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Biochemical and Clinicopathological study of phospholipase D1and Choline Kinase Alpha enzymes in Women with Invasive Ductal Carcinoma of the Breast in Thi- Qar Province- Iraq.

A Thesis
Submitted to the Council of the College of Science, University of Thi- Qar in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Clinical Biochemistry

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

﴿وَمَا تَوْفِيقِي إِلَّا بِاللَّهِ عَلَيْهِ تَوَكَّلْتُ

وَإِلَيْهِ أُنِيبُ﴾

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CERTIFICATE

We certify that this thesis, entitled ***Biochemical and Clinicopathological study of phospholipase D1and Choline Kinase Alpha enzymes in Women with Invasive Ductal Carcinoma of the Breast in Thi- Qar Province- Iraq***, was prepared by ***HADEEL RASHIDFARAJ*** under our supervision at the Department of Chemistry, College of Science, University of Thi-Qar (Iraq) in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Clinical Biochemistry.

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الاهداء

إلهي لا يطيب الليل إلا بشكرك... ولا يطيب النهار إلا بطاعتك... ولا تطيب اللحظات إلا بذكرك

"الله جل جلاله"

إلى صاحب السجدة الطويلة ... إلى راهب ال محمد ...وبابا للحوائج... ملاذي وأماني

الامام موسى بن جعفر الكاظم ...عليه السلام...

إلى الذين بذلوا مهجهم دون تراب الوطن. وسالت دماؤهم على السواتر .. وضحوا بالغالي والنفيس..

شهداؤنا الأبرار

إلى أبي العزيز... أطال الله بقاءه، وألبسه ثوب الصحة والعافية، ومتعني ببره ورد جميله...

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إلى من أرى التفاؤل بعيونهم... والسعادة في ضحكتهم... أخوتي

إلى زوجي الغالي ورفيق دربي في هذه الحياة... معك أكون أنا و بدونك أكون مثل أي شيء...
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List of Abbreviations

5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine
5-HTP	5-hydroxytryptophan
AA	Arachidonic acid
ACS	American Cancer Society
ADP	Human Adiponectin
AJCC	American Joint Committee on Cancer
ALDH	Aldehyde dehydrogenase
ALP	Alkaline phosphatase
ASIR	Age Standardized Incidence Rates
ATP	Adenosine tri phosphate
BC	Breast cancer
BMI	Body mass index
BRCA1/2	Breast cancer susceptibility gene one/two
BSA	Bovine serum albumin
BSE	Breast Self-Examination
C1q	Complement Factor
CBE	Clinical breast examination

ChoK	Choline kinase
CHKA	Choline kinase alpha
CNS	Central nervous system
CRP	C- reactive protein
DAB	3,3 Di Amino Benzidine
DAG	Diacylglycerol
DCIS	Ductal carcinoma in situ
df	Degree of freedom
DNA	Deoxy Ribonucleic Acid
DPX	Disteren Plasticizer Xylene
E2	Estradiol
ELISA	Enzyme-linked immune-sorbent assay
ER	Estrogen receptor
FA	Fatty acid
FAS	Fatty acid synthase
FFPE	Formalin-fixed , paraffin-embedded
FNAC	Fine needle aspiration cytology
H & E	Haematoxylin and Eosin
HER2	Human epidermal growth factor receptor 2
HIER	Heat induced epitope retrieval

HIOMT	Hydroxyindole –O- methyl transferase
HRP	Horseradish Peroxidase
HRT	Hormone replacement therapy
IHC	Immunohistochemistry
IGF-1	Insulin- like growth factor 1
IL-6	Interleukin 6
INCRC	Iraqi National Cancer Research Center
LCIS	Lobular carcinoma in situ
LPA	Lysophosphatidic acid
LPtdCho	Lysophosphatidylcholine
MRI	Magnetic resonance imaging
MT	Melatonin
nm	Nanometer
OD	Optical density
PA	Phosphatidic acid
PBS	Phosphate buffered saline
PC	Phosphocholine
PC-PLC	Phosphocholine – Phospholipase C
PLA1/2	Phospholipase A1/A2
PLD1 /2	Phospholipase D1/ D2

PtdCho	Phosphatidylcholine
PtdEtn	Phosphatidy lethanol amine
PTH	Para thyroid hormone
r	Correlation coefficient
ROS	Reactive oxygen species
SABC	Streptavidin Conjugate
SEEM	Selective estrogen enzyme modulators
SNAT	Serotonin N- acetyl - transferase
SPSS	Statistical package for social sciences
ST	Serotonin
TNM	Tumor size , lymph node , distant metastasis
TPH	Tryptophan hydroxylase
US	Breast ultrasound
X^2	Chi- square

Summary

Breast cancer is a complex and multi factorial disease resulting in abnormal cell growth that leads to malignant tumor formation. The present study is designed to determine the levels of {phospholipase D1enzyme **(PLD1)**, choline kinase alpha enzyme **(CHKA) }** , pineal gland hormones (Serotonin, Melatonin) **(ST , MT)**, Estradiol **(E2)** , asses the titer of C- reactive protein **(CRP)**, (Adiponectin hormone, Vitamin D3, Calcium) **(ADP,Vit.D3,Ca)**, and (Alkaline phosphtase enzyme) **(ALP)**, in women with invasive ductal carcinoma and healthy individuals.

The study includes **(160)** women who are divided into two groups: **(85)** women of invasive ductal carcinoma with age range (25—65) year and **(75)** supposed healthy subjects (control) with age range (25 −65)year. The study aims to shed a light on the possible correlation between phospholipase D1enzyme **(PLD1)** and each one of the studied parameters.

The results show that there is a significant increase in serum concentrations of each **(PLD1)**, **(CHKA)**, **(E2)** , **(CRP)**, **(Ca)** and **(ALP)** in all patients in comparison with control group ($P \leq 0.05$).

Whereas there is a significant decrease ($P \leq 0.05$) in concentrations of serum **(ST)**, **(MT)**, **(ADP),** and **(Vit.D3),** in all patients in comparison with (control) group .

In addition, the study reveals a positive correlation between **PLD1** and the levels of each (CHKA, E2, CRP, Ca ,and ALP) and a negative

correlation with the concentrations of (ST, MT, ADP, Vit.D3) in women with invasive ductal carcinoma.

Histopathologically, carcinoma was 50 cases (100%) invasive ductal carcinoma. Results showed that 2 cases (4%) were stage I, 16 cases (32%) were stage II, 28 cases (56%) were stage III and 4 cases (8%) were stage IV, also our results showed that the 4 cases (8%) were grade I, 21 cases (42%) were grade II and 25 cases (50%) were grade III.

Results of immunohistochemical analysis for phospholipase D1 were as followes: classified the cases of carcinoma, and control groups into different grades of intensity **(-, +, ++ and +++)** showed no significant difference ($P > 0.05$). In the 41 cases which were positive stain for phospholipase D1; staining with score +++ was seen in 14 (28%) cases, score ++ was seen in 18 cases (36%), and 9 (18%) cases were score + , and 9 (18%) cases of invasive ductal carcinoma were negative.

CHAPTER ONE

INTRODUCTION

1. Introduction

Breast cancer is the most frequently diagnosed cancer and is a common cause of cancer related death in women, accounting for 25% of cancer cases and 15% of cancer-related deaths worldwide (Torre L. *et al*., 2017).

In Iraq, breast cancer ranks the first among the top ten malignant neoplasms affecting the community; where it was comprising 19.5% of a total (4996 of cancer cases). On the other hand breast cancer women had been represented 34.3% of a total (4922 of female cancers). During **2016**, 897 women died from that disease which had been registered as the first cause of cancer related mortality among Iraqi females (23.6%) and the second overall among males and females (12.1%) after bronchogenic cancer. (Annual Statistical Report , 2016; Annual Report. Iraqi Cancer Registry, 2016). Notably, the incidence rate of breast cancer women in Thi-Qar Province basing on statistics of Iraqi Cancer Board / Ministry of Health and Environment from the **2013** to **2016** was as the following:

♦ (127cases) (10.65%) out of (1.930.835) population in **2013**(Annual Report. Iraqi Cancer Registry ,2013).

♦ (182 cases) (14.06%) out of (1.979.561) population in **2014**(Annual Report of Cancer Diseases in Iraq, 2014).

♦ (143 cases) (18.1%) out of (2.029.345) population in **2015** (Annual Report. Iraqi Cancer Registry ,2015).

♦ Lastly, (164 cases) (23.94%) out of (2.080.188) population in **2016** (Annual Report. Iraqi Cancer Registry , 2016). The development of invasive breast cancer involves multiple genetic alterations similar to other carcinomas of various anatomic sites, some of these molecular

alteration involves a wide range of biochemical and hormonal alterations will be detected in this study:

Phospholipase D1 and choline kinase alpha are interactive, such that choline kinase alpha silencing increases phospholipase D1 expression and phospholipase D1 silencing increases choline kinase alpha expression (Gadiya M.*etal*., 2014). Enhanced choline metabolism is emerging as a novel metabolic hallmark in cancer. Specifically, an increase in the synthesis of phosphatidylcholine, a major phospholipid of cellular membranes, can be detected in different cancers (Wu G. and Vance D. ,2010; andGlundeK.*etal*.,2011). It has been shown that breast cancer cells can synthesize serotonin , which are used to support tumor growth (Pai V. *et al*., 2009). It was linked to malignant progression in breast cancer (Pai V. *et al.*, 2009 ; Juhász C. *et al.* , 2012). Melatonin levels in cancer patients have been correlated with tumor aggressiveness and progression (Kajdaniuk D. *et al.* , 2002 ; Callaghan B.,2002). A high percentage of women with estrogen-receptor-positive breast cancer have low plasma melatonin levels (Brzezinski A.,1997). Conversely, melatonin inhibits human breast cancer cell growth (Cos S. and Sanchez-Barcelo E. , 2000). It has been suggested that melatonin acts as a naturally occurring anti-estrogen on tumor cells, as it down-regulates hormones responsible for the growth of hormone-dependent mammary tumors (Torres- Farfan C. *et al.* , 2003).

Researchers proposed that serum C- reactive protein (CRP) could be a marker of increased risk for breast cancer, estimation of CRP can be looked at as a simple, cost effective, easily available screening test to assess future risk of breast cancer (Guo L. *et al*., 2015). CRP is raised in circulation in response to acute inflammation, infection, and tissue damage, the rise in CRP levels is proportional to degree of tissue damage

(Mantovani A. *et al*. , 2008) . Calcium ions are required for ligand binding and stability of the molecule CRP (Allin K. and Nordestgaard B. , 2011).

Several reports have indicated association between low adiponectin levels and elevated risk of breast cancer (Dal Maso L. *et al.* ,2004; and Ishikawa M. *et al*. ,2005).

Being overweight or obese in adults is correlated with a greater risk of breast cancer (Neuhouser M. *et al.*, 2015). In addition, it has been demonstrated that breast cancer patients with higher body mass index (BMI) estimated as obesity or overweight have a worse prognosis regardless of tumor subtype (Calle E. *et al.* , 2003).

Vitamin D deficiency is also associated with secondary elevation in Para thyroid hormone (PTH) serum levels which has carcinogenic and tumor promoting effects. Hence, may lead to an increased risk of breast cancer.(Hoey R. *et al.* ,2003). Lin *et al.*: studied the effects of vitamin D and calcium intake from nutrient sources and supplements on breast cancer risk, they found that higher intakes of total calcium and vitamin D were associated with a lower risk of breast cancer (Lin J. *et al*. ,2007).

Increase serum level of elevated activity of alkaline phosphatase (ALP) in patients with malignancies including metastatic breast cancer (Ijaz A. *et al.* , 2006) .

In breast tissue: the classic isoform of PLD, PLD1 is highly expressed in various human tumors, including in breast (Noh D. *et al.*, 2000), and was stated to play a critical role in tumor progression (Park M., 2011).

1.1 Aims of the study

The present study aims were to estimate a new biochemical and clinicopathological features of Invasive ductal carcinoma patients. Such studies were important to look at the biochemical alteration in such disease which enable early diagnosis and treatment. More specifically, the purpose were:

1- Assessment of (phospholipase D1and choline kinase alpha enzymes) levels in serum of patients with Invasive ductal carcinoma.

2- Determination of the Pineal gland hormones levels (Serotonin and Melatonin) in serum of patients with Invasive ductal carcinoma.

3- Assessment of Estradiol hormone level in serum of patients with Invasive ductal carcinoma.

4- Investigation of the inflammation marker (C- reactive protein) in serum of patients with Invasive ductal carcinoma.

5- Evaluation of the relationship between Adiponectin hormone level and obesity in serum of patients with Invasive ductal carcinoma.

6- Determination of vitamin D3 and calcium concentrations in serum of patients with Invasive ductal carcinoma.

7- Investigation the effect of Invasive ductal carcinoma on the enzymatic activity of some hydrolytic enzyme, through measuring the activity of alkaline phosphatase.

8- Shedding a light on the possible correlation between phospholipase D1and each one of the studied parameters.

9- Immunohistochemical evaluation of phospholipase D1 in the malignant tissue of patients with Invasive ductal carcinoma.

CHAPTER TWO

LITERATURE REVIEW

2. Literature Review

2.1 Breast Cancer

Breast cancer is a complex and multi factorial disease resulting in abnormal cell growth that leads to malignant tumor formation. It is the most common female cancer, affecting one in eight women during their lifetime (Howlader N. *et al.*, 2013).

Tumor markers are biochemical indicators of the presence of a tumor, they include cell surface antigen, cytoplasmic proteins, enzymes and hormones that are important methods for diagnosis (Cotran R. *et al.*, 2000).

As to the first, we can improve the early detection through learning more about the extent and reasons behind diagnosis delay in breast cancer, in order to improve breast cancer outcome and survival, early detection remains the cornerstone of breast cancer control, early detection of cancer is important because delay is preventable and earlier treatment can lead to improved patient outcome, late detection has been associated with advanced stages and low survival as well as negative implication on cost and choice of treatment (Stapleton J. *et al.*, 2011; Chagpar A. *et al.*, 2011; and Kim J. *et al.*, 2012).

As to the second, because breast cancer is heterogeneous in respect to genetics, and variable in biological and clinical features, the identification of prognostic and predictive markers is clinically important. Biological markers have an important role as independent prognostic parameters in relation to the traditional clinic pathological variables which lead to the determination of tumor prognosis (Cancer Genome Atlas N., 2012).

2.2 Epidemiology of breast cancer

Breast cancer is the most common cancer and also the leading cause of cancer mortality in women worldwide, approximately 1.38 million

new breast cancer cases were diagnosed in 2008 with almost half of all breast cancer cases and nearly 60% of deaths occurring in lower income countries (Ferlay J.*et al*., 2010). There is a large variation in breast cancer survival rates around the world, with an estimated 5-year survival of 80% in high income countries to below 40% for low income countries (Coleman M. *et al*., 2008). According to a recent report published by the American Cancer Society, breast cancer is the most prevalent form of cancer in women, in the USA. In 2017 alone, study indicates that approximately 252,000 new cases of invasive breast cancer and 63,000 cases of in situ breast cancer are expected to be diagnosed, with 40,000 breast cancer-related deaths expected to occur (DeSantis *et al.* , 2017).

Incidence rates vary from 19.3 per 100,000 women in Eastern Africa to 89.7 per 100,000 women in Western Europe, and are high (greater than 80 per 100,000) in developed regions of the world (except Japan) and low (less than 40 per 100,000) in most of the developing regions (Ferlay J. *et al*., 2010). According to the International Agency for Cancer Research and Globocan 2008, the Age Standardized Incidence Rates (ASIR) in Iraq was (31.1/100,000), similar as compared to the countries surrounding Iraq, Kuwait (47.7), Saudi Arabia (22.4), Jordan (47.0), Syria (23.0), Iran (18.4), Turkey (28.3) (Globocan , 2008).

Iraqi National Cancer Research Center (INCRC) considered breast cancer as common type of Iraqi female cancer, account for approximately (1:3) of the registered female cancers in Iraq (INCRC, 2013).

2.3 Risk Factors

Several risk factors for the development of breast carcinoma have been established (Armstrong K. *et al* ., 2000). Risk factors for developing breast cancer include: (NCI, 2014).

1. Age and sex

The risk factor for being diagnosed with breast cancer is being a woman, followed closely by growing older. Breast cancer risk increases in women between the ages of 65-80 are at highest risk for breast cancer.

2. Genetics

About 5 to 10 percent of all breast cancers are due to genetic mutations, including the familial BRCA1 and BRCA2 mutations. While less than 1 percent of the general population carries these mutations.

3. Dense breasts

Breast tissue varies in density, woman to woman. Some women's breasts have a high percentage of fibrous and connective tissue compared to fat; this condition is known as "dense breasts." Studies have shown that women with dense breasts are significantly more likely to be diagnosed with breast cancer than those whose breasts have a higher ratio of fat to other tissues.

4. High estrogen levels

Women with higher levels of estrogen are more likely to develop breast cancer. Some common factors that increase estrogen include:

• Postmenopausal obesity.

• Bearing children after age 30 (or not at all).

• Use of oral contraceptives.

• Use of HRT (hormone replacement therapy).

5. Family history

Having two or more first-degree relatives (parent, sibling, or child) who've been diagnosed with breast cancer increases your risk, especially if any were diagnosed before age 50. One first-degree relative with breast cancer also increases your risk, though less significantly.

6. High-dose radiation to the chest

High-dose radiation therapy to the chest area before the age of 30, usually as a result of Hodgkin's disease, this increases your risk of developing breast cancer. The most common risk factors in table (2-1):

Table (2-1): The most common risk factors and their relative risk for breast cancer (modified from American Cancer Society: Breast Cancer Facts and Figures 2012).

Relative risk	Factor
>4.0	Advanced age Atypical hyperplasia BRCA1and/or BRCA2 gene mutation Mammography: dense breasts Family history of breast cancer (≥ 3 relatives with breast cancer)
2.1-4.0	High endogenous estrogen level High bone density (postmenopausal) Two first-degree relatives with breast cancer
1.1-2.0	Alcohol consumption Early menarche (<12 years) Tall patient Lack of exercise Radiation to chest or face before age 30 High socioeconomic status Late age at first pregnancy (>30 years) Late menopause Never breastfed a child No full-term pregnancies Obesity Personal history of endometrium , ovary or colon cancer Long-term use of menopausal hormone therapy with estrogen and progesterone analogs Recent oral contraceptive use

2.4 Types of Breast Cancer

Carcinomas can be non-invasive (confined to the ducts or lobules) or invasive tumors (extending into surrounding stroma).

-Non-invasive tumors

Ductal carcinoma in situ (DCIS)

DCIS is a non-invasive breast cancer, and consists of malignant epithelial cells confined to the mammary duct, without evidence of invasion to the surrounding tissue as shown in Fig (2-1). There are various histologic subtypes of DCIS, namely comedo, micropapillary, cribriform, papillary, mixed and solid. The comedo subtype is associated with high nuclear grade, and over expression of the HER2/neu oncogene (Pinder S. , 2010).

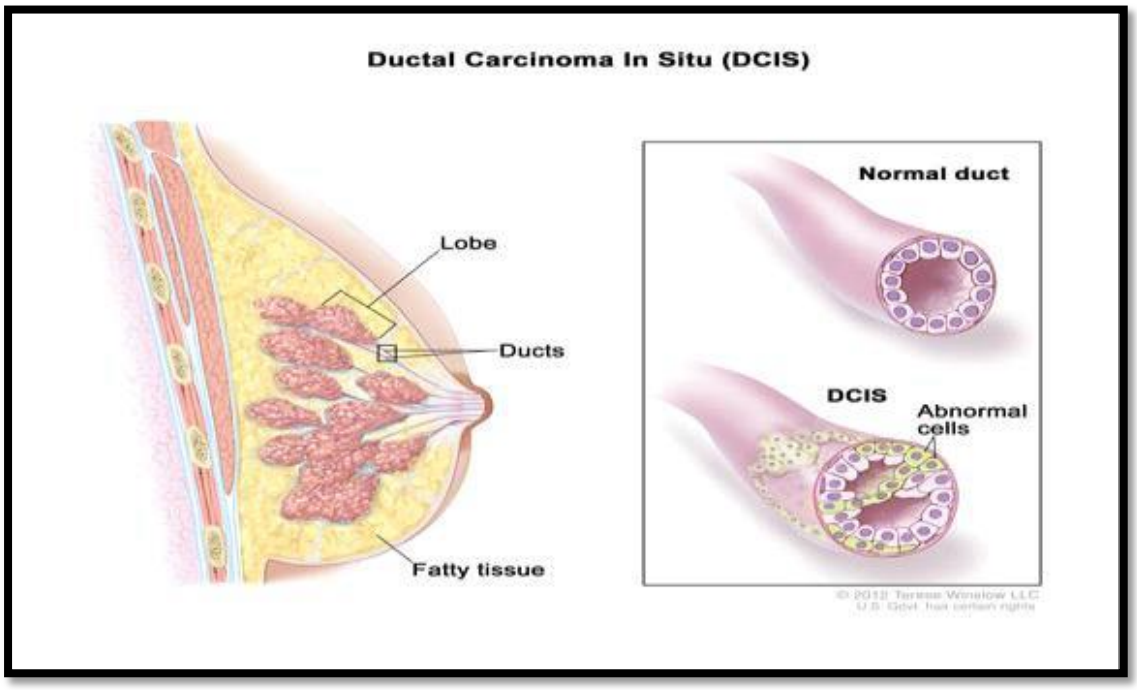

Figure (2-1) : Ductal carcinoma in situ (depicted from American cancer society, 2014).

Lobular carcinoma in situ (LCIS)

LCIS is a non-invasive tumor, and consists of abnormal cells growing inside the lobules of the breast, but have not spread to surrounding tissue as shown in Fig (2-1). LCIS increases the risk of invasive tumor, women who have LCIS are more likely to develop invasive cancer in either breast than women without LCIS (Kibride K. and Newman L. **,** 2010).

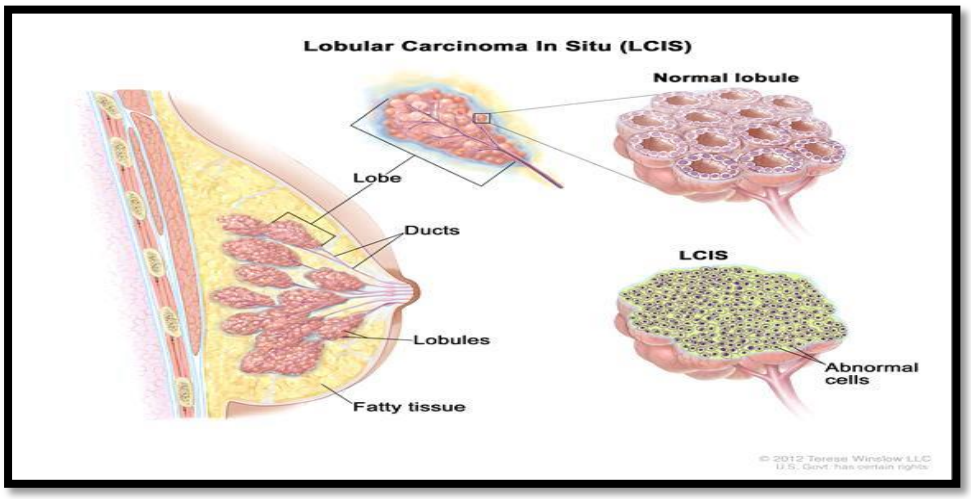

Figure (2-2): Lobular carcinoma in situ (depicted from American cancer society, 2014).

- Invasive tumors

Invasive tumors spread from the original site (either milk duct or lobules) into surrounding tissue of the breast and may spread to lymph nodes and/or other parts of body (Dillon D. *et al.* , 2010; and Merajver S. *et al.*, 2010). Invasive ductal carcinoma which is the most common type comprises 50- 75% of all breast cancers. Invasive lobular carcinoma is the next most common type and comprises about 10-15% of cases (Dillon D. *et al.*, 2010).There are less common types of invasive breast carcinomas that tend to have a better prognosis. They are mixed ductal lobular carcinoma, medullary carcinoma, mucinous (colloid) carcinoma, papillary carcinoma and tubular carcinoma (Dillon D. *et al.* , 2010 ; and Merajver S. *et al.* , 2010).

2.5 Hormonal Regulation

All changes that occur in the mammary gland, before and after puberty, pregnancy and lactation, are under hormonal control, estrogen is responsible for ductal development during puberty, while high level of progesterone (in the presence of estrogen) is responsible for alveoli (secretory part) development, it has been found that normal level of growth hormone, insulin and glucocorticoids are essential for estrogen and progesterone to influence mammary gland growth (Underwood E. , 2004).

The ovarian steroid hormones progesterone and estrogen play critical roles in the development and progression of breast cancer and endometriosis (Shao R. *et al*. , 2014). The estrogen receptor and the progesterone receptor, along with human epidermal growth factor receptor 2 (HER2), are used to classify phenotypes in breast cancers and to predict response to specific therapies (Cadoo K. *et al*. , 2013).

After delivery, the sudden decline in estrogen and progesterone level and the presence of high level of prolactin will exagurate lactogenesis in the mammary gland and milk 1et down. Later on, galactopoiesis is influenced by both prolactin and oxytocin which are stimulated by suckling. Lastly, cessation of breast feeding will eventually lead to involution of mammary gland (Ganong W., 1993).

2.6 Signs and Symptoms

The main symptoms of breast cancer collected from Cancer research UK (2014) and ACS (2016) may be:

- Presence in one or both breasts of one or more ”hard masses” lumps of any size, shape, texture, with smooth or non-smooth edges.
- Inflammation (redness, swelling, increase of temperature) of the entire breast, or some part of it.

- Change of the skin of breast: noticeable depressions, redness, or thickening.
- Prolonged pain in one or both breasts, the cause of which is not clear (in many cases, breast cancer develops painlessly).
- Nipple retraction (if before the nipple was another form).
- Redness, peeling, the appearance of sores on the nipple.
- Any discharge (including bleeding or transparent fluid) from the nipple, without a known reason.
- For a long time (over 2 weeks) swollen lymph nodes under or above the clavicle or in the armpit.

2.7 Diagnosis / Screening

Diagnosis of breast cancer involves :

- Breast Self-Examination (BSE).
- Clinical breast examination (CBE).
- Mammography.
- Breast ultrasound (US).
- Magnetic resonance imaging (MRI).
- Cytology.
- Surgical (open biopsy).
- Needle core biopsy.

2.7.1 Breast Self-Examination (BSE)

Screening for early detection and diagnosis of diseases and health conditions is an important public health principle (Bellgam H. and Buowari Y. , 2012). Breast self-examination (BSE) is a check-up that a woman does by herself at home to look for changes or problems affecting the breast tissue. BSE is still recommended as a general approach to increasing breast health awareness and thus potentially allow for early

detection of any anomalies because it is free, painless and easy to practice (Ginseng G. *et al.* , 2012).The American Cancer Society (ACS , 2014): also recommends that women, starting from the age of 20 years should be educated on the pros and cons of performing a monthly BSE.

2.7. 2 Clinical Breast Examination (CBE)

Clinical examination, particularly palpation , is the time honored method for the detection and evaluation of breast disease . It remains an extremely useful and practical technique , whether carried out by the physician or by the patient herself . only 60 % of the tumors detected by mammography are palpable (Ackerman , 2011).

2.7.3 Mammography

A mammogram is an x-ray of the breast. Screening mammograms are used to look for breast disease in women who have no signs or symptoms of a breast problem (Ciatto S. *et al* ., 2013). Not all breast cancer will be detected early by a mammogram, and some cancers that are screen-detected still have poor prognosis. Most women will never be diagnosed with breast cancer, but will undergo regular screening and may experience one or more “false alarms.” In an effort to maximize the benefits and minimize the harms of screening, some scientists are attempting to determine which factors could be used to individualize screening recommendations (e.g., which women could start screening at older ages and/or be screened less often) (Brawley O., 2012).

2.7.4 Breast ultrasound (US)

Breast ultrasound is sometimes used to evaluate abnormal findings from a mammogram or physical exam. Studies have shown that ultrasound detects more cancer than mammography alone when screening women with mammographically dense breast tissue; however, it also

increases the likelihood of false-positive results.(Tagliafico A. *et al.* ,2016; and Melnikow J.*et al.* , 2016).

