Biochemical Studies of the Binding of CA 19-9 to its Antibody in Patients with type 2 Diabetes Mellitus using Enzyme-Linked Immunosorbent Assay with some Modifications

A Thesis

Submitted to College of Medicine/Al-Nahrain University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Medical Chemistry

By

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Master of Science

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<u>Dedication</u>

To the sun that shine the way of my life
(My Mother).

It's impossible to thank you adequately

I am profoundly indebted to you

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Russul R. AL-Hamaoy.

Abstract

Back ground:-

Carbohydrate Antigen CA19-9 has been widely utilized for diagnosis of different kinds of cancer such as pancreatic cancer, cancer of upper gastrointestinal tract. It may also be used as an indicator of pancreatic tissue damage that may be caused by diabetes.

The method of enzyme linked immunosorbent assay (ELISA) for measurement CA19-9 in sera of patients with type 2 diabetes mellitus was developed using external CA19-9 antibody (competitive sandwich ELISA technique) and found to be suitable for the determination of a dissociation constant (K_d) of the binding of CA19-9 to its antibody in type 2 diabetic patients using scatchard plot.

Objectives:-

This study was performed to assess CA19-9 levels in sera of patients with type 2 diabetes mellitus with non-cancerous tissue by enzyme-linked immunosorbent assays (ELISA) and define the normal range (cutoff value) for the same marker in this patients also investigated the possible relationships of CA19-9 with fasting plasma glucose and HbA1c.

This study present a new method for determination the dissociation constant (K_d) of the binding of CA19-9 to its antibody in type 2 diabetic patients using scatchard plot through development of ELISA (competitive sandwich ELISA technique).

Different factors affecting this binding were extensively studied such as divalent cations, different halides, and Polyethylene (PEG-6000).

Methods:-

A case control study was conducted on eighty patients diagnosed as type 2 diabetes mellitus (T2DM) during the period from February 2015 to May 2015. Seven milliliters of blood sample was collected from each fasting subject (12 hour fast) with mean age (46.5625 ± 1.1054 years) were divided into two groups. Group Ⅰ, forty patients with type 2 diabetes mellitus have a standard error of mean duration 6.6± 0.9421 and take medication. Group Ⅱ consisted of forty patients were newly diagnosed type 2 diabetic and don't take medication, in addetion to forty healthy individuals with standard error of mean age (44.7± 1.4539) were classified as control group.

Glycate hemoglobin was measured in whole blood by NycoCard reader instrument, while FBS, lipid profile, Urea serum, serum ccreatinine, C-reactive protein, CA19-9 level, determination of Antigen Affinity, protein concentration using Bradford method were measured in serum by Chemistry Analyzer, NycoCard reader instrument ELISA technique and Spectrophotometer.

Results:-

The diabetic groups (with treatment & newly diagnosed) showed a significant elevation of serum CA19-9 protein in comparison with control group (p< 0.001), while there is no significant difference observed between the two diabetic groups (p=0.308). Serum CA19-9 concentration of 49.5 U/ml was used as cut off value for the diagnosis of type 2 diabetes mellitus, the sensitivity is 88.8% and specificity is 77.5%

The converter factor **1.010388** can be used to convert the concentration of carbohydrate antigen CA19-9 from (U/ml) to (µg/ml).

The dissociation constant (K_d) of CA19-9 for standard and patient was **15.6006 U/ml** and **12.5313 U/ml** respectively, while for healthy individual **is 4.1271 U/ml.**

The current study proves there is an effect for divalent cation which showed that the binding process is sensitive for the cations existence, it is found that Mn (II) at concentration 0.15M increases the binding more than the other divalent cations, whereas Cu (II) in the same concentration increased the binding less than other divalent cations.

The effect of different halides (NaI, NaCl and NaF) on CA19-9 Antigen-Antibody binding in incubation medium that contain sodium halides which causes activation in the percent of binding as the following sequence:

NaF > NaCl > NaI

Study the effect of divalent cations and different halides on the binding of carbohydrate antigen CA19-9 to its antibody is useful in the future work to obtain the maximum bind and create an optimum conditions for this binding.

Polyethylene (PEG-6000) affect negatively on CA19-9 Antigen-Antibody binding.

Conclusion:-

The CA19-9 level not only confined to identify the cancerous tissue but it rise in non-cancerous cases also such as type 2 diabetes mellitus as a result for pancreatic tissue damage that may be caused by diabetes. And the dissociation constant of the interaction between antibody and antigen for the same tumor marker can be calculated using a new method from the data of competitive sandwich enzyme linked immunosorbent assay (ELISA) by Scatchard plot.

List of Abbreviations:

DM	Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
BMI	Body Mass Index
FBS	Fasting Blood Sugar
HbA1c	Glycated Hemoglobin
TC	Total Cholesterol
TG	Triglyceride
c-HDL	Cholesterol High Density Lipoprotein
c-LDL	Cholesterol Low Density Lipoprotein
c-VLDL	Cholesterol Very Low Density Lipoprotein
CRP	C-reactive protein
CA19-9	Carbohydrate Antigen CA19-9
ELISA	Enzyme linked immunosorbent assay
Kd	Dissociation Constant
WHO	World Health Organization
ANOVA	One-way analysis of variance
p-value	Probability factor
R	Correlation coefficient
ROC	Receiver operator characteristic
SD	Standard deviation
SE	Standard error
SPSS	Statistical Package for the Social Sciences

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Introduction & Literature review

1.1   Diabetes mellitus:-

1.1.1 Definition and description:-

Diabetes mellitus is a metabolic disorder or can be expressed as a complex group of diseases with different causes. Worldwide, cancer ranked the second and diabetes mellitus ranked the 12th in the list of causes of death. (Lopez, et al., 2006), (Garg, et al., 2014), (Cromer, et al., 2015). It is a chronic disease that affects about 26.9% of U.S. population aged 65 year and older. every year 1.9 million diabetic person are diagnosed, in addition to 7.0 million undiagnosed and untreated (Centers for Disease Control, 2011).

The physician should depend in the diagnosis of diabetic patients on the following :

- HbA1c test which has a threshold of $\geq 6.5\%$.
- Fasting blood Glucose (FBG) ≥ 126 mg/dL.or Glucose Tolerance Test ; After two hours of intake of 75g glucose, a plasma glucose level ≥ 200 mg/dL (Redmon, et al., 2014).

Untreated diabetic patients have chronic hyperglycemia , i.e a level of blood glucose higher than normal range. The reason for that is either a low level of insulin (a polypeptide hormone secreted by the beta cell of the pancreas) , an abnormal function of insulin or both.
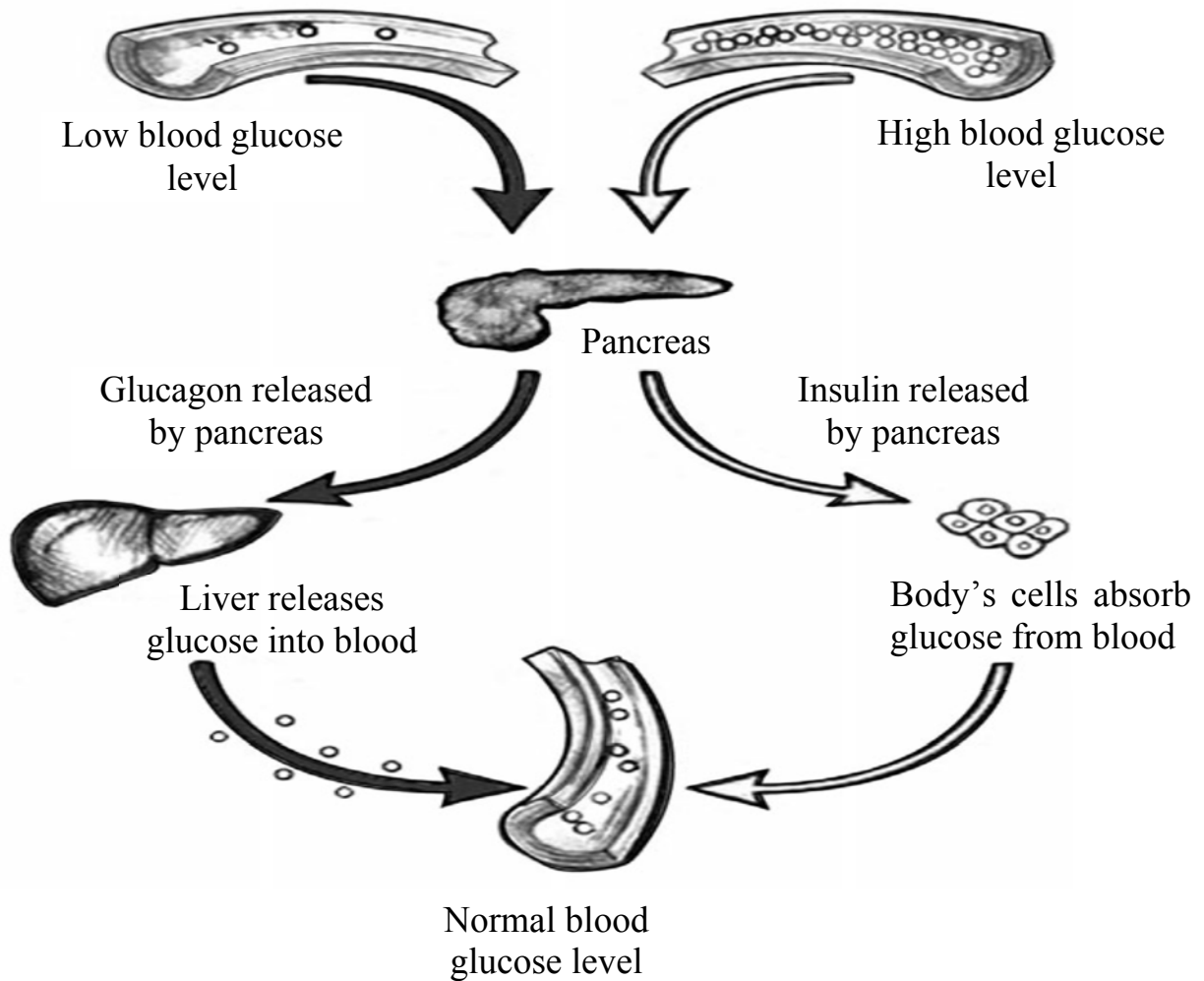
Figure (1-1) : glucagon and Insulin help to regulate the glucose level in blood.
(Publication, 2014)

1.1.2 Complications:-

Clinical data indicate this chronic hyperglycemia is the central initiating factor for any type of diabetes mellitus (Brownlee, et al., 2008).

