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GOLGI PROTEIN AND OTHER BIOCHEMICAL TUMOR MARKERS IN EARLY DETECTION OF BREAST CANCER IN IRAQI SUBJECTS

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We certify that we have read this thesis and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science

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ABSTRACT

GOLGI PROTEIN AND OTHER BIOCHEMICAL TUMOR MARKERS IN EARLY DETECTION OF BREAST CANCER IN IRAQI SUBJECTS

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Master of Science in Chemistry

Advisor: Assoc. Prof. Dr. Hakan ÇOLAK

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Breast cancer is a complicated, worldwide health issue. Clinical practice relies on tumor markers, biochemicals linked to cancers. Golgi protein 73 (GP73), a trans-membrane protein in the Golgi apparatus, is a promising diagnostic for breast and other malignancies. We investigated breast cancer tumor markers, including human Golgi protein 73. Blood samples were taken from 116 breast cancer patients (42 newly diagnosed and 74 under treatment), 26 benign tumor cases, and 34 seemingly healthy controls. An enzyme-linked immune-sorbent assay measured HER2, PR, ER, and Ca15-3 tumor markers and human Golgi protein 73 blood levels. The mean ages of breast cancer patients were benign tumor (39.54 ± 13.969), recently diagnosed (48.14 ± 7.754), and under treatment (48 ± 9.798), whereas healthy controls were 46.82 ± 12.413 years. Patients and healthy controls had similar serum human Golgi protein 73 assay levels. Tumor marker testing were promising and beneficial for monitoring breast cancer patients. Our work emphasizes tumor markers, notably human Golgi protein 73, in breast cancer diagnosis and prognosis. While blood GP73 levels did not change between patients and healthy controls, tumor marker testing indicated promise for monitoring breast cancer patients throughout therapy and follow-up.

2023, 67 pages

Keywords: Breast cancer, GP73, CA-15.3, Progesterone receptor, E2 receptor.

ÖZET

IRAK KONULARINDA MEME KANSERİNİN ERKEN TEŞHİSİNDE GOLGI PROTEİNİ VE DİĞER BİYOKİMYASAL TÜMÖR BELİRLEYİCİLERİ

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Meme kanseri karmaşık, dünya çapında bir sağlık sorunudur. Klinik uygulama, tümör belirteçlerine, kanserlerle bağlantılı biyokimyasallara dayanır. Golgi aparatındaki bir trans-membran proteini olan Golgi proteini 73 (GP73), meme ve diğer maligniteler için umut verici bir teşhistir. İnsan Golgi proteini 73 dahil meme kanseri tümör belirteçlerini araştırdık. 116 meme kanseri hastasından (42'si yeni teşhis edilmiş ve 74'ü tedavi altında), 26 iyi huylu tümör vakasından ve görünüşte sağlıklı 34 kontrolden kan örnekleri alındı. Enzime bağlı bir immün-sorbent deneyi, HER2, PR, ER ve Ca15-3 tümör belirteçlerini ve insan Golgi proteini 73 kan seviyelerini ölçtü. Meme kanserli hastaların ortalama yaşı iyi huylu tümör (39.54 ± 13.969), yeni teşhis (48.14 ± 7.754) ve tedavi altında (48 ± 9.798) iken, sağlıklı kontrollerin 46.82 ± 12.413 idi. Hastalar ve sağlıklı kontroller, benzer serum insan Golgi proteini 73 tahlil seviyelerine sahipti. Tümör belirteç testi, meme kanseri hastalarını izlemek için umut verici ve faydalıydı. Çalışmamız, meme kanseri teşhisi ve prognozunda tümör belirteçlerini, özellikle insan Golgi proteini 73'ü vurgulamaktadır. Kan GP73 seviyeleri hastalar ve sağlıklı kontroller arasında değişmezken, tümör belirteç testi, meme kanseri hastalarının tedavi ve takip boyunca izlenmesi için umut vaat ettiğini gösterdi.

2023, 67 sayfa

Anahtar Kelimeler: Meme kanseri, GP73, CA-15.3, Progesteron reseptörü, E2 reseptörü.

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CONTENTS

LIST OF SYMBOLS

=	Equals
<	Is less than
>	Is more than
L	Liter
Maxi	Maximum
µL	Microliter
Ml	Milliliters
mm	Millimeter
Mini	Minimum
Min	Minute
(-)	Negative or minus
%	Percent
pg/ml	Picograms per milliliter
(+)	Positive or plus
rpm	Revolutions per minute
S	Second
u/ml	Units per milliliter

LIST OF ABBREVIATIONS

ATM	Automated teller machine
BC	Breast cancer
BCI	Breast cancer index
BRCA1	Breast cancer gene
BRCA2	Breast cancer gene
C1	Unsatisfactory/no epithelia
C2	Benign
C3	Atypia probably benign
C4	Suspicious for malignancy
C5	Malignant
CA15-3	Carbohydrate antigen 15-3
CE	Contrast-enhanced digital mammography
CEA	Carcino-embryonic antigen
CF DNA	Cell-free tumor DNA
CHEK2	Checkpoint kinase 2
CRISPR-CAS9	Clustered Regularly Interspaced Short Palindromic
CT	Computed tomography
CT DNA	Circulating tumor DNA
CTCs	Circulating tumor cells
DNA	Deoxyribonucleic Acid
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
ER	Estrogen receptor
EU	European union
GBC	Gallbladder cancer
GP73	Golgi protein 73
GSLS	Glycosphingo lipids
HCC	Hepatocellular carcinoma
HER2	Human epidermal growth factor receptor
HER2	Human epidermal growth factor receptor 2
HRP	Horseradish peroxidase
HSP60	Heat shock protein 60
HSP90	Heat shock protein 90
Mi RNAs	Micro RNAs
MI	Microwave imaging
MRI	Magnetic resonance imaging
MUC1	Mucin1
P13K/AKT/MTOR	Signaling pathways of tumor cell
PALB2 gene	Partner and localizer of BRCA2

PCa	Prostate cancer
PET	Positron emission tomography
PR	Progesterone receptor
PTEN	Phosphatase
REs	Response elements
RNA	Ribonucleic Acid
TMB	Tetramethylbenzidin liquid substrate system for ELISA
TMEM165	Transmembrane protein 165
UK Rcpath	The royal College of pathologists
UTs	Ultrasound
WHO	World health organization

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1. INTRODUCTION

Malignant tumors, on the other hand, may metastasize (spread to other areas of the body) and infiltrate surrounding tissues (Hakomori 1996). Tumor cell adhesion is regulated by a number of variables, one of which is abnormal glycosphingolipids (GSLs), which contribute to tumor cell invasiveness and metastasis (Hakomori Handa 2002).

The clinical presentation, molecular characteristics, behavior, and therapeutic responses of breast cancer patients might differ widely from one another. To help with patient management and treatment decisions, various grading systems have been developed and recommended by international professional bodies, such as the WHO, AJCC, EU, and UK RCPath (Ellis *et al.* 2023, Tavassoli and Devilee 2003).

When it comes to female cancers, breast cancer is by far the most prevalent and deadly. (Elston and Ellis 1991) stressed the need of having accurate clinical and pathological prognostic and predictive indicators to direct patient care and improve treatment results. Tumor markers may help in cancer diagnosis, follow-up, and prognosis in a supplementary way. However, just because a person possesses a tumor marker does not guarantee they have cancer. Some tumor markers might be raised in illnesses other than cancer (Bhatt *et al.* 2010).

A biomarker is a substance or quantitative property that may be used as an indicator of a disease or physiological process in living organisms. Blood plasma, urine, cerebrospinal fluid, and saliva are only some of the bodily fluids that may be tested for biomarkers. They can be used in clinical practice for various purposes, such as diagnosing diseases, monitoring disease progression, assessing treatment efficacy, and predicting clinical outcomes (Virji *et al.* 1988).

Markers for tumors are biomolecules that may be used to detect or track the development of cancer. Tumor-derived cytokines are generated by tumor cells, whereas

tumor-associated cytokines are generated by the body in reaction to the presence of a tumor. Blood, urine, and cerebrospinal fluid are only some of the bodily fluids that may be tested for tumor markers (Virji *et al.* 1988).

Tumor markers are measurable proteins that may aid in the detection, management, and follow-up of cancer. The existence of a tumor or an increased risk of cancer recurrence after therapy might be suggested, for instance, by an elevated level of a particular tumor marker in the blood. Tumor markers are useful for detecting cancer, but it's crucial to remember that not all tumors produce tumor markers and not all raised tumor marker levels indicate malignancy. Since this is the case, tumor markers must always be considered with other clinical and diagnostic data (Sotiriou *et al.* 2004, Wu 2001).

In our earlier glyco-proteomic investigation (Zhao *et al.* 2012), Golgi membrane protein TMEM165 was shown to be associated with invasive ductal breast cancer. However, tumor markers should always be viewed in the context of the whole tumor. Mass spectrometry analysis revealed the presence of the TMEM165 protein in invasive breast cancer tissue but not in neighboring normal breast tissues from the same patients. Patients with congenital abnormalities of glycosylation have mutations in the TMEM165 gene, which encodes a putative ion transporter (Demaegd *et al.* 2013, Potelle *et al.* 2017).

Bone dysplasia and poor cartilage formation were among the phenotypic traits seen in a TMEM165-deficient zebrafish model (Ng and Freeze, 2018), mirroring those seen in the three patients with a homozygous splicing mutation. Glycosylation regulation Golgi protein TMEM165 was recently discovered by genome-wide screening of bacterial toxins using CRISPR-Cas9 (Demaegd *et al.* 2013, Potelle *et al.* 2017, Wu 2001, Ng and Freeze 2018).

Transmembrane protein 73 (GP73) is localized to the Golgi apparatus and is highly expressed in epithelial cells. Multiple pathways have been shown in which it plays a role in the onset and progression of breast cancer, making it a helpful marker for this illness (Bammens *et al.* 2015, Tian *et al.* 2018).

Prognostic significance has been shown for GP73 expression in a variety of malignancies, including gallbladder, lung, and prostate tumors, according to recent research. Evidence like this shows GP73 might be useful in the clinic for diagnosing various cancers (Sinha 2018, Graham *et al.* 2021).

Recent years have seen a flurry of research on GP73 as a possible biomarker for the diagnosis, follow-up, and prognosis of many different cancers. Although these studies have produced encouraging findings, more work is still required to completely clarify GP73's involvement in cancer formation and its diagnostic potential (Søndergaard *et al.* 2017).

1.1 Aim of Study

To assess the serum level of Golgi protein 73(GP73), Ca15.3, PR, ER and HER2 and the correlation between them in Iraqi women.

2. LITERATURE REVIEW

2.1 Breast Cancer

According to the World Health Organization (Baradaran *et al.* 2011), 22 percent of all female malignancies are caused by breast cancer. It is the second leading cause of death from cancer in women, behind lung cancer. Although males are not immune to developing breast cancer, women are at far higher risk (Saadat 2008).

In Iraq, breast cancer is the leading cause of cancer death among women, making it a major public health issue. It affects numerous areas, including Baghdad and Basra, and ranks sixth on the list of causes of mortality among Iraqi women. Survival rates and the likelihood of developing complications from breast cancer may be increased with early identification and treatment (Naghibi *et al.* 2013, Oldenburg *et al.* 2007.

Breast cancer is influenced not just by genetics but also by lifestyle and environmental factors. Breast cancer risk factors include age, gender, having a family history of the disease, having a BRCA1 or BRCA2 mutation, drinking excessively, being overweight, not getting enough exercise, being exposed to radiation, and having an irregular menstrual cycle or a late menopause. But having any of these risk factors does not guarantee that a person will get breast cancer, and conversely, not having any of these risk factors does not guarantee that a person will not develop breast cancer. Keep in mind that anybody may get breast cancer, so it's important to be screened regularly and get diagnosed early for the best chance of survival (Kushi *et al.* 2012, Gökbulak 2002).

Breast cancer has no known single cause, but researchers have found a number of risk factors, including genetics, hormones, and lifestyle choices, that might raise an individual's likelihood of acquiring the illness. Mutations in the BRCA1 and BRCA2 genes, for example, have been linked to an increased likelihood of developing breast cancer. Early menstruation, a prolonged menopause, and the use of hormone replacement therapy are all examples of hormonal conditions that may enhance

vulnerability. Breast cancer risk has been linked to lifestyle variables such heavy drinking, inactivity, and obesity. It's worth noting that even among those who have risk factors, not everyone will ultimately be diagnosed with breast cancer. Therefore, the best way to improve survival rates from breast cancer is by consistent screening and early diagnosis. Several researchers (Voutilainen *et al.* 2013) Breast cancer risk factors include both genetic and environmental variables. Factors including location, lifestyle, marital status, and weight all have a role (Anderson *et al.* 2005).

Having a family history of breast cancer is a well-established risk factor, and an individual's genetic composition plays a significant role in predicting their susceptibility to getting the disease. Women who have a first-degree relative (such as a mother, sister, or daughter) who has been diagnosed with breast cancer have a higher risk of developing the disease themselves than those who do not (Tyrer *et al.* 2004).

A person's vulnerability increases in proportion to the number of afflicted relatives and the closeness of those relatives to them. You're right that having even one first-degree family who has breast cancer raises a woman's chance of contracting the illness by around triple; having two or more such relatives raises that risk by tenfold. The degree to which a person's family history plays a role in their risk might vary depending on other circumstances, such as the age at which symptoms first appear or the existence of particular genetic abnormalities (Claus *et al.* 1993).

Not all breast cancers have a genetic component, and having a family history of the disease is no assurance that you will get it yourself. Women who have a high risk of developing breast cancer due to a family history of the disease should discuss their options with their doctor, which may include more frequent or earlier screenings, as well as genetic testing (Byrne *et al.* 2995).