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2.7.5 Magnetic Resonance Imaging (MRI)

MRI has long been known as an effective breast cancer screening modality that offers better sensitivity than mammography and ultrasound. Currently, guidelines reserve breast MRI screening for women who have a strong family history or other specific breast cancer risk factors. MRI screening has not been considered necessary for women at average risk, and there has been resistance to expansion of MRI into this population due, in part, to concern over higher costs (Christiane K. *et al.* , 2017).

2.7.6 Cytology

Fine needle aspiration cytology (FNAC) of the breast, as part of the triple approach to the diagnoses of palpable breast lesions, has become a valuable pre-operative tool. It is fast, inexpensive and minimally invasive and thus has gained wide acceptance in the pre-operative assessment of breast lesions. FNAC of the breast has two main goals; to confirm radiological and clinically benign lesions thus avoiding unnecessary surgery, and to confirm radiological and clinically malignant diagnoses thus enabling definitive treatment planning (Berner A. and Sauer T., 2011).

2.7.7 Needle Core Biopsy

A core biopsy uses a larger needle to sample breast changes which are felt by the doctor or pinpointed by ultrasound or mammogram. It removes a small cylinder (core) of tissue (about 1/16- to 1/8-inch in diameter and ½- inch long) from a breast abnormality (Ackerman , 2011).

2.7.8 Surgical (open biopsy)

Rarely, surgery needs to remove all or part of the lump for microscopic examination. This referrers to as a ***surgical biopsy*** or an ***open biopsy***. Most often, the surgeon removes the entire mass or abnormal area as well as a surrounding margin of normal-appearing breast tissue this calls an ***excisional biopsy*** . If the mass is too large to be removed easily, only part of it may be removed this calls an ***incisional biopsy*** (ACS , 2012).

2.8 Stages of Breast Cancer

The process of determining breast cancer stage has several important parameters of tumor development: its size, its distribution to the surrounding tissue, whether it has spread to the lymph nodes in the breast or around it, whether it has spread to other organs. On the basis of these data, the stage is found out as well as a treatment strategy. The survival rate is always calculated based on observations of people who have been treated for cancer 5 years ago. (American Cancer Society, 2016).

Breast cancers are staged on a scale from zero to four. The lower the number, the less the cancer has spread.

- **Stage 0** breast cancer is a non-invasive condition sometimes described as pre-cancerous. Stage 0 breast cancers include ductal carcinoma in situ (DCIS) and lobular carcinoma in situ (LCIS). These diseases are not considered life-threatening, but can become invasive over time if not appropriately treated. Both increase the risk of developing breast cancer in the future.
- **Stage I** cancer usually involves a tumor that is two centimeters or smaller and has not spread outside the breast. Stage I cancers are almost always curable.

- **Stage II and III** cancer includes medium- to larger-sized tumors as well as tumors with positive lymph nodes. With advances in breast care treatment, stage II and stage III cancers are often curable, but many require additional treatments to achieve this goal.
- In **Stage IV**, the cancer has spread to other organs of the body, most often the bones, lungs, or liver. Another name for Stage IV cancer is metastatic cancer.

Traditionally, staging has been based on three factors: the size of the tumor; the number and location of lymph nodes with tumor cells; and whether or not the cancer has spread to other parts of the body. This is known as the TNM system, in which **T** stands for **T**umor size, **N** stands for Lymph **N**ode status, and **M** stands for **M**etastases.

The American Joint Committee on Cancer (*AJCC*) implemented a new system in which stage designations also take into account biological information about the tumor cells, including tumor grade (how abnormal the cancer cells look under a microscope), presence of cell receptors for estrogen and progesterone (hormones that can drive cancer cell growth), and abnormally high levels of the protein HER2. (Dana-Farber Institute, 2018).

Staging is regarded as the most important prognostic factor, as stage increases the prognosis worsens, for example, the 5 year survival in stage I breast cancer is more than 90% while patients with stage IV disease have very poor prognosis and a 5 years survival is less than 30% (Edge S. *et al.* ,2010 and American Cancer Society: Breast cancer survival rates by stages, 2012).Considering all races, 5-year relative survival is 90% for localized disease and 23% for advanced stage disease (Howlader N. *et al*., 2012).

2.9 Tumors Grades

Grading systems differ depending on the type of cancer. In general, tumors are graded as 1, 2, 3, or 4, depending on the amount of abnormality. In Grade 1 tumors, the tumor cells and the organization of the tumor tissue appear close to normal. These tumors tend to grow and spread slowly. In contrast, the cells and tissue of Grade 3 and Grade 4 tumors do not look like normal cells and tissue. Grade 3 and Grade 4 tumors tend to grow rapidly and spread faster than tumors with a lower grade.

Breast cancer: Doctors most often use the Nottingham grading system (also called the Elston-Ellis modification of the Scarff-Bloom-Richardson grading system) for breast cancer , this system grades breast tumors based on the following features:

- Tubule formation: how much of the tumor tissue has normal breast (milk) duct structures
- Nuclear grade: an evaluation of the size and shape of the nucleus in the tumor cells
- Mitotic rate: how many dividing cells are present, which is a measure of how fast the tumor cells are growing and dividing

Each of the categories gets a score between 1 and 3; a score of "1" means the cells and tumor tissue look the most like normal cells and tissue, and a score of "3" means the cells and tissue look the most abnormal. The scores for the three categories are then added, yielding a total score of 3 to 9. Three grades are possible:

- Total score = 3–5: G1 (Low grade or well differentiated)
- Total score = 6–7: G2 (Intermediate grade or moderately differentiated)

- Total score = 8–9: G3 (High grade or poorly differentiated) (American Joint Committee on Cancer (*AJCC*) , 2010).

2.10 The Studying Parameters:

2.10.1.Metabolism of Phospholipids

Phospholipids, which form the bi-layer structures of all cellular membranes, are an essential component of all cells, the phospholipid content was shown to increase with cell transformation and tumor progression (Szachowicz –Petelska B. *et al*. , 2013, and Dobrzyńska I. *et al*.,2015). Lipid metabolism activation in BC cells is recognized as a hallmark of carcinogenesis (Hilvo M. *et al*. , 2011). Increased fatty acid (FA) synthesis due to increased levels of fatty acid synthase (FAS) has been observed in various cancers and is correlated with a poor prognosis in many instances (Menendez J. and Lupu R. ,2007).

Phospholipids play the dual role of being basic structural components of membranes and acting as substrates of reactions involved in key regulatory functions in mammalian cells (Fagone P. and Jackowski S. ,2013). Hydrolysis of PtdCho, the most abundant phospholipid in eukaryotic cell membranes, can generate second messengers, such as diacylglycerol (DAG), phosphatidic acid (PA), lysophosphatidic acid (LPA), arachidonic acid (AA), and lysophosphatidylcholine (LPtdCho). These PtdCho metabolites are produced through three major catabolic pathways, respectively, mediated by specific phospholipases of type C (PC-PLC) and D (PLD), which act at the two distinct phosphodiester bonds of the PtdCho headgroup, and by phospholipases of type A2 and A1 (PLA2 and PLA1), PCho accumulation either produced by ChoK in the first reaction of the three-step Kennedy biosynthetic pathway or by PLC-mediated PtdCho catabolism is associated with tumor growth and

progression (Beloueche-Babari M. *et al.* , 2010 ; Glunde K. *et al.* , 2011; Podo F. *et al.*,2011, and Podo F. *et al.* , 2016). Concentrations of the two major phospholipid components phosphatidylcholine (PtdCho) and phosphatidy lethanol amine (PtdEtn) increased with increasing breast cancer tumor grade (Beckonert O. *et al.* 2003), indicating that the phospholipid synthesis rate increases with oncogenesis and tumor progression as compared to normal tissue (Glunde K**.** *et al.* , 2011). phosphocholine (PC) was also reported as a useful imaging biomarker of tumor response to several targeted treatments of cancers (Beloueche-Babari M. *et al.* , 2012, and Ward C. *et al.* , 2013).

2.10.1.1. Phospholipase D1 (PLD1)

PLD1 (120 kDa) is an enzyme mainly found in the inner membranes of mammalian cells including the secretory granules, endosomes, lysosomes, and Golgi complex (Colley W. *et al*., 1997 ; and Corrotte M. *et al.*, 2006).

PLD (EC:3.1.4.4) is an enzyme that belongs to the phospholipase super family. Phospholipases occur widely and are found in a broad range of organisms such as viruses, yeast, bacteria, animals, and plants (Zhang Y. and Frohman M., 2014 ; Frohman M., 2015; and Nelson R. and Frohman M. , 2015). The primary substrate in PLD is phosphatidylcholine(PtdCho) which is one of the most abundant components found in the lipid bi-layer of the plasma membrane, as shown in Fig.(2- 3A), PtdCho is composed of a choline head, a phosphate, a glycerol and two fatty acids, PtdCho can be quickly hydrolyzed by PLD to generate soluble choline and a signaling molecule known as phosphatidic acid (PA) (Billah M. *et al.*, 1989). As shown in Fig.(2-3B), PLD can hydrolyze PtdCho into choline and lipid PA, as well as catalyze

transphosphatidylation in the presence of primary alcohols where the phosphatidyl group is transferred to the alcohol to generate phosphatidyl alcohol (Yang S. *et al.*, 1967). Previous studies have shown that mammalian cells encode two PLD isoforms; PLD1 and PLD2 (Colley W. *et al*., 1997, Nakashima S. *et al*., 1997). Since this discovery, PLDs have been studied intensively and it has been noted that these two isoforms play a vital role in physiological processes in the body, including receptor-mediated endocytosis, cell migration, and cytoskeletal reorganization (Kang D. *et al*., 2014). Furthermore, they have been implicated in the pathophysiology of various diseases such as the progression of cancer, Alzheimer's disease, and Parkinson's disease (Bae E. *et al*., 2014).

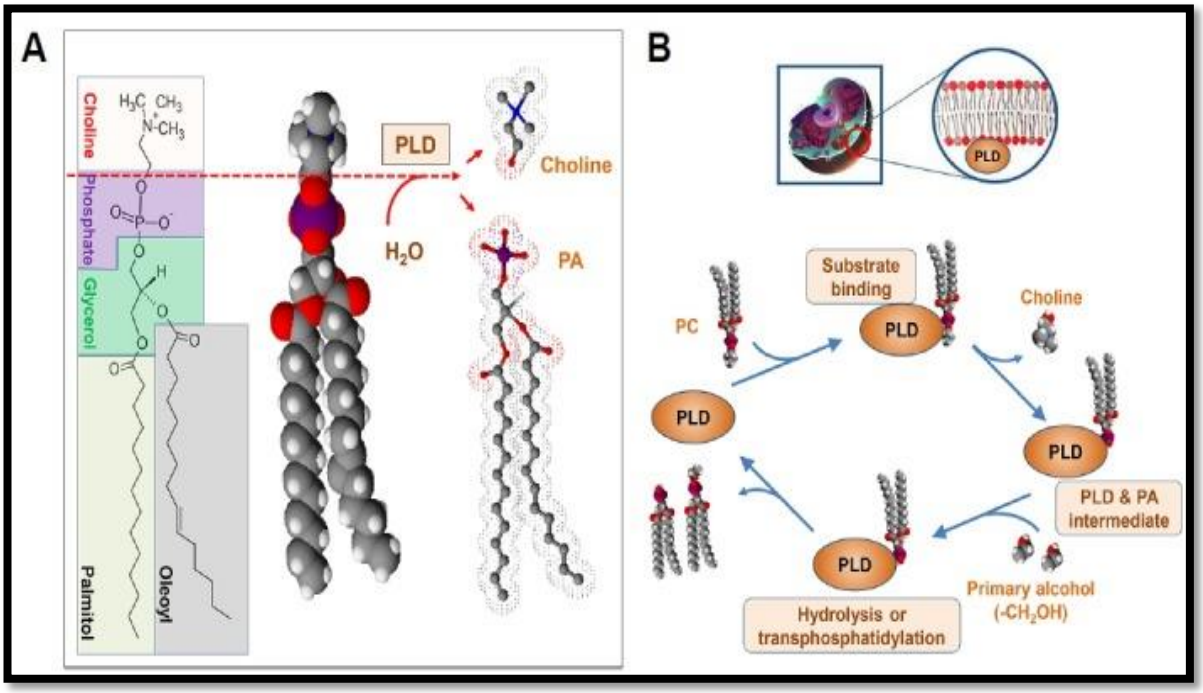


Figure (2- 3): Structure of a PC and enzymatic reaction of PLD with PC, hydrolysis or transphosphatidylation(A: depicted from Billah M. et al., 1989)(B: depicted from Yang S. et al., 1967).

(A) Phosphatidyl choline, a lipid formed from a choline head, a phosphate, a glycerol and two fatty acids. (Dark gray: carbon atoms; Light gray: hydrogen atoms; Red: oxygen atoms; Violet: phosphorus atom; Blue: nitrogen atom.). ***(B)*** The model summarizes of catalytic mechanism of PLD in biochemical reaction. Catalysis proceeds via the formation of PA (a covalent enzyme) intermediate. Hydrolysis or transphosphatidylation involves nucleophillic attack on the diester phosphate group of this intermediate by water or the hydroxyl group of a primary alcohol. PA, phosphatidic acid; PtdCho , phosphatidylcholine; PLD, phospholipase D.

2.10.1.2. Choline Kinase Alpha (CHKA)

Enhanced choline metabolism is emerging as a novel metabolic hallmark in cancer. Specifically, an increase in the synthesis of phosphatidylcholine, a major phospholipid of cellular membranes, can be detected in different cancers (Wu G. and Vance D. , 2010, and Glunde K. *et al*. ,2011) .

Choline kinase (ChoK) (EC:2.7.1.32) is an enzyme encoded by two genes, CHKA and CHKB, which catalyzes the phosphorylation of free choline in the cytoplasm using ATP to generate phosphocholine, three isoforms of ChoK are known in mammalian cells: ChoKα-1, ChoKα-2, and ChoKβ, and the active form of the enzyme consists of a hetero- or homo-dimer (Aoyama C. *et al.* , 2004). ChoK is the first enzyme in the Kennedy pathway, which is responsible for the synthesis of phosphatidylcholine and phosphatidyl ethanolamine, ChoKα expression and activity have been found up regulated in several types of cancer, including lung, prostate, colorectal, breast, ovarian, and bladder (Iorio E.

et al. , 2010; Li Z. *et al.* , 2014) , becoming an attractive potential therapeutic target in oncology (De la Cueva A. *et al.* , 2013).

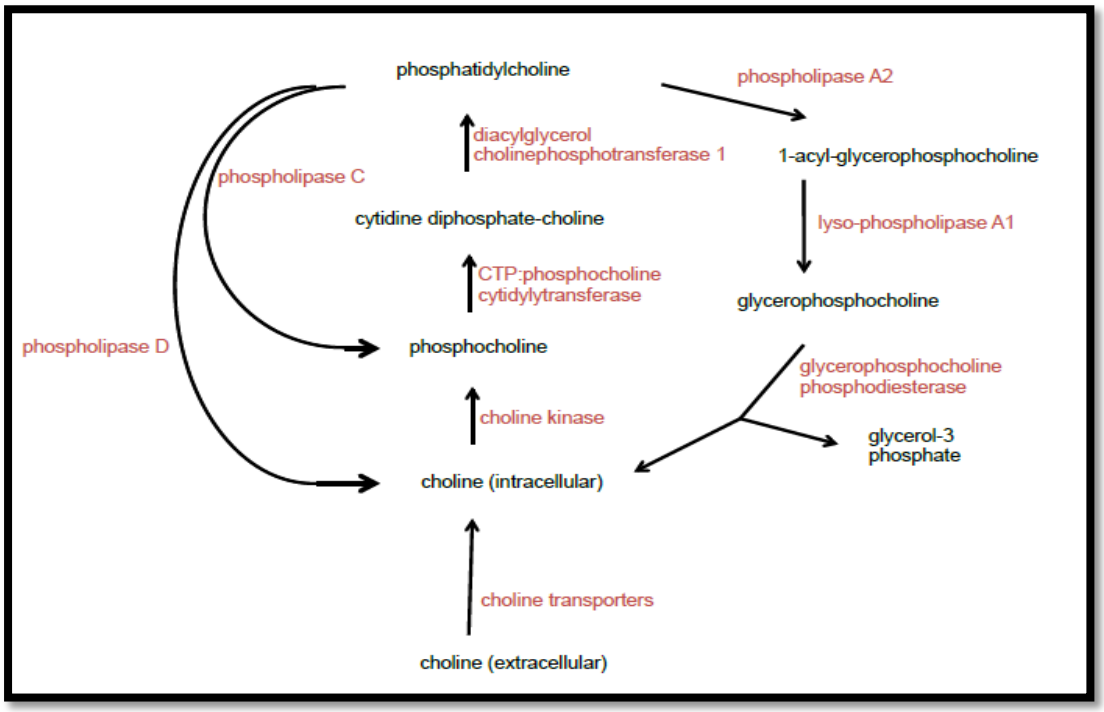


Figure (2- 4): Schematic diagram of choline and phosphatidylcholine metabolism(depicted from Glunde K. et al. 2011).

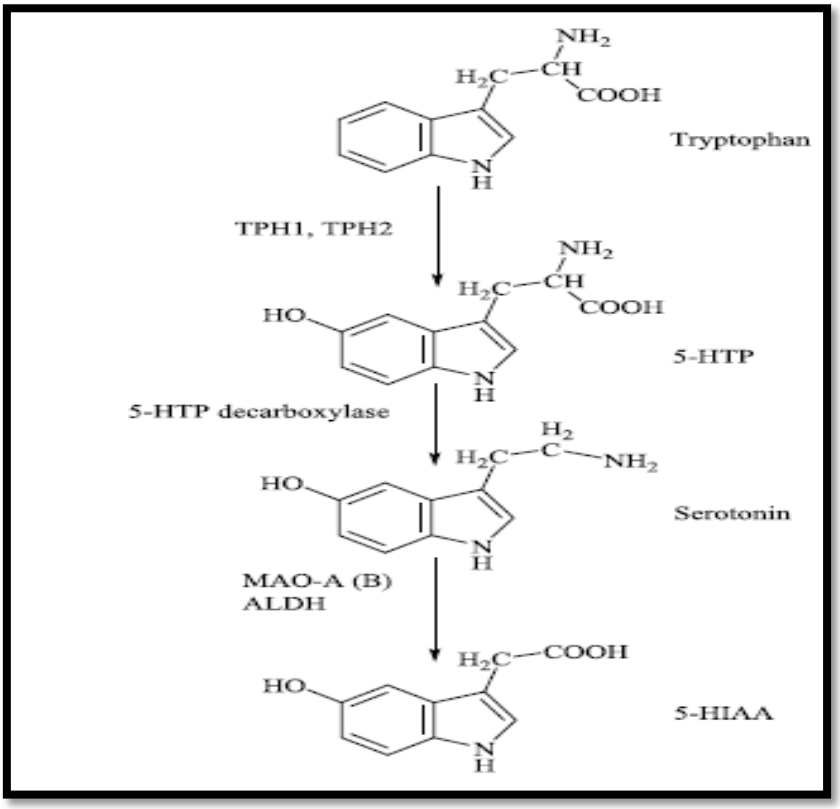
The above diagram is redrawn from a figure shown in (Glunde K. *et al.* 2011). Metabolites and enzymes are respectively shown in black and red respectively.

2.10. 2 Serotonin Hormone

Serotonin (5-hydroxytryptamine, 5-HT) is a biogenic monoamine that acts as a neurotransmitter in the central nervous system (CNS), local mediator in the gut and vaso active agent in the blood, serotonin is synthesized by a two step pathway from the essential amino-acid tryptophan (Fig.2- 5). (Bornstein J. , 2012).

Monoamine serotonin (5-hydroxytryptamine; 5HT) mediates a wide range of pathophysiological processes, including tumor growth and

differentiation (Siddiqui E. *et al.* , 2005). In clinical practice, study has examined the possible association of circulating serotonin with prognosis of several types of cancer, but to the best of this knowledge, only serotonin within platelets has been measured (Jungwirth N. *et al.* ,2008) , while no attempts were made to explore the potentially important pool of free serotonin in plasma, it has been shown that breast cancer cells can synthesize 5HT, which are used to support tumor growth (Pai V. *et al.* , 2009). It was suggested that increased tryptophan metabolism *via* the serotonin pathways could, therefore be linked to malignant progression in breast cancer (Pai V. *et al.* , 2009, Juhász C. *et al.* , 2012).



Figure(2- 5): Biosynthesis and metabolism of serotonin(depicted from Bornstein J. , 2012).

Serotonin is produced in two steps. Tryptophan hydroxylase (TPH) catalyzes tryptophan transformation to 5 hydroxytryptophan (5-HTP) , 5-HTP is further decarboxylated by 5-HTP decarboxylase (or aromatic L-amino acid decarboxylase) to form serotonin, after

biosynthesis, serotonin is concentrated in intracellular compartment, serotonin is metabolized mainly into 5-hydroxyindoleacetic acid (5-HIAA) by monoamine oxidase (essentially MAO-A) and aldehyde dehydrogenase (ALDH) and 5-HIAA is excreted in urine (Bornstein J. , 2012).

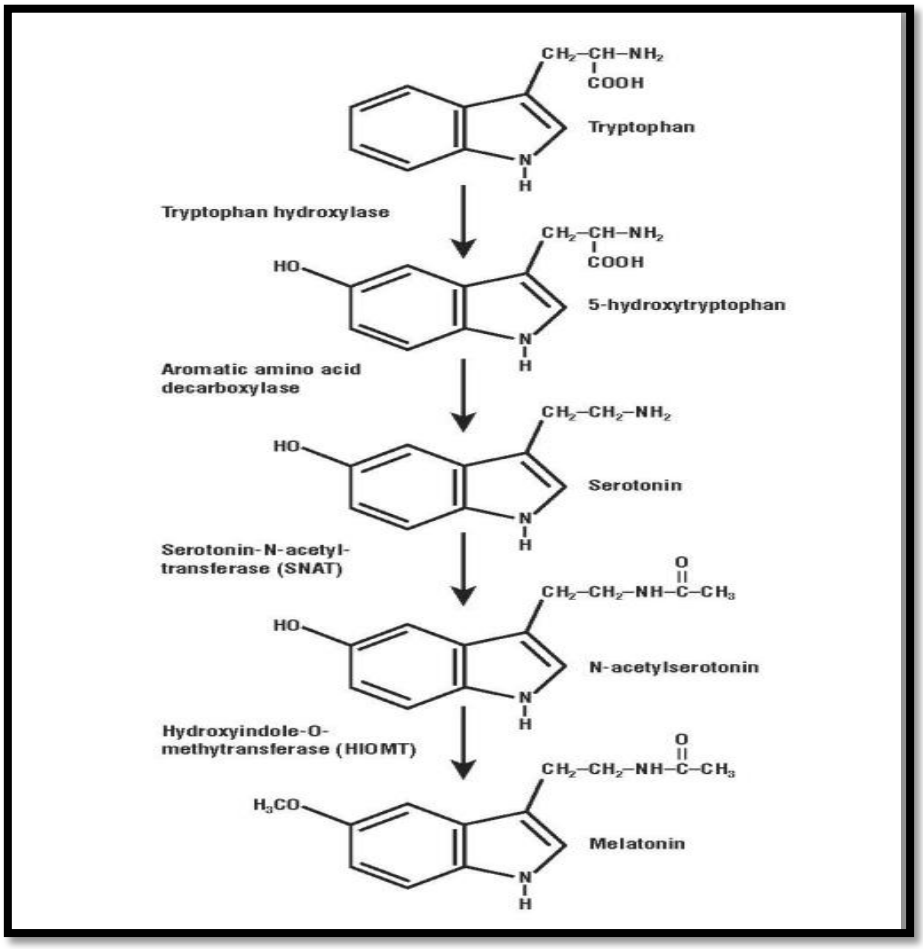
In mammals serotonin produced principally by enterochromaffin cells, it is found within the gut and stored within blood platelets (Nichols D. and Nichols C.,2008) . Serotonin has an effect on the number of physiologic and behavioral function, it plays a number of very important roles in normal brain function, which include modulation of mood states, memory, emotion, anxiety, endocrine effects appetite, hunger, aggression, cognition, gastrointestinal function, emesis, endocrine function, motor function, perception, neurotrophism, sensory function, sex, sleep and vascular function, and many others (Pytliak M. *et al.*, 2011, Chojnacki C. *et al.* ,2013). Serotonin exhibits a growth stimulatory effect on several types of carcinoma, carcinoid and other tumor cells, in contrast few data are available on serotonin involvement in cancer cell migration and metastatic processes (Sarrouilhe D. *et al.* , 2015) .

2.10.3. Melatonin Hormone

Melatonin is a small indolamine principally produced in vertebrates by the pineal gland and released in the bloodstream, melatonin shows a sharp peak of secretion at night, delivering the circadian message to the organism, in humans, it has several mechanisms of action, through its binding to G protein coupled melatonin receptors including circadian rhythm control, sleep cycle and many aspects of cancer inhibition, melatonin also exerts receptor-independent functions, such as detoxification of free radicals, protecting key molecules from oxidative stress under conditions of ischemia, drug toxicity or ionizing radiation

(Reiter R. *et al*., 2014). Melatonin interferes with cancer at all the phases of the illness: initiation, progression and spreading from the primary focus. Surprisingly, many molecular mechanisms have been proposed to explain its inhibitory actions (Reiter R. *et al.* , 2017).

Melatonin also exerts anti-oxidant effects, controlling the production of reactive oxygen species (ROS) modulating the intracellular free radical stress and therefore determining the progression of the tumor (Jaworek J. and Leja-Szpak A. , 2014). The protective role of melatonin as an anti-oxidant agent has been characterized in detail in other cancer types (Kimball S. *et al*., 2008).The synthesis of melatonin as shown in Fig (2-6).



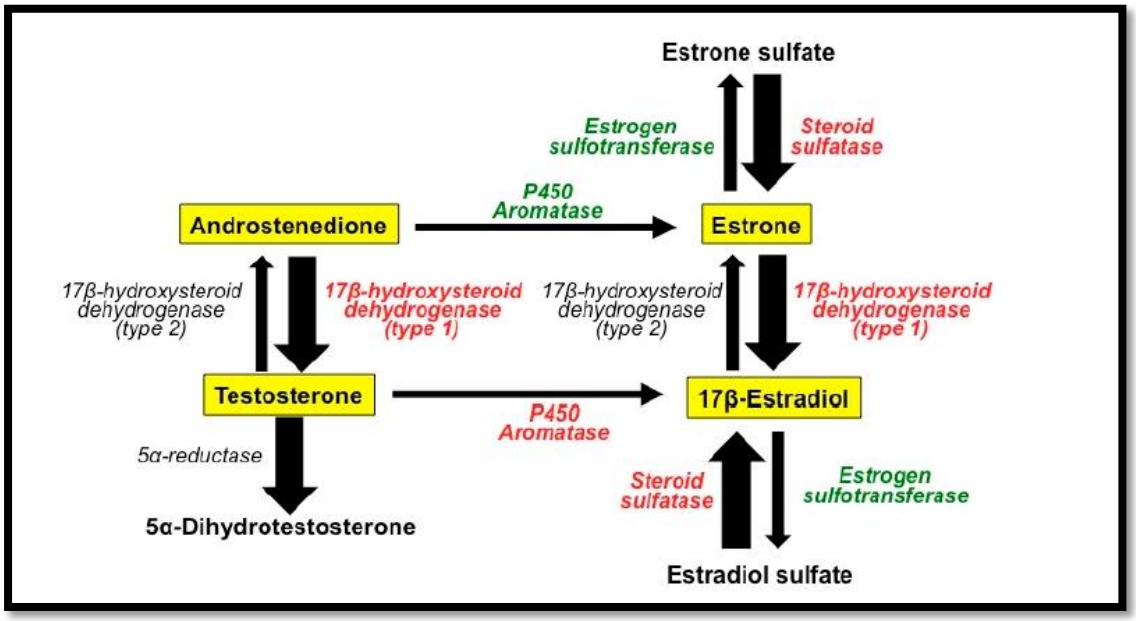
Figure(2- 6): This diagram provides a visual representation of the chemical reactions that take place during the production of melatonin in the human pineal gland (depicted from Harper and Kristin. , 2014).

- The Role of Estrogens in Breast Cancer:

About 70% of all breast cancers express estrogen-receptors (ER+) and circulating concentrations of estrogens are positively associated with an increased risk of BC in premenopausal women (Key T. *et al*. ,2013).