If diabetes mellitus isn't sufficiently controlled long term complications may occur such as damage, dysfunction and failure of various organs essentially the kidney, heart, nerves, eyes and blood vessels.

These complications include retinopathy (which can lead to blindness), cardiovascular disease (double risk compared to risk in

general population), and neuropathy with risk of foot ulcers. (Alberti, et al., 1998), (American Diabetes Association, 2010)

Foot ulcers is familiar complication of type 1 and type 2 diabetes mellitus. About (5-10) % of patients suffer from diabetes have had previously or existent foot ulceration and 1% have amputation (Brownlee, et al., 2008).

Diabetes mellitus is associated with acceleration of atherosclerotic macro vascular disease affecting arteries especially those supplying the brain, heart and lower extremities (American Diabetes Association, 2010), (American Diabetes Association, 2014).

Chronic hyperglycemia may result in an abnormal red blood cells formation due to impaired production of erythropoeiten , a hormone produced by the kidney and is essential for formation of red blood cells by the bone marrow. This abnormality , i.e. low erythropoeiten level , is the cause of early anaemia seen in diabetics. patients (Thomas, et al., 2004), (Thomass, et al., 2004), (Thomas, et al., 2003).

Patients who suffer from anemia and diabetes are more likely to die earlier than patients who suffer from diabetes without anemia.

Therefore anemia should be treated to reduce early mortality (McGill JB. and Bell DS., 2006).

Treatment of anaemia in diabetes is by controlling both serum glucose level as well as controlling high blood pressure and keeping the level of blood pressure at normal values. Such control of blood glucose and blood pressure delay the development of anaemia and kidney damage (American Diabetes Association, 2009)

1.1.3 Classification:-

The old classification systems of diabetes mellitus focused on the treatment instead of the causes of hyperglycemia while the American Diabetes Association (ADA) and World Health Organization (WHO) show simple classification system for different form of diabetes using four clinical categories according to the causes of hyperglycemia and named them (Fowler, 2007):-

- Type 1 diabetes (the main cause is b-cell devastation, usually result in absolute deficiency of insulin)
- Type 2 diabetes (Because there is a high insulin secretion, the defect is due to insulin resistance).
- Gestational diabetes mellitus "GDM" (pregnant women diagnosed with diabetes in the 2nd or 3rd trimester of pregnancy)
- Specific kinds of diabetes result from other causes, e.g., diseases of exocrine pancreatic disorders such as cystic fibrosis, monogenic diabetes syndromes such as maturity onset diabetes mellitus of the young (MODY) and neonatal diabetes and drugs or chemicals induced diabetes such as in the HIV/AIDS treatment or after organ transplantation.

1.1.3.1 *Type 1 diabetes mellitus:-*

Type 1 diabetes mellitus, previously known as Insulin-dependent diabetes mellitus (IDDM) or Juvenile diabetes, represent only (5-10) % of those with diabetes (American Diabetes Association, 2010). In this type , the pancreas fails to produce insulin and the main reason is autoimmune destruction of the pancreatic beta cells which usually leads to a complete lack of insulin (Goldman, et al., 2000)

In this diabetic kind, b-cell destruction rates is very variable, being slow in some individuals (mainly adults) while rapidly in other patients (mainly children and infants). In some individuals, particularly adolescents and children, the symptoms of ketoacidosis may be the first manifestation of this disease. Others have a slightly increase in fasting blood sugar that can rapidly alter to acute hyperglycemia and/or ketoacidosis with the presence of infections or other stress (American Diabetes Association, 2014)

The development of type 1 diabetes mellitus can occur at any age , but it mostly appears in those 40 year old and younger. It is most commonly seen in children who usually have distinctive symptoms of polyuria , polydypsia and failure to thrive.Sometimes the presentation is in the form of diabetic ketoacidosis . In adults with type 1 diabetic mellitus , the symptoms might be variable and different from those of children. (American Diabetes Association, 2015)

1.1.3.2 *Type 2 diabetes mellitus:-*

Type 2 diabetes mellitus , which represents 90-95% of all diabetics , is a major medical problem worldwide with a big economic impact. The prevalence of the disease has tripled in the last 30 years, (Makki, et al., 2013), (Wild, et al., 2004), (Aghili, et al., 2014), (American Diabetes Association, 2010), (Ajlan, et al., 2012), (Volek, et al., 2005).

Person in middle age or older and who are also overweight or obese are the common victim of type 2 or non-insulin dependent diabetes mellitus (NIDDM) also can develop in obese adolescents, the risk of development of this type of diabetes increase with age, lack of physical activity and with obesity. It occur more frequently in women with previous history of gestational diabetes and in individuals with

dyslipidemia or hypertension; it is often related with strong genetic preponderance more than autoimmune form seen in type 1 diabetes (Inzucchi, et al., 2015), (Hanis, et al., 1996), (Alruba'ae, 2011), (Tumoilehto, et al., 2001).

It is one of heterogeneous diseases characterized by chronic hyperglycemia. In this type the production of insulin is still constant, but the real problem comes from dual defect; impaired β-cell function (insulin secretion) and impaired insulin action. Impaired insulin action i.e. insulin resistance is reduced sensitivity of body tissues to the insulin action and that occurs when target tissue are incapable to respond to normal concentration of insulin as a result, β-cells in pancreas need to increase the amount of insulin secretion to keep euglycemia.

Over time,the functional defect in insulin secretion works to prevent the β-cell from keeping the high rate of insulin secretion. Subsequently, glucose tolerance is impaired and eventually type 2 diabetes develop (Reaven, 2005), (Lebovitz, 2001), (Who, et al., 2003), (Goldstein, 2002).

In fact (75-85)% of patients with type 2 diabetes have obesity ,dyslipidemia, insulin resistance, and hyperinsulinemia before developing to T2DM ,therefore insulin resistance is the best predictor for an individual will become diabetes later or not. (Lebovitz, 1999), (Warram, et al., 1990), (Lillioja, et al., 1988).

In brief we can summarize the development of insulin resistance and diabetes mellitus in the following points and figure :

- Have a parent, sister or brother suffers from type 2 diabetes (Tapia, et al., 2005).
- The age over 40 years old.
- Physically inactive person.
- Being overweight (Tapia, et al., 2005).
- Men have a waist measure more than 102 cm or women have a waist measure more than 88cm (Nwegbu, 2012).
- Level of Impaired fasting glucose more than 100 mg/dl and less than 125 mg/dl) (Karve, et al., 2010).
- Low level of good cholesterol HDL-c (35mg/dl or lower).
- High level of triglycerides reach to 150 mg/dl or more.
- The level of blood pressure more than 140/90 mmHg.
  (Vaag, 2008), (Mota, et al., 2011), (American Diabetes Association, 2004).
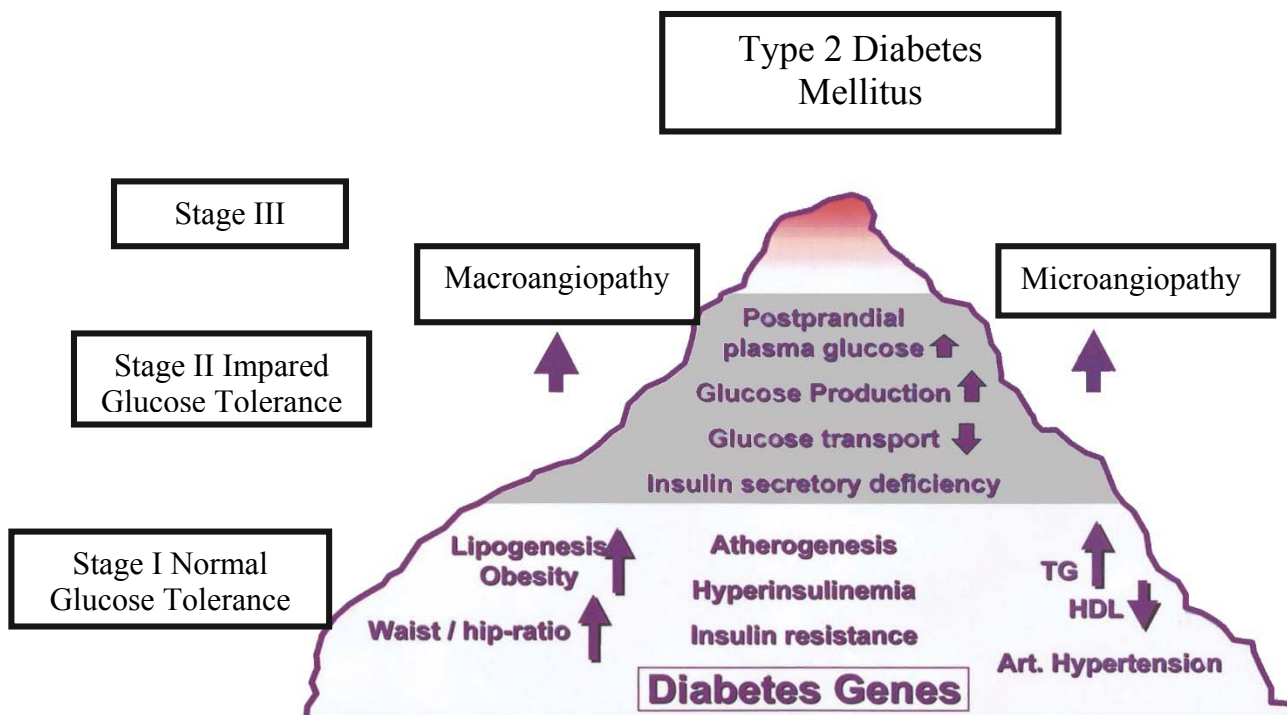
Figure (1-2): multiple risk factors involved in pathogenesis of Type 2 diabetes (Matthaei, et al., 2000)

