرطانية تساهم في تخليق الخلايا التائية المنظمة حيث يوجد في عدة انواع من السرطانات لان الخلايا السرطانية تنتج TGF β لتستحث الخلايا النخاعية والذي يلعب دور مهم في تطور بادئات بواسطه تحول الخلايا التائية الاصيلة الى المنظمة.

الهدف من الدراسة هو تقييم دور الخلايا التائية المنظمة والجزيئات التي تعبر عنها CD28 و CTLA-4 والتي كانت محسوبة بتقنية قياس التدفق الخلوي في سرطان الثدي وتقييم بعض الواسمات الورمية الموجودة في نسيج الثدي بتقنية الكيمياء النسجية المناعية. خضع للدراسة الحالية ٧٥ امرأة ، ٢٥ امراءة كانت غير مصابة وهي مجموعة السيطرة و٢٥ امرأة كانت مصابة بسرطان الثدي الخبيث فيما كان ٢٥ امرأة المتبقية فهي من النساء المصابات بسرطان الثدي الحميد.

تم جمع العينات كتشخيص اولي لنوعية سرطان الثدي سواء الحميد او الخبيث في محافظة كربلاء تم تصنيف المريضات حسب الفئة العمرية الى الفئة الشابة والتي تتراوح اعمارهن من ٢٩ الى ٤٩ والفئة الثانية الاكبر سنا هن من ٥٠ الى ٧٠ سنة.

اظهرت النتائج ان هناك زيادة واضحة ومعنوية للخلايا المنظمة في كلا الفئتين من المريضات بسرطان الثدي الحميد والخبيث (٤٥.٦١ و ٤٣.٤٢)% على التوالي مقارنة مع مجموعة السيطرة، وقد بلغت قيمة $P<0.0001$. اما بالنسبة للجزيئات CD28 وتعبيرها على خلايا المنظمة فقد وجد ان تعبيرها انخفض في كل من المجموعتين من المريضات بسرطان الثدي الحميد والخبيث (٨٧.٣٧ و ٩٠.٣٩)% على التوالي وكانت الفروق المعنويه قد وصلت الى ان $P< 0.001$. اما MFI فقد اظهرت مجموعة المرضى ارتفاعا مقارنة مع مجموعة السيطرة وخصوصا مجموعة سرطان الثدي الحميد(٤١.٢%) ، اما بالنسبة للتعبير لجزيئة CTLA-4 فكانت هناك انخفاض في نسبة الجزيئات هذه لمجموعتي سرطان الثدي الحميد والخبيث (٥٦.١٤ و ٤٩.٥٣)%على التوالي مقارنة مع مجموعة السيطرة .في حين ان MFI كان هناك انخفاض معنوي ملحوظ حيث ان $P<0.0001$ لكلا مجموعتي سرطان الثدي مقارنة مع مجموعة السيطرة.

وقد تم دراسة تاثير العمر على نسبة تعبير نسيج الثدي للواسمات الورمية(ER, PR , Her2\neo , BCL2 and KI67) لمجموعة سرطان الثدي الخبيث فكانت النتائج ان KI67 يزداد تعبيره في انسجة الثدي لفئة العمر الاقل من ٥٠ سنة وبفروق معنوية واضحة فكانتP<0.01. اما بقية الواسمات لم تظهر هناك فروق معنوية واضحة يبين تعبيرها في انسجة الثدي وعمر المريضات.

اما بالنسبه لتاثير العمر على الخلايا المنظمة فقد بينت النتائج ان هناك ارتفاع واضح لهذه الخلايا في مجموعة المرضى مقارنة مع مجموعة السيطرة لكل الفئتين العمريتين وان مجموعة السيطرة ذات الفئة العمرية الاكثر من ٥٠ سنه اظهرت فروق معنويه مقارنة مع الفئة الاقل من ٥٠ سنة في نفس المجموعة وبفروق معنوية $p<0.01$.اما المجموعة الاكثر نسبة من الخلايا المنظمة فهي مجموعة سرطان الثدي الحميد الاقل من ٥٠ سنة وبفرق معنوي واضح $p <0.0001$,بينما مجموعة سرطان الثدي الخبيث كان هناك ارتفاع للخلايا المنظمة وبالفارق المعنوي ذاته ايضا للفئة العمرية الاكثر من ٥٠ سنة مقارنة مع مجموعة السيطرة .