Women with one affected first-degree family have a 13% lifetime chance of acquiring breast cancer, while those with two affected first-degree relatives have a 21% lifetime risk, according to a research published in the Journal of Clinical Oncology. Women who

have a first-degree relative who has breast cancer have a 7.8% increased chance of developing the disease themselves (Volkow and Li 2005).

The BRCA1 and BRCA2 genes aren't the only ones implicated in an increased risk of breast cancer; there are many more. Cancers of the breast and ovaries are much more common among women who have these mutations than in the general population. The chance of getting breast cancer has also been linked to variations in the ATM, CHEK2, and PALB2 genes (Paul and Paul 2014).

The genes BRCA1 and BRCA2 play a critical role in the onset of breast cancer and other malignancies. These genes are vital for avoiding the accumulation of mutations that may lead to cancer by keeping the genome stable. Cancers of the breast and ovary, as well as those of the pancreas and the prostate, are more common in people with BRCA1 and BRCA2 gene mutations. Many cases of familial breast and ovarian cancer may be traced back to mutations in these genes. Compared to the general population, women with BRCA1 or BRCA2 mutations have a significantly greater lifetime risk of getting breast and ovarian cancer (Davis and Lin 2011).

DNA repair, in which the proteins BRCA1 and BRCA2 participate, is essential for preserving genomic stability and blocking the accumulation of mutations that might cause cancer. The double-stranded DNA breaks caused by radiation or other DNA-damaging agents are repaired in part by the proteins in question. BRCA1 and BRCA2 regulate gene transcription in addition to their involvement in DNA repair (Yoshida and Miki 2004). The expression of other genes involved in cell growth and division may be affected by mutations or abnormalities in these genes, which may then contribute to the development of cancer. The chance of developing cancer is increased when normal BRCA1 or BRCA2 function is lost, as shown by research. In particular, cancer formation may be aided by disruptions in BRCA1/2-mediated DNA repair (Konovalenko *et al.* 2022).

2.1.1 Breast cancer metastasis

The most common cause of death for cancer patients is metastasis, or the spread of cancer cells to other parts of the body. Breast cancer has the potential to metastasize to other organs. Metastasis happens when cancer cells escape from a primary tumor and spread to other organs through the blood or lymph system (Fares *et al.* 2020).

Up to 75% of individuals with metastatic breast cancer will have bone metastases at some point throughout their treatment. When breast cancer spreads to the bones, it can cause pain, weakness, and fractures, as well as other complications such as spinal cord compression. Effective treatment of metastatic breast cancer requires a comprehensive approach, including systemic therapy such as chemotherapy or hormone therapy, as well as targeted therapies such as bone-modifying agents to prevent bone complications. Additionally, supportive care such as pain management and physical therapy can help improve quality of life for individuals with metastatic breast cancer (Hermann *et al.* 2007, Chang and Chaudhuri 2019).

The ability of cancer cells to penetrate the basement membrane and reach the circulation or lymphatic system is one of the many phases in the process of cancer metastasis. The basement membrane is an extracellular matrix layer that acts as a barrier between the tissue's epithelial cells and the stroma underneath them. In order to get past this barrier and infiltrate the surrounding tissue, cancer cells must release enzymes that break down the basement membrane. Detaching cancer cells from the main tumor and allowing them to travel via the circulatory and lymphatic systems is just as important as the capacity to breakdown the basement membrane. Modifications in the expression of genes involved in cell adhesion and migration aid in this process (Benton *et al.* 2011).

Normally, cells are held together by adhesive proteins that allow them to form tissues and maintain their structure. To escape the confines of the original tumor and reach the bloodstream, cancer cells often lose their ability to adhere to one another due to mutations in genes that regulate the synthesis of sticky proteins. These alterations pave

the way for cancer cells to metastasize, or spread to other regions of the body and start new tumors in unrelated organs (Parsa *et al.* 2016, Lu *et al.* 2020, Park *et al.* 2013).

The loss of normal cell adhesion and the ability to invade through the basement membrane are key steps in the development of metastatic cancer. As cancer cells progress and become more aggressive, they often acquire changes in their genetic and molecular makeup that enable them to overcome the natural barriers that prevent invasion and metastasis. A decrease in normal function and an increase in cancer cells' capacity to migrate and invade have both been linked to changes in the expression of sticky molecules in cancer cells, as indicated by researchers. These changes can be particularly pronounced in advanced tumors, where cancer cells have undergone multiple rounds of genetic and molecular changes (Subbaram and DiPersio 2011).

Both cadherins and integrins belong to the large family of adhesive proteins that connect cells to one another and to the extracellular matrix. Cell migration, proliferation, and survival are just a few of the cellular processes that cadherins and integrins control. Transmembrane proteins called cadherins serve an essential role in cell-cell adhesion, tissue morphogenesis, and tissue homeostasis. Tumor growth, invasion, and metastasis in breast cancer have all been linked to alterations in cadherin expression and function. For instance, a poor prognosis is related with breast cancers in which E-cadherin, a key form of cadherin in epithelial tissues, is down-regulated (Aplin *et al.* 1998).

Transmembrane receptors called integrins facilitate communication between cells and the extracellular matrix. Alterations in integrin expression and function are associated with tumor development, angiogenesis, and metastasis in breast cancer. Overexpression of integrin v3 has been linked to a worse prognosis and increased breast cancer cell invasion and angiogenesis (Viera *et al.* 2021).

The mechanisms of metastasis can differ depending on the target tissue. For example, some cancers tend to metastasize to specific organs or tissues, such as breast cancer's tendency to metastasize to the bones. The microenvironment of different organs can

play a role in the metastatic process, as cancer cells must adapt to the unique environmental conditions of each site to establish new growth. Therefore, understanding the mechanisms of metastasis and the unique features of the microenvironments of different organs is critical for developing effective strategies to prevent and treat metastatic disease (Hejmadi 2014).

2.1.2 Breast cancer risk factors and risk-reduction strategies

Several variables increase a woman's likelihood of developing breast cancer:

1. Age: Women's breast cancer risk rises with age.
2. Gender: Breast cancer is more common in females than males.
3. A woman's risk of having breast cancer increases if she begins menstruating before the age of 12 or if she does not enter menopause until beyond the age of 55. Women who have never given birth or who wait until they are older than 30 to deliver their first child are also at a higher risk.
4. Family history: Women who have had a close family, such as a mother, sister, or daughter, diagnosed with breast cancer are at a higher risk of developing the disease themselves.
5. Breast density: Women who have dense breast tissue are at a higher risk for developing breast cancer.
6. Hormone therapy: The risk of breast cancer in postmenopausal women who have been treated with hormones is higher.

Radiation therapy: Breast cancer is more likely to occur in women who have had radiation treatment to the chest for another ailment, such as Hodgkin's lymphoma (O'Sullivan *et al.* 2018).

Mutations in genes like BRCA1 and BRCA2 may potentially enhance one's chance of developing breast cancer, in addition to the aforementioned variables. The chance of getting breast cancer is much increased in women who inherit a mutant copy of one of

these genes compared to women who do not inherit the mutation (O'Sullivan *et al.* 2018).

Identification of women at high risk for a genetic mutation, such as BRCA1/2, may be aided by validated risk assessment techniques. A woman's age at diagnosis, the kind of cancer, and the number of relatives who have had cancer are all common topics of inquiry in these sorts of instruments. The program determines a woman's likelihood of carrying a genetic mutation based on her responses to these questions and may then suggest further screening or genetic counseling (Mills and Fakolade 2020).

The National Cancer Institute's Breast Cancer probability Assessment Tool uses factors like age, family history of breast cancer, age at first menstrual period, and number of prior breast biopsies to estimate a woman's risk of developing breast cancer within the next five years. Women with BRCA1/2 gene mutations or other genetic concerns cannot be accurately assessed using this technique (Visvanathan *et al.* 2019).

Medications including tamoxifen, raloxifene, anastrozole, letrozole, and exemestane may be used to reduce the risk of breast cancer in women who are at an elevated risk of acquiring the illness. Some forms of breast cancer may be fueled by estrogen, which is why these drugs function. While aromatase inhibitors are normally only suggested for postmenopausal women, tamoxifen may be used in both premenopausal and postmenopausal women at high risk of developing breast cancer. A woman's risk factors, medical history, and personal beliefs will all play a role in determining which drug is best for her (Owens *et al.* 2019).

2.1.3 Breast cancer pathophysiology

Caused by both inherited and acquired causes, cancer cells interact intricately with their surroundings. In order to proliferate and survive, tumor cells may activate signaling pathways including PI3K/AKT/MTOR (Jakóbisiak *et al.* 2003). Activation of this

pathway is common in breast cancer and is crucial for proper cell growth and metabolic regulation (Fiers 1999).

Numerous cells and chemicals in the microenvironment around breast cancer cells may also encourage tumor development and invasion. For instance, macrophages that are found in tumors may generate cytokines and growth factors that aid in tumor cell proliferation and migration (Cheng *et al.* 2011). Likewise, tumor cells and their surrounding extracellular matrix (ECM) may be altered to facilitate tumor invasion and metastasis (Rich *et al.* 2015).

The microenvironment not only helps tumors grow and live, but it may also affect how breast cancer cells react to treatment. Low oxygen levels, or hypoxia, in the tumor microenvironment have been linked to increased resistance to chemotherapy and radiation treatment (Rich *et al.* 2015). Similarly, stromal cell–tumor cell interactions may alter how breast cancer cells respond to endocrine treatment, which blocks estrogen's effects (Apostolakis *et al.* 2001).

2.1.4 Genetic factors of breast cancer

In addition to BRCA1 and BRCA2, mutations in TP53, PTEN, CDH1, STK11, PALB2, CHEK2, ATM, and NBN have been shown to enhance the risk of breast cancer (Kobayashi *et al.* 2013, Ganten *et al.* 2009). DNA repair, cell cycle control, and tumor suppression are only some of the many biological processes in which these genes are engaged (Lundberg *et al.* 1987, Armstrong *et al.* 2000) These gene variants are associated with a higher risk of breast cancer, although they are far less prevalent than those in BRCA1 and BRCA2. Identifying women with these mutations via genetic testing helps direct screening and risk reduction efforts (Ford *et al.* 1998, Hellquist *et al.* 2015).

2.1.5 Clinical breast imaging techniques

Both the forms of cancer and the makeup of the breasts may affect the test's sensitivity and specificity. Breast cancer screening using mammography remains the gold standard, although it has limits, especially for younger women with denser breast tissue. In addition to its usefulness as an imaging modality, ultrasound suffers from drawbacks such as insufficient sensitivity and poor picture resolution. MRI is usually reserved for young women at high risk of breast cancer due to its high sensitivity but limited specificity (Table 2.1). However, CT has a limited sensitivity and substantial radiation risk when used to identify and image distant metastases. Injecting a radioactive tracer and exposing the patient to radiation are inherent risks of positron emission tomography (PET), which is used for functional imaging of biological processes and may aid in visualizing metastases or response to treatment. Microwave imaging (MI) methods provide a low-cost, radiation-free alternative to mammography for the detection of breast cancer, however additional study in this field is required. The purpose of most breast biopsies is to rule out cancer, but the procedure is costly and requires expert hands (Langsteger *et al.* 2016, Bai *et al.* 2023).

Table 2.1 Limitations of current breast screening methods

Type	Use	Sensitivity *	Specificity *	limitations	Time
Mammography	Widespread health checks. Think of a combination of bone, muscle, and blood vessels. Blocking light because of solid tissue	67.8%	75%	Ionizing radiation has poor sensitivity and specificity; as tissue density rises, it becomes less effective	few seconds
Ultrasound	Mammograms can detect and assess lumps; they are not intended for use on bones	83%	34%	Low sensitivity; evaluation by an expert operator is necessary; the resulting picture lacks detail;	10–20 min
MRI	High-risk young women; microscopic pictures of internal organs and tissues	94.4%	26.4%	The inability to diagnose malignancies such ductal and lobular carcinoma; high costs;	40–60 min
CT	A single check to detect and photograph distant metastases	91%	93%	Scanner is too costly, has low sensitivity, and poses radiation dangers;	5 min
PET	Imaging biological functions in real time. Metastasis imaging and therapeutic response imaging	61%	80%	Injection of radioactive tracers, exposure to ionizing radiation	90–240 min
*** Sensitivity and specificity are related to the types of cancer and breast composition.**					

Mammography

The most popular method of breast cancer screening, mammography, is not without its drawbacks. Women under the age of 40 and those with thick breast tissue may have a reduced response because dense breast tissue might obscure tiny cancers. Mammography is also less sensitive to small tumors, which may be missed in the screening. Additionally, mammography does not provide any information about the

eventual disease outcome, such as whether the tumor is aggressive or slow-growing (Onega *et al.* 2016, Lewis *et al.* 2017).

Contrast-enhanced (CE) digital mammography

Is a type of mammography that uses a contrast agent, typically iodine, to help highlight any abnormalities in the breast tissue. It's a novel method that helps detect breast cancer more precisely than mammography or ultrasound, particularly in people with thick breasts. However, it is not yet generally accessible because of its expensive price and greater radiation doses compared to conventional mammography. So, it is usually only utilized when there is a strong suspicion of breast cancer or when other imaging modalities have shown conflicting results (Ozmen *et al.* 2015).

Ultrasound

When assessing breast lumps or abnormalities detected by mammography, ultrasound is often utilized as a complementary imaging method, particularly for women with thick breast tissue. Ultrasound is a noninvasive, radiation-free method of imaging breast tissue using high-frequency sound waves. However, ultrasound has limitations in detecting small lesions and in differentiating between cancerous and benign masses, which is why it is often used in conjunction with other imaging techniques such as mammography or MRI (Roganovic *et al.* 2015).