Persons with Prediabetes and Insulin resistance usually have no symptoms. The person may suffer from one or both of the two conditions for many years without any complaint. Persons suffer from severe forms of insulin resistance may have dark spots on the skin, usually on axilla and the back of neck. Sometimes person have dark ring around the neck. Another possible sites for the dark spots include, knuckles, knees and elbows (Urrutia-Rojas, et al., 2004), (Stoddart, et al., 2002), (Yamazaki, et al., 2003), (Vangipurapu, et al., 2012) .

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┌─────────────────────────────┐
│  Assessment and diagnosis of│
│           T2DM              │
└─────────────────────────────┘
```

┌──────────────────────┐   ┌──────────────────────┐
│ Asymptomatic patient │   │ Symptomatic patient │
└──────────────────────┘   └──────────────────────┘

┌───────────────────────────┐
│ Does the patient have a   │   Yes
│ BMI ≥ 25 kg/m² and one or │
│ more additional risk   │
│ factors?   │
└───────────────────────────┘
              No

┌───────────────────────────┐
│ Does the patient have   │
│ increased cardiovascular │
│ risk?   │
└───────────────────────────┘
        No   Yes

┌───────────────────────────┐   ┌───────────────────────────┐
│ Asymptomatic patients with│   │ Diagnostic testing for   │
│ no risk factors should not│   │ T2DM – HbA1c, OGTT or FPG │
│ be screened for T2DM,   │   └───────────────────────────┘
│ regardless of age   │
└───────────────────────────┘

┌───────────────────────────┐   ┌───────────────────────────┐
│ Diagnosis of prediabetes │   │ Diagnosis of T2DM   │
│ •HbA1c between 5.7-6.4%   │   │ •HbA1c ≥ 6.5% • FPG ≥ 126 │
│ • FPG between 100-125 mg/dL│   │ mg/dL   │
│ • OGTT between 140-199 mg/dL│   │ • Symptomatic and casual │
└───────────────────────────┘   │ plasma glucose ≥ 200 mg/dL│
                                   └───────────────────────────┘

┌───────────────────────────┐   ┌───────────────────────────┐
│ Treatment to prevent or   │   │ Go to the Management   │
│ delay the progression to │   │ algorithm   │
│ T2DM   │   └───────────────────────────┘
└───────────────────────────┘

Figure(1-3): Diagnosis Algorithm Of Type 2 Diabetes Mellitus In Adult **(Redmon, et al., 2014)**

1.1.3.3 *Gestational diabetes mellitus :-*

This type of diabetes is diagnosed during pregnancy and usually disappears after delivery, the accurate cause of gestational diabetes is obscure but scientists think that during pregnancy, lady's body produced greater amount of some hormones such as estrogen, cortisol and placental lactogen. These produced by placenta and their function is to help to sustain the pregnancy. these hormons may interfere with the function of insulin.

With time and as the pregnancy progreses more placental hormones are produced and the amount of insulin secreted becomes insufficent to overcome the action of placental hormones leading to hyperglycemia and the development of gestational diabetes (AL-Dabagh, 2014), (Association, 2015).

A lady has a gestational diabetes if she has one of the following findings (Health, 2015):-

- Fasting blood glucose level is 5.6 mmol/litre (100 mg/dl) or more
- Two hour plasma postprandial glucose level is 7.8 mmol/litre (140mg/dl) or more.

In 2013, 21.4 million or 16.8% of live births worldwide had "some form of hyperglycemia according to the estimation of the international diabetes federation (IFD) (Federation, 2013). about 7% of pregnancies worldwide are diagnosed with gestational diabetes. Also as a result of the high rate of obesity in the world, it's expected that the incidence of gestational diabetes will rise. (Hunt, et al., 2007).

Ladies with Gestational diabetes mellitus (GDM) during the first pregnant have (30-50)% risk of developing GDM in another pregnant (Almario, et al., 2008) (Shah, et al., 2011) New born to women with

GDM will exhibit an increased risk of complications in addition to complications in   mother such as obesity, diabetes mellitus and cardiovascular disease (CVD) (Shah, et al., 2008) (Carr, et al., 2006) (Metzger, et al., 2007) (Damm, 2009)

Women who have had GDM have the single strongest predictor for development of T2DM compared with euglycemic pregnancy (Bellamy, et al., 2009).

Table 1-1): Guidelines for Diabetes prevention and care for women postnatal who suffer from gestational diabetes (O'Reilly 2014)

Guideline Topic	RACGP (Practitioners, 2012)	TG Ltd. (Group, 2014)	ADIPS (Nankervis, et al., 2014)	NICE (Excellence, 2008)	ADA (Association, 2014)
Postnatal screening	6–12 weeks after delivery; OGTT	6–12 weeks after delivery; 75g OGTT	6–12 weeks after delivery; OGTT	6 weeks after delivery; FPG	6–12 weeks after delivery; 75 g OGTT
Repeat screening	3 Yearly	Yearly (Alternatively 75 g OGTT every 2 years or if contemplating further pregnancy)	Yearly; OGTT if contemplating further pregnancy	Yearly	1–3 Yearly; yearly if IFG or IGT, otherwise 3 yearly
Repeat screening test	FPG	FBG or RBG	75 g OGTT or FPG	FPG	75 g OGTT
Lifestyle recommendations	General healthy eating Increase physical activity (30 min brisk walking 5 days a week) and/or weight loss. Encourage breastfeeding	Healthy diet and exercise	Weight control Healthy diet and exercise	Weight control Healthy diet and exercise Encourage breastfeeding	Weight loss 7% body weight Low fat Increase fibre at 14 g/1000 kcal and whole grains Increased PA to 150 min/week moderate activity

IFG = Impaired Fasting Glucose, IGT = Impaired Glucose Tolerance, FPG = Fasting Plasma Glucose, FBG = Fasting Blood Glucose, RBG = Random Blood Glucose, OGTT = Oral Glucose Tolerance Test

Multiple large studies of children born to mothers with GDM had shown that:

- Breastfeeding for 3> months lead to decrease the risk of T2DM by >40%.
- Breastfeeding for ≥3 months delayed T2DM onset by 10 years compared with <3 months breastfeeding (Ziegler, et al., 2012)
- Breastfeeding for >10 months make the glucose tolerance better and increase insulin sensitivity and insulin secretion. (Chouinard-Castonguay, et al., 2013)

1.1.3.4 *Other specific type of diabetes mellitus:-*

Other types of diabetes occur due to various reasons such as:-

A- Genetic defects in insulin action causing diabetes:-

1- Type A insulin resistance.

2- Lipoatrophic diabetes

3- Rabson-Mendenhall syndrome.

4- Leprechaunism. (an extremely rare form of insulin resistance)

B- Genetic defects in Beta cells function (Cleaver, et al., 2005), (Rhodes, et al., 2005), (Kahn, et al., 1998)
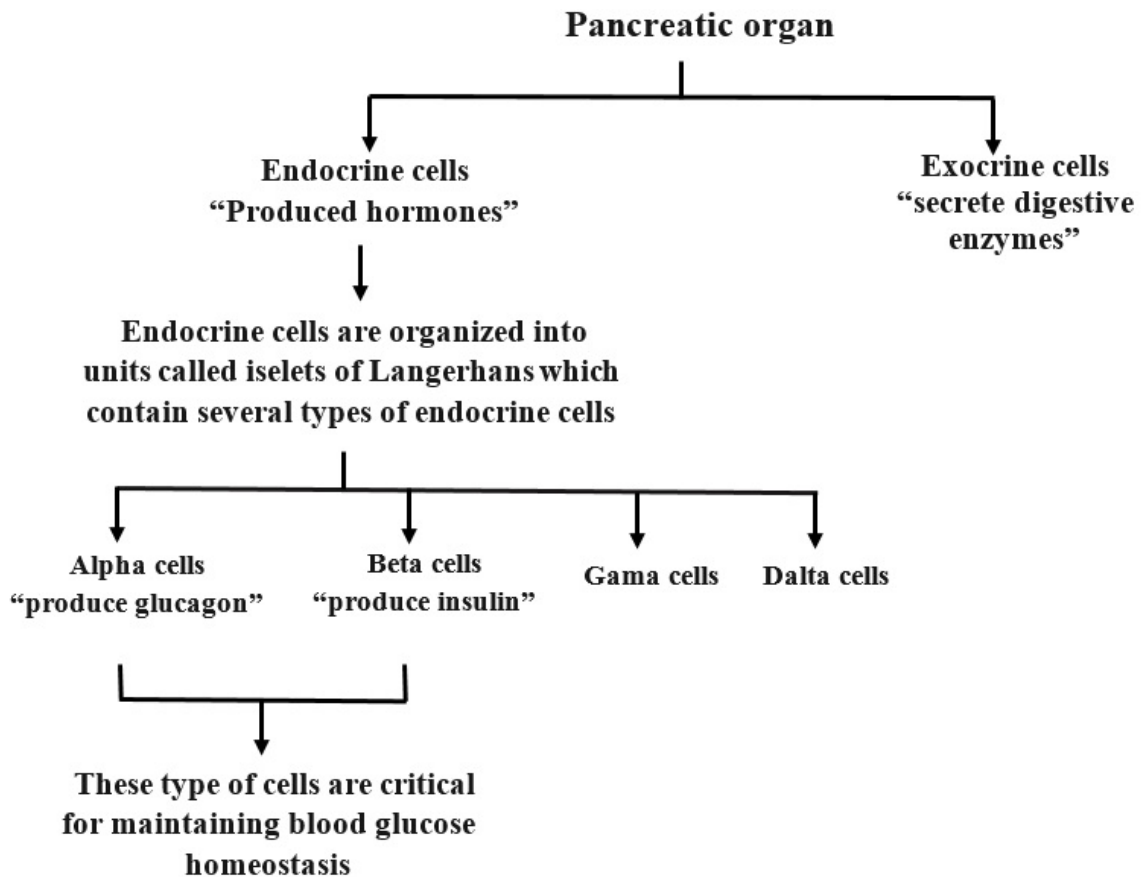
Pancreatic organ

Figure (1-4): Pancreatic organ

Any defect in Beta cell function produces any one of the following type of diabetes:-

1-MODY 1 (Chromosome 20 , HNF-4a)

2- MODY 2 (Chromosome 7 , glucokinase)

3- MODY 3 (Chromosome 12 , HNF-1a)

4-Other rare forms of MODY for example;

a-MODY 4 (Chromosome 13, insulin promoter factor-1)

b- MODY 6 (Chromosome 2, Neuro D1)

c- MODY 7(Chromosome 9,Carboxyl ester lipase)

5-Mitochonderial DNA

C- The exocrine pancreas diseases causing diabetes:-

    1- Cystic fibrosis

    2- Pancreatitis

    3- Fibrocalculous pancreatopathy

    4- Neoplasia

    5- Hemochromatiosis

    6- Trauma / Pancreatectomy

D- Endocrinopathies :-