كما تم دراسة علاقة الخلايا المنظمة مع الواسمات الورمية النسيجية لسرطان الثدي فقد لوحظ ان هناك ارتفاع لنسبة هذه الخلايا عند النساء اللاتي كانت انسجتهن تعبر تعبير ايجابي لهذه الواسمات الورمية لكنها لم تصل الى الفروقات المعنوية، اما بالنسبة للواسم الورمي ki 67 فكانت هناك علاقه طردية واضحة بينه وبين الخلايا المنظمة.

اما بالنسبة لفصيلة الدم للمريضات فقد تم دراسة تاثيرها على زيادة او نقصان الخلايا المنظمة فظهرت النتائج ان اعلى نسبة من هذه الخلايا تظهر في فصيلة الدم Bعند مجموعة المريضات المصابات بسرطان الثدي الحميد تليها فصيلة الدم O ثم فصيله الدم A واخيرا فصيلة الدم AB بينما كانت اكثر نسبة الخلايا المنظمة في فصيله الدم O وبعدها فصيله الدم A واقلها في فصيله الدم B في مجموعه سرطان الثدي الخبيث.

اما علاقة فصيلة الدم مع سرطان الثدي فقد تم التحري عنها واظهرت النتائج ان اعلى فصيلة دم مرتبطة مع سرطان الثدي الحميد هي A , فصيلة الدم O فكانت هي الاكثر قربا من فصيلة الدم A. اما في مجموعة سرطان الثدي الخبيث فكانت اكثر النساء المصابات بسرطان الثدي هن من فصيلة الدم A تليها فصيلة الدم O واخيرا فصيله الدم B .

بالنسبة للدراسة النسيجية فاظهرت النتائج ان نسبه تعبير اورام الثدي لمستقبل هرمون الاستروجين قد بلغ ٨٦% وهرمون البروجسترون كانت نسبته في الانسجة الورمية ٦٤% بينما her2-neu فقد كانت نسبة تعبيره الايجابي ٧% فقط بينما ٩٣% من المريضات لم تعبر انسجتهن لهذا الواسم اما فيما يخص bcl2 فكانت نسبه التعبير ما يقارب ٦٤% تعبير ايجابي

اما الواسم الورمي ki 67 فقد كانت انسجة المريضات مختلفة في نسبة التعبير عن هذا الواسم الورمي فكانت النسب من(١٢ الى ٨٠)% وقد قسمت الى فئتين الفئة الاولى هي الاقل تعبيرا من ٣٥% لهذا الواسم والفئة الاخرى هي كانت الاكثر من ٣٥%. النتائج اظهرت ان نسبة النساء اللاتي قد كانت انسجة الثدي لديهن تعبر عن الفئة الثانية وهي الاكثر من ٣٥% قد بلغن ٧٥%. كما درست ايضا نسبة علاقة المستقبلات الهرمونية مع سرطان الثدي فكانت النسبة الاكبر هي عندما يكون الورم ثنائي الايجابية لمستقبلي هرموني الاستروجين والبروجسترون وكانت نسبة النساء ٥٧% بينما علاقه العمر مع مزدوجات مستقبلات

الهرمونات فكانت النسبة الاعلى من النساء لمستقبلي مزدوج الايجابية لهرموني البروجسترون والاستروجين هن من النساء الاصغر سنا من ٥٠ سنة بينما علاقة مزدوج المستقبلات مع الواسم الورمي her2-neu فكانت نسبة التعبير الايجابي لهذا الواسم عند النساء ذات التعبير الايجابي لهرمون الاستروجين والسلبي لهرمون البروجسترون، اما بالنسبه bcl2 فكانت نسبة التعبير الاكبر عند النساء اللاتي اظهرت انسجتهن التعبير مزدوج ايجابي لهرموني البروجسترون والاستروجين.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة كربلاء / كلية العلوم
قسم علوم الحياة

أهمية الخلايا التائية التنظيمية في سرطان الثدي وعلاقتها مع الواسمات الورمية عند النساء في محافظة كربلاء \ العراق

اطروحة مقدمة إلى
مجلس كلية العلوم جامعة كربلاء
وهي جزء من متطلبات نيل درجة الدكتوراه في علوم الحياة

من قبل الطالبة

عتاب عبد الامير ابراهيم الموسوي

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