Magnetic resonance imaging (MRI)

The combination of a magnetic field and radio waves makes MRI a potent imaging method that can provide high-resolution pictures of breast tissue. It's especially helpful for women with thick breast tissue, since mammography may sometimes overlook minor tumors. However, MRI is expensive, takes a long time, and may be unpleasant for some patients since they have to lay motionless in a small room for up to an hour. Overdiagnosis and needless biopsies may result from MRI's inferior specificity

compared to other imaging modalities. Women who have a high lifetime risk of developing breast cancer or who have abnormal results from previous imaging tests may benefit from a breast MRI (Klemm *et al.* 2008, Xu *et al.* 2015).

Positron emission tomography (PET)

Is the gold standard for showing where cancer has spread and how it has responded to treatment (Chen *et al.* 2013).

Microwave imaging (MI)

Low-power microwave radiation is used in microwave imaging (MI), a relatively recent method, to obtain three-dimensional pictures of breast tissue. Women with thick breasts or those at a greater risk of developing breast cancer may benefit from MI since it is a safe and inexpensive alternative to mammography with the potential to identify early-stage breast cancer. More study is required to verify its efficacy and accuracy as a breast cancer screening tool, however (Yen *et al.* 2016).

A breast biopsy is performed when a doctor suspects cancer in the breast and wants to remove a sample of tissue for testing. Fine-needle aspiration biopsy, core-needle biopsy, and surgical biopsy are all examples of breast biopsies. Biopsies are generally recommended when mammography or other imaging tests reveal a suspicious area in the breast. While biopsies can be expensive and require trained personnel, they are necessary to confirm a breast cancer diagnosis and determine the appropriate treatment plan (Ritchie and Johnson 2009, Cheng 2016).

2.1.6 Biomarkers for breast cancer detection

Protein biomarkers for breast cancer include progesterone receptor (PR), estrogen receptor (ER), and human epidermal growth factor receptor 2 (HER2) (Table 2.2). The degree to which these indicators are expressed may be used to categorize breast cancer

and guide treatment. Protein biomarkers such as CA15-3, CA27.29, and carcino-embryonic antigen (CEA) have also been studied for their potential to aid in the diagnosis of breast cancer (Li *et al.* 2015, Mittal *et al.* 2017).

Breast cancer biomarkers range from DNA and protein sequences to circulating tumor cells (CTCs) and ctDNA for diagnosis and monitoring. The two forms of ctDNA released into the bloodstream by tumor cells are called circulating tumor cells (CTCs). Both CTCs and ctDNA have the potential to provide information on tumor burden and treatment response, making them valuable tools in the diagnosis and monitoring of early-stage breast cancer and disease recurrence (Ye *et al.* 2015, Chatterjee and Zetter 2005).

Breast cancer identification and treatment planning might benefit from the use of biomarkers. However, further research is needed to develop more sensitive and specific biomarkers and to optimize methods for their detection and measurement (Porto-Mascarenhas *et al.* 2017, Le Naour *et al.* 2007).

Table 2.2 Breast cancer biomarkers

Biomarker	Technology Used for Discovery	Type
RS/DJ-1	**Serum profiling**	**Serum protein**
CA15-3 Golgi protien		
CA27-29		
HER-2		
p53	**Humeral response**	**autoantibody**
HSP60		
HSP90		
MUC1		
α-2-HS-Glycoprotein	**Nipple aspirate fluid profiling**	**Ductal protein**
Lipophilin B		
β-Globin		
Hemopexin		
Vitamin D-binding protein		

Proteomic biomarkers

Proteomic biomarkers are proteins that can be measured in biological samples, such as blood or tissue, to aid in the diagnosis, prognosis, and treatment of breast cancer (Metcalfe *et al.* 2000).

Proteomic indicators such RS/DJ-1, p53, HSP60, HSP90, MUC1, and HER2 have been studied for potential therapeutic uses in breast cancer.Women with newly diagnosed breast cancer had considerably greater RS/DJ-1 levels compared to healthy people, but this finding is not exclusive to breast cancer since other forms of breast cancers were not studied (Jiang *et al.* 2015).

Around 15% of breast cancer patients also have P53, which is also present in individuals with various malignancies and inflammatory disorders. Having an autoantibody against p53 predicts a lower chance of survival. Autoantibodies against HSP60 and HSP90 have also been used to diagnose breast cancer, although they are correlated with a dismal outcome (Jung *et al.* 2001).

Traditional biomarkers, such as CA15-3, have high specificity for late-stage breast cancer but low sensitivity for early-stage disease. It's often used to track treatment progress and spot relapses in metastatic breast cancer. CA15-3 is a surface protein released from the cell by proteolytic cleavage of MUC1, which is expressed in normal and cancerous breast epithelium (Duffy 1999, Asif *et al.* 2016).

Blood samples from humans may be tested for the presence of HER2, an antigen linked to breast cancer. About 30% of breast cancer patients had considerably greater levels than healthy persons (Srensen *et al.* 2013). Treatment decisions, such as the use of Herceptin, for people with HER2-positive breast tumors may be aided by monitoring HER2 levels (Molina *et al.* 2012). Serum HER2 levels, tumor size, nodal involvement, and tumor markers are all important prognostic indicators for both disease-free and overall survival (De Mattos-Arruda *et al.* 2013).

Genomic biomarkers

The use of genomic indicators in the diagnosis of breast cancer has gained prominence in recent years. The molecular subtypes of breast cancer are linked with variable clinical outcomes, and gene expression profiling is an effective method for detecting these subtypes (Gracia-Aznarez *et al.* 2013). The Onco-type DX test is the gold standard for gene expression profiling because it can predict whether or not a patient with early-stage, hormone receptor-positive breast cancer will have a recurrence and how well she will respond to treatment (Rasheed and Sandhyarani 2014).

The Breast Cancer Index (BCI) is a genetic biomarker for determining whether or not a patient with hormone receptor-positive breast cancer will benefit from extended endocrine therapy and what their risk of late-distant recurrence will be (Yang *et al.* 2016).

There is also a strong correlation between BRCA1 and BRCA2 gene mutations and an elevated risk of breast cancer (Nygaard *et al.* 2003). Women who have been diagnosed with breast cancer at an early age or who have a strong family history of the disease should be tested for BRCA mutations (Sutton *et al.* 2020). Mutations in other genes, such PALB2, have also been linked to an increased chance of developing breast cancer (Matamala *et al.* 2015).

2.1.7 MicroRNAs (miRNAs)

MicroRNAs (miRNAs) have been shown to be useful in the diagnosis and monitoring of cancer (Zhang *et al.* 2020). Cancer-related alterations in gene expression may be detected by using these short RNA molecules since they are stable and very sensitive and specific (Jemal *et al.* 2011). MiR-21 is widely utilized as a biomarker for cancer diagnosis since it is the most stable miRNA. Sequence similarity with related RNAs and presence in various malignancies are two constraints of miR-21 that might compromise

its selectivity. Cancer is a huge public health problem globally, but miRNAs show promise for its early identification and treatment (Kladney *et al.* 2000).

Golgi glycoprotein 73 (Golgi protein-73, GP73)

Breast, gallbladder, lung, and prostate cancers are only some of the cancers for which GP73 shows promise as a biomarker (Mao *et al.* 2018). Researchers have discovered a correlation between its expression levels and cancer patient outcomes, suggesting it may have use in the clinic. The therapeutic value of GP73 as a cancer biomarker has not yet been completely explored, and further research and validation studies are required (Li, 2018). Proteins, lipids, and other biomolecules are all processed, modified, and transported by the Golgi apparatus (Bao *et al.* 2013). Breast cancer is only one kind of cancer in which the GP73 protein, which resides in the Golgi, is overexpressed (Yang *et al.* 2017, Ibrahim *et al.* Due to its high sensitivity and specificity in identifying breast cancer cells, GP73 has been proposed as a possible tumor marker for early detection of breast cancer (Liu *et al.* 2015). According to research by Kladney *et al.* (2000), aberrant expression of GP73 and other Golgi-resident proteins may have a role in carcinogenesis. In order to create new diagnostic and therapeutic techniques for cancer therapy, it is crucial to comprehend the function of GP73 and other Golgi-resident proteins (Kellokumpu *et al.* 2002).

Function of GP73

Hepatocellular carcinoma (HCC) has been linked to GP73 expression, as have breast and colon cancers (Zhang *et al.* 2019). Research suggests that GP73 may have a role in the development of drug resistance in cancer cells (Waidely *et al.* 2016, Lin *et al.* 2016), as well as in the control of tumor cell proliferation, apoptosis, and migration (Ozal *et al.* 2011). Since GP73 has been demonstrated to stimulate the production of cytokines and chemokines in liver cells, it also seems to have a role in the immune response. More study is required to reveal the molecular processes through which GP73 contributes to the onset and development of cancer.

GP73 in breast cancer

Breast cancer patients have been shown to have higher blood levels of GP73, suggesting that this protein may serve as a biomarker for the disease (Zhang *et al.* 2019). It's possible that GP73 helps breast cancer cells multiply and spread. It was first published in 2012 by Zhang and Cao. GP73 may have a role in regulating the p53 tumor suppressor pathway, since its overexpression has been linked to enhanced proliferation, reduced apoptosis, and decreased p53 mRNA expression in breast cancer cells (Shui and Tang 2018). These results provide light on the potential diagnostic and therapeutic use of GP73 in breast cancer. In order to completely comprehend the biological role of GP73 in breast cancer and its potential as a clinical biomarker, additional study is required (Pourzand *et al.* 2011).

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Laboratory equipment and materials used in the study

Specialized hormonal materials, Specialized biochemical proteins, and other laboratory equipment and materials were used in this investigation: Laboratory equipment used

Device name	Origin
Deep freezer	Germany
Centrifuge	Germany
Incubator	Germany
Pipette types (30- 1000) µL	China
Spectrophotometer	UK
Water bath	China
Pipette types (30- 1000) µL	China
Gel tubes	China
Eppendorf	China

Specialized hormonal receptors and protein kits used

Subject Name	The manufacture company
Golgi protein 73 receptor kit	USA
Estrogen hormone receptor kit	USA
Progesterone hormone receptor kit	UK
Human cancer antigen15.3 receptor kit	UK
Human epidermal growth factor receptor 2 kit	UK

3.1.2 Golgi Protien 73

Principle: This kit is designed to determine the concentration of Human GP-73 in a given sample by using a microtiter plate coated with a solid-phase antibody that was created by adding GP-73 to the wells of the plate. The GP-73 antibody and labeled HRP are mixed together to form an antigen-antibody-enzyme complex; after washing, the TMB substrate solution is added; the substrate turns blue when catalyzed by the HRP enzyme; the reaction is stopped with a stop solution; and the resulting color change is measured at 450 nm. After comparing sample and standard absorbance values (O.D.), we may calculate the concentration of GP-73 in the samples.

Procedure:

Step 1: Follow the instructions in the preceding sections to have your reagents, working standards, Blank, and samples ready to go.

Step 2: Once the desired number of wells has been determined using the Assay Layout Sheet, any unused wells should be returned to the pouch together with the desiccant, sealed, and stored at 4 degrees Celsius.

Step 3: Pipette 50 ml of the testing standard into the standard well. For each testing sample well, pipette 40 mL of sample diluent in, followed by 10 mL of testing sample (for a total sample dilution of 5 times).

Step 4: Incubate: Incubate for 30 minutes at 37 degrees and cover with the supplied adhesive strip.

Step 5: Configurate liquid: Washing solution should be diluted with distilled water 30 times.

Step 6: Washing: Remove the backing, pour off the liquid, and then pipette washing buffer into each well; let sit for 30 seconds before draining, and do this five times.

Step 7: Add enzyme: To each well, besides the blank well, pipet 50 l of HRP-Conjugate reagent.

Step 8: Incubate: Operation with 4

Step 9: Washing: Operation with 6.

Step 10: Color: Pipette 50ul of Chromogen Solution A and 50ul of Chromogen Solution B into each well, keeping them dark for 15 minutes at 37 degrees Celsius.

Step 11: Stop the reaction: Pipette Halt Remedy Add 50 ml of solution (the blue becoming yellow) to each well and halt the process. Step 12: In the calculation, a blank might be treated as a zero. After 15 minutes, check absorbance at 450 nm after pipetting Stop Solution.

Calculation of results:

Construct a standard curve by plotting the reference concentration along a horizontal axis and the optical density along a vertical axis. Solving the straight line regression equation of the standard curve with the standard concentration and the OD value, where OD value is the OD reading from the sample, yields the dilution factor, which is multiplied by the OD value to yield the sample concentration.

- Progesterone ELISA kiT:

Principle: The progesterone is a competitive ELAISA in the solid phase. Streptavidin-coated wells receive samples, an enzyme conjugate of active progesterone, and an anti-progesterone biotin reagent. The patient's progesterone levels are compared to those of a progesterone HRP conjugate. The compound of progesterone and the progesterone enzyme is removed by washing it with a buffer. The amount of Progesterone present in a sample is reflected in its color after being treated with the TMB substrate. Color intensity is shown as a function of Progesterone concentration on a standard curve.

Procedure: All reagents should be at room temperature before being used in an experiment. Before using, thoroughly combine all reagents.

1. Add as many coated strips as you want to the holder.
2. Standard, control, and patient serum samples of progesterone, pipetted at 20 I.