    1- Aldosteronoma

    2- Pheochromocytoma

    3- Acromegaly

    4- Somatostatinoma

    5- Glucagonoma

    6- Hyperthyroidism

    7- Cushing's syndrome

E- Uncommon forms of immune-mediated diabetes:-

    1- Anti-insulin receptor antibodies

    2- Stiff-man syndrome

F- Other genetic syndrome associated sometimes with diabetes:

    1- Porphyria

    2- Friedreich ataxia

    3- Down syndrome

    4- Prader-Willi syndrome

    5- Turner syndrome

    6- Laurence-Moon-Biedl syndrome

    7- Klinefelter syndrome

    8- Myotonic dystrophy

    9- Wolfram syndrome

    10- Huntington Chorea

G- Infections:-

      1- Cytomegalovirus

      2- Congenital rubella

H- Chemicals or drugs induced:-

      1- Diazoxide

      2- g-Interferon

      3- vacor

      4- Thiazides

      5- Thyroid hormone

      6- Dilantin

      7- Glucocorticoides

      8- Pentamidine

      9- Nicotinic acid

1.2 Tumor marker

1.2.1 Definition:-

The National Cancer Institute defines the tumor marker as a substance released by tumor cells or by another cells of the body indicating the presence of cancer or certain benign condition (Jilani, et al., 2012).

Anything produced by the cell including serum proteins encoded by genes, receptors, enzymes and metabolites can be used as a tumor markers. (Pamies, et al., 1996)

These tumor markers can be found in different tissue specimens or body fluids (Hussein, 2014), It can be measured qualitatively or quantitatively by molecular biological, chemical and immunological methods to look for the neoplasia presence., (Al-Said, 2004)

In cancer patient's specimen (blood or tissue) the quantity of tumor marker are higher than that found in benign tumor or normal individuals specimen, Therefore tumor markers used mainly to distinguish normal tissue from tumor (Nayak, et al., 2010).

Most tumor markers are present with various tumors of the same tissue type this is called "tumor associated markers" while "tumor specific markers " is the name given to few markers are specific for single individual tumor. (Sudbo, 2004)

1.2.2 History of tumor marker: (Gold, et al., 1980) (Manikantan, et al., 2014),Dhanya Balakrishnan, AD Manoj Kumar, Brijesh Shetty 2014), (Minton, et al., 1989), (Ablev, et al., 1963)

- The first attempts to detect tumor markers dates back to more than 2000 years ago when Egyptians tried to distinguish between

mastitis and breast cancer and was mentioned in Egyption papyrus. (Casiato, et al., 1983)

- In modern medicine the first tumor marker was set by Bence-Jones in 1846 by heat precipitate of acidified urine samples of patients with " Mollities ossium" .

- The period (1928-1963) is considered the second stage in the detection of tumor markers, which included the detection of proteins, hormones, enzymes and isoenzymes and it's applications in the diagnosis of tumor as below;

❖ Ectopic hormone syndrome was described by W H Brown in 1928.

❖ Human Chronic Gonadotropin "HCG" was detect by B Zondek in 1930.

❖ Adrenocoticotropic hormone "ACTH" was detect by H Cushing in 1932.

❖ Deletions of blood group antigens by K.oh-Vti in 1949.

❖ Isoenzymes was detected by C Markert in 1959.

❖ Alpha-Feto protein "AFP" was detected by GI Abelv in 1963.

- Carcinoembryogenic antigen (CEA) was detected by S Freeman and P Gold in 1965.

- Oncogenes was detected by G Todaro and R Heubner in 1969.

- In 1975 began the next phase in tumor marker detection. In this phase monoclonal antibodies were recognizwd

- Monoclonal antibodies were detect by G Milsein and H Kohler in 1975.

- Oncogene probes and transfection were invented by M Bishop, G Cooper and R Weinber in 1980.

- Suppressor gene was detected by A Knudson, H Harris and R Sager in 1985.

- In the past few decades, the sensitivity and specificity of presently used tumor markers are significantly improved

1.2.3 The ideal tumor marker concept (Nayak, et al., 2010):-

Ideally tumor marker to be beneficial for final diagnosis of cancer , it needs to have 100% specific and 100% sensitive and should have the following ideal criteria (Malati, 2007)(3TM), (Roulston, et al., 1993) (Schrohl, et al., 2003) (Amayo, et al., 2009):-

1- Be specific for the tumor under study and usually associated with it.
2- Be inexpensive and easy to measure in available fluids of body.
3- Have urine levels, plasma levels or both, that are not subject for wide fluctuations and it should be stable. If it found in healthy individuals plasma, it is present in a lower concentration than that exists in association with all cancer stages.
4- Have stoichiometric relationship between the associated mass of tumor and the marker plasma level.
5- Have an abnormal urine levels, plasma levels or both with the existence of micrometastases.

In addition to the foregoing, Pesce and Kaplan (Kaplan, et al., 1984)have stated that ideal tumor marker must be related to the clinical status and comply with the following:-

1- It must precede and prophesy repetition before the tumor is clinically detectable.
2- It must prognosticate a lower or higher risk for definitive development of repetition.

3- It must change with the current clinical situation of the tumor which usually changes with passage of time.

1.2.4 Classification of tumor markers:-

The tumor markers can be classified by several ways such their biological function, biochemical properties, chemical structure, etc. (Novaković, et al., 1992), (Nayak, et al., 2010), (Rassekh, et al., 1994)

- **In 1993, Scully and Burkhardt classified the tumor markers according to their type of tissue interaction** (Manikantan, et al., 2014)**:**

Table (1-2): Classification of tumor markers

1st classification	2nd classification	Markers of tumor invasion and metastatic potential
Cell surface markers	Another way of classifying tumor markers	
1-Carbohydrates particularly blood group antigens 2-Squamous carcinoma antigens Ca-1, TA-4, SQMI and 3H-1 3-Histocompatibility antigens –HLA class Ⅰ and HLA class Ⅱ 4-Growth factors and receptors. Intracellular markers 1-Cytoskeletal components 2-Cytokeratins Markers of abnormal keratinization-filaggrin, involucrin, desmosomal proteins 3-Carcinoma antigen 17, 13 4-Silver binding nucleolar organizing regions 5-Oncogenes 6-Tumor suppressor genes 7-Arachidonic acid products-PEG2, leukotriene B4 and 5, 12 and 15	Tumor growth markers 1- Epithelial growth factor (EGF) 2-Cyclins 3-Nuclear cell proliferation antigens 4-AgNORs (argyrophilic nucleolar 5-Organizer region) 6-Skp2 (S-ohase kinase-interacting protein 2) 7-HSP 27 and 70 (heat shock proteins) 8-Telomerase Markers of tumor suppression and anti-tumor response 1- Retinoblastoma protein (pRb) 2- Cyclin dependent kinase inhibitors 3- P53 4- Bax 5- Fas/FasL Angiogenesis markers 1- VEGF/VEGF-R (vascular endothelial growth factor/rece	1- MMPs (matrix metalloproteinases 2- Cathepsins 3- Cadherins and catenins 4- Desmoplakin Cell surface markers 1- Carbohydrates 2- Histocompatibility antigen (HLA) 3- CD57 antigen Intracellular markers Cytokeratins Markers of anomalous keratinization 1- Filaggrins 2- Involucrin 3- Desmosomal proteins 4- Intercellular substance antigen 5- Nuclear analysis Arachidonic acid products 1- Prostaglandin E2 2- Hydroxyeicosatetraenoic acid 3-Leukotriene B4 Enzymes