The influence of estrogens on mammary tissue depends on the circulating levels of estradiol and other steroids as well as on their local concentration in mammary tissue, while the main source of estrogens in premenopausal women is the ovaries, in postmenopausal women adrenal androgens and sulfated estrogens are the primary circulating steroids, and these steroids are converted into active estrogens by enzymatic processes in the mammary tissue.(Fig. 2-7) depicts the basic enzymatic mechanisms involved in the transformation of steroids in the mammary gland (Pasqualin J. and Chetrite G., 2005). Aromatases are enzymes that transform androgens (testosterone and androstenedione) into estrogens (estrone and 17β-estradiol) (Conley A. and Hinshelwood M., 2001).These estrogens, due to the action of estrogen sulfatases and estrogen sulfotransferases (Suzuki T. *et al*., 2003). Another family of enzymes, the 17β-hydroxysteroid dehydogenases types I and II, catalyzes the conversion of low activity steroids (androstenedione and estrone) into high activity steroids (testosterone and 17β-estradiol respectively) (Hilborn E. *et al.* , 2017). Contrary to what happens in normal mammary tissue, in breast cancer, the local production of steroids is biased toward the production of the more active forms (a fact indicated by the thickness of the arrows in Fig. 2- 7), melatonin acts as a selective estrogen enzyme modulators (SEEM) by inhibiting the expression and activity of the enzymes (labeled in red) responsible for the formation of biologically active estrogens from steroids with low biological activity, whereas it increases the expression and activity of the enzymes (labeled in green)

involved in the transformation of estrogens into their inactive sulfo conjugates, in breast cancer tissue, the local production of steroids is biased towards production of the more active forms (wide arrows) (Pasqualin J. and Chetrite G., 2005).



Figure(2- 7): The basic enzymatic mechanisms involved in the transformation of steroids in the normal mammary gland and in breast cancer tissue (depicted from Pasqualin J. and Chetrite G., 2005).

2.10.4. C- Reactive protein (CRP)

Inflammatory pathways play an important role in the causation of breast cancer, there is a bidirectional link between chronic inflammation and carcinogenesis: tumor originates and progresses at the site of chronic inflammation while tumor cells attract immune cells and promote the production of cytokines and chemokine creating tumor microenvironment. Hence, cancer is associated with the persistent inflammatory state, there is a vicious cycle and complex interplay between cancer and inflammation , so researchers proposed that serum CRP could be a marker of increased risk for breast cancer(Hanahan D. and Weinberg R., 2000).

CRP is raised in circulation in response to acute inflammation, infection, and tissue damage, the rise in CRP levels is proportional to degree of tissue damage, its plasma half-life is 19 hours and is catabolized by hepatocytes (Mantovani A. *et al.*, 2008).

Numerous prospective epidemiological studies have observed the association of CRP at the time of diagnosis of breast cancer with the prognosis of the disease , CRP is synthesized in hepatocytes in response to cytokines, particularly IL-6, released from leucocytes within the tumor microenvironment. IL-6 also helps in binding CRP to phospholipids on tumor cells that results in activation of classic C1q complement pathway. Here it acts as opsonin leading to tumor cell lysis (Cha-Molstad H. *et al.*, 2007).

2.10.5.Adiponectin Hormone

The circulating adiponectin levels are inversely associated with the risk of obesity-related malignancies, including breast cancer (Tworoger S. *et al.*, 2007*)*. Low blood concentrations of adiponectin are associated with high incidence and poor prognosis of breast cancer (Fu Y. *et al.*, 2005). Adipose tissue serves as the site of peripheral aromatization of adrenal androgens to estrogens, which induce mitogenic activity in mammary tissue by binding to estrogen receptors, adiponectin has been inversely associated with estrogen levels, remains possible that adiponectin may influence breast cancer risk by altering circulating estrogen levels (Nabablou M. *et al.*, 2014).Interventions of obesity related provide an important idea for breast cancer prevention. As important adipokines, adiponectin is considered to be one of the key factors for obesity carcinogenic (Dalamaga M. *et al.*, 2012) .

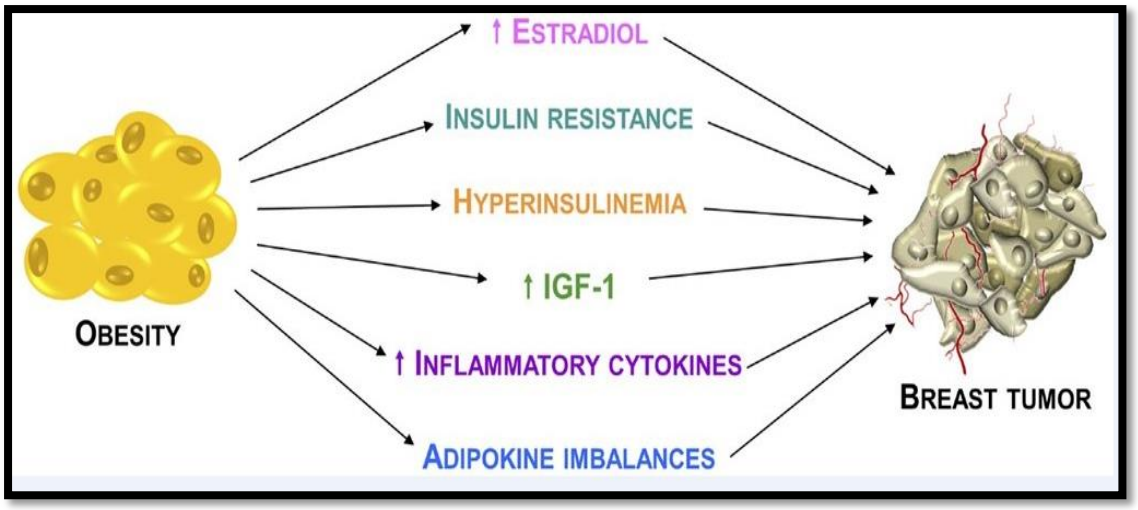


Figure (2- 8) : Relationship between obesity and breast cancer. (depicted from McTiernan A. et.al., 2003).

Principal mechanisms through which the obesity condition may promote breast cancer development and progression.

2.10.6. Body Mass Index (BMI)

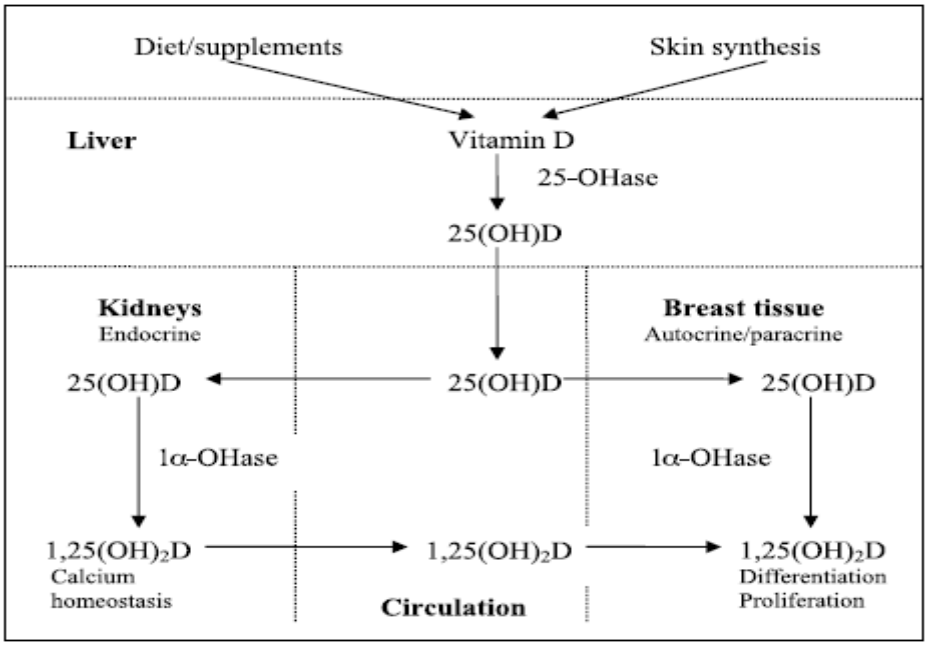
The usage of body mass index (BMI) to characterize the different body/obesity types has been commonplace for decades, yet limitations persist in its use. BMI is a calculated value [body weight (kg) divided by square height (m^2)] and exists as an easy and simple tool in the clinic and in research to differentiate and categorize patients as underweight (BMI < 18.5), normal weight (18.5–24.99), overweight (25–29.99), and obese (>30) (Bhaskaran K. *et al*., 2014).

Compared with people of normal weight, those who are overweight or obese are at greater risk for many cancers (Flegal K. *et al*., 2013 , Kitahara C. *et al*., 2014). Among postmenopausal women, those who are obese have a 20% to 40% increase in risk of developing breast cancer compared with normal-weight women (Munsell M. *et al*., 2014). The higher risks are seen mainly in women who have used menopausal hormone therapy and for tumors that express hormone receptors. Obesity is also a risk factor for breast cancer in men (Brinton L. *et al*., 2014). In

premenopausal women, by contrast, overweight and obesity have been found to be associated with a 20% decreased risk of breast tumors that express hormone receptors (Munsell M. *et al*., 2014).

2.10.7. Vitamin D3

Vitamin D is a seco-steroid hormone also known as sunshine vitamin as more than 90% of vitamin D is synthesized in skin in response to ultraviolet (UV) radiations from sunlight exposure (wavelength,290- 315 nm) (Bolland M. *et al.*, 2011). However, calcitriol, or 1,25-dyhydroxy-vitamin D3, is a multifunctional steroid hormone with many extra skeletal actions and may regulate signaling pathways related to cancer development and progression, the major circulating form of vitamin D is 25-hydroxyvitamin D [25(OH)D] formed after the first hydroxylation of vitamin D3 or vitamin D2 in the liver, in the kidneys, 25(OH)D is converted to the active metabolite $1,25(OH)_2D$ after second hydroxylation. $1,25(OH)_2D$ has a short half life and tight homeostatic control (Kulie T. *et al.*, 2009).Once $1,25(OH)_2D$ completes the task of maintaining normal cellular proliferation, inhibition of angiogenesis, differentiation and apoptosis in Fig (2-9) , it induces expression of the enzyme 24-hydroxylase (24-OHase) which enhances the catabolism of $1,25(OH)_2D$ (Rose A. *et al*., 2013). 25(OH)D is the best indicator of vitamin D status and is related to maintaining the $1,25(OH)_2D$ homeostatic control which is thought to be potentially relevant to BC risk (Holick M. , 2007). The local production of $1,25(OH)_2D$ in the breast, colon, prostate and other tissues is supposed to regulate a variety of genes that control proliferation, inhibit angiogenesis and induce differentiation and apoptosis (Bertone-Johnson E. *et al*., 2005).

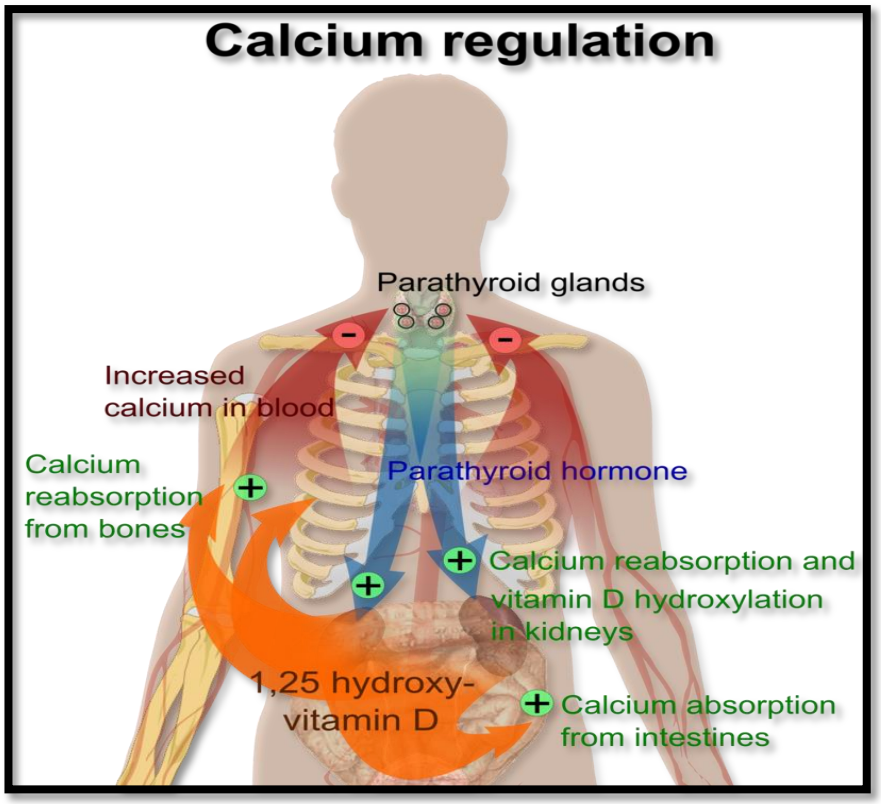


Figure(2- 9): A simplified diagram of vitamin D metabolism(depicted from Colston K., 2002).

2.10.8. Calcium

The total amount of calcium in the human body ranges from 1000 to 1200 g. Approximately 99% of body calcium resides in the skeleton; the other 1% is present in the extracellular and intracellular spaces. Although >99% of the total body calcium is located in bone, calcium is a critical cation in both the extracellular and intracellular spaces (Hebert S. and Brown E. , 1996).

Calcium balance is tightly regulated by the concerted action of calcium absorption in the intestine, reabsorption in the kidney, and exchange from bone, which are all under the control of the calciotropic hormones that are released upon a demand for calcium, although serum calcium levels can be maintained in the normal range by bone reabsorption, dietary intake is the only source by which the body can replenish stores of calcium in bone in Fig (2-10) (Johnson J. and Kumar R. , 1994).



Figure(2- 10): Calcium regulation in the human body(depicted from Boron - Walter F. and Boulpaep- Emile L. , 2003).

Calcium is involved in many cellular process including those involved in the process of carcinogenesis, as gene transcription, cell motility, angiogenesis**,** calcium regulates various cellular processes, including those relevant to tumorgenesis, such as cell motility, angiogenesis, gene transcription, apoptosis and proliferation (Dickinson H. *et al.* , 2006).

Hypocalcemia may be implicated in malignancy as resistance to apoptosis is accompanied by reduction in the calcium content of the lumen of the endoplasmic reticulum (Brame L. *et al*., 2004). On the other hand, the presence of hypercalcemia in cancer patients confers a poor prognosis. Depending on the type of malignancy, hypercalcemia can result from production of circulating factors that stimulate osteoclastic

resorption of bone, and form direct invasion of bone due to metastatic disease (Hofbauer L. and Heufelder A., 2001).

2.10.9.Alkaline Phosphatase (ALP)

Alkaline phosphatase [ALP; orthophosphoric monoester phosphohydrolase (alkaline optimum) EC 3.1.3.1] are plasma membrane-bound glycoproteins (Tsai L. *et al.* , 2000). Mammalian alkaline phosphatases (ALPs) are zinc-containing metalloenzymes encoded by a multigene family and function as dimeric molecules. Three metal ions including two Zn^{2+} and one Mg^{2+} in the active site are essential for enzymatic activity. However, these metal ions also contribute substantially to the conformation of the ALP monomer and indirectly regulate subunit–subunit interactions (Hoylaerts M. *et al.* , 1997).The increased in total alkaline phosphatase activity is either due to physiological causes, or is caused by disease of the liver or bone. Also its activity may be elevated in one or more forms of cancer such as multiple myeloma and breast tumors (Al-Gazally M. , 2007).

2.11.Immunohistochemistry (IHC)

Immunohistochemistry (IHC) studies are a major source of data on protein expression and location (Guillaud M. *et al.*, 2005).

- Basic Immunohistochemistry

We begin with formalin-fixed, paraffin-embedded (FFPE) tissues, processed into unstained slides. These are treated with hydrogen peroxide to quench endogenous peroxidase, which may be seen in erythrocytes and granulocytes and can be a common cause of background staining (Ramos-Vera J. ,2005 ; Key M. ,2006). The next step has had a great impact on recent advances in IHC. Formalin fixation, especially over extended

periods, causes the blockage of antigen epitopes (antigenic determinants) on the tissue surface via cross-linking, and historically this has severely limited the utility of IHC. Heat induced epitope retrieval (HIER) acts to remove the formalin blockage, freeing the targeted epitopes to allow for successful binding (Ramos-Vera J. ,2005). The amount of heat and the method by which it is applied varies by tissue type, by conditions under which the tissue was fixed, and by antibody specifications. The longer the time the tissue has been stored in formalin, the more intense a retrieval method may be required. However, there is an upper limit above which overheating the tissue renders IHC results unreliable. Although tissues preserved in formalin for an extended period of time may no longer have identifiable epitopes even with HIER, archival paraffin-embedded tissues may retain sufficient antigen preservation for IHC studies. After HIER, the primary antibody is applied, and ideally the Fab region (area other than the tail of the ‘‘Y’’-shaped protein) of the antibody attaches to the recently uncloaked epitope of the antigen on the target tissue. The primary antibody is usually raised in an animal species different from the species of the tissue to be examined. The next step is to apply the secondary antibody, which reacts against the exposed Fc region (the tail of the ‘‘Y’’) of the primary antibody. This secondary antibody is often referred to as the ‘‘linking’’ antibody, and in this procedural example, an avidin-biotin complex (known as the label) is applied, and it binds to the Fc region of the secondary antibody. Next, a chromagen dye is used to visualize the antibody-antigen reaction, and a hematoxylin counter stain is applied as the last step. Now the slides are ready for viewing. Again, there are numerous methods by which IHC is accomplished and this example illustrates one of the most frequently used detection methods, the Avidin-Biotin Complex detection system (Key M. ,2006). The use of positive and negative controls is paramount and should be routine in IHC

(Ward J. ,2004). Negative controls confirm that the staining being reported is due to the antibody binding, and positive controls confirm that the antibody is working and that the suspected target of the antibody is the actual target. Internal controls, normal tissues on the stained slide known to be either positive or negative for the marker in question, are excellent confirmatory evidence. While actual images of those controls need not necessarily be presented, discussion of the choice of positive and negative controls should be included in the materials and methods section of the manuscript. All of the results reported in the present document, as well as the images included, were compared to positive and negative controls.

CHAPTER THREE

MATERIALS AND METHODS

3. Materials and Methods

3.1 Chemicals

Chemicals that were used in this study with their companies are showed in table (3-1).

Table (3-1): Chemicals and Their Suppliers

NO.	Chemicals	Supplied Company
1	Alkaline phosphatase kit	Biomerieux , France
2	Adiponectin kit	Elabscinece , USA
3	C- reactive protein kit	Boditech , Korea
4	Calcium kit	BioLabo , France
5	Choline kinase alpha kit	Al- Shkairate, Jordon
6	DPX	Germany
7	Estradiol kit	Elabscinece , USA
8	Eosin	India
9	Ethanol	UK
10	Formalin	UK
11	Hematoxylin	Fluka Chemical AG Switzerland

12	Melatonin kit	Elabscinece , USA
13	Paraffin wax	Merck (Germany)
14	Phospholipase D1 kit	Elabscinece , USA
15	Phospholipase D1primary antibody	Biorbyt , UK
16	Super sensitive IHC detection system kit	Biorbyt , UK
17	Serotonin kit	Elabscinece , USA
18	Vitamin D3 kit	Boditech , Korea
19	Xylene	(FlukaGarnatie Switzerland)

3.2 Collection of Blood Sample

About (5mL) of blood samples of breast caner patients and controls were taken and allowed to clot at room temperature in empty disposable tubes, centrifuged to separate it in the centrifuge at 3000 xg for 10min ,the serum samples were separated and stored at (-20^{o}C) until use unless used immediately to analyze biochemical parameters.

3.3 Design of Study

This study was designed as prospective study, all samples were taken from patients who attended the oncology center in Al-Habooby Hospital, Al-Hussain teaching Hospital and specialist clinics. During the period between 1/3/2018 to 15/1/2019.The study was included (85) blood samples from patients with breast cancer, (75) blood samples are collected from healthy women as a control group. For histopathological

and immunohistochemical analysis (50) tissue samples are collected from patients with breast cancer who undergoing surgical resection (mastectomy) and normal breast tissues: (control group, which include normal breast tissue from patients with benign lesions , and normal breast tissue from the same women with invasive ductal carcinoma).

The details of the numbers and age of the two groups are illustrated in table (3.2).

Table (3-2): Details of numbers and age of the studied groups

Groups	*No.*	*Range of Age (years)*
patients	85	25-65
Control	75	25-65
Total	160	25-65

The clinical chart of this study describe experimental parameters and immunohistochemical analysis in the table (3-3).

Table (3-3): The Clinical Chart of the Study

Patient Profile
Name:-
Age:-
Occupation:-
Address:-
Risk factors:-

BMI (kg/m²):-
Drug:-
Others:-
Biochemical Test
Enzymes: PLD1, CHKA , ALP
Hormones: Serotonin , Melatonin , Estradiol , Adiponectin .
CRP , Vit.D3 , Ca.
Immunohistochemical Test
Enzyme: PLD1 primary antibody.

3.4 Instruments

The instruments which were used in this study and their suppliers shown in Table (3-4).

Table (3-4): The Instruments and their manufacturers

NO.	Instruments	Manufacturers
1	Centrifuge	Universal 16A , Germany
2	Cover slide 24*50mm	China
3	Cool plate KEDEE	China Jin Hua KEDI CO.
4	Digital camera	Canon

5	Elisa	Bio Tek , P.N 7331000 , USA
6	Ground edge slide	China
7	Hot plate KEDEE	China Jin Hua KEDI CO.
8	I Chroma	Boditech Med Inc , Korea
9	Incubator	Jard , Japan
10	Light Microscope	China Meji Techno CO.
11	Manual Microtome KEDEE	China Jin Hua KEDI
12	Microtome blade	Germany
13	Positive charge slide	USA
14	Refrigerator	China
15	Tissue Floating bath	Japan
16	UV/VIS spectrophotometer	UV-7804C , Japan
17	UV/VIS spectrophotometer	T60 , PG Instruments Ltd , Germany

3.5 Methods

3.5.1 Biochemical Parameters:

Several considerable methods were used to measure the studied parameters. It is notable that all measurements were duplicated for each sample.

3.5.1.1 Determination of Serum phospholipase D1(PLD1) Activity:

Principle:

This ELISA kit was used the Sandwich-ELISA as the method. The micro ELISA plate provided in this kit has been pre-coated with an antibody specific to Human PLD1. Standards or samples were added to the micro ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for Human PLD1 and Avidin-Horseradish Peroxidase (HRP) conjugate were added to each micro plate well successively and incubated. Free components are washed away. The substrate solution was added to each well. Only those wells that contain Human PLD1, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color. The enzyme-substrate reaction is terminated by the addition of stop solution and the color turns yellow. The optical density (OD) was measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The OD value was proportional to the concentration of Human PLD1. You can calculate the concentration of Human PLD1 in the samples by comparing the OD of the samples to the standard curve.

Reagent preparation:

1.The kit was taken out from the refrigerator 20 minutes in advance to bring all reagents to room temperature (18-25 °C) before use.

2.The samples were brought to room temperature (18-25 °C) before use, mixed fully , avoided foaming.

3.The 25xConcentrated wash buffer was diluted to 1x working solution.

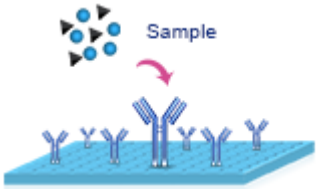
4.The reference standard was diluted to different concentrations.

5.The 100xBiotinylated detection Ab was diluted to 1x working solution 15 minutes earlier before step 1 finished.

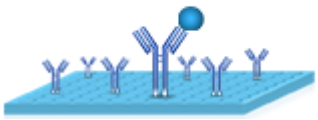
6.The 100xConcentrated HRP conjugate was diluted to 1x working solution 15 minutes earlier before step 2 finished.

7.The micro plate reader was preheated when step 6 starts.

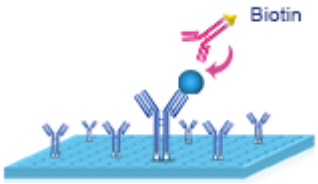
Assay Procedure:



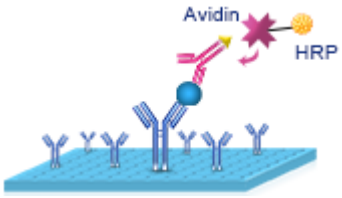
1. 100 µL standard or sample was added to each well. Incubated for 90 min at 37℃.



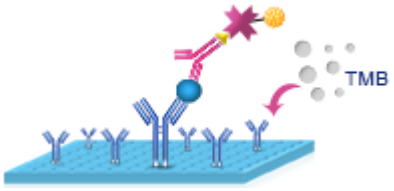
2. The liquid was removed.



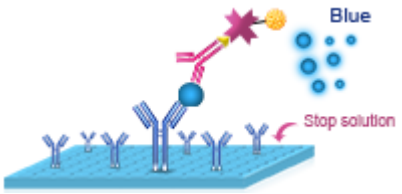
3. 100 µL Biotinylated Detection Ab was added . Incubated for 1 hour at 37℃. Aspirated and washed 3 times.



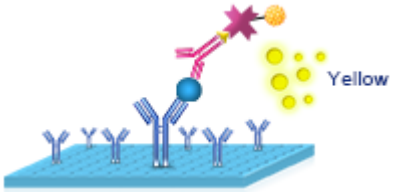
4. 100 µL HRP Conjugate was added. Incubated for 30 min at 37℃. Aspirated and washed 5 times.



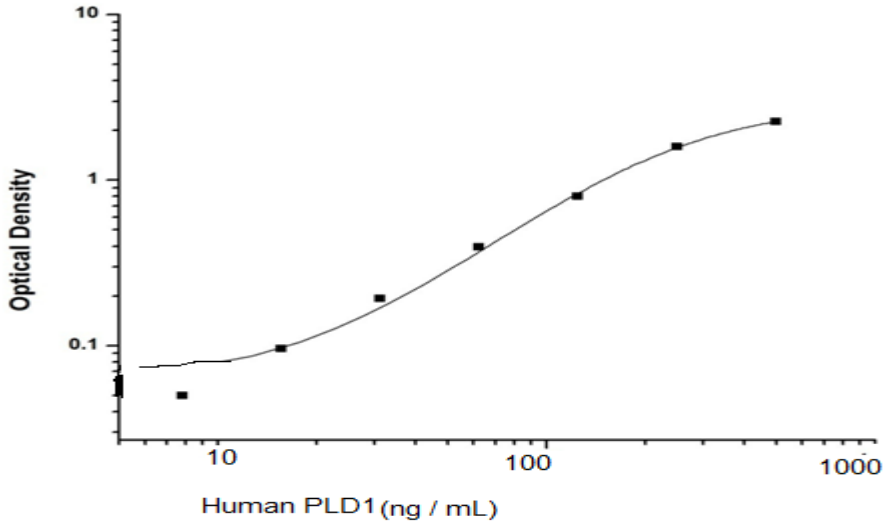
5. 90 µL of Substrate Reagent was added. Incubated for 15 min at 37℃.



6. 50 µL Stop Solution was add ed.



7. At 450 nm immediately was read.
Calculation of results.



3.5.1.2Determination of Serum Choline Kinase Alpha (CHKA) Activity:

Principle:

This kit was based on sandwich enzyme-linked immune-sorbent assay technology. Anti- CHKA antibody was pre-coated onto 96-well plates. And the biotin conjugated anti- CHKA antibody was used as detection

antibodies. The standards, test samples and biotin conjugated detection antibody were added to the wells subsequently, and washed with wash buffer. HRP-Streptavidin was added and unbound conjugates were washed away with wash buffer. TMB substrates were used to visualize HRP enzymatic reaction. TMB was catalyzed by HRP to produce a blue color product that changed into yellow after adding acidic stop solution. The density of yellow is proportional to the CHKA amount of sample captured in plate. Read the O.D. absorbance at 450nm in a microplate reader, and then the concentration of CHKA can be calculated.

Reagent Preparation:

The kit was Put at room temperature for 20 minutes before use.