3. To each well, add 1 OOI of active progesterone enzyme conjugate.
4.
5. To all of the wells, add 50 LI of Progesterone Biotin Conjugate.
6. 60 minutes of incubation time at 20-25 degrees Celsius.
7. Drain the water from every well. Three times through with 300 cc of 1X wash buffer, wash the wells. Remove excess liquid with paper towels.
8. TMB substrate, 100 LI per well.
9. Hold at room temperature for 15 minutes to incubate.
10. To all wells, inject 50 j..ll of the stop solution. Gently shake the plate to combine the contents.
11. 15 minutes after adding the stop solution, check the absorbance at 450 nm using the ELISA Reader.

Calculation of results:

This is how the standard curve is built:

1. Verify the Progesterone value on each vial of standard solution. Lot to lot, this figure might shift. Every kit should have its value verified. Attached is a sample of the required format.
2. The absorbance of the Progesterone standards (the vertical axis) is graphed against the concentrations of the Progesterone standards (the horizontal axis) to produce the standard curve. Find the best line to connect the spots.
3. Take the absorbance values off the curve for the control and unknown samples. Every sample, control or otherwise, should have its value recorded. The Typical Curve.

   Standard Optical Units (450 nm

- Human estrogen ELISA kiT:

Detection principle: This kit employs the Double Antibody Sandwich ELISA technique, as was previously mentioned. The human estrogen-specific monoclonal antibody serves as the main antibody, while the biotinylated polyclonal antibody serves as the secondary. The wells of the ELISA plate are washed with PBS or TBS after the addition of samples and biotinylated antibodies. Conjugates of Avidin and peroxidase will then be added to the wells. Wells are stained with a TMB substrate after enzyme conjugate is washed off with PBS or TBS. TMB reacts due to peroxidase activity, yielding a blue product that is converted to yellow by the addition of a stop solution (Color Reagent C). The concentration of the analyte of interest correlates positively with the intensity of the color.

Procedure

1. Once the ELISA Kit has been brought to room temperature (after being removed from the fridge), the test may begin.
2. Washing buffer is concentrated, thus diluting it with double-distilled water (1:25) is recommended. Put back any leftovers in the box.
3. Standard: To a lyophilized standard vial, add 1.0 ml of Standard Diluent and let it rest for 30 minutes. Label the tube once the standard has been dissolved and given a gentle stir. The following concentrations are suggested for use in constructing a standard curve: 1000, 500, 250, 125, 62.5, 31.2, 15.6pg/ml. Be sure that the lyophilized standard has fully dissolved and is well blended.
4. Sample dilution according to industry standards: Prepare the following seven clean tubes with the indicated concentrations: (500), (250), (125), (62.5), (31.2), (15.6), and (0). Each tube needs 300 mL of Standard. Diluent. The reconstituted standard requires 300 l of diluent, which may be pipetted into a tube marked 500 pg/mL and combined well. Further Take 300 l of the diluent out of the 500 pg/mL tube and add it to the 250 pg/mL solution. Proceed with this procedure until you reach the 15.6pg/mL threshold. The 0 pg/mL tube of Standard Diluent serves as a negative control.

5. Biotinylated Antibody: Take the amount of solution needed for the number of wells in your experiment, multiply it by 100, and then dilute it with the Antibody Diluent. We strongly advise against reusing this for future assays and suggest starting 30 minutes in advance.
6. Take off enough of the enzyme conjugate solution to cover the number of wells you want to test, and dilute it with the enzyme diluent such that the final concentration is 1:100. We strongly advise against reusing this for future assays and suggest starting 30 minutes in advance.
7. Colorant: Mix 9 parts Colorant A to 1 part Colorant B 30 minutes in advance to make Colorant solution.

Result

1. Subtract the blank well's OD value from each sample's and the standard's. Standard curve will be drawn manually. Use OD measurements as the Y-coordinate and standard concentration values (X-coordinate). Draw a straight line between the reference points of the normative values.
2. Third, the OD values of the samples may be entered into the line equation for the standard curve to get the concentration of the samples.
3. Professional curve software (like curve expert 1.3) should be used for analysis and computation of the findings.
4. The sample should be diluted (or further diluted) and the test rerun if its optical density (OD) is greater than that of the highest standard on the standard curve. To get the unidentified variable, multiply the known variables by the dilution factor.

CA15-3 ELISA KIT:

Principle: The CA15-3 ELISA is a modified version of a solid phase sequential sandwich ELISA. Streptavidin-coated wells are used to add samples and biotinylated monoclonal antibody. Biotinylated capture antibody binds CA15-3 from patient sample. At the same time, the biotinylated antibody binds to the streptavidin-coated plate. After

washing, the trapped CA15-3 is sandwiched between two layers of anti-CA15-3-HRP enzyme conjugate. Washing removes unbound antibodies. When the TMB substrate is introduced, a bluish hue is produced. The intensity of the resulting color is related to the CA15-3 concentration. The concentration of CA15-3 is shown as a function of color intensity on a standard curve.

Procedure

Bring all specimens and kit reagents to room temperature (20-25c) and gently mix.

1. In order to utilize patient samples, they must first be diluted 10-fold. Don't lower the bar.
2. After inserting the necessary number of coated wells into the holder, dispensing 25 ml of CA15.3 standards, diluted samples, and diluted controls into the correct wells, and securing the holder, you're done!
3. Put 10 ml of the antibody-biotin conjugate reagent (the blue solution) into each well. Slowly combine at 500-600 rpm for 20-30 seconds.
4. Let stand at room temperature for 60 minutes.
5. Fifth, drain all the water from the wells. Each well should be washed with 350 µL of 1x wash buffer three times. After each wash, place the plate upside down on absorbency paper and give it a quick, forceful tap to eliminate any remaining drips.
6. Place 100 µl of enzyme conjugate (red solution) in each well, and then proceed to.
7. Let it sit out at room temperature for an hour.
8. Empty the plate and wash it three times before using it again.
9. Fill each well with 100 µL of the TMB solution.
10. Let sit for 15 minutes at room temperature to incubate.
11. To halt the reaction, add 50 µl of the stop solution to each well at step.
12. Microtiter plate absorbance at 450 nm must be read within 15 minutes.

Calculations and Results

1. The first step is to determine the mean absorbance of each group of standards, controls, and samples.
2. Using linear graph paper, with absorbance on the vertical (y) axis and concentration on the horizontal (x) axis, construct a standard curve by graphing the mean absorbance obtained for each reference standard against its concentration in U/ml.
3. From the standard curve, calculate the concentration of - CA15.3 in U/ml using the mean absorbance value for each sample.

Human epidermal receptor 2[HER2]:

Principle: Included in this set is a microplate that has been pre-coated with an antibody specific for EGFR2. Next, a biotin-conjugated antibody specific for EGFR2 is incubated with the microplate wells containing the standards or samples. Avidin conjugated to Horseradish Peroxidase (HRP) is applied to microplate wells that have been pretreated. The addition of TMB substrate solution will cause a change in coloration only in the wells that initially contained EGFR2, biotin-conjugated antibody, and enzyme-conjugated Avidin. After the enzyme-substrate reaction has been halted with a sulphuric acid solution, the color change is measured spectrophotometrically at a wavelength of 450 nm 10 nm. The concentration of EGFR2 in the samples may be determined by comparing their optical densities to those of the reference curve.

Procedure

1. Create dilution wells, blank wells, and sample wells. Get ready 7 standard wells and 1 blank well.
2. Pour 100 μL of standard (see to Reagent Preparation), blank, and sample dilutions into their respective wells. Place the Plate sealer on top. Let it sit at 37 degrees for an hour.
3. Third, drain each well without washing.
4. Reagent for Detection (100 μL) Incubate the plate at 37 degrees Celsius for one hour after putting a working solution in each well.

5. Transfer 350 µL of 1 Wash Solution to each well using a squirt bottle, multichannel pipette, manifold dispenser, or autowasher. Any remaining liquid may be collected by snapping the plate into absorbent paper. Scrub the area thoroughly three times. After the last wash, you should aspirate or decant off any remaining Wash Buffer. Invert the dish over a sheet of absorbent paper to drain any remaining liquid.
6. After the wells have been sealed with the plate sealer, add 100 µL of the working solution of Detection Reagent B to each well and incubate at 37 °C for 30 minutes.
7. Perform the aspiration and wash procedure five more times.
8. Calls for the addition of Substrate Solution (90 µL) to each well. You should cover it with some new Plate sealant. Incubate for 10-20 minutes (no more than 30) at 37 degrees Celsius. Filter out the light. Substrate Solution turns liquids a dark blue when added to them.
9. A volume of 50 µL of the Stop Solution should be added to each well. The addition of the Stop solution causes the fluid to change color. The liquids may be mixed by tapping the plate's edge. Give the plate a light slap to make sure everything is well-combined if the change in color is abrupt.
10. Verify that no air bubbles have formed on the liquid's surface by drying the plate's base. Then, quickly after, use a microplate reader to take readings at 450nm.

4. RESULTS AND DISCUSSION

Take the mean of the replicated readings and subtract the mean zero to get the average for the standard, control, and samples. Construct a standard curve by plotting the mean O.D. and concentration for each standard and drawing a best fit curve through the points on the graph, or generate a standard curve using log-log graph paper with EGFR2 concentration on the y-axis and absorbance on the x-axis. In addition, you need use plot software such as curve expert 1.30. Standard curve concentrations need to be corrected for sample dilution when dealing with diluted samples.

4.1 Design of Study

After obtaining consent from each participant, 150 female cases were included. As shown in the following unit, these totes are separated into four categories: Grouping patients: Participants were 116 women seeking diagnosis for breast cancer at an oncology teaching hospital (42 with newly diagnosed disease, 74 under treatment, 26 with benign disease, and 34 without the disease).

This research was conducted at the Biochemistry labs of the cancer teaching Hospital in the medical city of Baghdad, Iraq. From January 1st to April 1st, 2023, the observer will be present for a total of three months.

4.2 Sample Collection

After hysterectomy, venous blood from women in the four groups (healthy, benign, newly diagnosed breast cancer, and breast cancer under treatment) pooled in gel tubes to a depth of five millimeters. After taking blood samples in a gel tube, we centrifuge them at 4000 rpm for 10 minutes. After using a tiny pipette to separate the serum from the opposing cells, it is placed in three EPPENDORF tubes and stored at -20 ° C until it is needed for the test.

4.3 Results

In this chapter we will review the statistical analysis results and their discussion for women afflicted by breast cancer with different groups as follows: 116 women with breast cancer 42 newly diagnosis, aged between 35 and 61 years & 74 under treatment, between 30 and 75 years; In addition to, 26 benign tumors, between 20 and 62 years, were involved in the study, a 34 apparently healthy women formed control subjects; their ages ranged from 23 to 62 years.

From (Table 4.1 and Figure 4.1), showed there were a statistically significant difference (P = 0.034 at P<0.05), when compared between age groups / Year of studied groups; with (41 – 60 Year) predominant in {Healthy control 24, (70.6%), newly diagnosis 30, (71.4%) & under treatment 54, (73%)}, except benign tumor {20 – 40 year 16, (61.5%)}.

While within groups, documented that a highly significant difference (P = 0.00, P = 0.003, P = 0.00 & P = 0.00 respectively at P<0.01).

Table 4.1 Age groups / Year distributions according to studied groups

Age groups / Year		**Studied groups**				**P - value**
		Healthy control	**Benign tumor**	**Newly diagnosis BC**	**Under treatment BC**	
20 - 40	N	6	16	10	10	**P = 0.034 Sign. (P<0.05)**
	%	17.6%	61.5%	23.8%	13.5%	
41 - 60	N	24	8	30	54	
	%	70.6%	30.8%	71.4%	73%	
61 - 80	N	4	2	2	10	
	%	11.8%	7.7%	4.8%	13.5%	
Total	N	34	26	42	74	
	%	100%	100%	100%	100%	
P - value		P = 0.00	P= 0.003	P = 0.00	P = 0.00	

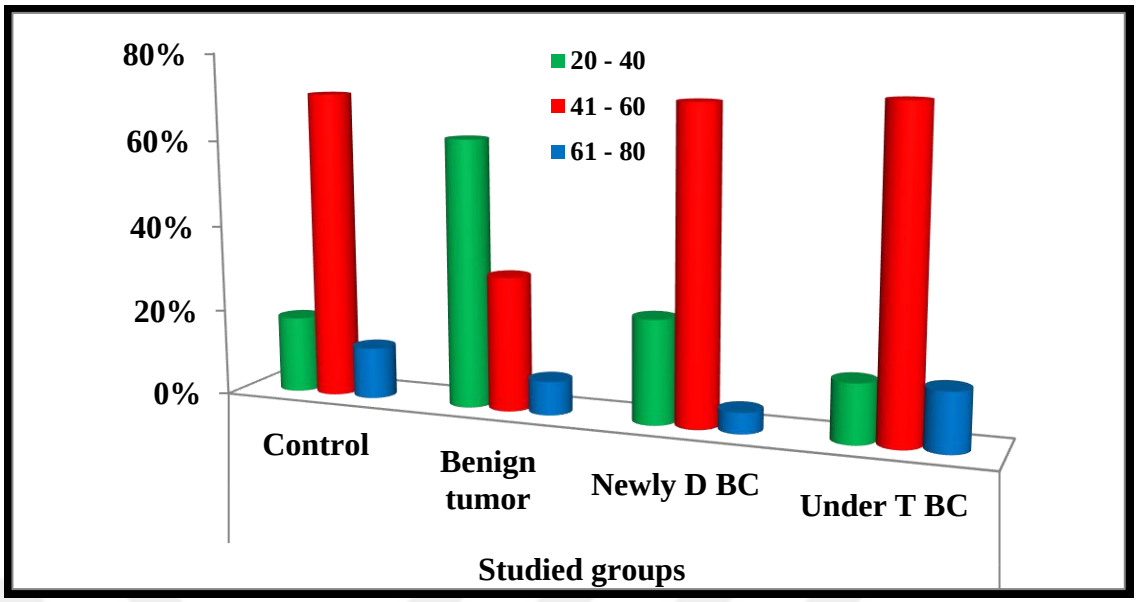

Figure 4.1 Age groups / Year distributions according to studied groups

Moreover, (Table 4.2 and Figure 4.2), noted that there was a similarity between studied groups mean age / Year; healthy control (46.82 ± 12.413), benign tumor (39.54 ± 13.969), newly diagnosis (48.14 ± 7.754) & under treatment (48 ± 9.798), with non-significant difference (P = 0.091 at P>0.05). Although, within groups Table 4.3, presented that non-significant difference (P = 0.091 at P>0.05); except the comparison between (Benign tumor Vs Newly diagnosis: P = 0.026) & (Benign tumor Vs under treatment: P = 0.017) were significant difference (at P<0.05).