hydroxyeicosatetraenoic acids 8-Enzymes-gamma-glutamyl transpeptidase-LDH 9-Basement membrane markers-fibronectin, laminin 10-Matrix markers-tenascin	2- PD-ECGF (platelet-derived endothelial cell growth factor) 3- FGFs (fibroblast growth factor)	Glutathione S-transferase

- **Classification of tumor markers in another way as follow:-**

  A- Mensenchymal markers

  1. Vasculator antigen

     - CD 31

     - CD 34

  2. Muscle antigens

     - Myoglobin

     - Desmin

     - Myosin

     - Actin

  3. Neural antigens

     - Synaptophysin

     - Neuron specific enolase (NSE)

     - Never growth factor receptor

     - S 100

     - Glial fibrillary acidic protein (GFAP)

  B- Biochemical markers

  1- Protein

     - Beta protein

     - Ferritin

     - Immunoglobulins

     - Glycoprotein

2- Enzymes and isoenzymes

- Placental alkaline phosphatase (PALP)
- Prostatic acid phosphatase (PAP)
- Lysozyme
- Prostate specific antigen (PSA)

C- Epithetical markers

1- Desmoplakin

2- Epithetical membrane antigen (EMA)

3- Cytokeratins (CK)

4- Oncofetal antigens

- Carcinoembryonic antigen (CEA)
- Alph-fetoprotein (AFP)

D- Hormone receptors

1. Pogesterone receptor (PR)

2. Estrogen receptor (ER)

E- Prognostic markers

1- Cell adhesion molecules

- Selectins
- Cadherins
- Intearins

2- Proliferation markers

- Ki67
- AgNORs
- PCNA

F- Epithelial markers

1.2.5 **Tumor marker applications :- (Gold, et al., 1980)**

Theoretically, there is numerous possibilities of tumor markers applications in oncology field . But the uses depend on the specificity and sensitivity of marker and depend also on the reliability of other methods that used for the same purpose (Novaković, 2004). various markers are utilised for various purposes, some markers are   more suitable for follow up of diseases while other markers are suitable for early detection of the illness recurrence. (Oncology, 1996), (Bast, et al., 2001)

Sell and Chan give summary of potential tumor marker uses as below (Chan, et al., 1994):-

1- In symptomatic patients it used for differential diagnosis.
2- In general it is used for screening.
3- Used for estimating tumor volume.
4- Clinical staging of cancers.
5- Evaluation the treatment success.
6- Prognostic index for disease progression.
7- Monitoring the responses for therapy.
8- For detecting the recurrences of disease.
9- Determine the usefullness of immunotherapy.
10- It is used in Radioimmunolocalization of masses tumor.

Table 1.3): contain the common tumor marker applications (A. A. AMAYO and J. G. KURIA 2009)

Marker	Reference Range	Main malignancy and uses	Other malignancies with raised value	Non neoplastic causes of raised level
CEA	Nonsmoke: 0-3.5ug/L Smokers: 0-5ug/L	Colorectal carcinoma P, TM, R	Other gastrointestinal malignancies, breast and ovarian carcinoma	Hepatitis, cirrhosis, obstruction, biliary, Crohn's disease, bronchitis
AFP	0 – 10U/L	Hepatocellular carcinoma (S, D,TM) Hepatoblastoma (D) Non-semitomatous germ cell tumours (P,TM)	Not recommended for other tumors	Hepatitis, cirrhosis, biliary obstruction
PSA	0-4 ug/L	Prostate cancer (S, D, P, TM)	Not recommended for other tumors	Benign prostate hyperplasia, prostatitis
CA 125	0-35U/L	Ovarian cancer	Not recommended, in males or for non-ovarian cancers	Endometriosis, peritonitis, ascites, pelvic inflammatory Disease
CA 19-9	0-37U/L	Pancreatic cancer	Gastric,colorectal and cholangiocarcinoma	Pancreatitis, cholangitis, hepatitis, cirrhosis.

P=Prognosis, D=Diagnosis, TM=Treatment monitoring, S=Screening

1.2.6 Conditions Associated with the Elevation of Tumor Marker Levels (Greg, et al., 2003):-

Table 1.4): Conditions Associated with the Elevation of Tumor Marker Levels

Tumor marker	Normal value	Primary tumor(s)	Additional associated malignancies	Benign conditions
CA 27.29	< 38 units per mL	Breast cancer	Colon, gastric, hepatic, lung, pancreatic, ovarian, and prostate cancers	Breast, liver, and kidney disorders ovarian cysts
CEA	nonsmokers < 2.5 ng per mL in smokers <5 ng per mL	Colorectal cancer	Breast, lung, gastric, pancreatic, bladder, medullary thyroid, head and neck, cervical, and hepatic cancers, lymphoma, melanoma	Cigarette smoking, peptic ulcer disease, inflammatory bowel disease, pancreatitis, hypothyroidism, cirrhosis, biliary obstruction
CA 19-9	< 37 units per mL	Pancreatic cancer, biliary tract cancers	Colon, esophageal, and hepatic cancers	Pancreatitis, biliary disease, cirrhosis
AFP	< 5.4 ng per mL	Hepatocellular carcinoma nonseminomatous germ cell tumors	Gastric, biliary, and pancreatic cancers	Cirrhosis, viral hepatitis, pregnancy
B-hCG	<5 mIU per mL	Nonseminomatous germ cell tumors, gestational trophoblastic disease	Rarely, gastrointestinal cancers	Hypogonadal states, marijuana use
CA 125	< 35 units per mL	Ovarian cancer	Endometrial, fallopian tube, breast, lung, esophageal, gastric, hepatic, and pancreatic cancers	Menstruation, pregnancy, fibroids, ovarian cysts, pelvic inflammation, cirrhosis, ascites, pleural and pericardial effusions, endometriosis
PSA	<4 ng per mL for screening	Prostate cancer	None	Prostatitis, benign prostatic hypertrophy,

	Undetectab le level after radical prostatecto my			prostatic trauma, after ejaculation

1.2.7 Limitations:-

The tests of tumor markers provide a great useful information but unfortunately, there are some limitations such as:-

- Not all cancers have a tumor marker specific to it.
- Several tumor markers are special for certain types of cancer .
- Level of some tumor markers may be elevated in persons suffering from illnesses other than cancer.
- Not all person with a certain kind of cancer will have high level of the tumor corresponding marker.
- Therefore, tumor marker alone is not enough to diagnose some kinds of cancer but they give additional information which can considered in conjunction with the physical exam and medical history of the patient as well as imaging teste and/ or other laboratory tests.

The following figure summarizes some of prevalent factors which affect on the concentration of tumor marker in serum:- (Novaković, 2004)

insufficient expression of a certain antigenic determinant or production in only some of tumor cells

Insufficient blood circulation in the tumor

False negative result

Completly absence of production e.g. CA 19-9 for Le(a-b-) person

Production of immune complexes with autoantibodies

Rapid degradation and clearance of antigens

As a consequence of different physiological conditions (Pregnancy - sHCG, CA 125, CA 15-3, MCA, AFP, menstrual cycle - CA 125)

As a consequence of diagnostic and therapeutic procedures (digitorectal examination, mamography, surgery, radio and chemotherapy)

False positive results

Disturbancies of renal function (beta-2 microglobulin, calcitonin, PSA, CEA, CA 19-9, CA 15-3)

Benign liver diseases and consequential disturbancies in metabolism and excretion (AFP, TPA, CEA, CA 19-9, CA 15-3)

Presence of inflammatory processes

Extensive tumor necrosis

Figure (1-5): some of prevalent factors which affect the concentration of tumor marker in serum

1.3 Carbohydrate Antigen CA 19-9

1.3.1 Definition of CA19-9:-