1.Wash Buffer:

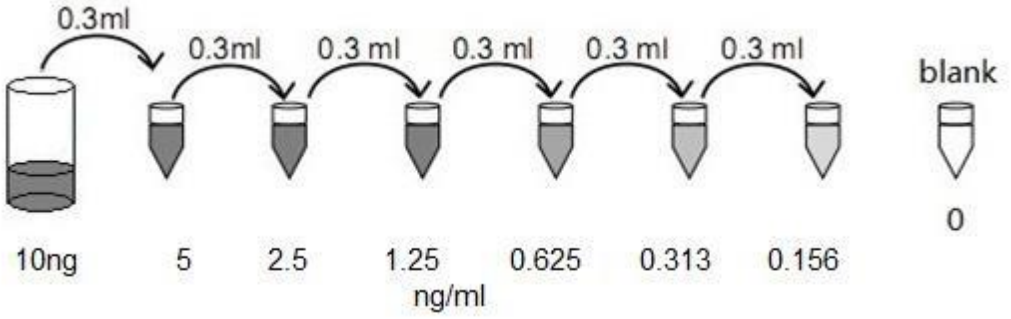
30mL Concentrated Wash Buffer was diluted into 750 mL Wash Buffer with deionized or distilled water. Put unused solution back at 4°C. If crystals have formed in the concentrate, you can warm it with 40°C water bath (Heating temperature should not exceed 50°C) and mixed it gently until the crystals have completely been dissolved. The solution should be cooled to room temperature before use.

2. Standard:

1). 10ng/ml of standard solution : 1 ml Sample / Standard dilution buffer was added into one Standard tube, kept the tube at room temperature for 10 minutes and mixed them thoroughly.

2).5ng/ml→0.156ng/ml of standard solutions: 6 Eppendorf tubes with (5, 2.5, 1.25, 0.625, 0.312, 0.156) ng/ml, was Labelled respectively. 0.3 ml of the Sample/Standard dilution buffer was added into each tube. 0.3 ml of the above 10ng/ml standard solution was added into 1st tube and mixed them thoroughly. 0.3 ml from 1st tube was transfered to 2nd tube

and mixed them thoroughly. 0.3 ml from 2nd tube was transferred to 3rd tube and mixed them thoroughly, and so on.



3. Preparation of Biotin-labeled Antibody Working Solution

It was prepared within 1 hour before experiment.

1) Total volume of the working solution was Calculated required: 0.1 ml / well × quantity of wells. (Allowed 0.1-0.2 ml more than the total volume).

2) The Biotin-detection antibody with antibody dilution buffer were diluted at 1:100 and mixed them thoroughly. (i.e. 1µl Biotin-labeled antibody was added into 99µl Antibody Dilution Buffer) .

4.Preparation of HRP-Streptavidin Conjugate (SABC) Working Solution:

It was prepared within 30 minutes before experiment.

1) Total volume of the working solution was Calculated required: 0.1 ml / well × quantity of wells. (Allowed 0.1- 0.2 ml more than the total volume).

2) the SABC with SABC dilution buffer were diluted at 1:100 and mixed them thoroughly. (i.e. 1µl of SABC was added into 99µl of SABC Dilution Buffer) .

Assay Procedure:

1. Standard, test sample and control (zero) wells were set on the pre-coated plate respectively, and then, recorded their positions. It was recommended to measure each standard and sample in duplicate. Washed plate 2 times before adding standard, sample and control (zero) wells.

2. 0.1ml of (10, 5, 2.5, 1.25, 0.625, 0.312, 0.156) ng/ml, standard solutions were aliquotted into the standard wells.

3. 0.1 ml of Sample/Standard dilution buffer was added into the control (zero) well.

4. 0.1 ml of properly diluted sample (Human serum) was added into test sample wells.

5. The plate was sealed with a cover and incubated at 37 °C for 90 minutes.

6. The cover was removed and discarded the plate content, and washed plate 2 times with wash buffer.

7. 0.1 ml Biotin-labeled antibody working solution was added into above wells (standard, test sample & zero wells). the solution was added at the bottom of each well without touching the sidewall.

8. The plate was sealed with a cover and incubated at 37°C for 60 min.

9. The cover was removed , and washed plate 3 times with wash buffer, and let the wash buffer stay in the wells for 1 minute each time.

10. 0.1 ml of SABC Working Solution was added into each well, cover the plate and incubated at 37°C for 30 minutes.

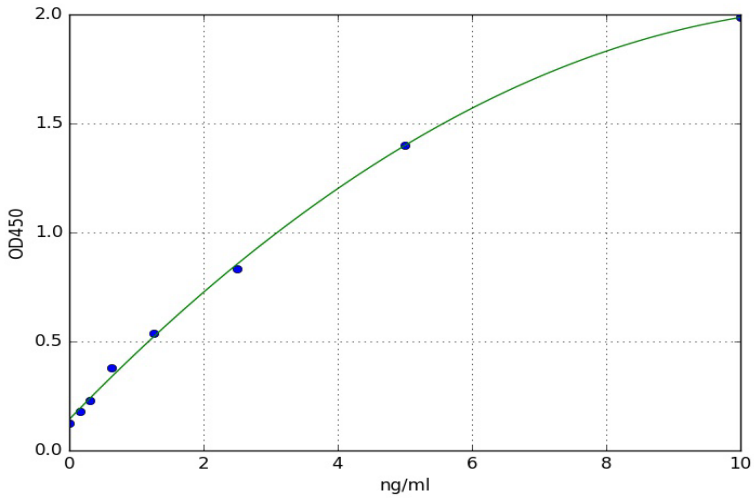
11. The cover was removed and washed plate 5 times with Wash Buffer, and let the wash buffer stay in the wells for 1-2 minute each time.

12. 90µl TMB substrate was added into each well, cover the plate and incubated at 37°C in dark within 15-30 minutes. (Note: This incubation

time is for reference only, end user shall determine the optimal time.) It will turn blue in the first 3-4 wells (with most concentrated CHKA standard solutions), the other wells may not display obvious color.

13. 50µl stop solution was added into each well and mixed them thoroughly. The color changes to yellow immediately.

14. The O.D. absorbance was read at 450 nm in microplate reader immediately after adding the stop solution.



3.5.1.3Determination of Serum Serotonin Hormone:

Principle:

This ELISA kit was used the Competitive-ELISA principle. The micro ELISA plate provided in this kit has been pre-coated with ST/5-HT. During the reaction, ST/5-HT in the sample or standard competes with a fixed amount of ST/5-HT on the solid phase supporter for sites on the biotinylated detection Ab specific to ST/5-HT. Excess conjugate and unbound sample or standard were washed from the plate, and avidin conjugated to Horseradish Peroxidase (HRP) were added to each microplate well and incubated. Then a TMB substrate solution was added

to each well. The enzyme-substrate reaction was terminated by the addition of stop solution and the color change was measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The concentration of ST/5-HT in the samples was then determined by comparing the OD of the samples to the standard curve.

Reagent preparation:

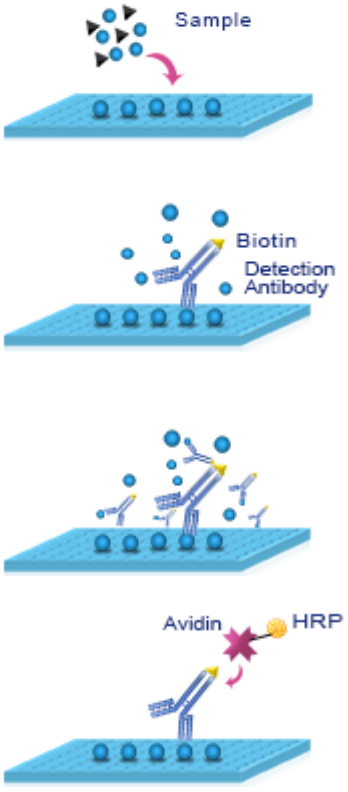
1.The concentrated wash buffer was diluted (1:25).

2.The reference standard was diluted to different concentrations.

3.The Biotinylated detection Ab was diluted (1:100) 15 minutes earlier before step1.

4.The concentrated HRP conjugate was diluted (1:100) 15 minutes earlier before step 5.

Assay Procedure :



1. 50 µL standard or sample was added to each well.

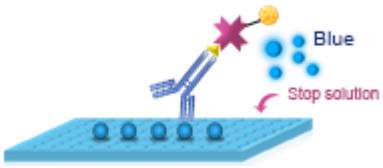
2.Immediately 50 µL Biotinylated Detection Ab was added to each well.

3. For 45 min at 37℃ was Incubated. Aspirated and washed 3 times.

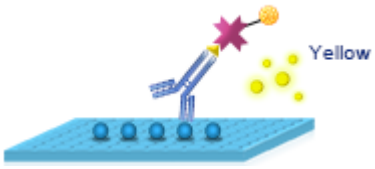
4. 100 µL HRP Conjugate was added to each

well. Incubated for 30 min at 37℃. Aspirated and washed 5 times.

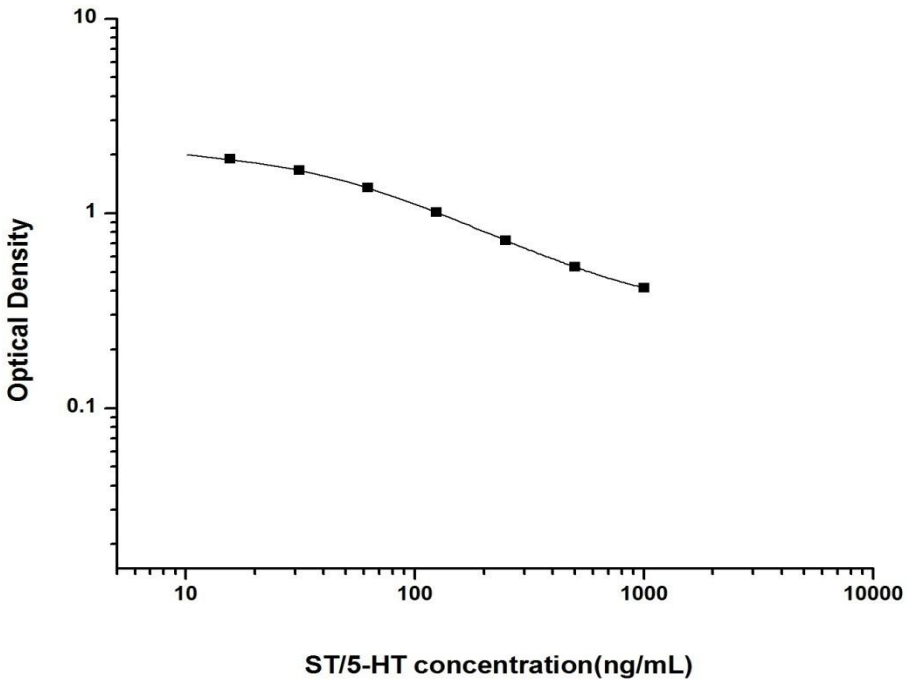
5. 90 µL Substrate Reagent was added . Incubated for 15 min at 37℃.



6. 50 µL Stop Solution was added.



7. At 450 nm immediately was read. Calculation of results.



3.5.1.4Determination of Serum Melatonin Hormone:

Principle:

This ELISA kit was used the Competitive-ELISA principle. The micro ELISA plate provided in this kit has been pre-coated with Human MT. During the reaction, Human MT in the sample or standard competes with a fixed amount of Human MT on the solid phase supporter for sites on the biotinylated detection Ab specific to Human MT. Excess conjugate and unbound sample or standard were washed from the plate, and avidin conjugated to Horseradish Peroxidase (HRP) were added to each microplate well and incubated. Then a TMB substrate solution was added to each well. The enzyme-substrate reaction was terminated by the addition of stop solution and the color change was measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The concentration of Human MT in the samples was then determined by comparing the OD of the samples to the standard curve.

Reagent preparation:

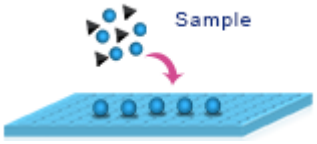
1.The concentrated wash buffer was diluted (1:25).

2.The reference standard was diluted to different concentrations.

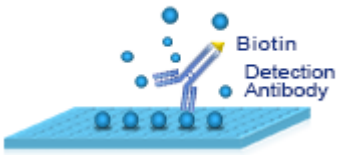
3.The Biotinylated detection Ab was diluted (1:100) 15 minutes earlier before step1.

4.The concentrated HRP conjugate was diluted (1:100) 15 minutes earlier before step 5.

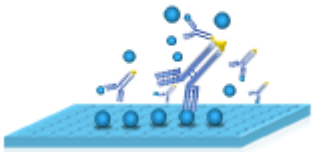
Assay Procedure :



1. 50 µL standard or sample was added to each well.

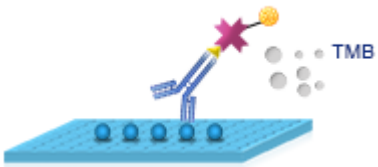


2.Immediately 50 µL Biotinylated Detection Ab was added to each well.

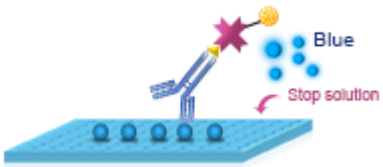


3. For 45 min at 37℃ was Incubated. Aspirated and washed 3 times.

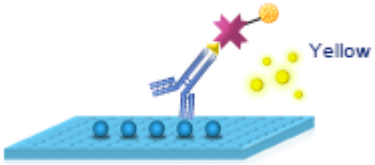
4. 100 µL HRP Conjugate was added to each well. Incubated for 30 min at 37℃. Aspirated and washed 5 times.



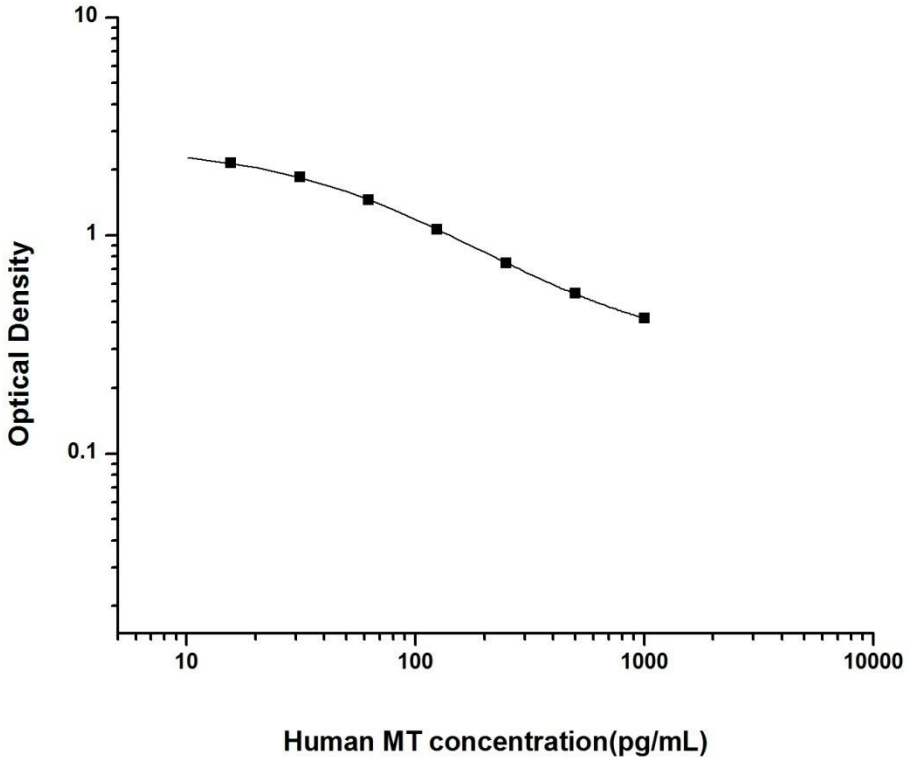
5. 90 µL Substrate Reagent was added . Incubated for 15 min at 37℃.



6. 50 µL Stop Solution was added.



7. At 450 nm immediately was read. Calculation of results.



3.5.1.5 Determination of Serum Estradiol Hormone:

Principle:

This ELISA kit was used Competitive-ELISA as the method. The microtiter plate provided in this kit has been pre-coated with Goat Anti-Rabbit IgG, make solid-phase secondary antibody. And then add samples, horseradish peroxidase-labeled Estradiol and anti- Estradiol antibody, so as to form a coated secondary antibody - anti- Estradiol antibody - HRP-labeled Estradiol complex. The amount of bound labeled Estradiol was inversely proportional to that of Estradiol in the samples. The TMB substrate solution was added to each well. The enzyme-substrate reaction was terminated by the addition of stop solution and the color change was measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The

concentration of Estradiol in the samples was then determined by comparing the OD of the samples to the standard curve.

Reagent preparation:

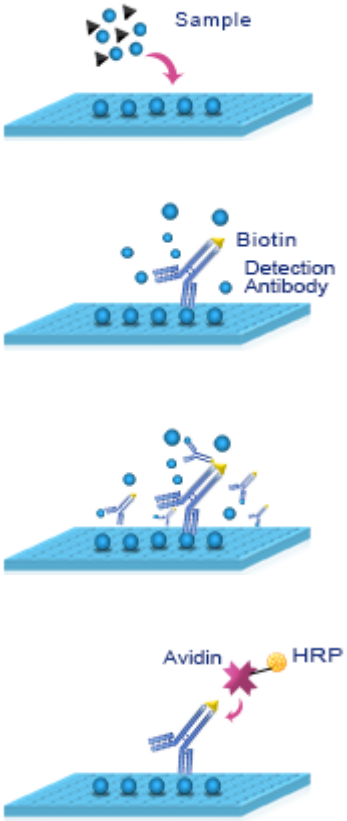
1.The concentrated wash buffer was diluted (1:25).

2.The reference standard was diluted to different concentrations.

3.The Biotinylated detection Ab was diluted (1:100) 15 minutes earlier before step1.

4.The concentrated HRP conjugate was diluted (1:100) 15 minutes earlier before step 5.

Assay Procedure :

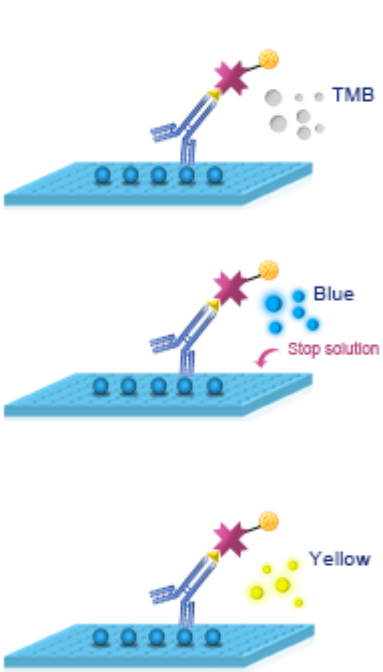


1. 50 µL standard or sample was added to each well.

2.Immediately 50 µL Biotinylated Detection Ab was added to each well.

3. For 45 min at 37℃ was Incubated. Aspirated and washed 3 times.

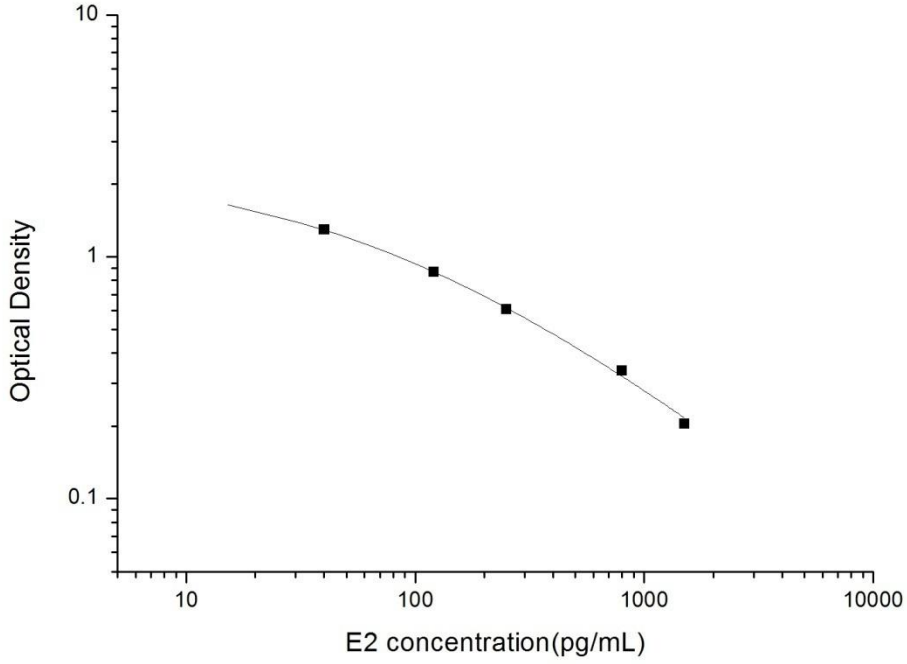
4. 100 µL HRP Conjugate was added to each well. Incubated for 30 min at 37℃. Aspirated and washed 5 times.



5. 90 µL Substrate Reagent was added . Incubated for 15 min at 37℃.

6. 50 µL Stop Solution was added.

7. At 450 nm immediately was read. Calculation of results.



3.5.1.6 Determination of Serum C- Reactive Protein:

Principle:

The test was used a sandwich immune detection method ; the detector antibody in buffer binds to antigen in sample , forming antigen – antibody

complexes, and migrates onto nitrocellulose matrix to be captured by the other immobilized – antibody on test strip.

The more antigen in sample forms the more antigen- antibody complex and leads to stronger intensity of fluorescence signal on detector antibody , which is processed by instrument for I chroma ™ test to show CRP concentration in sample.(Pepys M. and Hirschfield G., 2003).

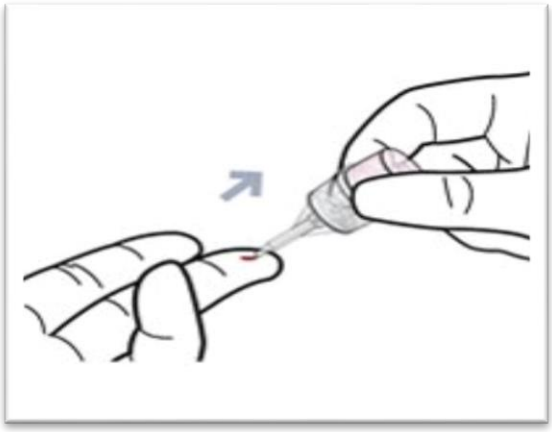
Test components

The **ichroma™** CRP consists of Cartridges , Detection buffer tubes, sample collectors, and an ID chip.

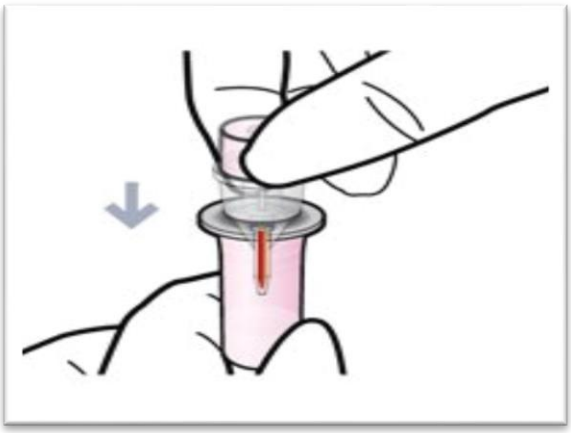
- The cartridge contains a test strip, the membrane which has anti human CRP at the test line, while rabbit IgG at the control line.
- Each cartridge is individually sealed in an aluminum foil pouch containing a desiccant. 25 sealed cartridges are packed in a box which also contains an ID chip.
- The detection buffer contains anti human CRP-fluorescence conjugate, anti rabbit IgG - fluorescence conjugate, bovine serum albumin (BSA) as a stabilizer and sodium azide in phosphate buffered saline (PBS) as a preservative.
- The detection buffer is pre-dispensed in a tube. 25 detection buffer tubes are packaged in a box and further packed in a Styrofoam box with ice- pack for the shipment.

Procedure :

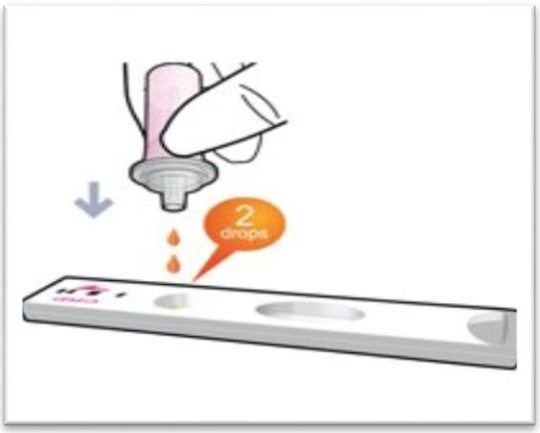
Here is a short instruction manual for the **ichromα** CRP test. For further details, refer to the information card on sampling and CRP test administration.



Step 1: sampling

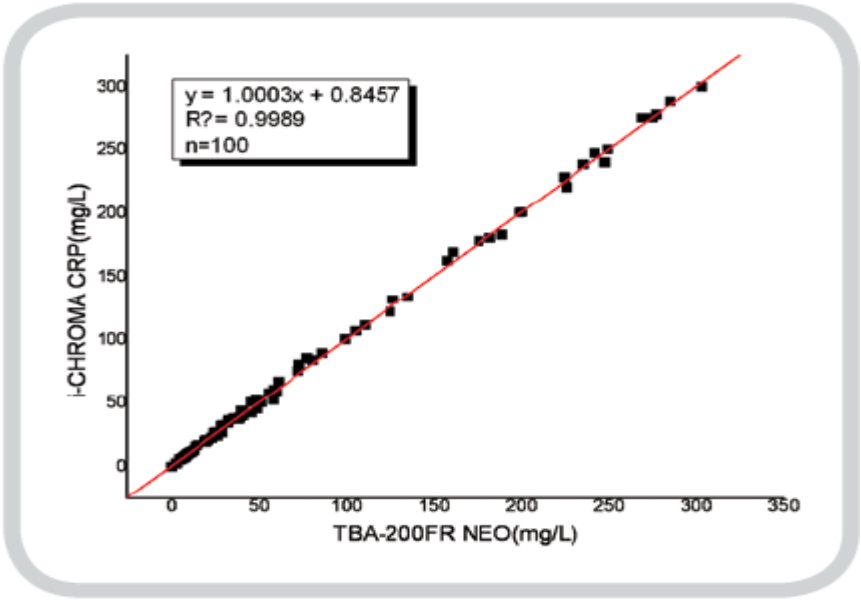


Step 2: sample preparation



Step 3: filling the test strip

Step 4: analyzing the test strip



3.5.1.7 Determination of Serum Adiponectin Hormone:

Principle:

This ELISA kit was used the Sandwich-ELISA principle. The micro ELISA plate provided in this kit has been pre-coated with an antibody specific to Human ADP/Acrp30. Standards or samples were added to the micro ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for Human ADP/Acrp30 and Avidin-Horseradish Peroxidase (HRP) conjugate were added successively to each micro plate well and incubated. Free components were washed

away. The substrate solution was added to each well. Only those wells that contain Human ADP/Acrp30, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color. The enzyme-substrate reaction was terminated by the addition of stop solution and the color turns yellow. The optical density (OD) was measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The OD value was proportional to the concentration of Human ADP/Acrp30. You can calculate the concentration of Human ADP/Acrp30 in the samples by comparing the OD of the samples to the standard curve.

Reagent preparation:

1.The kit was taken out from the refrigerator 20 minutes in advance to bring all reagents to room temperature (18-25 °C) before use.

2.The samples were brought to room temperature (18-25 °C) before use, mixed fully , avoided foaming.

3.The 25xConcentrated wash buffer was diluted to 1x working solution.

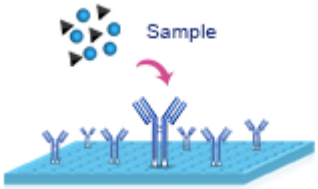
4.The reference standard was diluted to different concentrations.

5.The 100xBiotinylated detection Ab was diluted to 1x working solution 15 minutes earlier before step 1 finished.

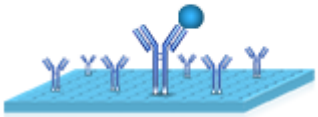
6.The 100xConcentrated HRP conjugate was diluted to 1x working solution 15 minutes earlier before step 2 finished.

7.The micro plate reader was preheated when step 6 starts.