Table 4.2 Mean age / Year distributions among studied groups

Age / Year							
Studied groups	**N**	**Mean**	**Std. Deviation**	**Std. Error**	**Range**		**P - value**
					Mini.	**Maxi.**	
Healthy control	**34**	**46.82**	**12.413**	**2.129**	**23**	**62**	**P = 0.091 Non sign. (P>0.05)**
Benign tumor	**26**	**39.54**	**13.969**	**2.740**	**20**	**62**	
Newly diagnosis BC	**42**	**48.14**	**7.754**	**1.196**	**35**	**61**	
Under treatment BC	**74**	**48**	**9.798**	**1.139**	**30**	**75**	
Total	**176**						

Table 4.3 Multi comparison of mean age / Year among studied groups

Age / Year		
Studied groups		**P - value**
Healthy controls	Benign tumor	0.071
	Newly diagnosis BC	0.708
	Under treatment BC	0.711
Benign tumor	Newly diagnosis BC	0.026
	Under treatment BC	0.017
Newly diagnosis BC	Under treatment BC	0.961

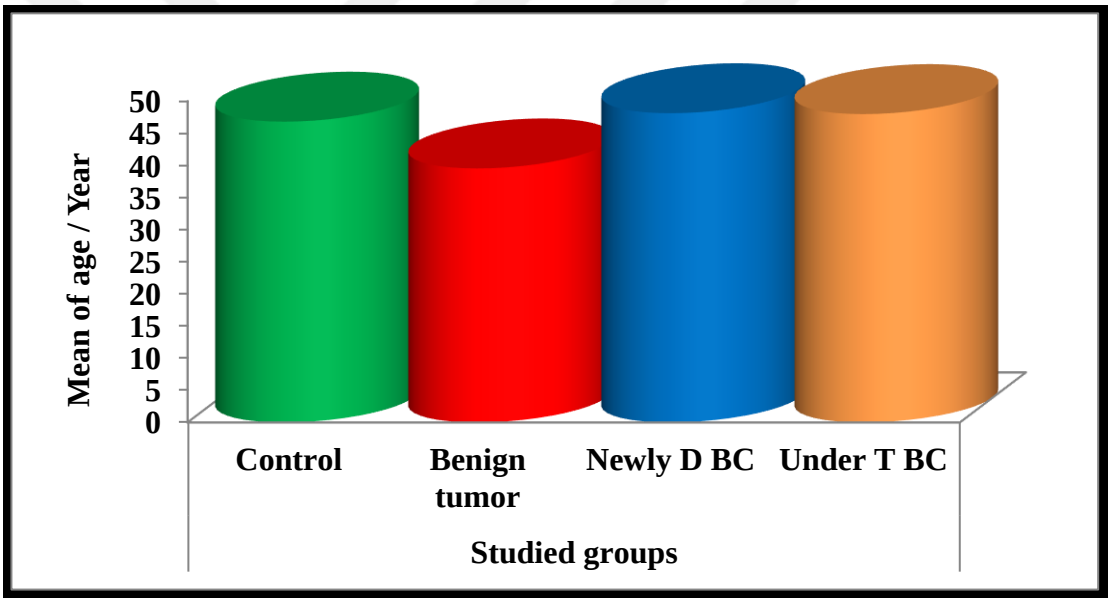

Figure 4.2 Mean age / Year distributions among studied groups

Study (Table 4.4 and Figure 4.3), presented that a significant difference (P = 0.031 at P<0.05), when compared between studied groups of family history breast cancer; without history of the disease was high frequency in healthy control 30, (88.2%) than with history 4, (11.8%), but in other groups was comparable frequency even with or without history of the disease; benign tumor: without history 14, (53.8%) & with history 12, (46.2%), newly diagnosis: without history 24, (57.1%) & with history 18, (42.9%) & finally, under treatment: without history 34, (45.9%) & with history 40, (54.1%).

Whereas, within groups, showed that a highly significant difference (Healthy control: P = 0.00, at P<0.01) & non-significant difference (Benign tumor: P = 0.845, newly diagnosis: P = 0.441 & under treatment: P = 0.561 at P>0.05).

Table 4.4 Family history distributions according to studied groups

Family History		Studied groups				P - value
		Healthy control	Benign tumor	Newly diagnosis BC	Under treatment BC	
With	N	4	12	18	40	P = 0.031 Sign. (P<0.05)
	%	11.8%	46.2%	42.9%	54.1%	
Without	N	30	14	24	34	
	%	88.2%	53.8%	57.1%	45.9%	
Total	N	34	26	42	74	
	%	100%	100%	100%	100%	
P - value		P = 0.00	P=0.845	P = 0.441	P = 0.561	

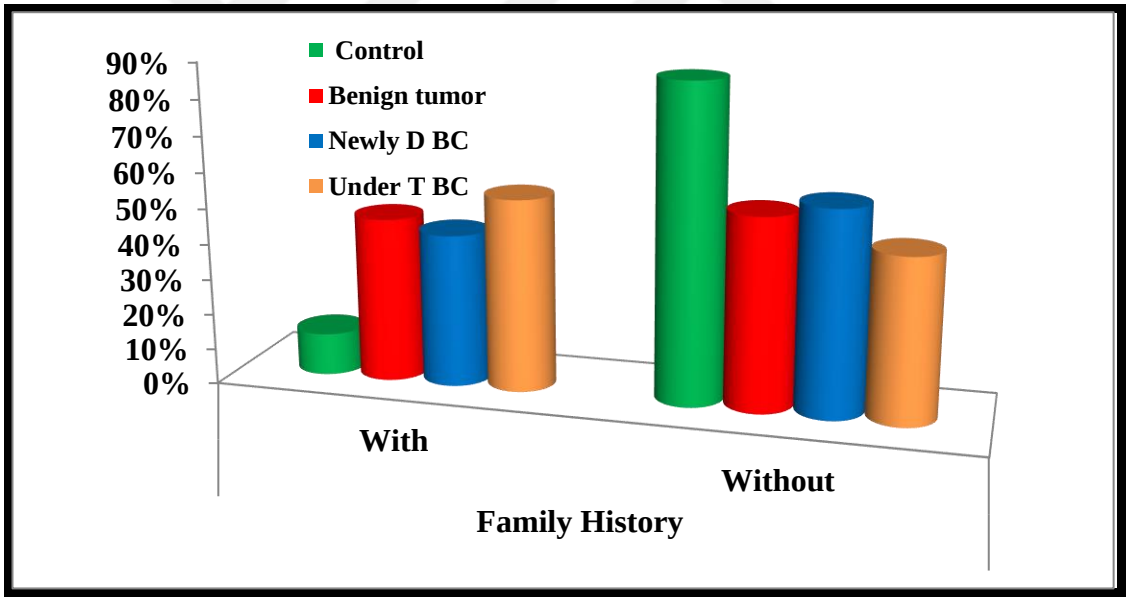

Figure 4.3 Family history distributions according to studied groups

Table 4.5 and Figure 4.4, presented that a non-significant (P = 0.299 at P>0.05), when compared between studied groups of social status with breast cancer disease; married {Healthy control 28, (82.4%), benign tumor 18, (69.2%), newly diagnosis 38, (90.5%) & under treatment 66, (89.2%)}; more than single {Healthy control 6, (17.6%), benign tumor 8, (30.8%), newly diagnosis 4, (9.5%) & under treatment 8, (10.8%)}. However,

within groups, showed that a non-significant difference (Benign tumor: P = 0.076, at P>0.05) & highly significant difference (P = 0.00, at P<0.01) for other groups.

Table 4.5 Social status distributions according to studied groups

Social Status		Studied groups				P - value
		Healthy control	Benign tumor	Newly diagnosis BC	Under treatment BC	
Married	N	28	18	38	66	P = 0.299 Non sign. (P>0.05)
	%	82.4%	69.2%	90.5%	89.2%	
single	N	6	8	4	8	
	%	17.6%	30.8%	9.5%	10.8%	
Total	N	34	26	42	74	
	%	100%	100%	100%	100%	
P - value		P = 0.00	P=0.076	P = 0.00	P = 0.00	

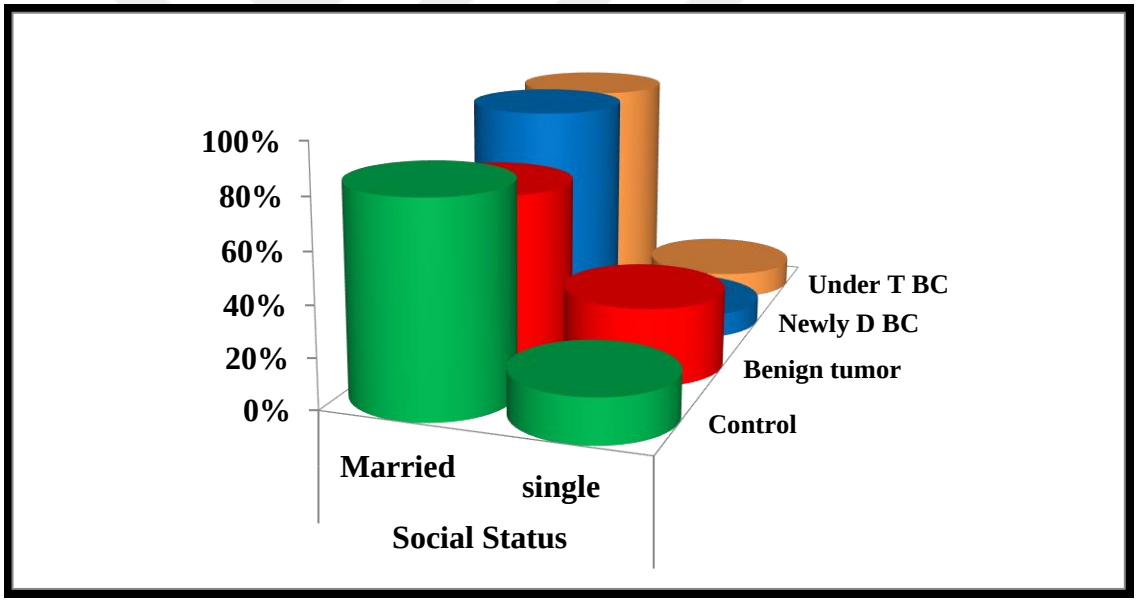

Figure 4.4 Social status distributions according to studied groups

Additionally, grade of breast cancer disease reviewed in study (Table 4.6 and Figure 4.5), renowned that a non-significant (P = 0.141 at P>0.05), when compared between studied groups {newly diagnosis: grade II = 16, (38.1%) & grade III = 14, (33.3%)} & {under treatment: grade II = 36, (48.6%) & grade III = 24, (32.4%)}; larger than other grades.

Within groups, showed that a significant difference (Benign tumor: P = 0.012, at P<0.05) & highly significant difference (P = 0.00, at P<0.01) for under treatment.

Table 4.6 Grade of breast cancer disease distributions according to studied groups

Grade		Studied groups		P - value
		Newly diagnosis BC	Under treatment BC	
I	N	10	6	P = 0.141 Non sign. (P>0.05)
	%	23.8%	8.1%	
II	N	16	36	
	%	38.1%	48.6%	
III	N	14	24	
	%	33.3%	32.4%	
IIII	N	2	8	
	%	4.8%	10.8%	
Total	N	42	74	
	%	100%	100%	
P - value		P = 0.012	P = 0.00	

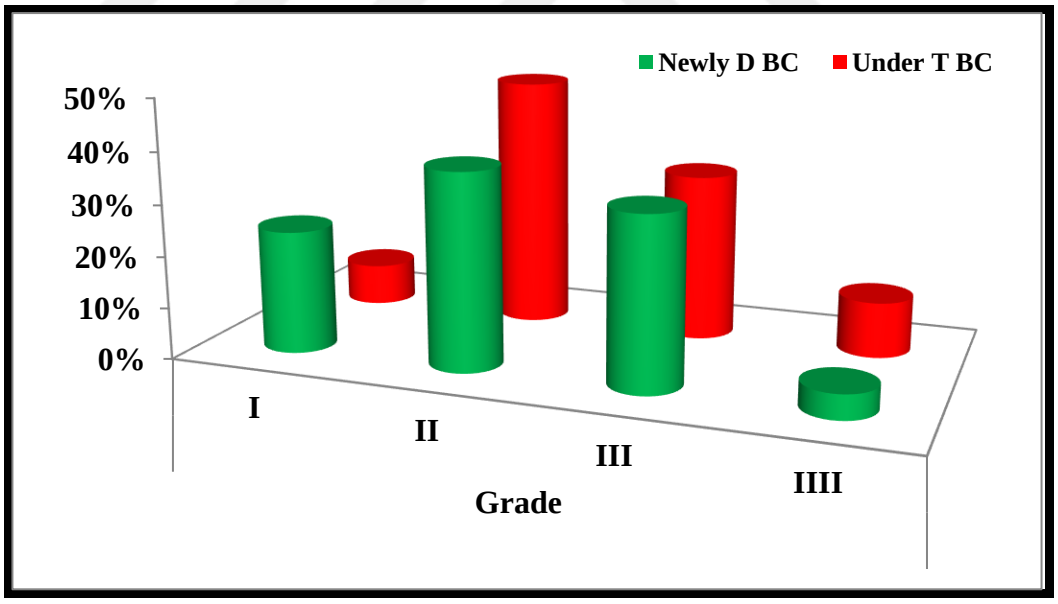

Figure 4.5 Grade of breast cancer disease distributions according to studied groups

Also, a highly significant (P = 0.004 at P<0.01), when studied the results of HER2 tumor marker test in sera of breast cancer patients {newly diagnosis: Positive: 22, (52.4%) & Negative: 20, (47.6%)} & {under treatment: Positive: 58, (78.4%) more than Negative: 16, (21.6%)}.