CA 19-9 is a type of Tumor Associated Antigens "TAA", molecularly defined as Cytokeratins, glycoproteins, carbohydrate antigens and mucins. This marker has a molecular weight of 210 KD with a half-life of (4-8) days, correlated with glycoprotein antigen described as carbohydrate determinant on glycoprotein and glycolipid CA19-9 antigen defined by Koprowski and Colleagues in 1979 as "IgG1 mouse monoclonal antibody raised against the human colonic carcinoma cell line SW 1116". This monoclonal antibody reacts with carbohydrate antigenic (CA19-9) which is defined as sialylated lacto–Nfucopentaose II an oligosaccharide sharing the structural characteristics with substances of Lewis blood group. The antigen localized immunohistologically on fetus epithelium of colon, liver, stomach, small intestine and pancreas and very small concentration in adult lung tissue and gastrointestinal tract. Tangible concentrations of CA19-9 are also found in ovarian cyst fluid, duodenal secretions, mucin rich saliva, urine, seminal fluid, amniotic fluid, gall bladder, pancreatic and gastric juice.

In 99.6 % of healthy adult, serum level of CA 19-9 is lower than 37 U per ml. while if the serum level is less than 100 U/ml it is considered as value in benign and malignant diseases which may overlap. In malignant tumors the value of CA 19-9 may be higher than 100,000 U/ml. CA19-9 is neither organ specific nor tumor specific. However, the diagnostic specificity (95%) and sensitivity (85%) of CA-19-9 are the highest for the pancreatic adenocarcinoma. seventy percent is the Sensitivity that observed in gallbladder carcinomas and cholangiocarcinomas. while the sensitivity for mucinous ovarian, stomach, colorectal, uterus, primary liver, bronchial, and mammary

carcinoma was very low, therefore the CA19-9 marker used mainly for pancreatic adenocarcinoma. (Eskelinen, et al., 1999), (Rückert, et al., 2010), (Harmenberg, et al., 1988), (Magnani, et al., 1983), (Malati, et al., 1996), (Shukla, et al., 2006), (Sturgeon, et al., 2009) , (Duffy, et al., 2000).

Figure 1-6): Comparison of cyst fluid CA19 9 levels in patients with benign and
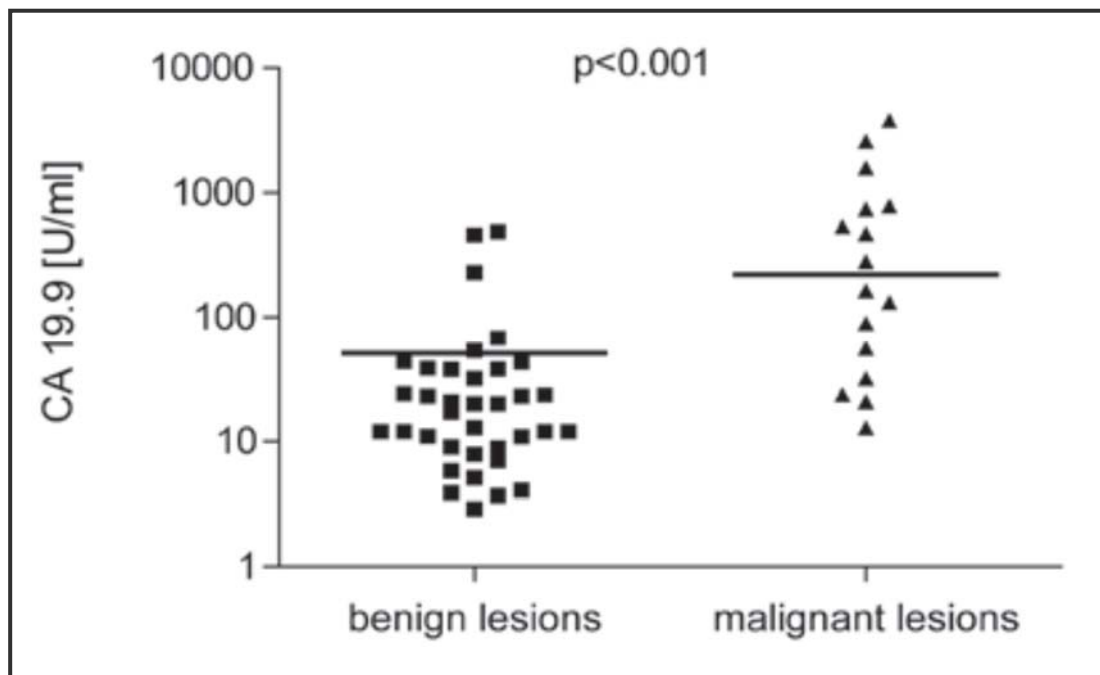
malignant pancreatic lesions (results presented in logarithmic scale)
(Talar-Wojnarowska, et al., 2013)

1.3.2 Application of CA19-9 :-

CA19-9 has been widely utilized for diagnosis of different kinds of cancer e.g. pancreatic cancer, cancer of upper gastrointestinal tract, hepatocellular cancer, liver cancer, colorectal cancer, ovarian cancer and even urothelial cancers in addition to inflammatory conditions of hepatobiliary system (such the rising of this marker in benign jaundice), thyroid diseases, (Zheng, et al., 2013), (Uygur-Bayramiçli, et al., 2007) , (ESTEGHAMATI, et al., 2014), pancreaticobilliary disorders and bronchioalveolar pathologies (Perkins, et al., 2003), (Kim, et al., 2009) , (Giannini, et al., 2000), (Mann, et al., 2000), (Korkmaz, et al., 2010),

(Ventrucci, et al., 2009) , (Howaizi, et al., 2003) with a 37 U/mL as the upper limit of normal CA19-9 serum concentration. (Lin, et al., 2014)

Cancer antigen CA 19-9 is the most beneficial serologic marker to diagnose the pancreatic carcinoma, and it's important for detection of frequent disease and follow up of patients after surgery .This marker is a dependable diagnostic test when it's evaluated by CT, US or endoscopic US. (DelMaschio, et al., 1991), (Tomazic, et al., 2000), (Safi, et al., 1998), (Yeh, et al., 1998), (Pleskow, et al., 1989), (Cerwenka, et al., 1997), (GrzegorzC´ wik, et al., 2006), (Wu, et al., 2007), (Aljebreen, et al., 2007), (Wu, et al., 2007).

It is presently one of the most beneficial blood test to differentiate between pancreatic cancer and recurring or chronic pancreatitis with a specificity ranges from 68–91% and a sensitivity from 70–90 % (Audisio, et al., 1996), (Aoki, et al., 2006), (Okusaka, et al., 1996), (Tanaka, et al., 2006).

False positive elevation in CA 19-9 serum level has been observed in a variety of pathological conditions, most notably in the presence of obstructive jaundice. As such, CA 19-9 serum levels can be used to differentiate benign from malignant pancreatic diseases.

Table (1.5): False positive elevation of CA 19-9 serum level has been noted in variety of pathological condition, most notably in presence of obstructive jaundice **(Ballehaninna, et al., 2012)**

Organ/system	Pathologic condition	CA 19-9 range (U/mL)
Pancreatic diseases (Steinberg, 1990), (Bedi, et al., 2009) , (Ulla Rocha, et al., 2007)	Acute pancreatitis Chronic pancreatitis Pancreatic abscess Pseudo-pancreatic cyst	3-22
Hepato-biliary diseases (Kim, et al., 1999), (Steinberg, 1990) , (Paganuzzi, et al., 1988), (Marcouizos, et al., 2009)	Cholangio-carcinoma Cholangitis Choledocholithiasis Cholelithiasis Cirrhosis of liver Hepatitis Hepatocellular carcinoma Liver cyst Liver abscess Polycystic liver disease	50-99000
GI malignancies (Duffy, et al., 2010), (Kim, et al., 2004), (Satake, et al., 1994), (Chang, et al., 2006), (Tessler, et al., 2006)	Colorectal cancer Esophageal cancer Gastric cancer	37-100
Miscellaneous (Kim, et al., 2009), (Ventrucci, et al., 2009)	Bronchitis Congestive heart failure Cystic fibrosis Diverticulitis Hashimoto's thyroiditis Lung cancer Ovarian cyst Pleural effusion Renal cyst Rheumatoid arthritis	112-1338

1.3.3 CA19-9 and diabetes mellitus

In addition to their use in the diagnosis of pancreatic cancer it is also used as an indicator of pancreatic tissue damage that may be caused by diabetes (Uygur-Bayramiçli, et al., 2007).

The relationship between diabetes mellitus and pancreatic cancer still controversial, a Meta-analysis was conducted on 20 studies and reached to two-fold increase peril of pancreatic cancer in patients with diabetes for 5 years duration, Which indicates that diabetes mellitus is one of risk factor for the tumor Incidence (Rosewicz, et al., 1997)While other studies concluded that the pancreatic cancer precedes and caused diabetes mellitus (Everhart, et al., 1995). There are even other studies which suggest that diabetes protects against this type of cancer (Gullo, 1999).So, the elevation of level of CA19-9 in patients with diabetes mellitus makes it imperative to find normal range for CA 19-9 level in patients with type 2 diabetes to eliminate on the additional interventional approaches (Uygur-Bayramiçli, et al., 2007).

Diabetes even was depicted as the last stage of the chronic pancreatitis with new developing concept of pathogenesis (Göke, et al., 2005) and the Chronic pancreatitis is the risk factor for threatening pancreatic cancer (Lowenfels, et al., 2005)Therefore chronic pancreatitis and pancreatic cancer associated with diabetes are recorded to be accompanied with a high levels of CA19-9, In these case, the association of the presenting diabetes with CA19-9 is clear (Mann, et al., 2000), (Guo, et al., 2010) precedent reports have indicated higher levels of CA19-9 in diabetic cases despite the lack of malignant conditions (Kim, et al., 2009) (Ventrucci, et al., 2009), but with poor glycemic control and deprived metabolic compensation (Nakamura, et al., 1986), (Shimojo, et al., 1990), (ESTEGHAMATI, et al., 2014)