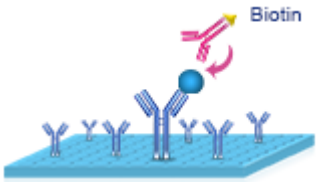
Assay Procedure:



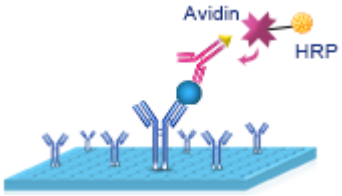
1. 100 µL standard or sample was added to each well. Incubated for 90 min at 37℃.



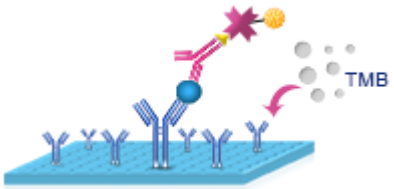
2. The liquid was removed.



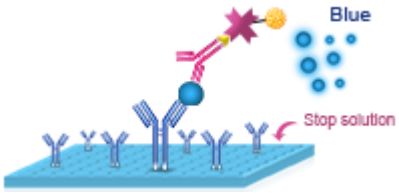
3. 100 µL Biotinylated Detection Ab was added . Incubated for 1 hour at 37℃. Aspirated and washed 3 times.



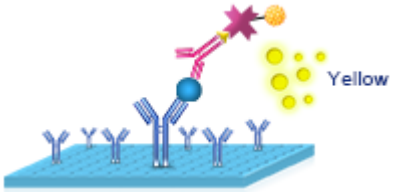
4. 100 µL HRP Conjugate was added. Incubated for 30 min at 37℃. Aspirated and washed 5 times.



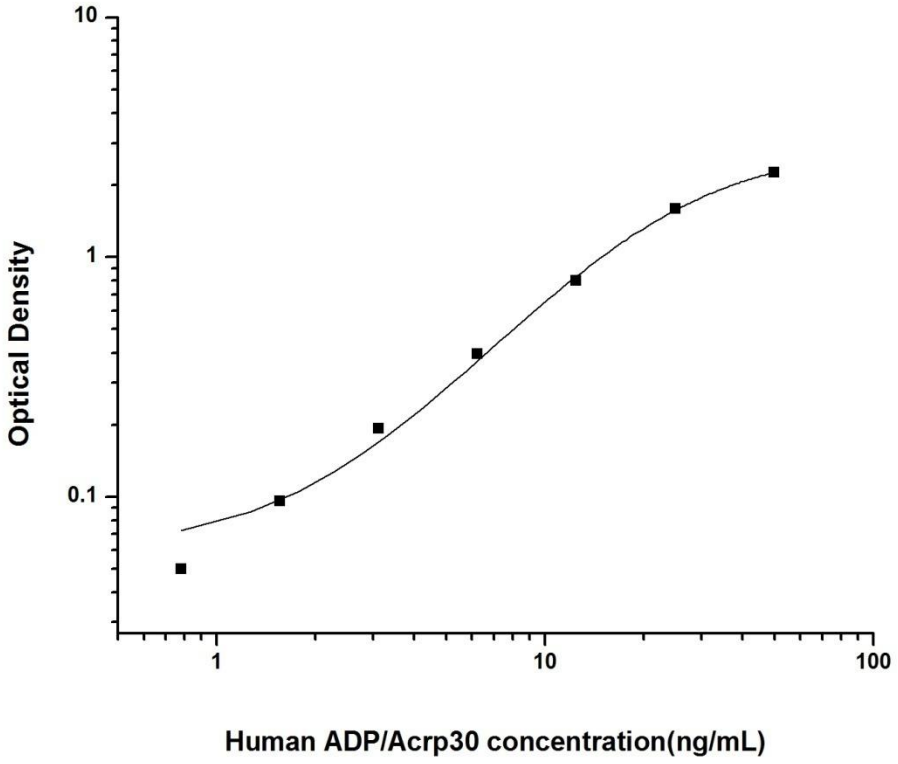
5. 90 µL of Substrate Reagent was added. Incubated for 15 min at 37℃.



6. 50 µL Stop Solution was add ed.



7. At 450 nm immediately was read. Calculation of results.



3.5.1.8 Determination of Serum Vitamin D3:

Principle:

The test was used a competitive immune detection method. In this method , the target material in the sample binds to the fluorescence (FL)-labeled detection antibody in detection buffer , to form the complex as sample mixture. This complex was loaded to migrate onto the nitrocellulose matrix , where the covalent couple of 25(OH)D3 and bovine serum albumin (BSA) was immobilized on a test strip, and interferes with the binding of target material and FL-labeled antibody. If the more target material exists in blood , the less detection antibody was accumulated, resulting in the less fluorescence signal (Bilinski K. and Boyages S. ,2012 , Lu Chuanyi M.,2012).

Test components

The **ichroma ™** Vitamin D consists of Cartridges , Detection buffer Vial , Releasing buffer Vial, sample Mixing tubes, and an ID chip.

- The cartridge contains a test strip, the membrane which has 25(OH)D3- BSA conjugate at the test line, while rabbit IgG at the control line.
- Each cartridge is individually sealed in an aluminum foil pouch containing a desiccant. 25 sealed cartridges are packed in a box which also contains an ID chip.
- The releasing buffer contains NaOH and DMSO.
- The detection buffer contains 25(OH)D3 -fluorescence conjugate, anti rabbit IgG - fluorescence conjugate, gelatin as a stabilizer and sodium azide in Tris- HCl buffer as a preservative.
- The releasing buffer and the detection buffer are dispensed in a vial. Releasing buffer vial and the detection buffer vial are packed in a Styrofoam box with ice- pack for the shipment.

Procedure:

1.The test cartridge was put into the I chamber slot.

2.50 µL of releasing buffer using a transfer pipette was transferred to a sample mixing tube.

3.50 µL sample (serum) using a transfer pipette was added to a sample mixing tube containing releasing buffer and mixed well by pipetting 10 times.

4.The sample mixing tube was inserted into the inserting tube block and left the tube in the inserting tube block at 35℃ for 5 min.

5.100 µL of detection buffer using a transfer pipette with tip was added to the sample mixing tube containing releasing buffer and sample mixture.

6.Well by pipetting 10 times was mixed and left it in the inserting tube block again at 35℃ for 15 min.

7.The half of test cartridge from i-chamber was taken out, pipette out 75 µL of incubated mixture and loaded it into the sample well on the test cartridge. Then the test cartridge was pushed into the i-chamber slot fully.

8.The sample – loaded test cartridge was left in i-chamber for 8 min.

9.The test result was read on the display screen of the instrument for i chroma ™ tests.

Calculations:

25(OH)D		***status***
<10 ng / mL	< 25nmol/L	Deficiency
10- 30 ng / mL	25-75 nmol/L	Insufficiency
30-100 ng / mL	75-250 nmol/L	sufficiency

3.5.1.9 Determination of Serum Calcium Concentration:

Principle:

Moorehead and Briggs derived CPC (o- cresol phtalein complexone) method allows to determinate total Calcium concentration in serum , plasma.

In alkaline solution CPC reacts with calcium to form a dark- red colored complex which absorbance measured at 570 nm is proportional to the amount of calcium in the specimen (Moorehead W. and Briggs H., 1974).

Reagents:

Vial R1 buffer	Amino – 2-methyl- 2-propanol-1 PH 11.0 at 20°C Hydrochloric acid	1.70 mol/L 210 mmol/L
Vial R2 Chromogen	O- cresolphtalein complexone (CPC) Hydroxy -8-Quinoline Hydrochloric acid	78µmol/L 3.36mmol/L 25 mmol/L
Vial R3 EDTA	EDTA	10 mmol/L
Vial R4 Standard	Calcium	10 mg/dL (2.5mmol/L)

Procedure:

	Blank	**Standard**	**Assay**
Reagent	1 ml	1 ml	1 ml
Demineralised water	25µl	------	-------
Standard	------	25µl	-------
Specimen	-------	-------	25µl

Mixed well . Incubated for 5 minutes at room temperature.

Read absorbances at 570 nm(550 – 590) against reagent blank.

The coloration is stable for 1 hour away from light.

Calculation:

$$\text{Result} = \frac{\text{Abs(Assay)}}{\text{Abs (Standard)}} * \text{Standard concentration}$$

3.5.1.10 Determination of Serum Alkaline phosphatase (ALP) Activity:

Principle:

Colorimetric determination of alkaline phosphatise activity according to the following reaction :

phenylphosphate $\xrightarrow[\text{PH 10}]{\text{ALP}}$ phenol + phosphate

The liberated phenol was measured in the presence of 4-aminoantipyrine and potassium ferricyanide. The presence of sodium arsenate in the reagent stops the enzymatic reaction.(Kind P. and King E., 1954, Belfielda A. and Gold Berg D. , 1971).

Reagent Composition:

Reagent 1	Disodium phenylphosphate Carbonate-bicarbonate	5 mmol/L
Substrate	buffer PH 10	50 mmol/L
buffer	Sodium merthiolate	0.1 g/L
Reagent 2	Phenol equal to 20 kind and kig U	
standard		

Reagent 3 Blocking reagent	4-aminoantipyrine Sodium arsenate TOXIC reagent R 45 : may cause cancer R 23/25 : toxic by inhalation and if swallowed. S 28 : after contact with skin , wash immediately with plenty of water. S 45 : in case of accident or if you feel unwell ,seek medical advice immediately (show the label when possible).	60 mmol/L 75 g/L
Reagent 4 Color reagent	Potassium ferricyanide	150 mmol/L

Procedure:

	Serum sample	**Serum blank**	**Standard**	**Reagent blank**
Reagent 1	2 ml	2 ml	2 ml	2 ml
Incubate for 5 minutes at 37 ºC.				
Serum	50µl	------	-------	------
Reagent 2	------	--------	50µl	--------
Incubate for exactly 15 minutes at 37 ºC.				
Reagent 3	0.5 ml	0.5 ml	0.5 ml	0.5 ml
Mix well or preferably vortex.				
Reagent 4	0.5 ml	0.5 ml	0.5 ml	0.5 ml
Serum	-------	50µl	------	-------
Distilled water	-------	--------	-------	50µl
Mix. Let stand for 10 minutes in the dark. Measure absorbance at 510 nm.				

Calculation:

$$\text{Calculation} = \frac{A \text{ serum sample} - A \text{ serum blank}}{A \text{ standard}} * n$$

Kind and King U/100 ml : n= 20

U/L : n= 142

3.5.2. Histological Preparations:

The histological technique is used in the preparation of tissue (Luna L. , 1969)according to Microscopic examination including:

3.5.2 .1.Sectioning :

Each tissue breast was sectioned to make it ready for further steps. The ideal thickness of each specimen was in thick between 4-5 mm, which make the fixation satisfactory.

3.5.2.2 . Histological Technique :

3.5.2.2.1. Fixation :

All samples from patients with breast cancer instantly fixed in solution of 10% buffered neutral formalin .The fixation time ranges between 18-22 hours at room temperature. Prolong fixation periods (over 24 hours) usually yielded less accurate immunohistochemical results.

3.5.2.2.2 .Dehydration :

Tissues were dehydrating in an ascending grade of ethanol (30%, 50%, 70%, 80%, 90% and100%) for 2hours.

3.5.2.2.3 . Clearing :

After dehydration, tissues were clearing with xylene for 12-24 hours.

3.5.2.2.4 .Impregnation :

This process was done by using paraffin wax at 60 C for 6 hours. In the first two hours the wax was twice renewed to riddance xylene .

3.5.2.2.5. Embedding :

Firstly, cleaned mold was filled with molten paraffin wax. Then, by using warm forceps, the selected tissue was placed in the mold according to the slide of sectioning , this slide should be facing down against the mold orienting the tissue and firming it into the wax with warmed forceps. This ensures that the correct orientation was maintained and the tissue surface to be sectioned was kept flat. Followed by positioning the labeled cassette onto the mold and molten paraffin wax was added up to the surface. Using the forceps chilled the mold on the cold plate to allow the wax to cool down and form a tissue block. As final stage, the cassette with wax block was removed from the mold and kept in freeze overnight. Plain , solid, no crack tissue would be the best structure of the tissue block.

3.5.2.2.6 .Sectioning:

By using rotary microtome, blocks were first trimmed for removing excessive wax and then sectioned (5µm thickness).Ribbons resulting from sectioning process were floated in a warm water bath and mounted on clean slides covered with a thin layer of Mayer's albumin after their extension in water bath and left to dry at room temperature.

3.5.2.2.7 . Mounting and Staining :

To obtain stained section, first paraffin must be removed by xylene for 10 minutes. The deparaffinized sections were passed by descending concentration of ethyl alcohol (absolute,90%,80%,70%,50%,30%) for 2 minutes for each concentration ,stained with haematoxylin stain for 10 minutes and extensively washed with tap water .after that they were passed by ascending grade of ethanol (30%,50%,70%,80%,90%,100%) for 2 minutes each, stained with alcoholic eosin Stain for 2 minutes and then passed by absolute ethanol for 5 min. finally sections were cleared by xylene (5-10) min. mounted by DPX ,covered with cover slides and removed to dry at room temperature to be ready for microscopic examination by using light microscope**.**

3.6. Immunohistochemistry (IHC):

Before working note that

1. The primary antibody was divided into small volumes (5 microliters) in each Eppendorf tube and put them in -20 °C until assay. (use one for each working day) to avoid freezing and thawing cycles.
2. The required quantity for primary antibody was calculated (for each slide 50-75 microliters) for 5 slide you can prepare 250-375 microliter from diluted primary antibody (2 microliters diluted in

400 microliters of antibody diluent) or 3 microliters in 400 microliters of antibody diluent.

Principles of the test

This technique based on the detection of the antigen using specific rabbit antibodies, that binds to specific targeted protein. The bound primary antibody then detected by secondary antibody conjugated with horse raddish peroxidase polymer which catalyze the substrate H_2O_2 into free oxygen and water (H_2O). The free oxygen then oxidize 3, 3`-diaminobenzidine (DAB) into dark brown precipitate. A positive reaction will have indicated as a brown-colored precipitate at the antigen site counter stained with hematoxylin and tested for immunoreactivity.

Materials

Materials used in this study have been handled, stored, diluted, and processed according to manufacturer instructions.

1. Primary Antibody: all primary antibodies were listed in table (3-5).
2. Immunohistochemistry detection kit Super Sensitive IHC Detection System Kit (Mouse/Rabbit).

Super Sensitive IHC Detection System Kit is the latest technology in polymeric labeling. Polymer detection methods have been shown to provide increased sensitivity. This innovative polymer technology has major advantages than conventional IHC systems. Super Sensitive IHC Detection System amplifies the signal with both mouse and rabbit primary antibodies. The Super Sensitive IHC Detection System Kit provides the user with a rapid, easy to use, and versatile IHC detection system.

Immunohistochemical Procedure:

Pre-staining steps:

These steps include preparation of slides for staining steps this can be accomplished by the following steps:

A. Slide preparation: Paraffin embedded sections were cut into 5µm thickness, then the sections were carried by adhesive positively charged slides, sections were left to dry to facilitate adhesion between the section and the charged glass surface.

B. Deparaffinization and rehydration:this step involves:

1. Dewaxing of paraffin embedded sections were placed inside hot air oven at 65°C for 30 minutes.
2. Deparaffinization was done by immersing the slides in xylene for 5 minutes then in fresh xylene for 5 minutes.
3. Rehydration of tissue section accomplished through immersing of slides in sequential dilutions of ethanol as the following order:

I. Absolute ethanol for 5 minutes.
II. 95% ethanol for 5 minutes.
III. 90% ethanol for 5 minutes.
IV. 70% ethanol for 5 minutes.
V. 50% ethanol for 5 minutes.
VI. Distilled water for 5 minutes.

C. Peroxidase block: Slide encircled with Pap pen. Hydrogen peroxide was applied to cover the tissue and incubated for 20 minutes. Then the slides were rinsed with distilled water, drained and blotted gently.

D. Protein blocking of Non-specific binding of primary antibody:

Before adding the primary antibodies, slides were ready for blocking step, to block endogenous Fc receptor, incubate sections for 20 minto prevent

any unspecific binding of primary antibody (FC region) with tissue section, this with prevent false positive results. then slides were drained and blotted without washing.

Immunostaining steps: these multi steps specific interaction between primary antibodies reacts with target antigen and ended by colored designation of target:

1. Fifty µl of primary antibody was placed onto the tissue section and incubated for 1 hour at 37˚C in humid chamber. After incubation, the slides were drained and blotted gently. Then Slides were placed in washing buffer bath for 5 minutes twice, drained and blotted gently.

2. Fifty µl of HRP polymer was placed onto the tissue section and incubated for30 minutes at 37˚C in humid chamber, then slides were placed in washing buffer bath for 5 minutes, drained and blotted gently.

Table (3-5)*:*List of Materials (kits) used in the study

No.	Item name	Manufacturer company	Quantity	Catalogue No.	Host	Target	Clonality
1	**PLD1 antibody**	Biorbyt (UK)	50 µL	**Orb96001**	Rabbit	Human	Polyclonal
2	**Super Sensitive IHC Detection System Kit (Mouse/Rabbit)**	**Biorbyt (UK)**	**1kits**	**orb219874**	-	**Rabbit and mouse**	-

3. For preparation of 1ml of substrate chromogen complex adequate for 15-20 slides. mix 50µl DAB Buffer, 50 µl DAB Substrate Reagent and 50 µl DAB Chromogen into 850 µl of distilled water, mix by swirling and apply to tissue (50 µl for each slice), incubated for about (3-5 min) and then slides were rinsed in distilled water, drained and blotted gently.

4. Counter-stain: the slides immersed in a bath of Mayer's Hematoxylin

for 1 minute. Slides were washed three times in distilled water, 1 minute each; then drained and blotted gently.

5. Counterstain using Mayer's hematoxylene for 30 seconds or 1 minute then wash with distilled water for 5 minutes for two times.

Post staining steps: include preparation of stained slides for microscopic examination:

1. Slides were dehydrated by placing them in Ethanol and Xylene containing jars in the following order:

a) 50% ethanol for 5 minute.
b) 70% ethanol for 5 minute.
c) 90% ethanol for 5 minute.
d) Absolute ethanol for 5 minutes.
e) Xylene for 5 minutes.
f) Fresh Xylene for 5 minutes

2. A drop of mounting media placed onto section and the tissue section was quickly covered with cover slip and slides were left to dry.

Negative control was included for each run of immunohistochemistry.

The slides were not allowed to dry in any step of the immunostaining.

3.7. Evaluation of the Immunostaining

Evaluation of the immunostaining was done through the histological observation as positive and negative cases, in which breast tissue samples immunostaining will give nuclear dark brown granules.

The presence of a brown chromagen complexes at the site of the target antigen is indicative of positive reactivity. Counter stain will be pale to

dark blue coloration of the cell nuclei (Allred D. and Moshin S. , 2005).

3.8.Scoring:

To assess the fraction of immunolabeled cells in specimens from each patient case, the labeling index defined as the percentage of positive cells of the total number of tumor cells counted was determined as follow:

(-) indicates negative immunoreactivity.

(+) indicates 1-30% cell positivity.

(++) indicates 31-60% cell positivity.

(+++) indicates > 61% cell positivity (Allred D. and Moshin S. , 2005).

3.9. Statistical Analysis :

Statistical analysis was done using the software **SPSS** version 20.0.The results were expressed as mean ± standard deviations (mean ± SD) with LSD. One way ANOVA-test was used to compare parameters in different studied groups. P-values ($P \leq 0.05$) and Chi-square test Categorical data was described as percentage ; comparison done by using Chi-square test. P value of ($P > 0.05$) were used as the level of significance.

Person correlation coefficient (r) was used to test the correlation among the different parameters in each patients group.

CHAPTER FOUR

RESULTS AND DISCUSSION

4. Results and Discussion

4.1. Clinical and Characteristic Features of the Studies Groups:

There are 160 women included in the present study, divided into two groups: invasive ductal carcinoma patients group (eighty five women) were compared with group of apparently healthy control (seventy five women) without significant difference in Age.

Characteristic data for all studied groups shown in table (4-1).

Table (4-1): Characteristic data for studied groups

Groups	NO	Age year mean± SD	BMI(Kg/m^2) mean± SD	Social status				Home Address	
				Single	Married	Divorced	Widow	City	Countryside
Control	75	44.67±8.22	23.68±2.32	10	15	20	30	75	----
Patients	85	47.63±9.94	31.62±2.42	14	19	28	24	50	35

4.2 Biochemical study For Women with invasive ductal carcinoma :

4.2.1. Serum Phospholipase D1(PLD1) enzyme activity:

Table (4-2) and figure (4.1) show a significant increase in the activity of PLD1 enzyme in patients group in comparison with the control group ($P \leq 0.05$). This result matched with the results of study of (Bruntz R. *et al*., 2014a): posits that PLD1 activity and expression are usually increased in various types of cancers.

The role of PLD in the development of diseases such as cancer and thrombotic diseases has been an area of focus for researchers. In particular, cellular studies have examined how cancer influences PLD expression (Stegner D. *et al*., 2013 ;Schonberger T. *et al*., 2014).

A growing body of research evidence indicates that PLD activity is increased significantly in cancer tissues and cells, showing that it may play a critical role such as signal transduction, cell proliferation, and anti-apoptotic processes (Choi H. *et al*., 2009).

Table (4 –2):- *Serum PLD1 enzyme activity of control and invasive ductal carcinoma*

Groups	No.	PLD1 activity (ng/ml) Mean ± SD
Control	75	75.24+17.06 [b]
patients	85	283.68+58.79 [a]
LSD		11.66

Note: Each value represents mean ± SD values with non-identical superscript (a , b or c…etc.) , were considered significantly differences (P≤ 0.05).

-No: Number of subjects.

-SD: Standard deviation.

-LSD: Least Significant Difference.

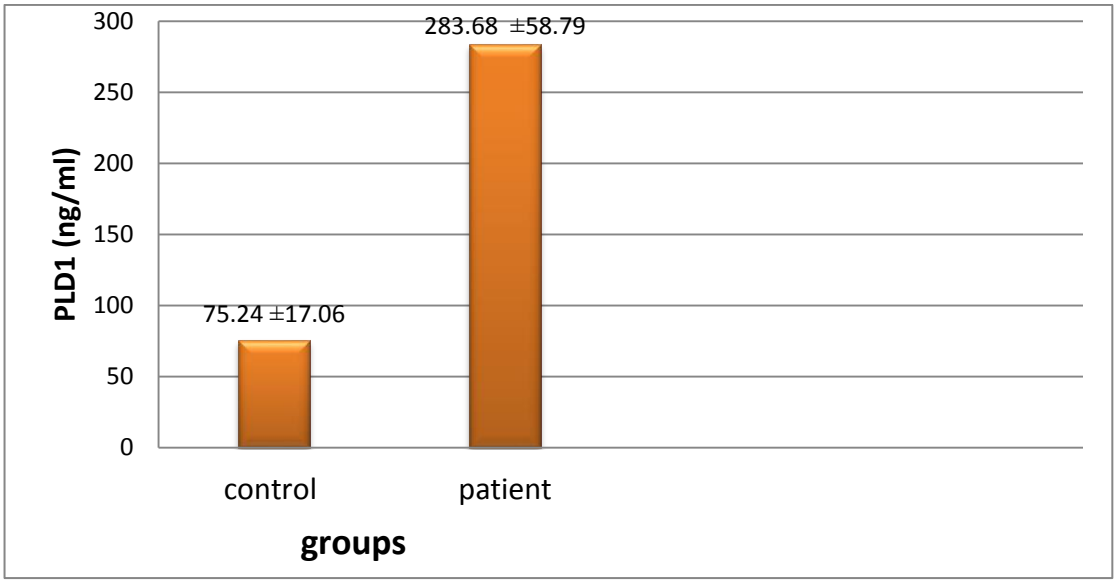


Figure (4.1): *Serum PLD1 activity in control and invasive ductal carcinoma*

4.2.2. Serum Choline Kinase Alpha (CHKA) enzyme activity:

Table (4-3) and figure (4.2) show a significant increase in the activity of CHKA enzyme in patients group in comparison with the control group

(P≤0.05). This result matched with the results of study of (Podo F. *et al.*, 2011).

Over expression and increased activity of ChoK have been for instance reported in breast cancer compared with the respective normal counterparts, major contributions to PCho accumulation in tumor cells may derive from activation of choline kinase (ChoK) in the Kennedy pathway, as well as from activation of phosphalipases in the PC degradation pathways, choline kinase, the first enzyme involved in the Kennedy pathway, is a cytosolic enzyme expressed ubiquitously in all tissues, which catalyzes the phosphorylation of free choline, yielding PCho in the presence of ATP and Mg^{2+}, a substantial evidence points to the activation of ChoK and subsequent PCho accumulation in the progression of several cancers(Glunde K. *et al*., 2011b).

Figure (4.3) shows the positive correlation between PLD1 and CHKA in invasive ductal carcinoma patients group with correlation coefficient (r = 0.08).

Table (4–3):- *Serum CHKA enzyme activity of control and invasive ductal carcinoma*

Groups	No.	CHKA activity (ng/ml) Mean ± SD
Control	75	8.51 ± 2.20^{b}
patients	85	27.68 ± 5.28^{a}
LSD		1.10

- Legend as in table (4-2).

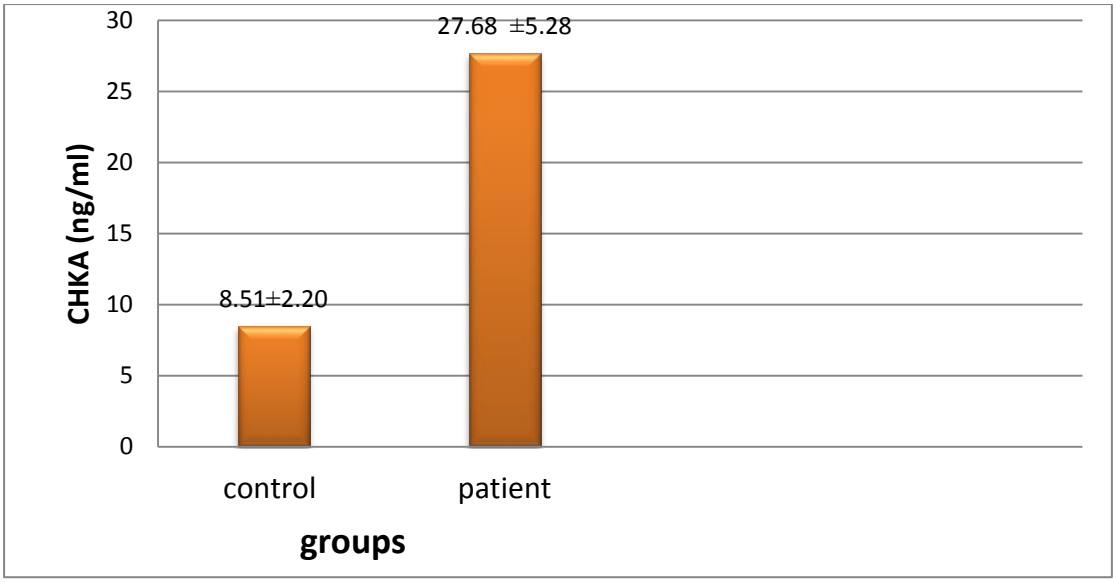


Figure (4.2): *Serum CHKA activity in control and invasive ductal carcinoma*

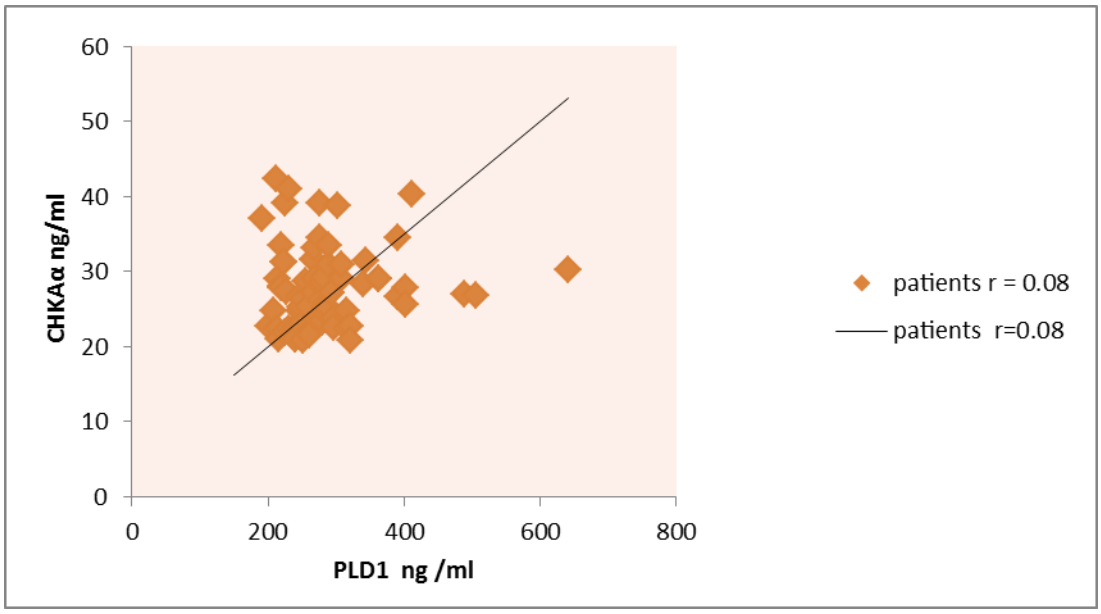


Figure (4.3): *Correlation between PLD1 and CHKA in invasive ductal carcinoma patients group*

4.2.3. Serum Serotonin Hormone(ST)Concentration:

Table (4-4) and figure (4.4) show a significant decrease in the levels of ST hormone in patients group in comparison with the control group ($P \leq 0.05$). We found that lowest serotonin concentrations were recorded in patients with invasive ductal carcinoma in comparison with control group, the cause of reduction may be related to genetic alteration in genes

responsible on serotonin synthetic enzymes, which need further study. After treatment, serotonin levels continue to be decrease in the serum of all patients with invasive ductal carcinoma, the reduction of serum serotonin levels in patients with breast cancer after treatment with chemotherapy or radiotherapy may be explained by the reduction in the abnormal (cancerous) cells in response to chemotherapy or radiotherapy. Consequently, the reduction in the serotonin concentration may reduce the vascularity of malignant tissue leading to necrosis that finally leads to increase of cancer cell mortality.