Within groups, displayed that a non-significant difference (newly diagnosis: P = 0.878, at P>0.05) & highly significant difference (under treatment: P = 0.00, at P<0.01), (Table 4.7 and Figure 4.6).

Table 4.7 HER2 tumor marker result distributions according to studied groups

HER2		**Studied groups**		**P - value**
		Newly diagnosis BC	**Under treatment BC**	
Positive	**N**	22	58	**P = 0.004 Highly sign. (P<0.01)**
	%	52.4%	78.4%	
Negative	**N**	20	16	
	%	47.6%	21.6%	
Total	**N**	42	74	
	%	100%	100%	
P - value		**P = 0.878**	**P = 0.00**	

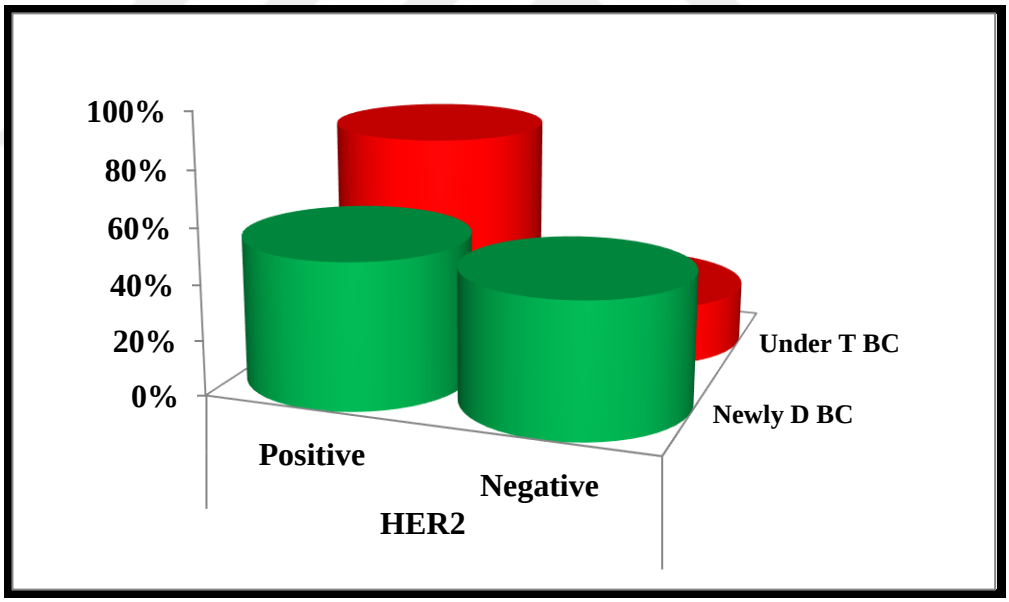

Figure 4.6 HER2 tumor marker result distributions according to studied groups

Furthermore, a non-significant (P = 0.811 at P>0.05), when studied the results of progesterone receptor (PR) tumor marker test in sera of breast cancer patients {newly diagnosis: Positive: 38, (90.5%) raised than Negative: 4, (9.5%)} & {under treatment: Positive 72, (97.3%) increased than Negative: 2, (2.7%)}.

Within groups, exhibited that a highly significant difference (P = 0.00, at P<0.01) for both newly diagnosis & under treatment; (Table 4.8 and Figure 4.7).

Table 4.8 progesterone receptor (PR) tumor marker result distributions according to studied groups

progesterone receptor (PR)		Studied groups		P - value
		Newly diagnosis BC	Under treatment BC	
Positive	**N**	38	72	**P = 0.811 Non sign. (P>0.05)**
	%	90.5%	97.3%	
Negative	**N**	4	2	
	%	9.5%	2.7%	
Total	**N**	42	74	
	%	100%	100%	
P - value		**P = 0.00**	**P = 0.00**	

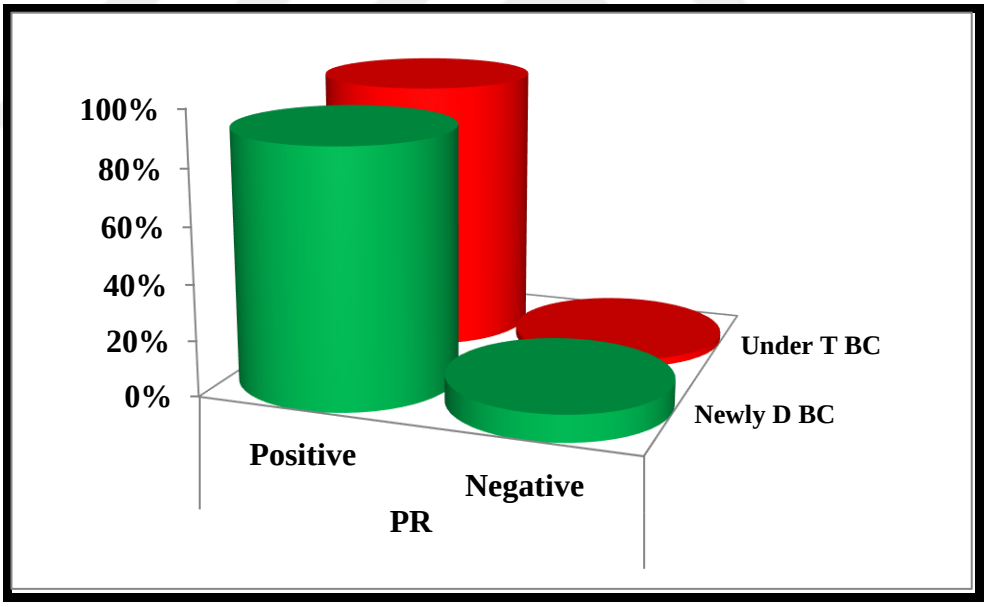

Figure 4.7 progesterone receptor (PR) tumor marker result distributions according to studied groups

Furthermore, a non-significant (P = 0.783 at P>0.05), when projected the results of estrogen receptor (ER) tumor marker test in sera of breast cancer patients {newly diagnosis: Positive: 36, (85.7%) higher than Negative: 6, (14.3%)} & {under treatment: Positive 62, (83.8%) bigger than Negative: 12, (16.2%)}.

Within groups, showed that a highly significant difference (P = 0.00, at P<0.01) for both newly diagnosis & under treatment; (Table 4.9 and Figure 4.8).

Table 4.9 Estrogen receptor (ER) tumor marker result distributions according to studied groups

Estrogen receptor (ER)		Studied groups		P - value
		Newly diagnosis BC	Under treatment BC	
Positive	N	36	62	P = 0.783 Non sign. (P>0.05)
	%	85.7%	83.8%	
Negative	N	6	12	
	%	14.3%	16.2%	
Total	N	42	74	
	%	100%	100%	
P - value		P = 0.00	P = 0.00	

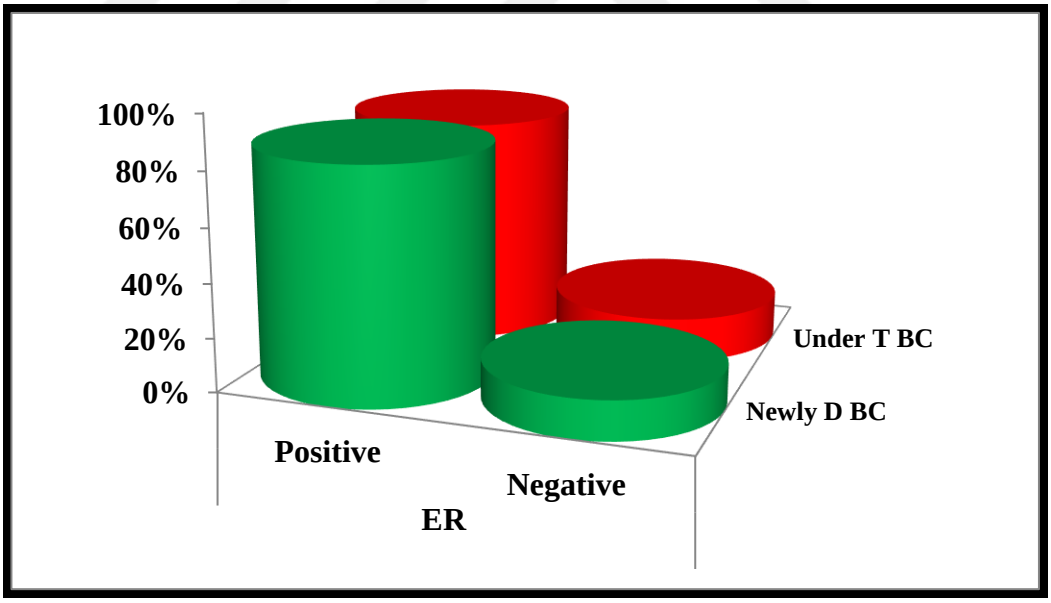

Figure 4.8 Estrogen receptor (ER) tumor marker result distributions according to studied groups

Besides, a non-significant (P = 0.891 at P>0.05), when estimated the results of Ca15 -3 tumor marker test in sera of breast cancer patients {newly diagnosis: Positive: 38,

(90.5%) elevated than Negative: 4, (9.5%)} & {under treatment: Positive 72, (97.3%) increased than Negative: 2, (2.7%)}.

Within groups, showed that a highly significant difference (P = 0.00, at P<0.01) for both newly diagnosis & under treatment; (Table 4.10 and Figure 4.9).

Table 4.10 CA15- 3 tumor marker result distributions according to studied groups

CA15 - 3		Studied groups		P - value
		Newly diagnosis BC	Under treatment BC	
Positive	**N**	38	72	**P = 0.891 Non sign. (P>0.05)**
	%	90.5%	97.3%	
Negative	**N**	4	2	
	%	9.5%	2.7%	
Total	**N**	42	74	
	%	100%	100%	
P - value		**P = 0.00**	**P = 0.00**	

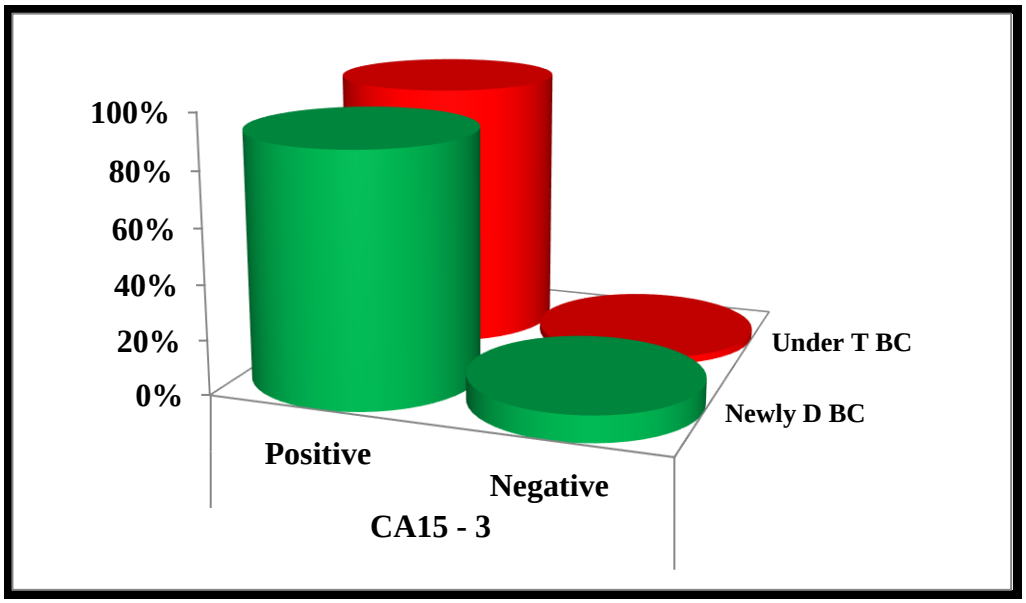

Figure 4.9 CA15- 3 tumor marker result distributions according to studied groups

Eventually, the (Table 4.11, Table 4.12, Table 4.13, Table 4.14, Table 4.15, Table 4.16, Table 4.17 and Table 4.18) and (Figure 4.10, Figure 4.11, Figure 4.12, Figure 4.13, Figure 4.14 and Figure 4.15), proved that there was a similar mean of human Golgi protein 73 assay in sera of breast cancer patients & their healthy controls with low levels in all above studies.

With non-significant difference (at P>0.05); except a significant difference (P = 0.028 at P<0.05) when comparison between grade I Vs grade III; in (Table 4.14 and Figure 4.11).