So, investigating the rising of CA19-9 level and the corresponding diabetic status is crucial to detect a more accurate interpretation.

Benhamou et al studied the association between CA19-9 and pancreatic cancer and investigated the relationship between metabolic control of diabetic patients and CA 19-9 in 51 adults. From that they concluded that CA19-9 in diabetic patients is elevated in acute metabolic conditions, which is correlated very well with the concentration of blood glucose (Benhamou, et al., 1991).
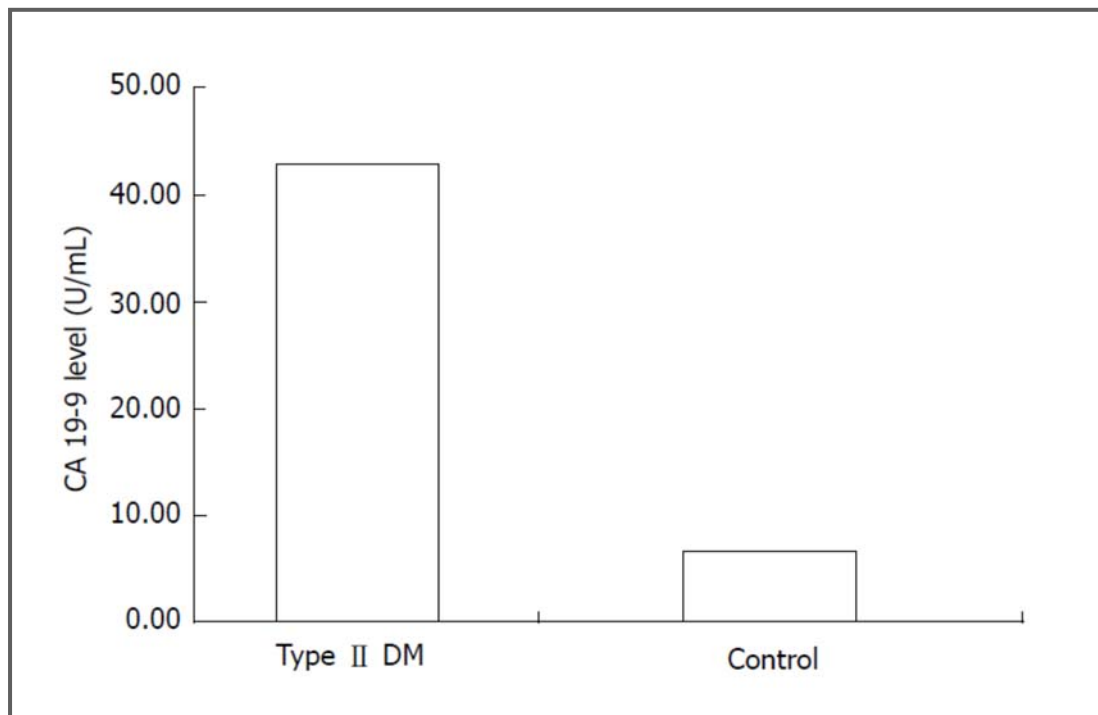
Figure (1-7): Mean CA 19-9 levels in Type 2 DM and Control group
(Uygur-Bayramiçli, et al., 2007).

THE AIM OF THE STUDY

1. Determination of CA19-9 levels in sera of patients with type 2 diabetes mellitus by enzyme-linked immunosorbent assays (ELISA).

2. Define the normal range (cutoff value) of CA 19-9 in type 2 diabetic patients.

3. Investigate the possible relationships of CA19-9 with fasting plasma glucose, HbA1c.

4. Present a new method for the determination of a dissociation constant (Kd) of the binding of CA19-9 to its antibody in type 2 diabetic patients using Scatchard plot through development of ELISA (competitive sandwich ELISA technique).

5. Study the effects of different chemicals [divalent cations, different halides, and Polyethylene (PEG-6000)] on the binding of CA19-9 to its antibody in type 2 diabetic patients.

Subjects, Materials and Methods

2.1 Subjects and Study protocol:-

The study was executed during the term from February 2015 to May 2015. The study included 80 patients (34 male and 46 female) with type II diabetes mellitus have a standard error of mean age (mean ± SE) 46.562 ± 1.105 years, The patients were divided into two groups:-

Group I :-Forty patients with type 2 diabetes mellitus have a standard error of mean duration (mean ± SE) 6.6± 0.9421 years and take medication.

Group II:- Forty patients were newly diagnosed with type 2 diabetes mellitus and don't take medication.

Forty individuals were classified as control group (19 male and 21 female) with a standard error of mean age 44.7 ± 1.454 years. All patients volunteered from the Center of Diabetes and outpatient diabetes clinic of Al-Imamain Al-Kadhimain Medical City.

The exclusion criteria were represented as follow:-

♦ Patients suffered from cancer of the upper gastrointestinal tract, pancreatic, ovarian, colorectal cancer and any other malignancy.
♦ Patients suffering from pancreatic, thyroid, liver, and renal diseases (S Pavai,MD,S FYap,FRCPath, 2003).
♦ Pregnant women.
♦ Smokers.

In this study, there were no statistical significant difference in age between groups ($p > 0.05$).The standard error of mean age (Mean ±SE) of patients with type 2 diabetes mellitus who take medication (group Ⅰ) was 48.15 ± 1.731 years, group Ⅱ or patients newly diagnosis with type 2 diabetes mellitus who don't take medication was 44.975 ± 1.348 years, while in control group (group Ⅲ) was 44.7 ± 1.454 years as shown in table (2.1).

Table (2-1): The host information of type 2 diabetic patients and healthy subjects studied.

Characteristics	Control	Group (Ⅰ)	Group (Ⅱ)
Number	40	40	40
Male/Female	19/21	18/22	16/24
Age (year)	44.75 ± 1.45	48.25 ± 1.72	44.975 ± 1.35
Duration (year)	—	6.6± 0.942	—
BMI (Kg/m^2)	27.53 ± 0.323	31.051 ± 0.993	33.915 ± 1.066
FBS (mg/dl)	98.23 ± 1.35	223.83± 16.27	171.65 ± 9.91
HbA1c (%)	5.86± 0.078	9.4 ± 0.34	8.60 ± 0.316

2.2 Collection and preparation of Blood samples:-

1. Seven milliliters of blood was obtained from each fasting subject (12 hour fast).
2. Each blood samples divided into two parts
   a. Part one; two milliliter was collected into EDTA containing tube and was sent to an external laboratory for measuring Glycated hemoglobin (HbA1c) using NycoCard reader instrument.

  b. Part two; the blood sample was left for 20 minutes into plane tube at room temperature. After coagulation, sera were separated by centrifugation at 3000 rpm for 10 min.

3. serum was separated and divided into small amounts in eppendorf tubes for:-

  a. Immediate measurement of serum glucose (FBS), lipid profile (total cholesterol, Triglyceride c-TG and High Density Lipoprotein c-HDL), serum urea and serum creatinine. This was done using appropriate enzymatic colorimetric method (Chemistry Analyzer) in addition to C - reactive protein which done using NycoCard reader instrument.

  b. The rest was stored at -20°C until assayed for CA19-9. This was measured using enzyme-linked immunosorbent assay (ELISA) kits, determine the antigen affinity and the effect of some chemicals on the antigen affinity.

2.3   Materials:

The following sections include the instruments and chemicals used in this study:-

2.3.1 Instruments:-

Table (2.2) The instruments and their companies

No.	Instrument	Company
1	Centrifuge	Hettich EBA 20, DJBC2002, UK
2	Centrifuge eppendrof	Germany
3	Adjustable micro pipettes SLAMED (100\1000 µl) , (5\50 µl)	Germany
4	NycoCard reader	Alere, Norway
5	Clinical Chemistry Analyzer	Abbott diagnostics, USA
6	Refrigerator	Germany
7	Vortex	Scientific Industries, Inc. U.S.A
8	(UV/Vis) Spectrophotometer	Biotech Engineering Management Co. Ltd.[UK]
9	pH-meter	HANNA pH/ORP meter, U.S.A
10	ELISA reader and washer	Bio-teck ELx800, U.S.A
11	Incubator (20-25)°C	Germany

2.3.2 Chemicals:-

The Chemicals and biochemical kits used in this study are listed below:-

Table (2.3): List of chemicals and their companies

No.	Chemicals	Companies
1	Sodium chloride NaCl	EDUTECH, India
2	Monobasic sodium phosphate NaH_2PO_4	Qualikems, India
3	Sodium hydroxide NaOH	Panreac, spain
4	Tris Hydroxymethyl aminomethane $C_4H_{11}NO_3$	SCRC, China
5	Hydrochloric acid HCL	Central Drag House, India
6	Coomasie Blue (G250)	Himedia, India
7	Ethanol 95%	Central Drag House, India
8	Phosphoric acid 85%	GCC, UK
9	Bovine serum albumin	Himedia, India
10	Copper (II) Chloride $CuCl_2$	FLUKA, Germany
11	Calcium Chloride $CaCl_2$	Panreac, spain
12	Magnesium Chloride $MgCl_2$	CARLO ERBA, Italy
13	Manganese Chloride $MnCl_2$	GCC, UK
14	Zinc chloride $ZnCl_2$.	Qualikems, India
15	Sodium Fluoride NaF	Riedel-de Haën, Germany
16	Sodium Iodide NaI	Thomas Baker, India
17	Polyethylene Glycol PEG 6000	BDH, England

Table (2-4): List of Biochemical kits and their companies

No.	Biochemical kits	Company
1	Glucose kit	ABBOTT, Germany
2	HbA1c kit	NycoCard, Alere, Norway
3	CRP kit	NycoCard, Alere, Norway
4	Total Cholesterol kit	ABBOTT, Germany
5	High Density Lipoprotein kit	ABBOTT, Germany
6	Triglyceride kit	ABBOTT, Germany
7	Urea kit	ABBOTT, Germany
8	Creatinine Kit	ABBOTT, Germany
9	CA19-9 Antigen ELISA kit	Human, Germany
10	Anti-CA19-9 antibody	Catalog#MBS850288,MyBioSource, U.S.A.

2.4 Measurement of Body Mass Index (BMI):

Body Mass Index is a simple index of weight-for-height that is ordinarily used to distinguish underweight, normal, overweight and obesity. It is calculated by dividing the weight in kilograms on the height square in meters (kg/m^2) according to the following equation:-

$$\textbf{BMI = Weight (Kg) /(Height)}^2 \textbf{ (m}^2\textbf{)}$$

BMI was categorized into the following groups: (WHO, 2015)

Table (2-5): BMI classification according to World Health Organization 2015

Classification	BMI(kg/m2)	
	Principal cut-off points	**Additional cut-off points**