An elevation in the serotonin levels in the cancer cases recorded by (American Cancer Society, 2016) **and** (Frobe A. *et al*., 2014) were explained through different hypothesis: During malignant transformation process, DNA translocation and amplification lead to an increased expression of oncogene proteins and loss of tumor suppressor gene proteins. The outcome of these phenomena are alteration in the cellular proteins amounts so as nature, according to that an increase in the concentration of serotonin synthetic enzymes may be altered as result of activation of genes expression.

Recently it was suggested that 5HT-receptor sub-types expressed in breast cancer cells may have utility as diagnostic/prognostic markers (Kopparapu P. *et al*., 2013).

Figure (4.5) shows the negative correlation between PLD1 and ST in invasive ductal carcinoma patients group with correlation coefficient (r = -0.10).

Table (4–4):- *Serum ST Hormone levels of control and invasive ductal carcinoma*

Groups	No.	ST levels (ng/ml) Mean ± SD
Control	75	51.29±14.76[a]
patients	85	11.84±2.17[b]
LSD		2.17

- Legend as in table (4-2).

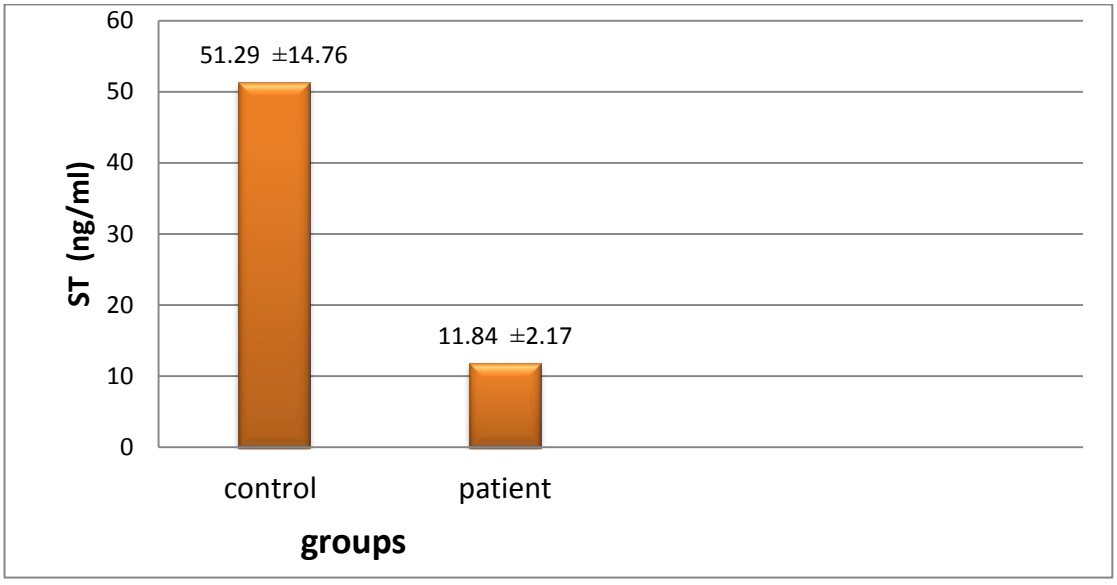


Figure (4.4): *Serum ST levels in control and invasive ductal carcinoma*

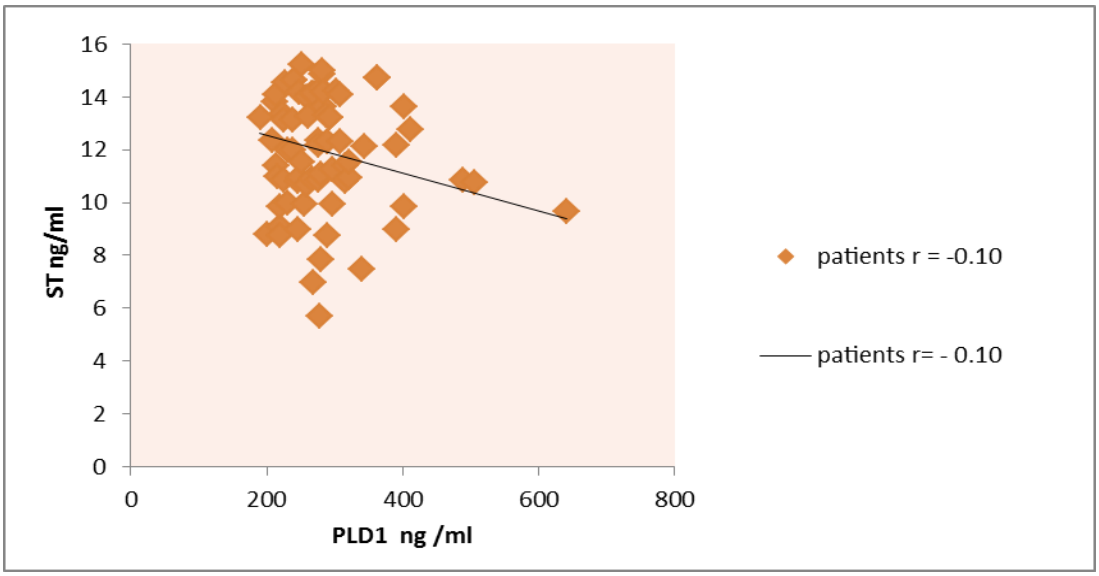


Figure (4.5): *Correlation between PLD1 and ST in invasive ductal carcinoma patients group*

4.2.4. Serum Melatonin Hormone(MT)Concentration:

Table (4-5) and figure (4.6) show a significant decrease in the levels of MT hormone in patients group in comparison with the control group (P≤0.05). The results of this study support other similar studies in this field regarding disruption of diurnal sleep– wakefulness rhythms, especially through night-time shift work, having greater chance of breast cancer (Jia Y. *et al*., 2013 ; Wang F. *et al*. ,2013).

The study suggest that low melatonin levels may be associated with a higher risk for breast cancer, women with breast cancer tend to have lower levels of melatonin than those without the disease, low levels of melatonin stimulate the growth of certain types of breast cancer cells (Ehrlich and Stephen D. , 2016).

Although melatonin is most known for its connection to sleep, many studies have demonstrated the presence of antioxidant properties in melatonin, melatonin is effective in counteracting damage from one small cell to the entire organism, melatonin directly scavenges a multitude of free radicals, when melatonin interacts with free radicals, it forms metabolites, the metabolites formed by melatonin are actually capable of scavenging free radicals as well (Tan D. *et al*. ,2003). However, it is one of the last molecules used by the human body to defend against damage from oxidation, as it is utilized even after Vitamin E. (Johns Jeffrey R. and James Platts A. ,2014).

Regarding the influence of decreased melatonin secretion, a relationship between low plasma melatonin concentration and breast cancer, women with ER+ breast cancer had nocturnal plasmatic concentrations of melatonin significantly lower than both healthy women and women suffering ER-breast cancer, these authors coined the expression "relative hyperestrogenism" to define the hormonal situation

of women with low melatonin secretion (Gómez-Acebo I. *et al*., 2015; Papantoniou K. *et al*., 2016).

In spite of the fact that melatonin has a circadian rhythm which reaches the peak during the night in normal this time individuals and the difficulty of sampling at that time where, the blood samples of our study have been collected in the morning at 8:00 O'clock then the melatonin levels have been analyzed and compared with it's normal levels at the morning time to provide a reliable evaluation.

Figure (4.7) shows the negative correlation between PLD1 and MT in invasive ductal carcinoma patients group with coefficient correlation (r =-0.09).

Table (4–5):- *Serum MT Hormone levels of control and invasive ductal carcinoma*

Groups	No.	MT levels (pg/ml) Mean ± SD
Control	75	22.71±4.13[a]
patients	85	10.72±2.29[b]
LSD		0.83

- Legend as in table (4-2).

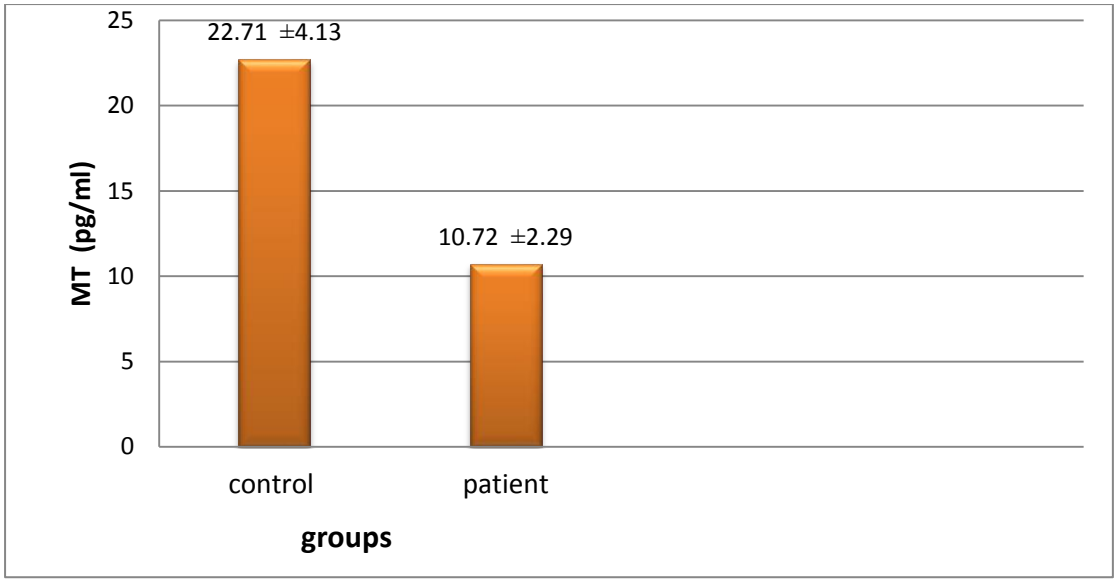


Figure (4.6): *Serum MT levels in control and invasive ductal carcinoma*

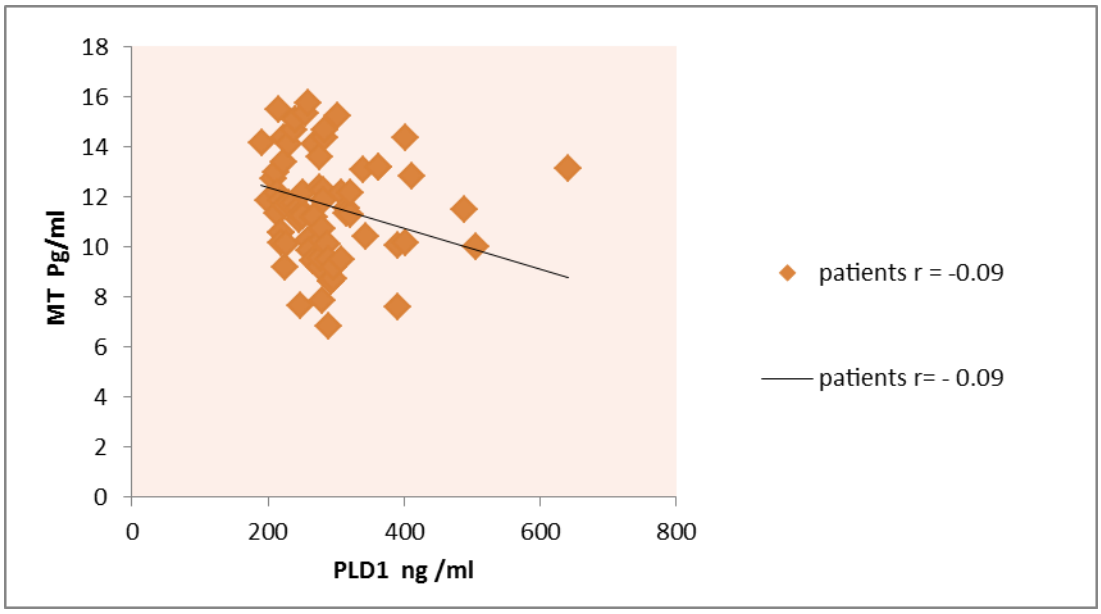


Figure (4.7): *Correlation between PLD1 and MT in invasive ductal carcinoma patients group*

4.2.5. Serum Estradiol Hormone(E2)Concentration:

Table (4-6) and figure (4.8) show a significant increase in the levels of E2 hormone in patients group in comparison with the control group ($P \leq 0.05$). This result matched with the results of study of (Yip C. *et al.*, 2011). High circulating E2 levels in pre- and postmenopausal women, are

associated with an enhanced incidence of breast cancer (Kaaks R. *et al*., 2005 ; Chen W. *et al*., 2006).

In this study, we found that the obesity among postmenopausal women increases breast cancer risk which consistent with study done by (Irwin M. *et al.*, 2013) who stated that for each 5 kg of weight gain from the lowest adult weight, breast cancer risk increases by 8%. One possible mechanism by which postmenopausal obesity increases the risk for developing breast cancer is through higher levels of endogenous estrogen in obese women, as adipose tissue is an important source of estrogens (Anthony H. *et al.*, 2014).

Figure (4.9) shows the positive correlation between PLD1 and E2 in invasive ductal carcinoma patients group with correlation coefficient (r = 0.21).

Table (4–6):- *Serum E2 Hormone levels of control and invasive ductal carcinoma*

Groups	**No.**	**E2 levels (pg/ml) Mean ± SD**
Control	75	113.84±14.53[b]
patients	85	1581.79±2.29 [a]
LSD		6.33

- Legend as in table (4-2).

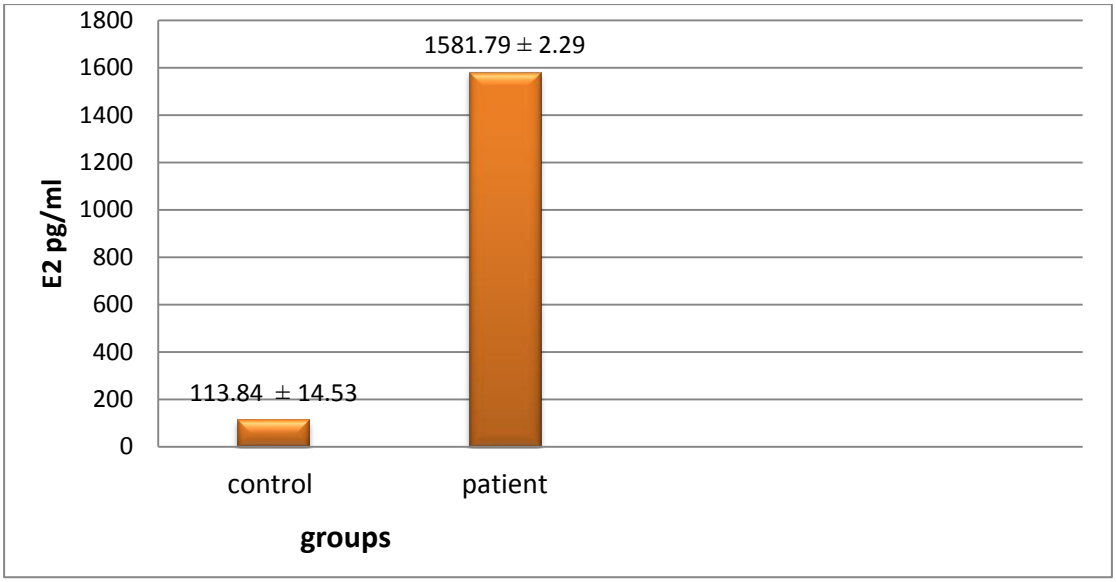


Figure (4.8): *Serum E2 levels in control and invasive ductal carcinoma*

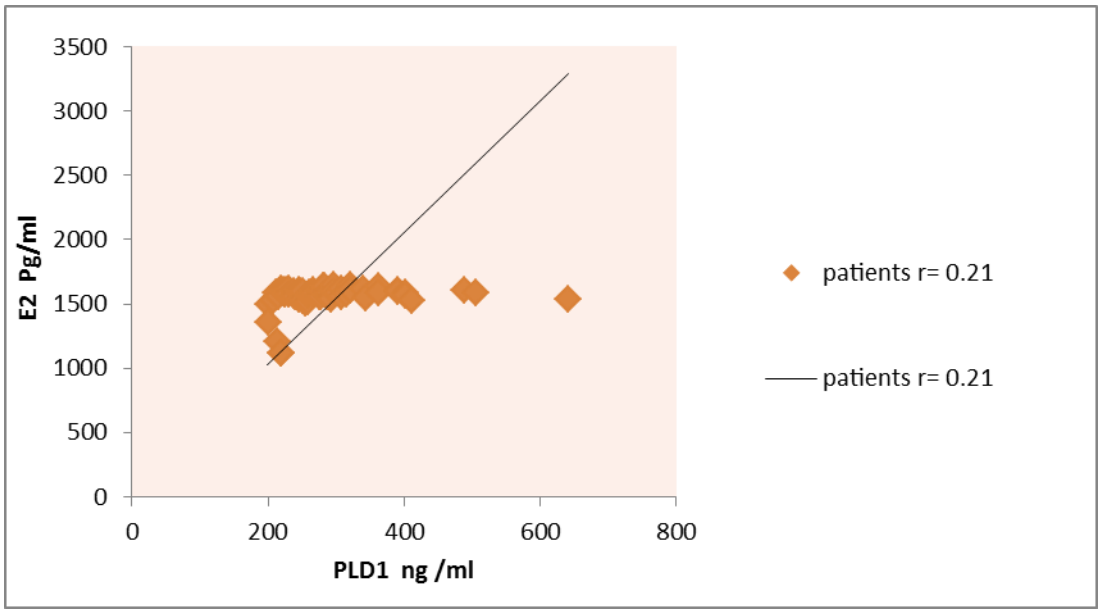


Figure (4.9): *Correlation between PLD1 and E2 in invasive ductal carcinoma patients group*

4.2.6. Serum C- reactive protein (CRP)Concentration:

Table (4-7) and figure (4.10) show a significant increase in the levels of CRP in patients group in comparison with the control group (P≤0.05). This study matched with the results of study of (Ferlay J. *et al.*, 2013).

CRP is an important biomarker of chronic inflammatory processes that may explain health challenges among women following breast cancer

diagnosis, for example, higher CRP levels have been implicated in reduced disease-free survival and higher risk of mortality among breast cancer patients (Allin K. *et al.* , 2011;Villaseñor A. *et al.* , 2014).

Epidemiological prospective study proved CRP as a well-established independent prognostic marker in breast cancer (Sicking I.*et al.*, 2014). Inflammatory pathways play an important role in the causation of breast cancer, there is a bidirectional link between chronic inflammation and carcinogenesis: tumor originates and progresses at the site of chronic inflammation while tumor cells attract immune cells and promote the production of cytokines and chemokine creating tumor microenvironment. Hence, cancer is associated with the persistent inflammatory state, there is a vicious cycle and complex interplay between cancer and inflammation (Hanahan D. and Weinberg R. ,2000).

Figure (4.11) shows the positive correlation between PLD1 and CRP in invasive ductal carcinoma patients group with correlation coefficient (r = 0.13).

Table (4–7):- *Serum CRP levels of control and invasive ductal carcinoma*

Groups	No.	CRP levels (mg/L) Mean ± SD
Control	75	6.84±1.76 [b]
patients	85	11.81± 1.18 [a]
LSD		0.36

- Legend as in table (4-2).

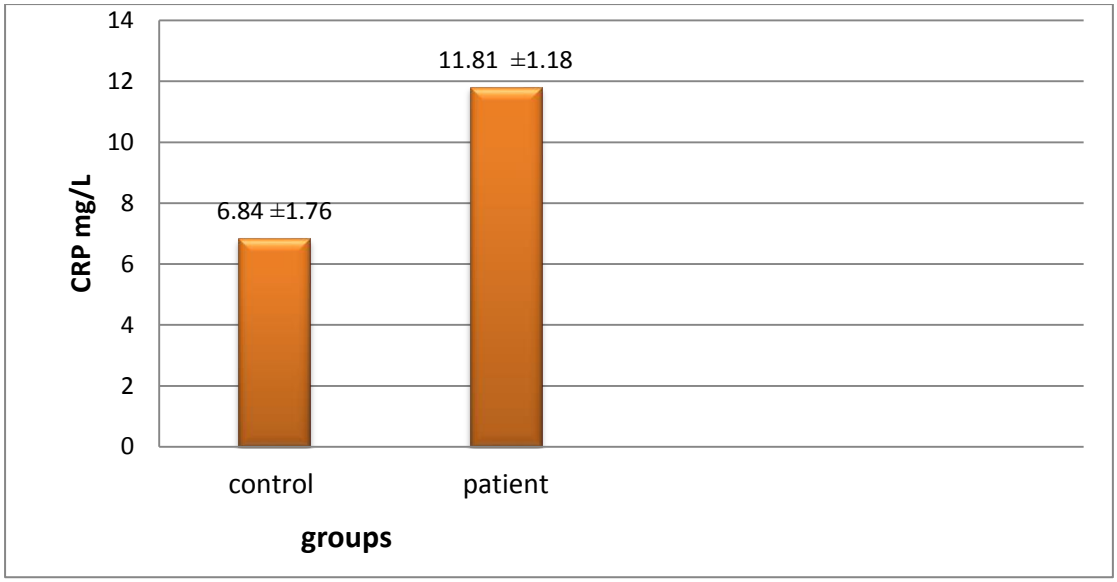


Figure (4.10): *Serum CRP levels in control and invasive ductal carcinoma*

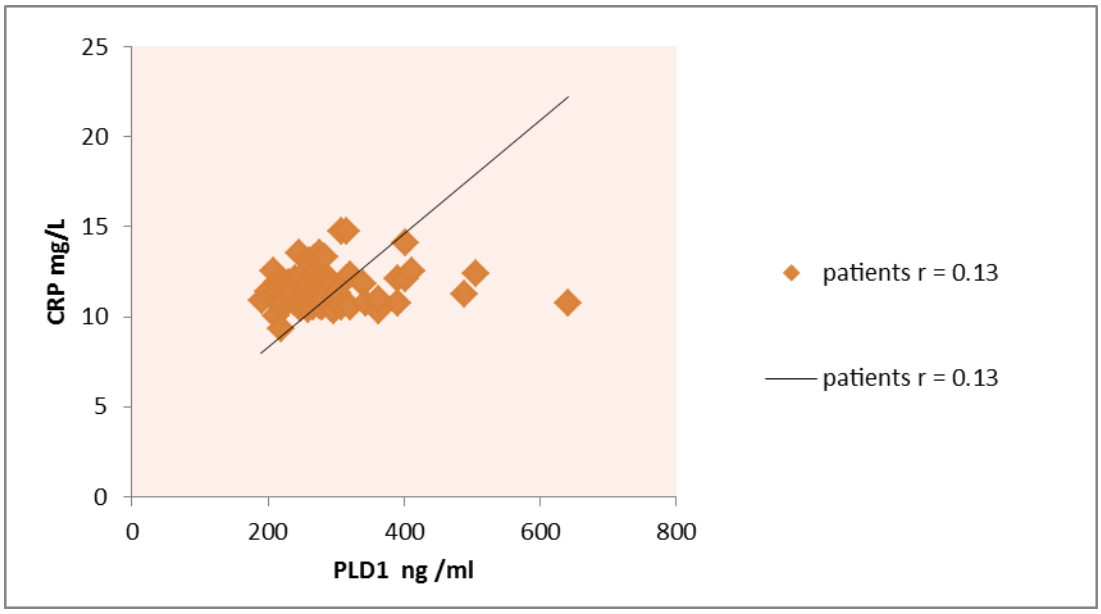


Figure (4.11): *Correlation between PLD1 and CRP in invasive ductal carcinoma patients group*

4.2.7. Serum Adiponectin Hormone(ADP)Concentration:

Table (4-8) and figure (4.12) show a significant decrease in the levels of ADP hormone in patients group in comparison with the control group ($P \leq 0.05$). This result matched with the results of study of (Tabaan *et al* ., 2014). Our finding also agree with (Ahmed S. *et al*., 2015) studied 175 diagnosed breast cancer patients and 175 healthy controls .They found

serum adiponectin was significantly decreased in breast cancer cases when compared to control subjects, had a statistically significant difference (P< 0.05) . Several studies have demonstrated that low serum adiponectin levels are associated with increased risk for breast cancer (Chlebowski R. *et al*., 2005 ; Mohan R. *et al*., 2012).

Adiponectin has been inversely associated with estrogen levels, it remains possible that adiponectin may influence breast cancer risk by modifying flowing estrogen levels (Nalabou *et al*., 2014).

Obesity is significantly associated with hypoadiponectinemia agrees with a study done by (Haluzik M. *et al*., 2004). Obesity has been linked to an increased risk of developing breast cancer by many scientific studies. There is evidence to suggest that excess body fat at the time of breast cancer diagnosis is associated with higher rates of cancer recurrence and death (Emily E., 2011 ; Hojan K. *et al*., 2013).

Figure (4.13) shows the negative correlation between PLD1 and ADP in invasive ductal carcinoma patients group with correlation coefficient (r = -0.07).

Table (4-8):- *Serum ADP Hormone levels of control and invasive ductal carcinoma*

Groups	No.	ADP levels (ng/ml) Mean ± SD
Control	75	6.74 ± 1.69^{a}
patients	85	0.50 ± 0.11^{b}
LSD		0.23

- Legend as in table (4-2).