Table 4.11 Mean human Golgi protein 73 distributions among studied groups

Human Golgi protein 73							
Studied groups	**N**	**Mean**	**Std. Deviation**	**Std. Error**	**Range**		**P - value**
					Mini.	**Maxi.**	
Healthy control	**34**	**0.3973**	**0.33002**	**0.0566**	**0.03**	**1.38**	**P = 0.607 Non sign. (P>0.05)**
Benign tumor	**26**	**0.3480**	**0.26481**	**0.0519**	**0.03**	**0.95**	
Newly diagnosis BC	**42**	**0.4074**	**0.35999**	**0.0555**	**0.03**	**1.33**	
Under treatment BC	**74**	**0.3148**	**0.19577**	**0.0227**	**0.02**	**0.80**	
Total	**176**						

Table 4.12 Multi comparison of mean human Golgi protein 73 among studied groups

Human Golgi protein 73		
Studied groups		**P - value**
Healthy controls	Belgian tumor	**0.638**
	Newly diagnosis BC	**0.913**
	Under treatment BC	**0.323**
Belgian tumor	Newly diagnosis BC	**0.553**
	Under treatment BC	**0.717**
Newly diagnosis BC	Under treatment BC	**0.235**

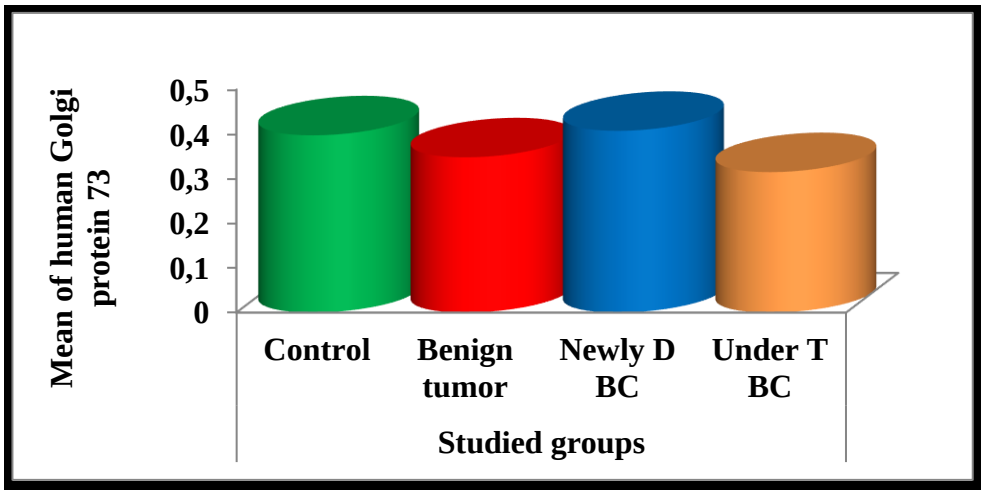

Figure 4.10 Mean human Golgi protein 73 distributions among studied groups

Table 4.13 Mean human Golgi protein 73 distributions among breast cancer grade

Human Golgi protein 73							
Grade	N	Mean	Std. Deviation	Std. Error	Range		P - value
					Mini.	Maxi.	
I	16	0.4149	0.41530	0.1038	0.03	1.33	P = 0.119 Non sign. (P>0.05)
II	52	0.3540	0.27201	0.0377	0.02	1.07	
III	38	0.3462	0.21221	0.0344	0.05	0.79	
IIII	10	0.2210	0.09383	0.0296	0.13	0.39	
Total	116						

Table 4.14 Multi comparison of mean human Golgi protein 73 among breast cancer grade

Human Golgi protein 73		
Grades		P - value
I	II	0.121
	III	0.028
	IIII	0.069
II	III	0.303
	IIII	0.331
III	IIII	0.689

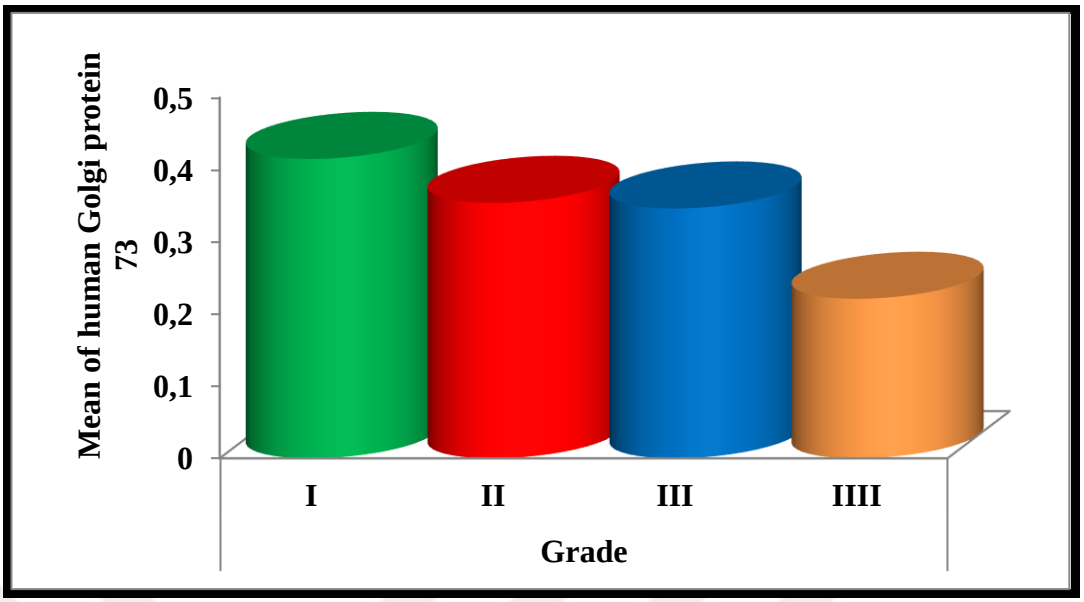

Figure 4.11 Breast cancer grade. Mean human Golgi protein 73 distributions among

Table 4.15 Mean human Golgi protein 73 distributions among HER2 tumor marker results

Human Golgi protein 73							
HER2	N	Mean	Std. Deviation	Std. Error	Range		P - value
					Mini.	Maxi.	
Positive	80	0.3383	0.20806	0.02326	0.02	0.89	P = 0.551 Non sign. (P>0.05)
Negative	36	0.3707	0.37397	0.06233	0.05	1.33	
Total	116						

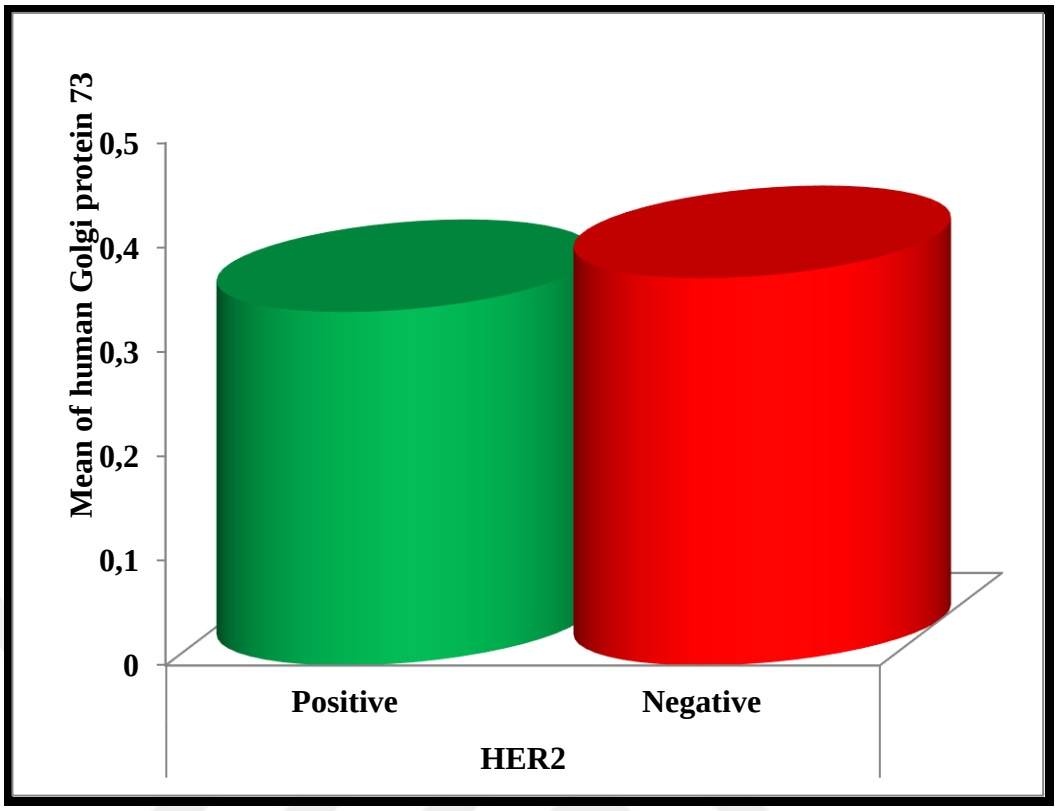

Figure 4.12 Mean human Golgi protein 73 distributions among HER2 tumor marker results

Table 4.16 Mean human Golgi protein 73 distributions among Progesterone receptor (PR) tumor marker results

Human Golgi protein 73							
Progesterone receptor (PR)	N	Mean	Std. Deviation	Std. Error	Range		P - value
					Mini.	Maxi.	
Positive	110	0.3484	0.27370	0.02610	0.02	1.33	P = 0.995
Negative	6	0.3477	0.18748	0.07654	0.14	0.56	Non sign.
Total	116						(P>0.05)

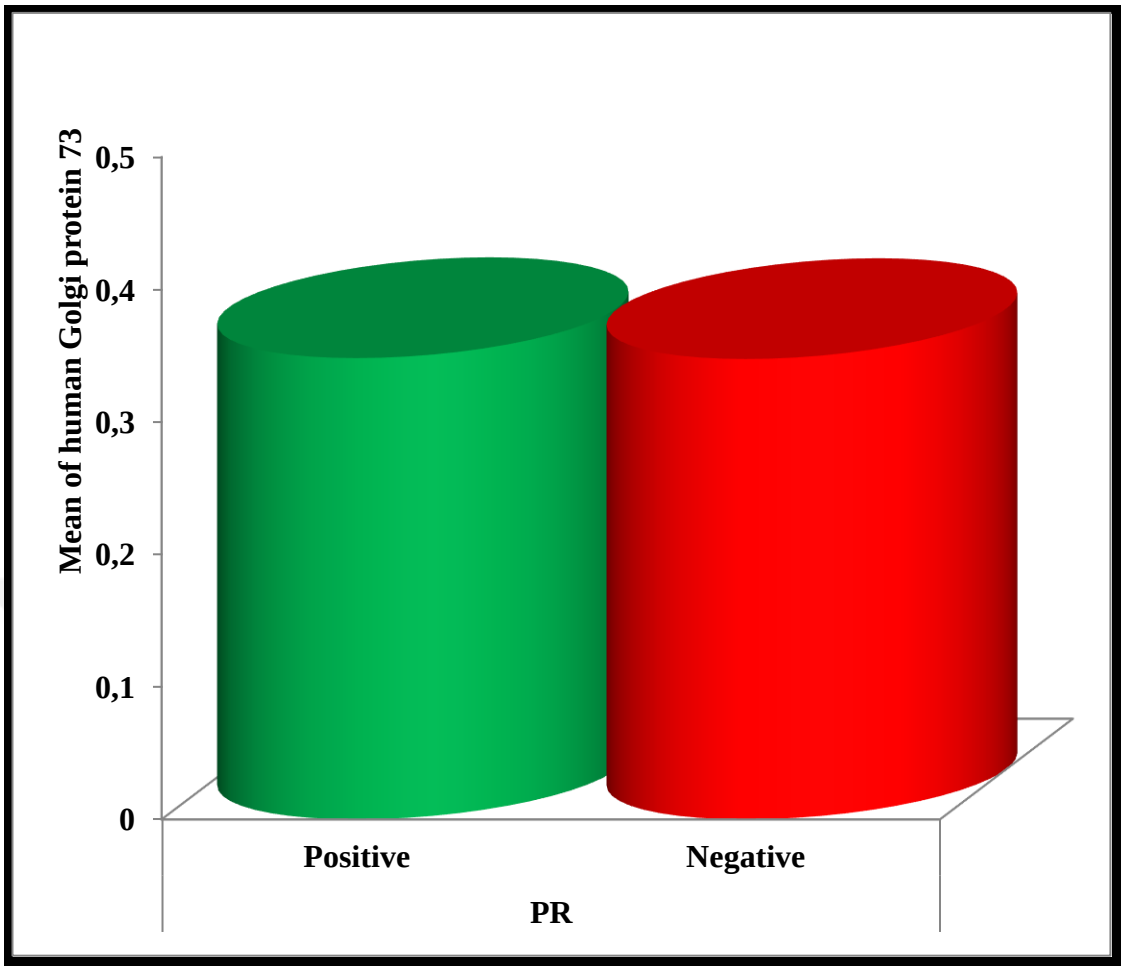

Figure 4.13 Mean human Golgi protein 73 distributions among Progesterone receptor (PR) tumor marker results

Table 4.17 Mean human Golgi protein 73 distributions among Estrogen receptor (ER) tumor marker results

Human Golgi protein 73							
Estrogen receptor (ER)	N	Mean	Std. Deviation	Std. Error	Range		P - value
					Mini.	Maxi.	
Positive	98	0.3411	0.28258	0.02855	0.02	1.33	P = 0.492
Negative	18	0.3880	0.18175	0.04284	0.17	0.65	Non sign.
Total	116						(P>0.05)