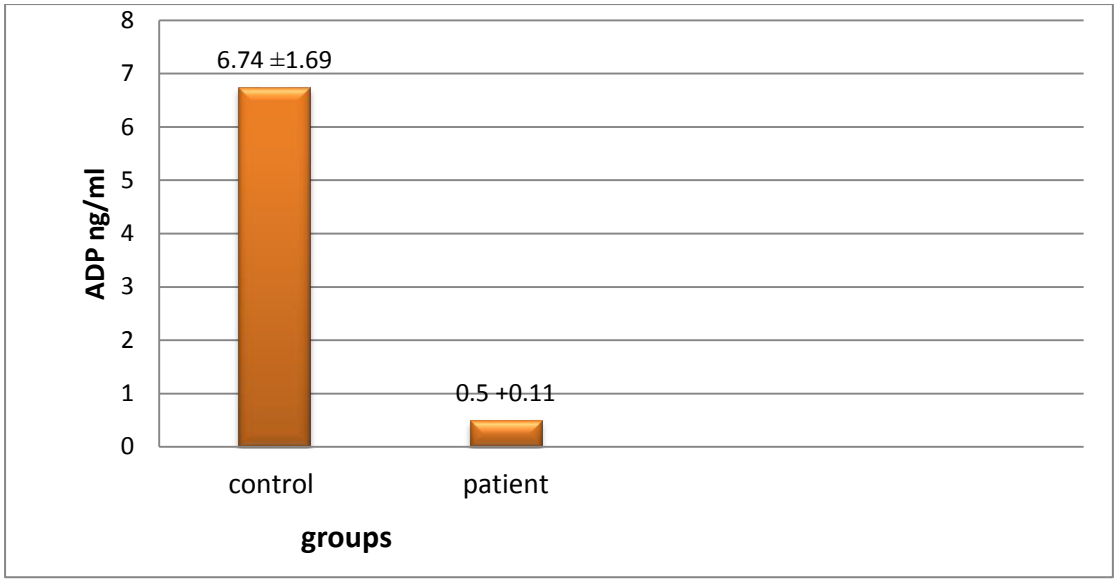


Figure (4.12): *Serum ADP levels in control and invasive ductal carcinoma*

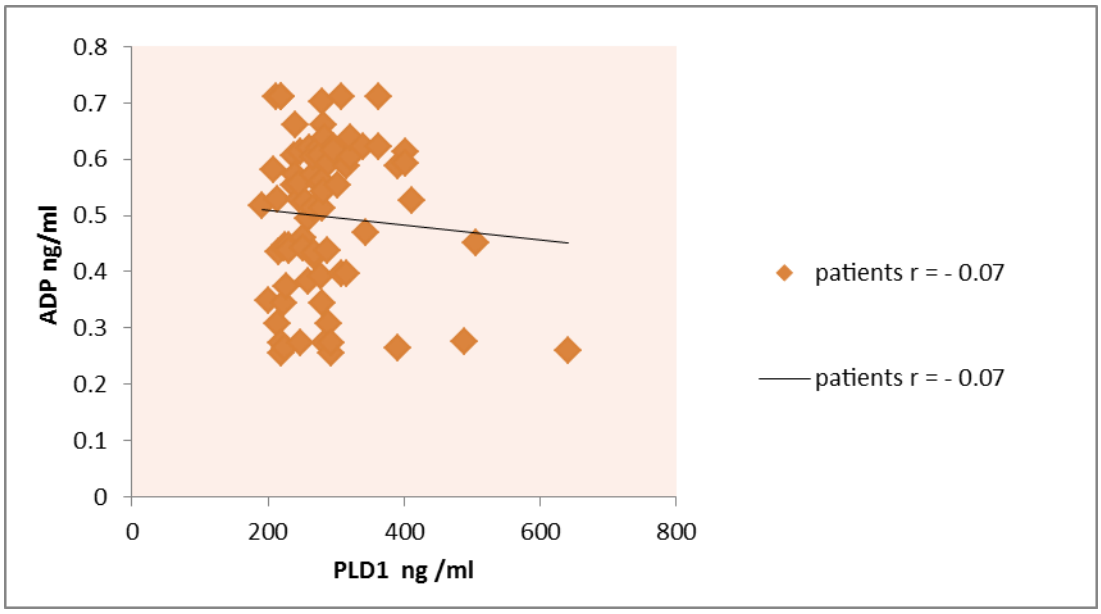


Figure (4.13): *Correlation between PLD1 and ADP in invasive ductal carcinoma patients group*

4.2.8. Serum Vitamin D_3 Concentration:

Table (4-9) and figure (4.14) show a significant decrease in the levels of Vit. D3 in patients group in comparison with the control group ($P \leq 0.05$). Similar results have been reported by other investigato (Botros R. *et al*., 2015).Laboratory studies have demonstrated that vitamin D_3 and

its analogs inhibit cell proliferation and promote apoptosis in cancer cells (Sergeev I., 2012 ; Balasubramanian S. and Rotti S., 2013).

Recent research study on the nutritional risk factors in breast cancer revealed that more than 90% of women with breast cancer were suffering from vitamin D deficiency, the higher risk of BC was found to be associated with low serum 25(OH)D levels (Sofi N. *et al*., 2016). Anti-carcinogenic effects of vitamin D are mediated via the estrogen pathway by down regulation of the estrogen receptor (ER), which inhibits cancer cell proliferation, induces cell apoptosis, and prevents carcinogenesis in vitro (Swami S. *et al*., 2000 ; Welsh J. , 2004).

Figure (4.15) shows the negative correlation between PLD1 and Vit.D3 in invasive ductal carcinoma patients group with correlation coefficient (r = - 0.15)

Table (4–9):- *Serum Vit. D3 levels of control and invasive ductal carcinoma*

Groups	**No.**	**Vit D3 levels (nmol/L)** **Mean ± SD**
Control	75	30.50±2.40 [a]
patients	85	21.98±1.20 [b]
LSD		0.43

- Legend as in table (4-2).

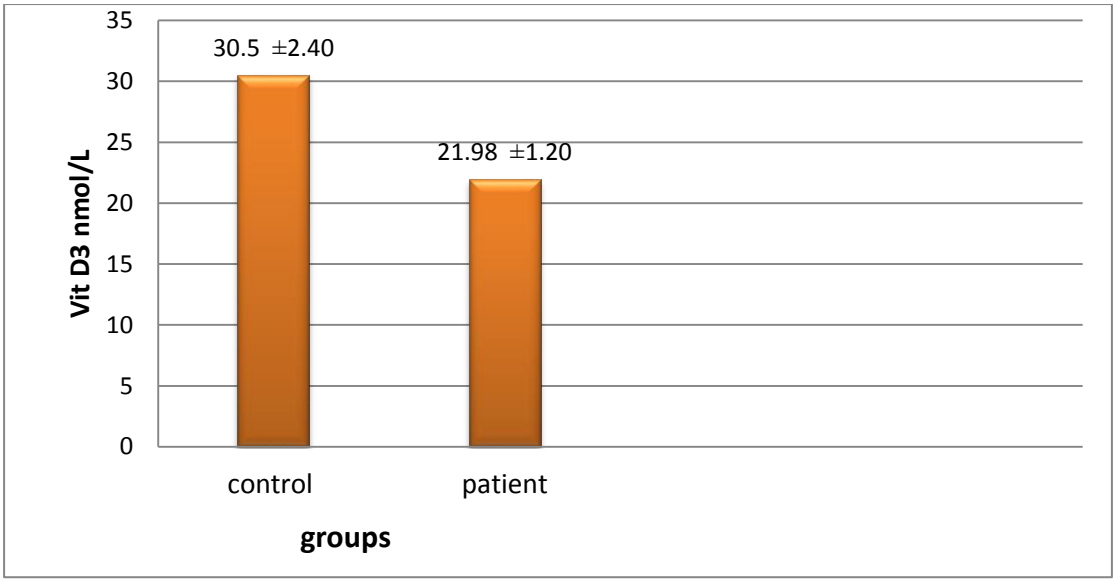


Figure (4.14): *Serum Vit. D3 levels in control and invasive ductal carcinoma*

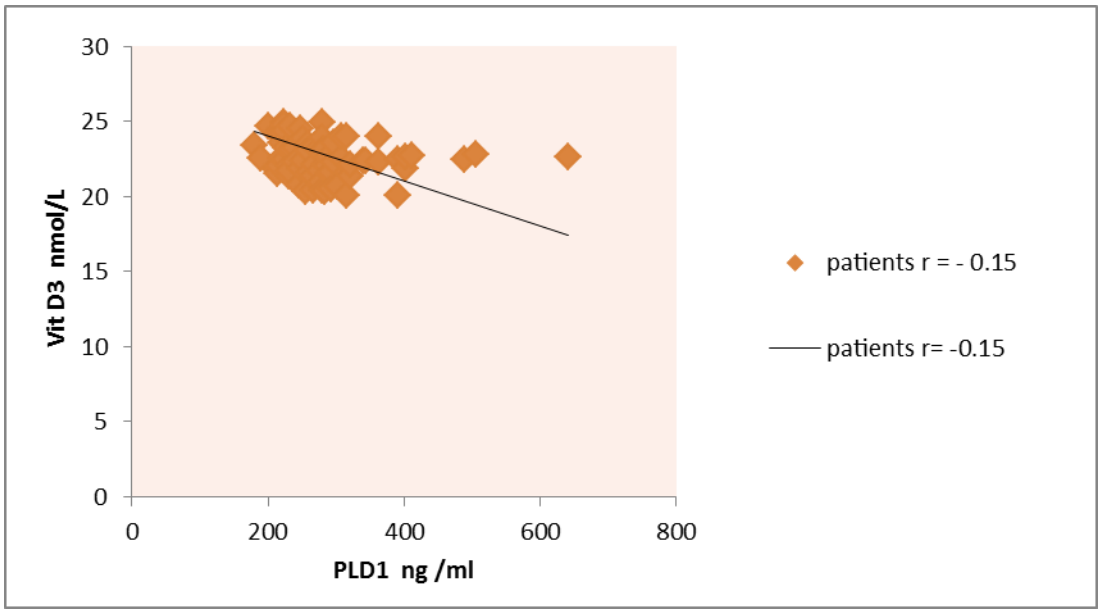


Figure (4.15): *Correlation between PLD1 and Vit.D3 in invasive ductal carcinoma patients group*

4.2.9. Serum Calcium Concentration:

Table (4-10) and figure (4.16) show a significant increase in the levels of serum Calcium in patients group in comparison with the control group ($P \leq 0.05$). This findings agree with the results of study of (Usoro N. *et al.*, 2010).

The importance of calcium in carcinogenesis derives from its participation in regulating cell proliferation, differentiation, and apoptosis (Sergeev I. , 2004). Increasing the concentration of calcium decreases cell proliferation and induces differentiation of mammary cells (Russo J. and Russo I., 2001). calcium are strongly correlated and share similar anti carcinogenic effects on mammary gland. Hence, any apparent effect of vitamin D on breast cancer risk might be due in part to an effect of calcium and vice versa. However, few epidemiologic studies have investigated the joint and independent effects of vitamin D and calcium on breast cancer risk (McCullough M. *et al.*, 2005).

Figure (4.17) shows the positive correlation between PLD1 and Ca in invasive ductal carcinoma patients group with correlation coefficient (r = 0.59).

Table (4–10):- *Serum Calcium levels of control and invasive ductal carcinoma*

Groups	No.	Calcium levels (mmol/L) Mean ± SD
Control	75	2.27 ± 0.11^{b}
patients	85	2.73 ± 0.19^{a}
LSD		0.12

- Legend as in table (4-2).

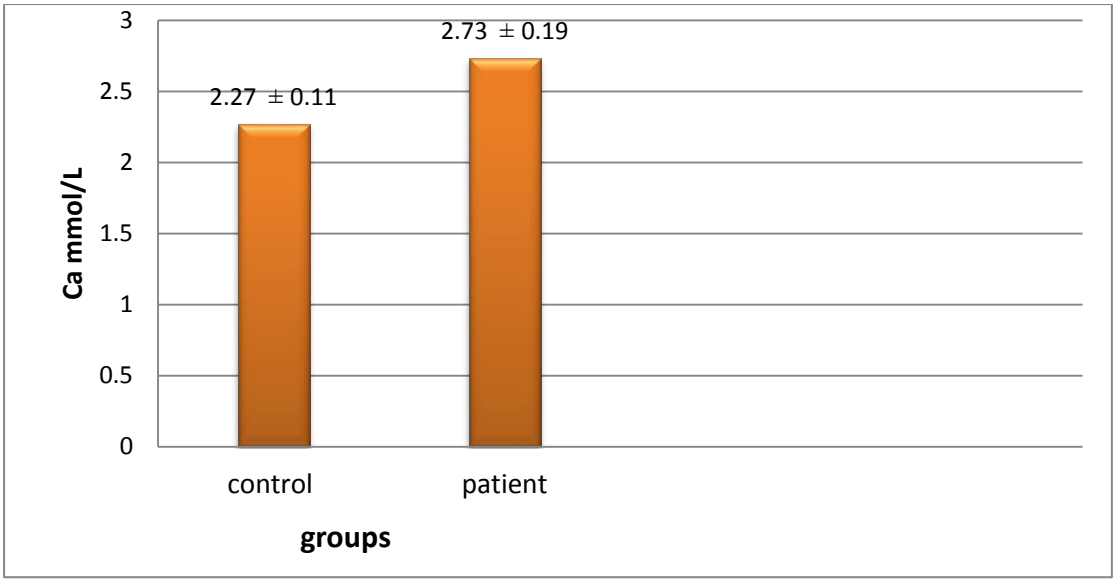


Figure (4.16): *Serum Calcium levels in control and invasive ductal carcinoma*

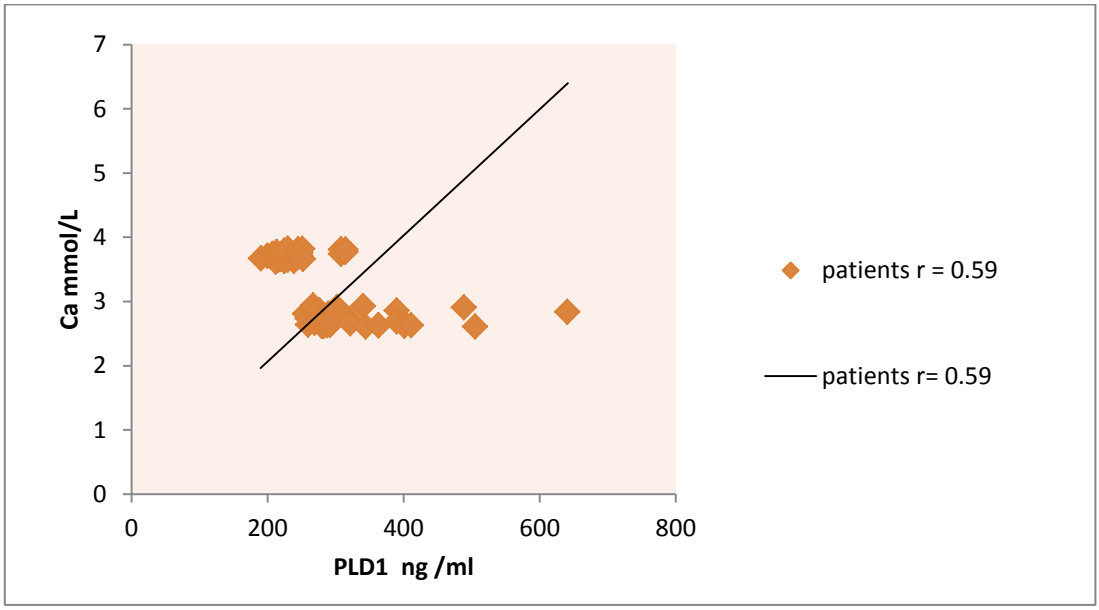


Figure (4.17): *Correlation between PLD1 and Ca in invasive ductal carcinoma patients group*

4.2.10. Serum Alkaline phosphatase (ALP)Enzyme activity:

Table (4-11) and figure (4.18) show a significant increase in the levels of ALP enzyme in patients group in comparison with the control group ($P \leq 0.05$).This result is agreement with (Singh A. *et al*., 2013).Many scientists reported elevated level of ALP in breast, bone and liver metastasis (Morcos N. *et al*., 2013 ; Chauhan P.*et al.* , 2016).

The progressive increase in the serum alkaline phosphatase (ALP) activity in breast cancer patients is an indication of metastasis (Ramaswamy G. *et al*., 2000; Mishra **S**. *et al*., 2004). Elevation of serum ALP occurs because of the accelerated de novo synthesis of the enzyme and subsequent regurgitation into the serum, the serum alkaline phosphatase level is a better predictor for metastatic breast cancer in comparison to other parameters activities as its level gradually increases as the stages advances.

Figure (4.19) shows the positive correlation between PLD1 and ALP in invasive ductal carcinoma patients group with correlation coefficient (r = 0.05).

Table (4–11):- *Serum ALP activity of control and invasive ductal carcinoma*

Groups	No.	ALP activity (U/L) Mean ± SD
Control	75	67.32± 13.84[b]
Breast cancer	85	132.89±21.50 [a]
LSD		4.68

- Legend as in table (4-2).

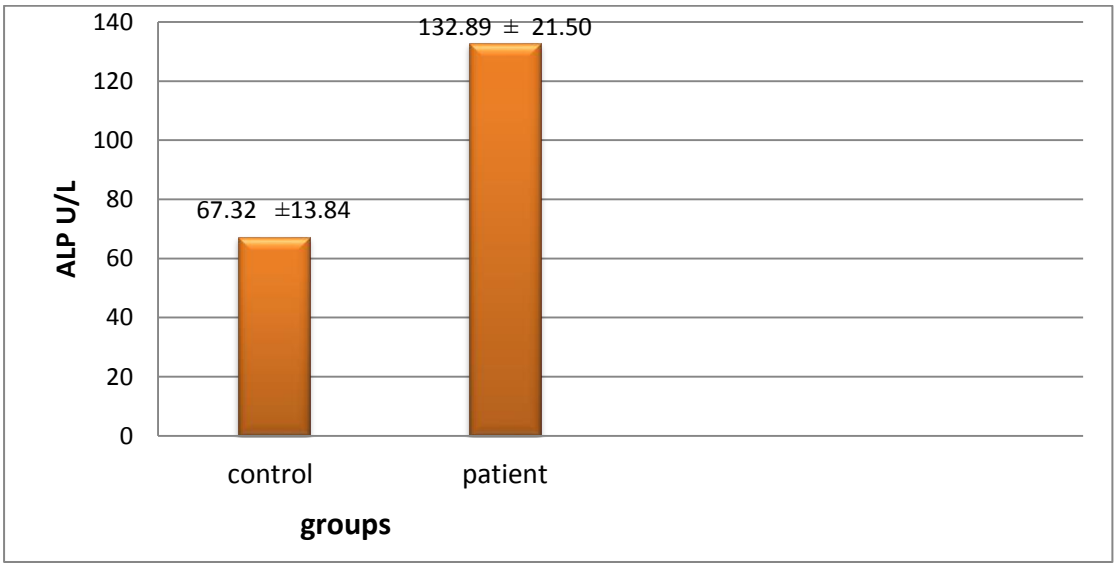


Figure (4.18): *Serum ALP activity in control and invasive ductal carcinoma*

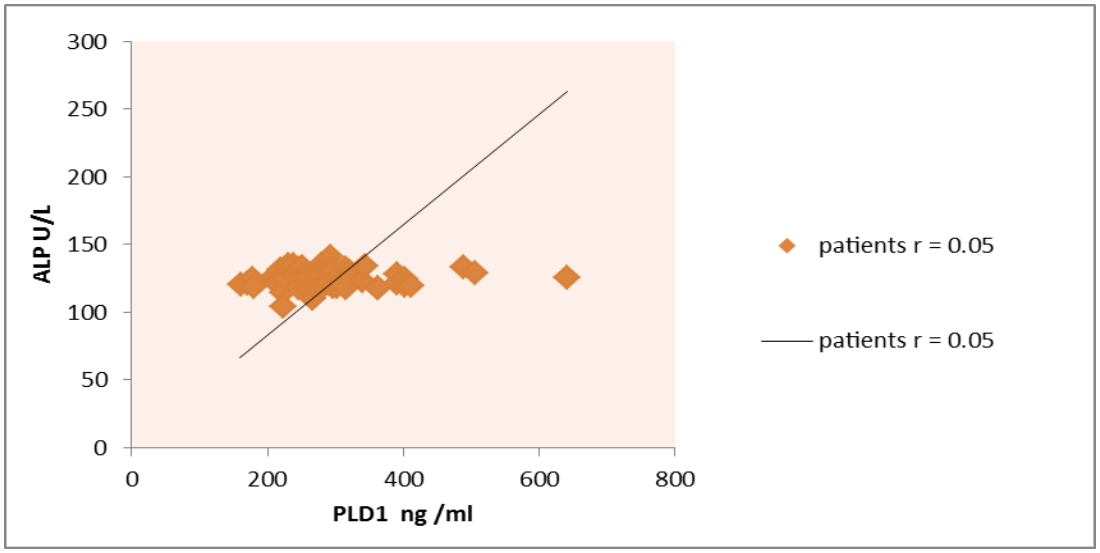


Figure (4.19): *Correlation between PLD1 and ALP in invasive ductal carcinoma patients group*

4.3. Histopathological studies:

Histological type of breast cancer

All studied cases (50 case) were invasive ductal carcinoma .

4.3.1.Staging of breast cancer:

Out of 50 cases , we found that 2 cases (4%) were stage I, 16 cases (32%) were stage II, 28 cases (56%) were stage III and 4 cases (8%) were stage IV. There was no significant difference among different stages (P > 0.05), table (4-12). This result agree with Iraqi study which is done by (Al-hamadawi *et al* . , 2015) who observes that (43%) of patients are diagnose in stages III, and also this result agrees with Iranian in a study done by (Pourzand *et al*. , 2011) who shows that the stage III find in (44.7%) of cases and stage II present in (38.1%) of cases.

Table (4-12): *Distribution of patients according to stage*

Stage	Number of patients	%
Stage I	2	4
Stage II	16	32

Stage III	28	56
Stage IV	4	8
Total	50	100

(X^2 =12 , df = 9, P > 0.05)

4.3.2.Grading of breast cancer:

Out of 50 cases, we found that 4 cases (8%) were grade I, 21 cases (42%) were grade II, and 25 cases (50%) were grade III. There was no significant difference among different grades (P > 0.05), table (4-13), and Fig.(4-20),(4-21),(4-22). This result agrees with a study which is done by (Fayaza M. *et al* . , 2014) who observe that the grade III find in (46.4 %) of patients follows by grad II that find in (36.2%).

We conclude most cases in present study are advance stage and grade. These observations obviously reflect the poor health education of the general population and their ignorance regarding the significance of clinical breast examination, breast self examination and early medical consultation, this conclusion agree with (Etzioni R. *et al*. , 2003).

***Table (4-13)**: distribution of patients according to grade*

Grade	Number of patients	%
Grad I	4	8
Grade II	21	42
Grade III	25	50
Total	50	100

(X^2 = 6 , df = 4, P > 0.05)

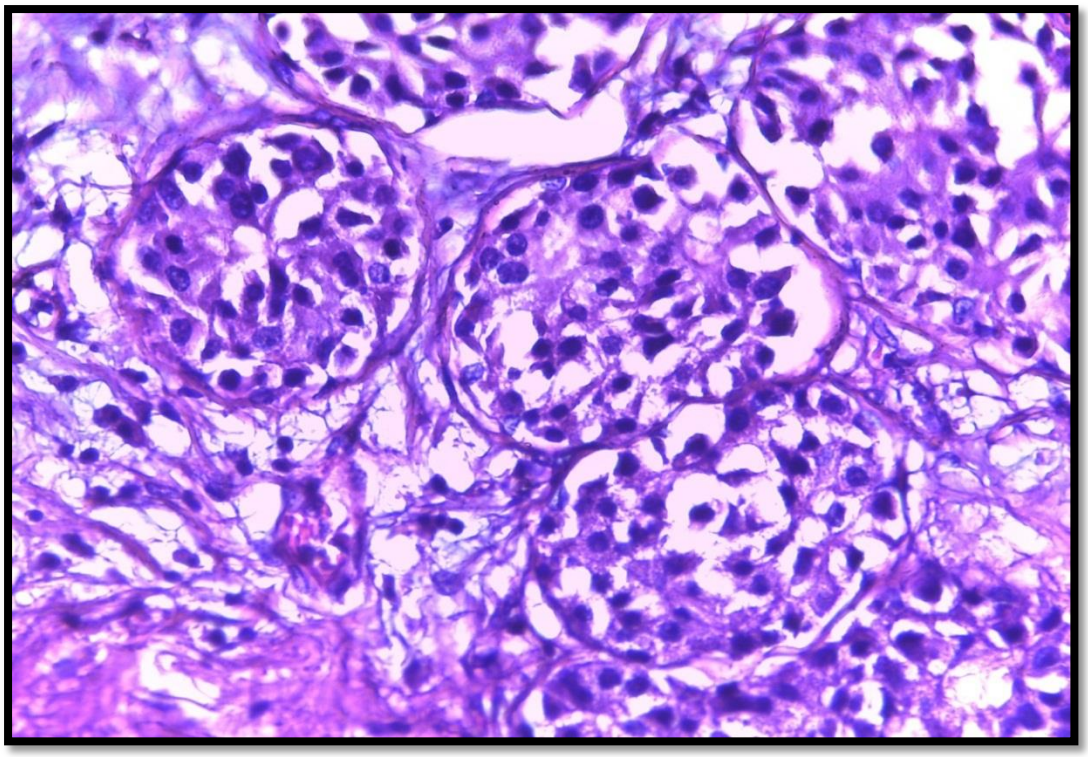


Figure (4.20): Invasive ductal carcinoma, grade I, (40X)

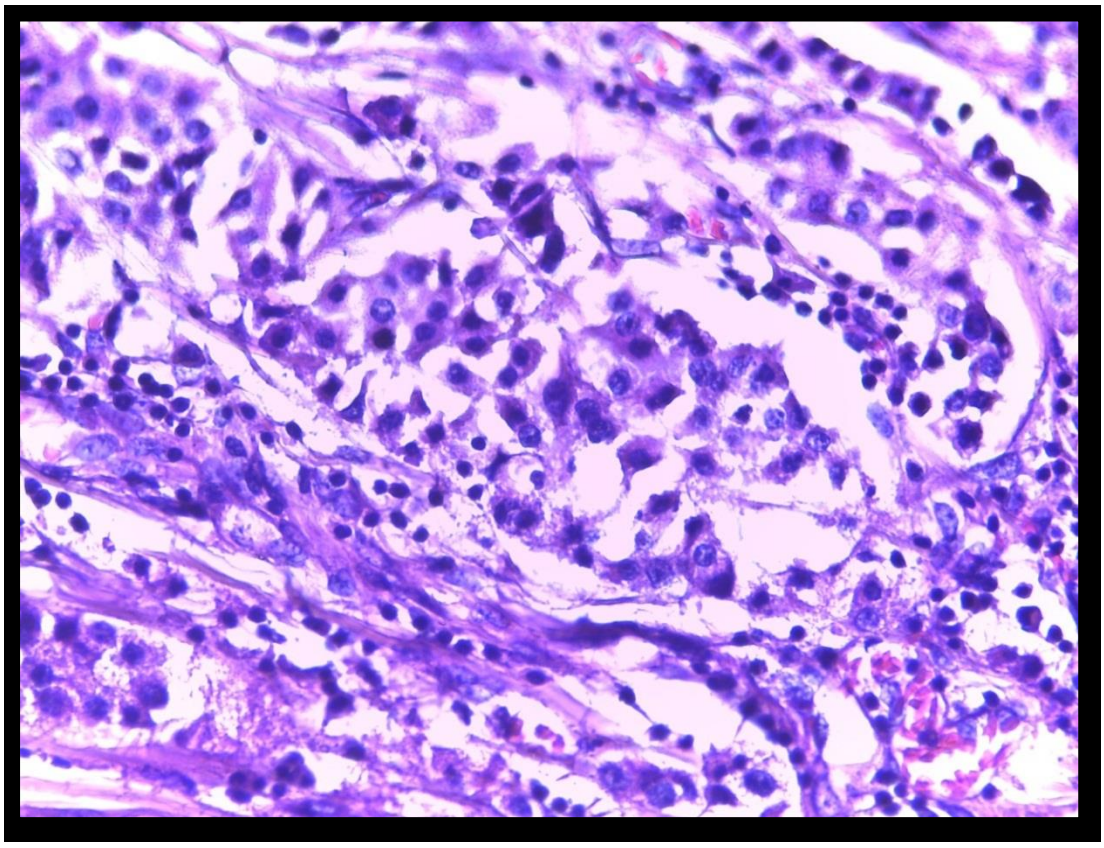


Figure (4.21): Invasive ductal carcinoma, grade II, (40X)

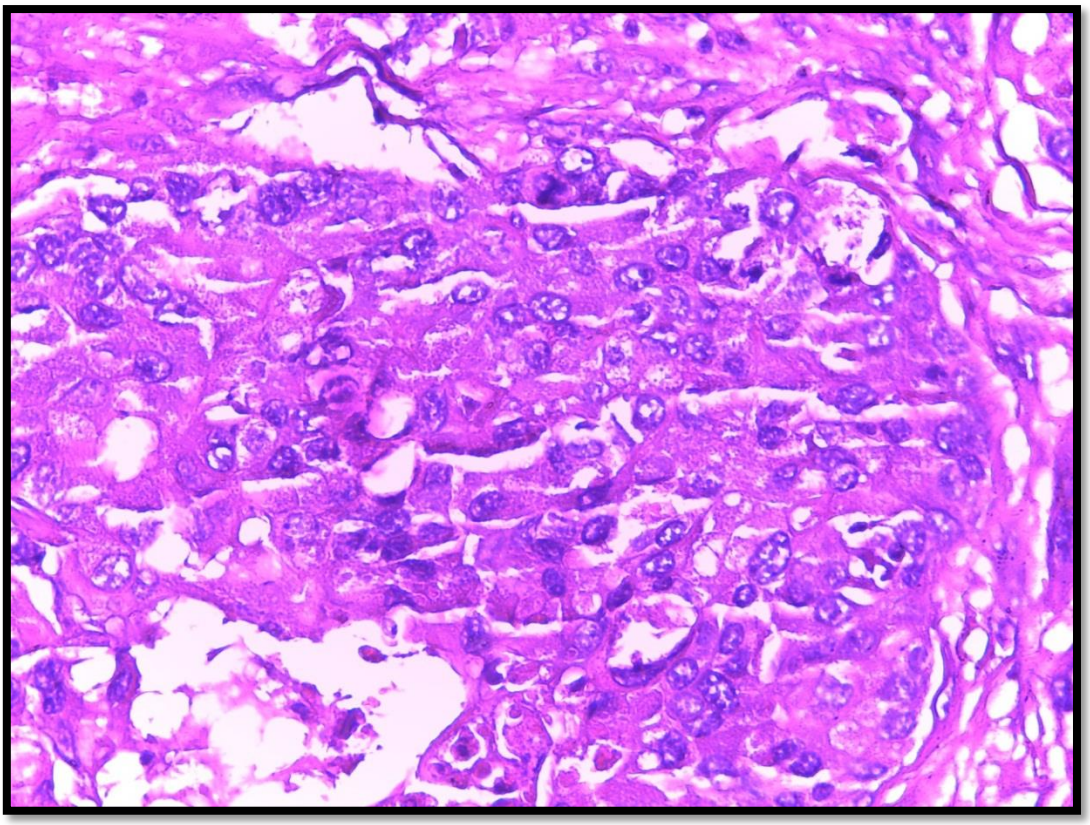


Figure (4.22): Invasive ductal carcinoma, grade III, (40X)

4.4. Immunohistochemistry staining for phospholipase D1:

We found that 41 cases (82%) were **phospholipase D1** positive results, and 9 cases (18%) were negative, figure (4.23). There was no expression of **phospholipase D1** in all normal breast tissues (control group, which include normal breast tissue from patient with benign lesion, and normal breast tissues from the same studied group with breast cancer).

4.4.1. Immunohistochemical staining of phospholipase D1 according to different grades :

In this study we found that 25 cases (which are positively stained with phospholipase D1) were grade III and the remaining 21 cases were grade II breast cancer. All four cases which were grade I was negative immunohistochemical staining for phospholipase D1.

4.4.2.Staining Scoring :

The scoring of expression of phospholipase D1 in the current study classified into **(-, +, ++ and +++)** according to the percentage of cells positively stained with phospholipase D1. Brown cytoplasmic staining was considered positive reaction and compared with the cytoplasmic staining of the positive control slide that used in this study which is normal splenic tissue fig. (4.24).

Classification of the cases of carcinoma, and control groups into different grades of intensity **(-, +, ++ and +++)** showed no significant difference ($P > 0.05$). In the 41 cases which were positive stain for phospholipase D1; staining with score +++ was seen in 14 (28%) cases, score ++ was seen in 18 cases (36%), and 9 (18%) cases were score + , and 9 (18%) cases of invasive ductal carcinoma were negative, table (4-14), figures(4.25,4.26, 4.27).

Interestingly, the positive result and high severity of the disease with the rise the level of phospholipase D1 can reflect the suspected relationship between this biomarker and tumor stage or grade.

This result suggests that the overexpression of PLD1 play an important role in the human breast tumorigenesis and PLD1 useful as a marker for malignant disease in the breast.

Table (4-14): *Frequency distribution of immunohistochemical expression of Phospholipase D1 in invasive ductal carcinoma, and control groups*

		Study groups		
		Patients N (%)	**Control 1 N (%)**	**Control 2 N (%)**
IHC score	**-**	9 (18%)	50 (100%)	35(70%)
	+	9 (18%)	0 (0%)	0 (0%)
	++	18 (36%)	0 (0%)	0 (0%)
	+++	14 (28%)	0 (0%)	0 (0%)
	Total	50 (100%)	50 (100%)	50 (100%)

(X^2 =1.33, df = 2, P > 0.05)

****Note: Control 1**(normal breast tissue from the same women with invasive ductal carcinoma).

Control 2 (normal breast tissue from patients with benign lesions).

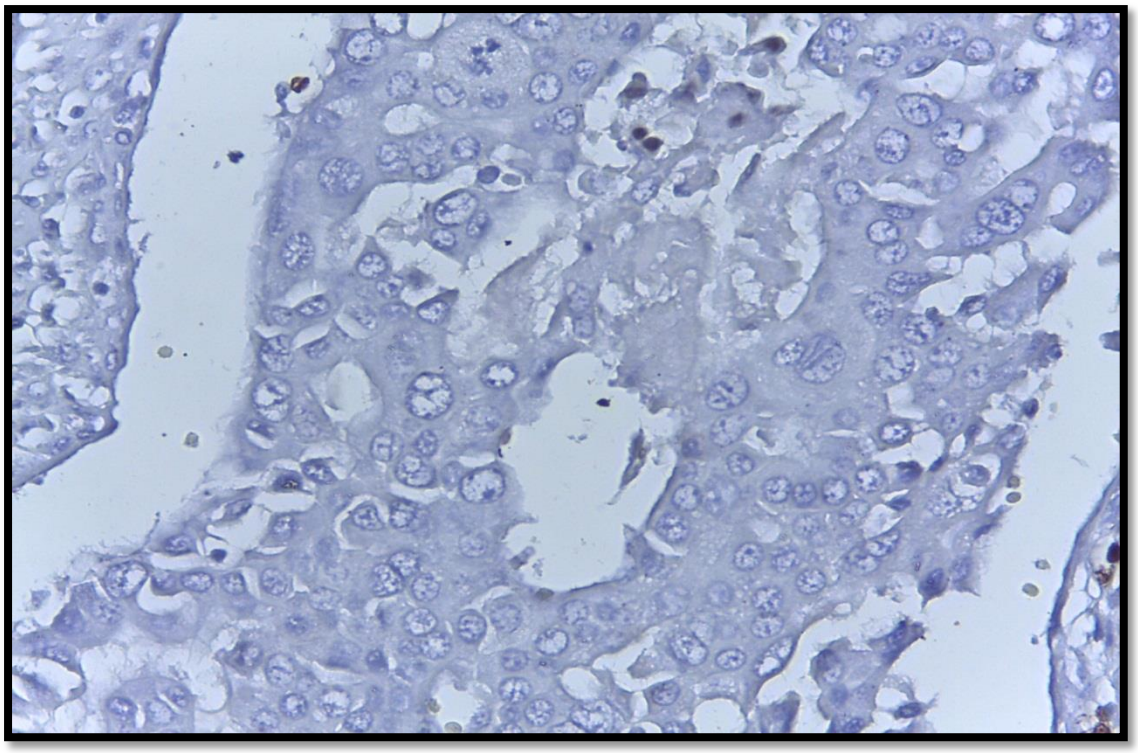


Figure (4.23): Invasive ductal carcinoma, showing negative expression of phospholipase D1, negative control (40X)

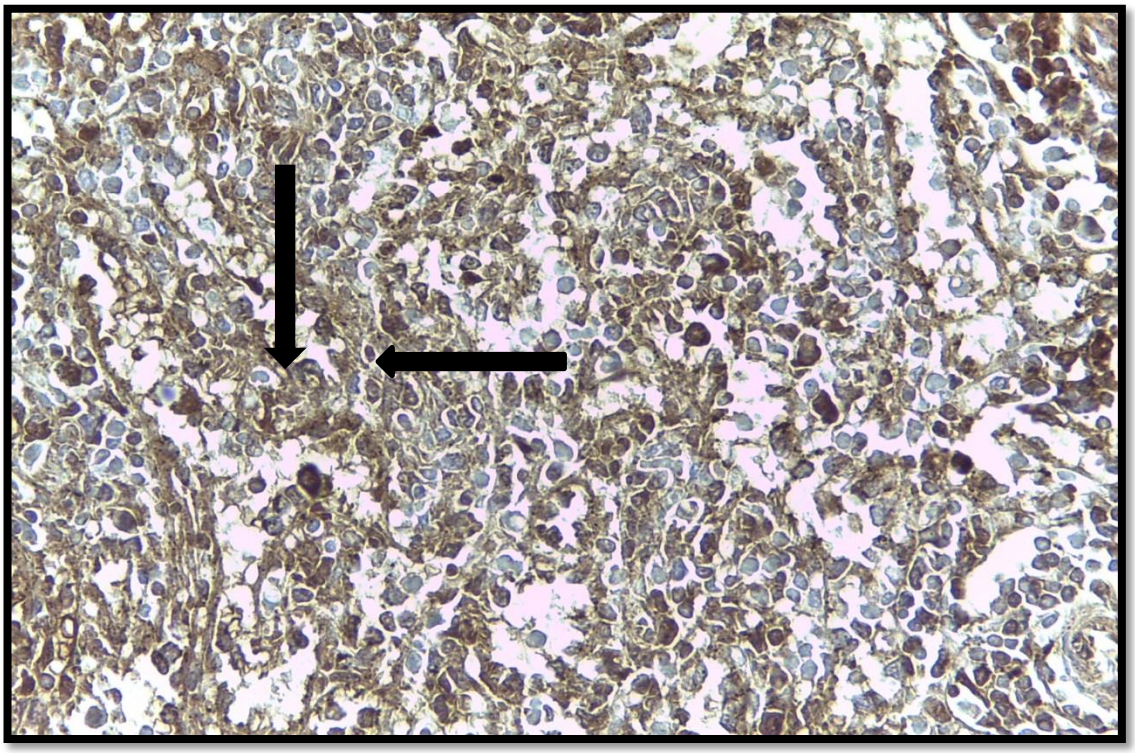


Figure (4.24): positive control, splenic tissue showing positive (arrows) expression of phospholipase D1 (10X)

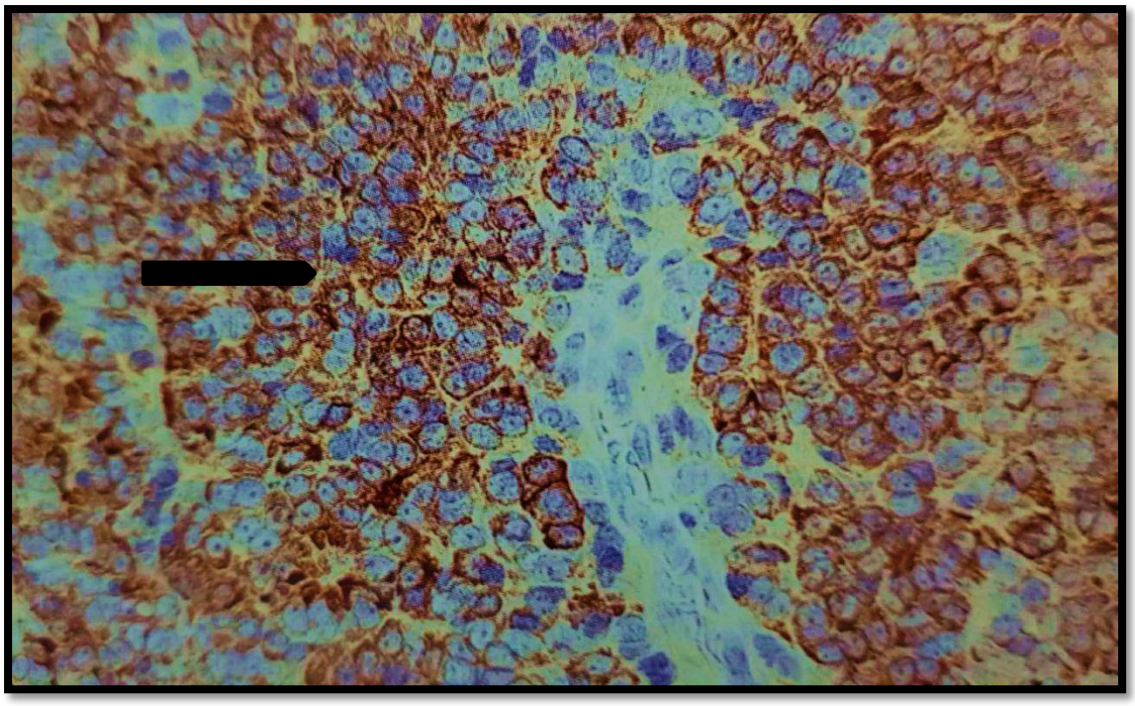


Figure (4.25): Invasive ductal carcinoma, showing strong positive (arrow) expression of phospholipase D1 ***(+++)*** *(10X)*

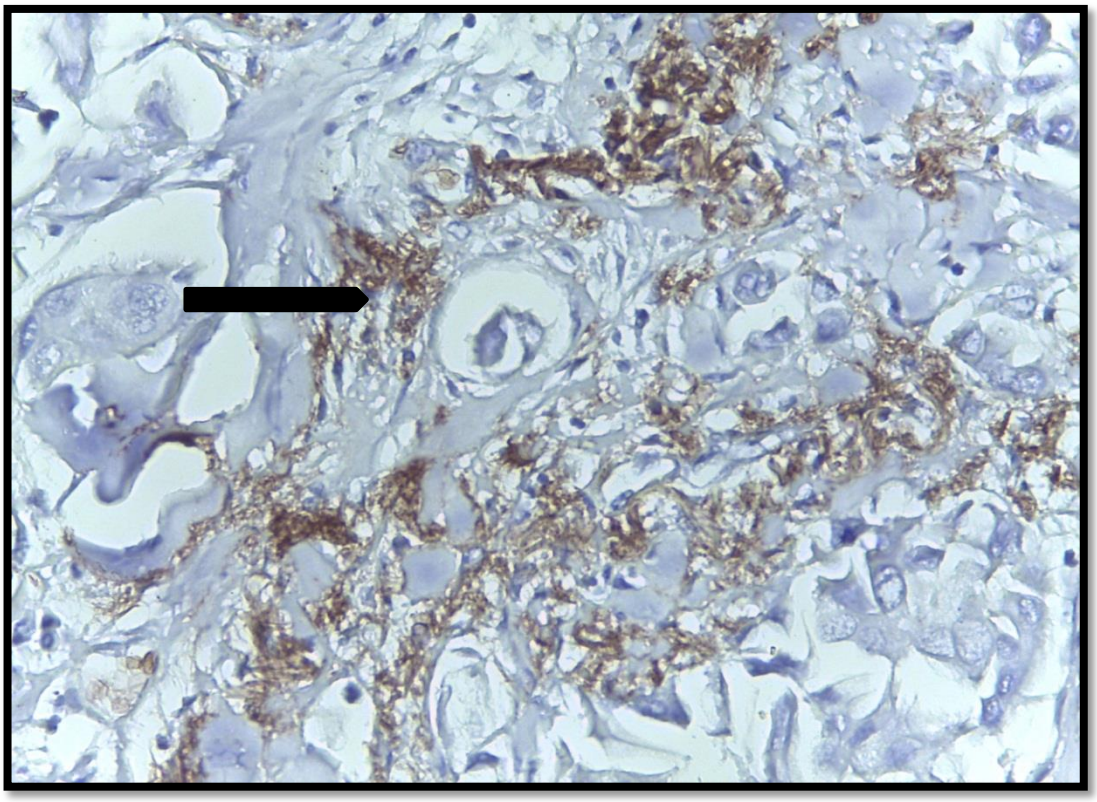


*Figure (4.26): Invasive ductal carcinoma, showing moderate positive (arrow) expression of phospholipase D1****(++)*** *(40X)*

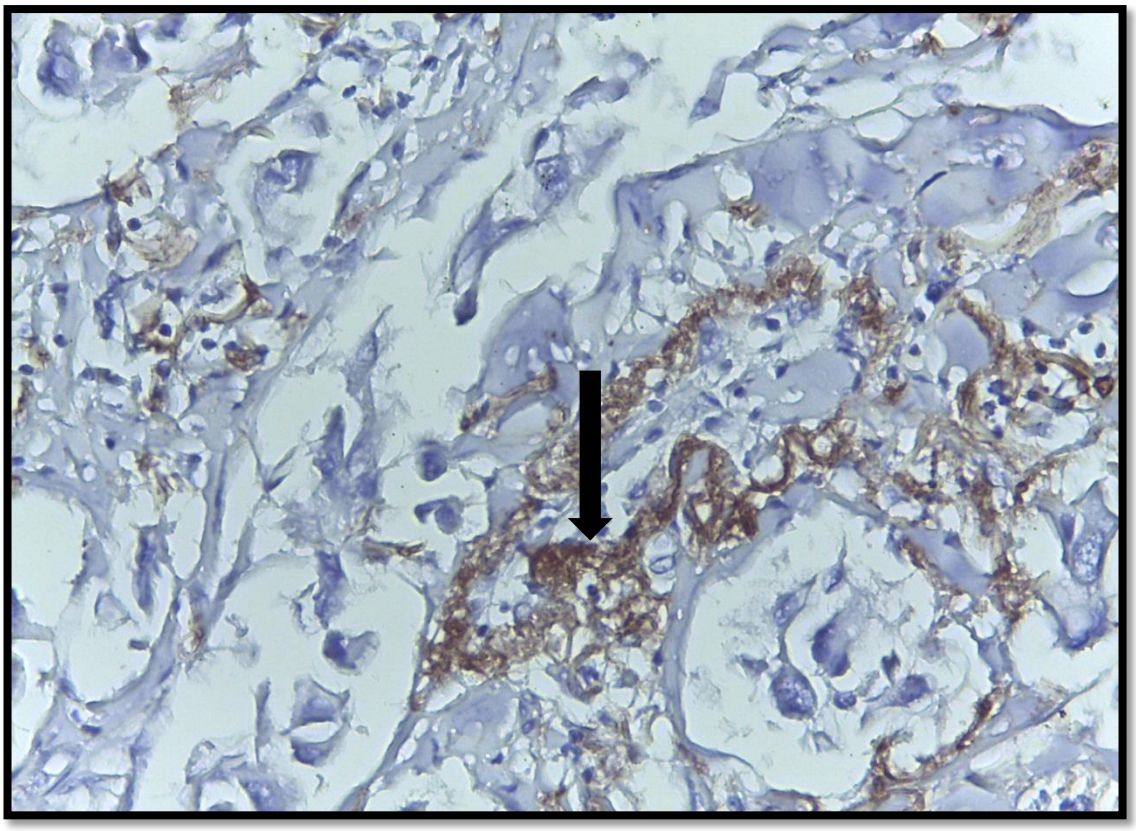


Figure (4.27): Invasive ductal carcinoma, showing weak positive (arrow) expression of phospholipase D1 ***(+)*** *(40X)*

Conclusions

The conclusions which were achieved out of the results obtained in the current study are as follows:

1. The present results suggest a relationship between highly increased PLD1 and and CHKA enzymes activity and E2 hormone concentration in sera of patients with invasive ductal carcinoma as compared with that of the normal breast ($P \leq 0.05$).

2. The pineal gland hormones (ST and MT) concentrations appear to be associated with invasive ductal carcinoma women.

3. Serum calcium levels were positively associated with breast cancer risk in premenopausal and/or overweight women.

4. A clear relationship between elevated CRP levels and prognoses indicates that CRP could be useful in predicting prognoses in advanced cancer patients.

5. This present study indicated that low blood adiponectin level was associated with the risk of obesity-related malignancies, including breast cancer.

6. Based on the findings in this study, especially low levels of Vit. D3, we can conclude that deficient levels of Vit. D3 may contribute to the process of carcinogenesis among the breast cancer patients.

7. An increase in alkaline phosphatase activity was found in sera with patients with invasive ductal carcinoma.

8. Biochemical parameters affected by breast cancer of (PLD1) and there is a significant correlation between them and each one of studied biochemical parameters.

9. We also observed increased expression by immunohistochemistry of PLD1 activity in human breast cancer. PLD1 expression was elevated in human breast cancer compared with normal breast tissues. These results implicate a possible role of PLD1 in human breast tumorigenesis and suggest that PLD1 may be useful as a marker for malignant disease in the breast.

The overall conclusions of the present study summarized in the following:

Parameters	Serum	Breast tissue
PLD1 activity (ng/ml)	↑	
CHKA activity (ng/ml)	↑	
ST conc. (ng/ml)	↓	
MT conc. (pg/ml)	↓	
E2 conc. (Pg/ml)	↑	
ADP conc. (ng/ml)	↓	
CRP conc. (mg/L)	↑	
Vit.D3 conc. (nmol/L)	↓	
Ca conc. (mmol /L)	↑	
ALP activity (U/L)	↑	

Immunohistochemistry of PLD1		↑

Note:

↑ **: Highly significant increase**

↓ **: Significant decrease**

↑ **: Significant increase**

Recommendations

The Following points are suggested as *Recommendations*:

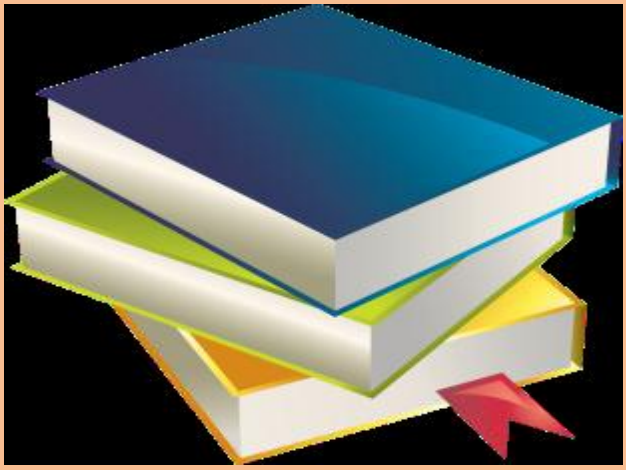
1- Study of others metabolites (choline transporter, phosphocholine cytidylyltransferase, glycerophosphocholine , and lysophocphocholine).

2- Genetic study of all phospholipids metabolites genes.

3- Immunohistochemistry study of others metabolites expect (PLD1 enzyme).

4- Follow- up of the patients with breast cancer for further analysis of the role of lipids in breast cancer and its level variation with progression of the disease and in relation to treatment.

5- Studying of larger sample to correlate between the level of phospholipase D1 with stage and grade of tumor.



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APPENDICES

Appendix 1: Questionnaire List

1. Patient name.
2. Patient age.
3. Occupation.
4. Address.
5. Marital status.
6. Type of Carcinoma.
7. Stage of breast cancer.
8. Grade of breast cancer.

Patient Signature

Appendix 2: Hematoxylin-Eosin Stain (H.&E.)/(Harri's Haematoxylin)

Hematoxylin crystals (5 gm)

Alcohol, absolute (50ml)

Potassium Alum (100gm)

Distilled water (1000ml)

Mercuric oxide (red) (2.5gm)

Hematoxylin had dissolved in alcohol; the alum in the water by the aid of heat. Then removed from heat and the two solutions mixed and; boiled for less than 1 minute with stirring (bringing to boiling should be rapid as soon as possible). Then; removed from heat and, the mercuric oxide added slowly. Reheated to a simmer until it becomes dark purple, removed from heat and, the vessel plunged into a basin of cold water till cooled. 2-4ml of glacial acetic acid per 100ml added (to increase the precision of nuclear stain). Filtered before use.

Eosin as counter stain for Hematoxylin

1% stock alcoholic Eosin:

Eosin Y, water soluble (1gm)

Distilled Water (20ml)

Dissolved , and add Alcohol , 95% (80ml)

Working Eosin solution :

Eosin stock solution (1 part)

Alcohol, 80% (3 parts)

0.5ml/100ml of glacial acetic acid was added just before use with stirring.

الخلاصة

سرطان الثدي هو مرض معقد ومتعدد العوامل يؤدي الى نمو الخلايا غير الطبيعية التي تؤدي الى تكوين ورم خبيث. وقد تم تصميم الدراسة لتحديد مستويات {انزيم الفوسفولايبيز دي 1**(PLD1)** و انزيم كولين كاينيز الفا **(CHKA)**} و هورمونات الغدة الصنوبرية (السيروتونين والميلاتونين) **(ST,MT)** والاسترادايول **(E2)** وتقويم معيار بروتين سي الفعال **(CRP)** و(هورمون الاديبونكتين وفيتامين دي 3 والكالسيوم) (**ADP,Vit.D3, Ca**) و(انزيم الفوسفاتيز القاعدي) **(ALP)** في النساء المصابات بسرطان الاقنية المنتشر والنساء من الاصحاء.

شملت الدراسة (160) امرأة وزعت الى مجموعتين : (85) امرأة مصابة بسرطان الاقنية المنتشر تتراوح اعمارهم (25-65) سنة و (75) امرأة من الاصحاء (مجموعة السيطرة) تتراوح اعمارهم (25-65) سنة . هدفت الدراسة الى تسليط الضوء على العلاقات الترابطية بين انزيم الفوسفولايبيز دي 1**(PLD1)** وكل واحد من المعايير التي درست والمذكورة اعلاه .

بينت النتائج ان هناك زيادة معنوية في تراكيز كل من انزيم الفوسفولايبيز دي 1**(PLD1)** و انزيم كولين كاينيز الفا **(CHKA)** والاسترادايول **(E2)** و بروتين سي الفعال **(CRP)** والكالسيوم**(Ca)** وانزيم الفوسفاتيز القاعدي **(ALP)** في كل مجاميع المرضى مقارنة مع مجموعة السيطرة .

بينما نقصان معنوي في تراكيز(السيروتونين والميلاتونين) **(ST,MT)** و(هورمون الاديبونكتين وفيتامين دي 3) **(ADP and Vit.D3)** في كل مجاميع المرضى مقارنة مع مجموعة السيطرة.

كما أظهرت الدراسة وجود علاقة ترابطية موجبة بين (**PLD1**) وكل من (CHKA وE2 و CRP وCa وALP) علاقة ترابطية سالبة مع (ST, MT ADP,Vit.D3) في النساء المصابات بسرطان الاقنية المنتشر.

اعتمادا على التشخيص النسيجي للحالات السرطانية (50 حالة) (100%) من سرطان الاقنية المتسرب او المنتشر).

اظهرت النتائج الى ان حالتين (4 %) من المريضات كانوا في المرحلة الاولى من المرض، 16 حالة (32%) كانوا في المرحلة الثانية ، 28 حالة (56%) كانوا في المرحلة الثالثة،4 حالات (8%) كانوا في المرحلة الرابعة.

وأيضا النتائج التي توصلنا إليها أظهرت 4 حالات (8%) كانوا من الدرجة الاولى، 21 حالة (42 %) كانوا من الدرجة الثانية ، 25 حالة (50%) كانوا من الدرجة الثالثة.

نتائج التحليل المناعي النسيجي للفوسفولاييز دي 1. قسمت الى مجموعة سرطان ومجاميع سيطرة حسب كثافة او شدة الgrades المختلفة **(- ،+ ،++ و+++)** لم تظهر أي فرق إحصائي كبير. في 41 حالة كانت صباغتها موجبة لل**PLD1** ، صبغت مع +++ score في 14 حالة (28%) و++ score في 18 حالة (36%) و9 حالات (18%)مع + score و و9 حالات (18%) من سرطان الاقنية المنتشر كانت سالبة.

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة ذي قار

كلية العلوم

دراسة مرضية سريرية وكيموحيوية لأنزيمات الفوسفولايبيز دي 1 و كولين كاينيز الفا لدى النساء المصابات بسرطان الأقنية المنتشر في الثدي في محافظة ذي قار- العراق

الأطروحة

مقدمة إلى مجلس كلية العلوم /جامعة ذي قار كجزء من متطلبات نيل درجة الدكتوراه فلسفة في الكيمياء الحياتية السريرية

من قبل

هديل رشيد فرج السنيد

بكالوريوس جامعة ذي قار (2006)

ماجستير جامعة ذي قار (2013)

بإشراف

أ.د. حسام محمد كريدي

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