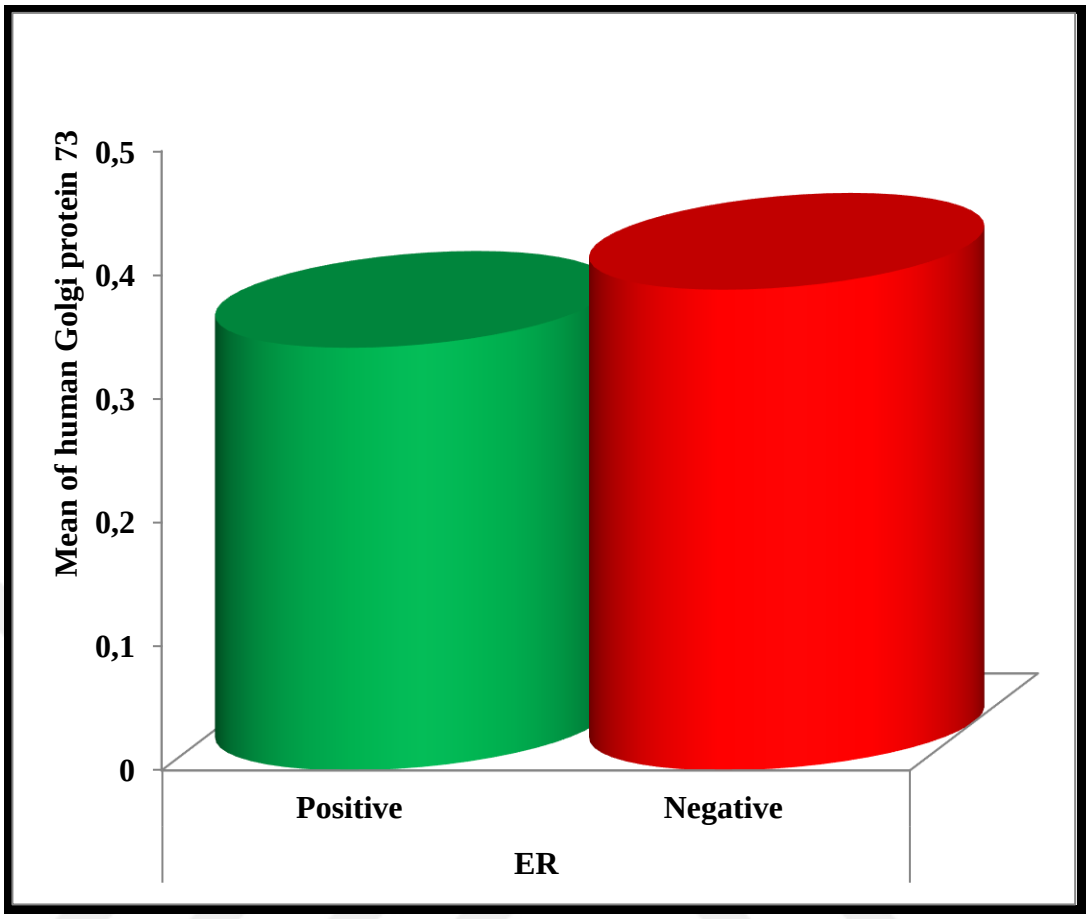

Figure 4.14 Mean human Golgi protein 73 distributions among Estrogen receptor (ER) tumor marker results

Table 4.18 Mean human Golgi protein 73 distributions among CA15 - 3 tumor marker results

Human Golgi protein 73							
CA15 - 3	N	Mean	Std. Deviation	Std. Error	Range		P - value
					Mini.	Maxi.	
Positive	110	0.3389	0.26635	0.02540	0.02	1.33	P = 0.105
Negative	6	0.5227	0.28832	0.11771	0.17	0.80	Non sign.
Total	116						(P>0.05)

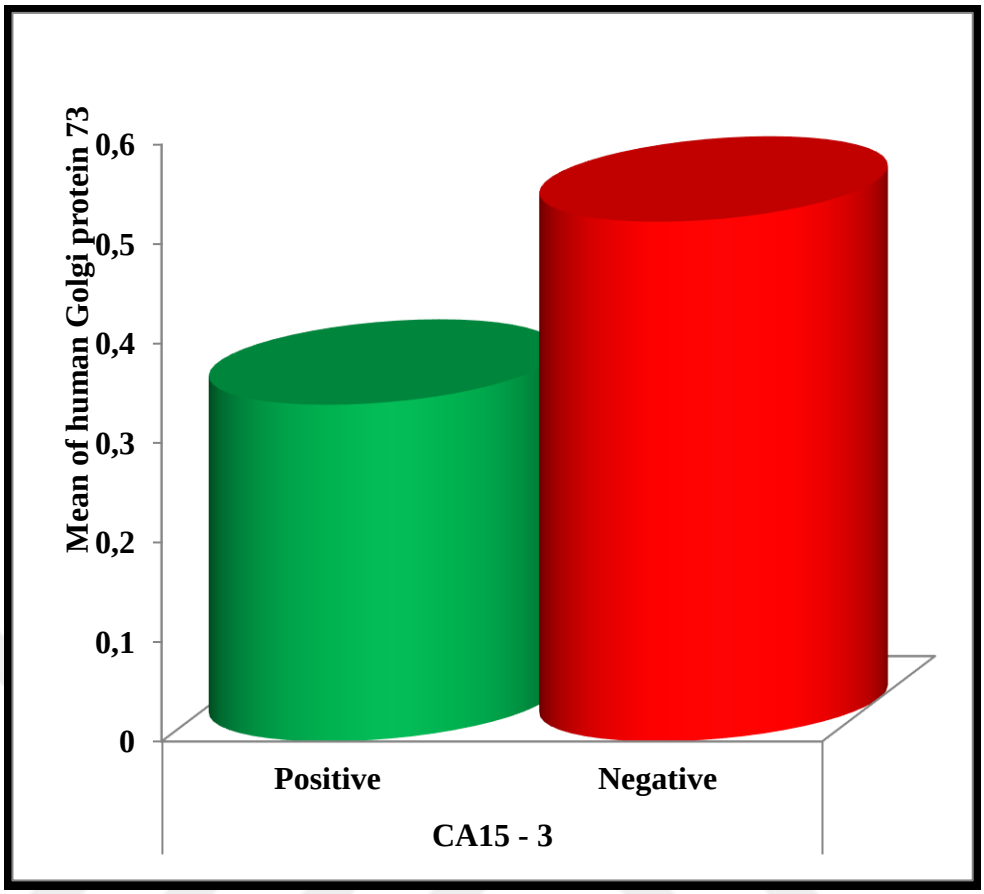

Figure 4.15 Mean human Golgi protein 73 distributions among CA15 - 3 tumor marker results

4.2 Discussion

Age is a major risk factor for developing breast cancer. Although breast cancer may affect women of any age, its occurrence rises dramatically after the age of 50. The global trend of fewer cases of breast cancer in younger women (23.8% of newly diagnosed cases and 13.5% of under-treated cases) is in line with findings from other regions. Although breast cancer is more common in older women, it may still occur in younger women, making screening and early diagnosis even more crucial for this demographic.

The reduced rate of breast cancer in younger women is supported by data from a similar research conducted in Iran, where 17.1% of patients were under 40 years old (Hassoon *et al.* 2021). Possibly attributable to demographic differences and/or variations in risk factors and/or access to healthcare, the greater incidence of breast cancer in younger women observed in the research from Bahrain (43%).

Younger women's risk of developing breast cancer varies widely between populations. It has been estimated that 5-7% of American women under the age of 40 would get breast cancer in their lifetimes (Zaemey *et al.* 2012).

However, variations in the age structure of the population, in addition to genetic and environmental influences, might account for this discrepancy. Some research, for instance, has linked a higher risk of breast cancer in younger women to a specific set of genetic abnormalities that may be more common in particular groups.

Middle-aged patients (41-60 years old) were the most prevalent in this research, accounting for 71.4% of newly diagnosed patients and 73% of under-treated patients. This conforms to the overall trend in breast cancer incidence, which shows an increased chance of acquiring breast cancer with age, reaching a peak between the ages of 60 and 70.

The overall trend of breast cancer incidence is supported by the findings of other studies, which revealed that around 57% (Bland *et al.* 1998) or 50% (Zaemey *et al.* But different demographic and societal conditions might lead to a shift in the typical age range of breast cancer patients.

This research, like others in the Arab area, found that the average age of breast cancer diagnosis increased with age, which is in line with the overall trend of breast cancer incidence.

Similar to previous research from the Arab area, your study found that the average age of patients who were newly diagnosed and receiving treatment was 48.14 7.754 years. In Yemen, the average age of diagnosis was found to be 47.7 years (Abood 2018), whereas in Bahrain it was 49.4 (Arkoob *et al.* 2010), in Jordan it was 51.4 (Mehdi *et al.* 2014), in Oman it was 49.5 (Chahine *et al.* 2015), and in Lebanon it was 52 (Lakkis *et al.* 2010).

According to the research (Rakha *et al.* 2010), the average age of breast cancer diagnosis in the United States and Western Europe is much older than in the Arab area, at 61 and 63 years old respectively.

Having a close relative with breast cancer increases your chances of having the illness. Researchers have shown that a woman's risk of developing breast cancer increases by a factor of two to three if she has a first-degree relative (mother, sister, or daughter) who has the illness.

It is in line with global trends that 42.9% of newly diagnosed and 54.1% of under-treated patients in this research had a positive family history of breast cancer. It is true that some women who have no family history of breast cancer end up getting it nevertheless, and it is also true that having a positive family history is no guarantee that a person will acquire breast cancer.

Therefore, it is essential that all women, regardless of family history, understand their individual risk factors for breast cancer and adhere to established screening protocols to catch the disease at an early stage when it is most treatable.

When deciding on a course of therapy for breast cancer, the patient's histological subtype and degree of differentiation are crucial considerations. The tumor's histological subtype is defined by the predominant cellular type inside it; breast cancer comes in a variety of subtypes, each with its own set of symptoms and therapeutic options. Higher-grade tumors have less differentiated and more aggressive cancer cells and more closely resemble normal breast cells under the microscope.

Breast cancer is often graded using the Nottingham Histological Grading System (NGS), which evaluates tumors for tubule development, nuclear pleomorphism, and mitotic rate. This approach aids in providing more precise information for treatment choices by standardizing the grading process. The significance of the NGS in directing breast cancer therapy is shown in its support by many professional groups throughout the globe (Alwan, 2014).

Grade II and III malignancies accounted for 38.1% and 48.6% of new diagnoses and 33.3% and 32.4% of new diagnoses and treatments in our analysis, respectively.

These findings corroborate those of a research conducted in Iraq, which also found a comparatively high prevalence of high-grade tumors (about 40% of all cases). In 2013, Shomaf *et al.* Similarly, a Jordanian research (Diab *et al.* 2000) found that grade II breast cancer accounted for 43.5% of all cases, whereas grade III accounted for 39.9%.

To learn more about the frequency of each histological subtype and differentiation grade among patients diagnosed with breast cancer in this area, however, larger-scale investigations are required.

The majority of the patients described had a tumor grade of II, which is consistent with a research conducted in the United Kingdom (Orucevic *et al.* 2015).

Treatment choices for breast cancer patients depend on a number of characteristics, including the patient's histological subtype and degree of differentiation as well as their hormone receptor status, Ca 15 - 3, and HER2 status. Individualized cancer treatment plans are generally developed via a collaborative effort by oncologists, pathologists, and other medical experts.

The prognosis and treatment of breast cancer patients may be improved with the use of biomarkers like estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), and ca 15-3. There was a drop in the number of negative receptors and a rise in the number of positive ones (ER, PR, Ca 15-3, and HER2) in our research. This agrees with the results of previous research that have drawn similar conclusions. Patients with breast cancer whose tumors tested positive for ER and PR had a higher chance of survival. Overall survival rates were lower for HER2-positive than HER2-negative breast cancer patients.

Ca 15-3 biomarker has also been demonstrated to be helpful in determining whether or not a patient's breast cancer has returned after first therapy (Zhao *et al.* 2012).

Biomarkers' importance in improving breast cancer patient outcomes has grown substantially in recent years, and this trend is only expected to increase.

Despite being a novel protein and having a small number of studies reporting that it has been studied in breast cancer, the levels of human Golgi 73 protein (GP73) remain low across all groups studied. This includes newly diagnosed patients, those undergoing treatment, those with benign tumors, and their healthy controls. Instead, GP73 is well recognized for its link to liver illness and its use as a biomarker for doing so (Bammens *et al.* 2015, Tian *et al.* 2018).

However, GP73 expression has been detected in other cancers, including as colon, stomach, and prostate cancers, suggesting a potential function for this gene in the pathogenesis of these illnesses. More study is necessary to determine GP73's function in carcinogenesis and its use as a diagnostic marker for cancers (Sinha 2018, Graham *et al.* 2021, Søndergaard *et al.* 2017).

5. CONCLUSIONS AND RECOMMENDATION

5.1 Conclusions

1. Given the fact that tumor marker tests (HER2, PR, ER and Ca 15 - 3) are best tests results for breast cancer patients with highly positive percent.
2. Human Golgi protein 37 blood levels were also tested between breast cancer patients and patients with benign tumors and healthy controls, and the findings indicated no significant changes.
3. The present study indicates no significant differences in levels of Human Golgi protein 37 between patients with breast cancer regarding tumor marker tests (HER2, PR, ER and Ca 15 - 3).

5.2 Recommendations

We recommend the researchers perform the flowing in future studies:

1. Further studies are needed to be investigation Human Golgi protein 37 in sera of patients afflicted by others cancers or inflammatory diseases.
2. Future studies should focus on measuring Human Golgi protein 165 levels in sera of breast cancer patients.
3. Assessment of liver disease activity with Human Golgi protein 37 as a prospective follow-up study.

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