Underweight	<18.50	<18.50
Severe thinness	<16.00	<16.00
Moderate thinness	16.00 - 16.99	16.00 - 16.99
Mild thinness	17.00 - 18.49	17.00 - 18.49
Normal range	18.50 - 24.99	18.50 - 22.99
		23.00 - 24.99
Overweight	≥25.00	≥25.00
Pre-obese	25.00 - 29.99	25.00 - 27.49
		27.50 - 29.99
Obese	≥30.00	≥30.00
Obese class I	30.00 - 34.99	30.00 - 32.49
		32.50 - 34.99
Obese class II	35.00 - 39.99	35.00 - 37.49
		37.50 - 39.99
Obese class III	≥40.00	≥40.00

2.5 Methods:-

2.5.1 Determination of Serum Glucose:-

a- Principle of procedure (Burtis CA and Ashwood ER, 1994) (World Health Organization, 2004)**:-**

Glucose is phosphorylated by hexokinase (HK) in the presence of adenosine triphosphate (ATP) and magnesium ions to produce glucose-6-phosphate (G-6-P) and adenosine diphosphate (ADP).

Glucose-6-phosphate dehydrogenase (G-6-PDH) specifically oxidizes G-6-P to 6-phosphogluconate with the concurrent reduction of nicotinamide adenine dinucleotide (NAD) to nicotinamide adenine dinucleotide reduced (NADH). One micromole of NADH is produced for each micromole of glucose consumed.

$$\text{Glucose + ATP} \xrightarrow[\text{Mg}^{+2}]{\text{Hexokinase}} \text{G-6-P + ADP}$$

$$\text{G-6-P + NAD} \xrightarrow{\text{G-6-PDH}} \text{6-phosphogluconate + NADH}$$
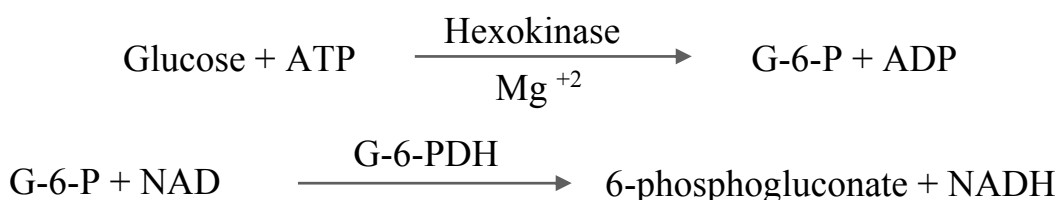
The NADH produced absorbs light at 340 nm and can be detected spectrophotometrically as an increased absorbance.

b- Reference Range (Burtis CA and Ashwood ER, 1994)**:-**

Table 2-6): Reference range of Fasting Serum/Plasma Glucose

Fasting	Range (mg/dl)	Range (mmol/L)*
Cord	45 – 96	2.50 – 5.33
Premature	20 – 60	1.11 – 3.33
Neonate	30 – 60	1.67 – 3.33
Newborn, 1 day	40 – 60	2.22 – 3.33
Newborn, > 1 day	50 – 80	2.78 – 4.44
Child	60 – 100	3.33 – 5.55

Adult	70 – 105	3.89 – 5.83
>60 years	80 – 115	4.44 – 6.38
>70 years	83 – 110	4.61 – 6.10

*To convert results from mg/dl to mmol/L multiply mg/dl by 0.0555.

2.5.2 Determination of Glycated hemoglobin (HbA1c) (Lenzi S et al., 1987), (Sacks BD etal., 2011):-

a. Principle of the assay :-

NycoCard HbA1c is a boronate affinity assay. The kit contains test devices with a porous membrane filter, test tubes prefilled with reagent and a washing solution. The reagent contains agents that lyse erythrocytes and precipitate hemoglobin specifically, as well as a blue boronic acid conjugate that binds cis-diols of glycated hemoglobin. When blood is added to the reagent, the erythrocytes immediately lyse. All hemoglobin precipitates. The boronic acid conjugate binds to the cis-diol configuration of glycated hemoglobin. An aliquot of the reaction mixture is added to the test device, and all the precipitated hemoglobin, conjugate-bound and unbound, remains on top of the filter. Any excess of colored conjugate is removed with the washing solution. The precipitate is evaluated by measuring the blue (glycated hemoglobin) and the red (total hemoglobin) colour intensity with the Nycocard READER Ⅱ, the ratio between them being proportional to the percentage of HbA1c in the sample.

b. Test procedure :-

Five microliters of blood was added to the test tube with R1/Reagent, mixed well, and the tube was left for minimum 2 minutes, maximum 3 minutes (using a timer).

The test tube was remixed to obtain a homogenous suspension, and 25 µl of the mixture was applied to a TD/Test Device. The mixture

was allowed to soak completely into membrane (waiting for minimum 10 Seconds).

1. Twenty five microliters of R2/Washing Solution was applied to the TD/Test Device. The reagent was allowed to soak completely into the membrane (waiting for minimum 10 Seconds).

2. The test result was read using the NycoCard READER Ⅱ.

c- Reference Range (The International Expert Committee, 2009) (American Diabetes Association, 2011)**:-**

The upper limit of non-diabetic reference range is 6.4% HbA1c.

2.5.3 Determination of C-reactive protein (Kushner I and Rzewnicki DL., 1994), (Pepys MB. and Hirschfield GM., 2003):-

C-reactive protein (CRP) is one of the cytokine induced acute-phase proteins, the levels of which rise during a general, unspecific response to infections and non-infections inflammatory processes. In healthy persons the serum or plasma CRP levels are below 5 mg/l. This threshold is often exceeded within four to eight hours after an acute inflammatory event, with CRP values reaching approximately 20 to 500mg/l. As elevated CRP levels are always associated with pathological changes, the CRP assay provides information for the diagnosis, therapy and monitoring of inflammatory diseases.

a. Test principle:-

NycoCard CRP single test is a solid phase, Sandwich-Format, immunometric assay. In the test well of the devise there is a membrane coated with immobilised CRP-specific monoclonal antibodies. A diluted sample is applied to the test device. When the sample flows through the membrane, the C-reactive proteins are captured by the antibodies. CRP trapped on the membrane will then bind the gold- antibody conjugated

added, in a sandwich-type reaction. Unbound conjugate is removed from the membrane by the washing solution.

A paper layer underneath the membrane absorbs excess liquid. In the presence of a pathological level of CRP in the sample, the membrane appears red-brown with color intensity proportional to the CRP concentration of the sample. The color intensity is measured quantitatively with the NycoCard™ READER Ⅱ.

b. Test procedure:-
   1. Five microliters of patient sample was added to the tube with R1/Dilution Liquid. The tube was closed and mixed well for 10 seconds.
   2. Fifty microliters of diluted sample was applied to the TD/Test Device. The sample was allowed to soak into the membrane (waiting for 30 second).
   3. One drop R2/Conjugate was applied to the TD/Test Device. The reagent was allowed to soak into the membrane (waiting for 30 second).
   4. One drop R3/Washing solution was applied to the TD/Test Device. The reagent was allowed to soak into the membrane (waiting for 20 second).
   5. Read the result using NycoCard™ READER Ⅱ.
   c- **Reference Range** (Dati F., 2001)**:-** < 5 mg/L

2.5.4 Determination of serum lipid profile:-

2.5.4.1 Determination of serum total Cholesterol (Flegg HM., 1973), (Richmond W., 1973), (Allain CC, 1974), (Roeschlau P, 1974)**:-**
   a- **Principle of procedure:-**

The use of enzymes to assay cholesterol has been studied by many investigators. This reagent is based on the formulation of Allain, et al. and the modification of Roeschlau with further improvements to render the reagent stable in solution.

Cholesterol esters are enzymatically hydrolyzed by cholesterol esterase to cholesterol and free fatty acids. Free cholesterol, including that originally present, is then oxidized by cholesterol oxidase to cholest-4-ene-one and hydrogen peroxide. The hydrogen peroxide combines with hydroxybenzoic acid (HBA) and 4-aminoantipyrine to form a chromophore (quinoneimine dye) which is quantitated at 500 nm.

b- Reference Range (American Academy of Pediatrics, 1998)**,** (The National Cholesterol Education Program (NCEP), 2001)**:-**

Table 2-7): Reference range of Serum/Plasma total Cholesterol

Age	Diagnosis	Range (mg/dl)	Range(mmol/L)*
Adult	Desirable	< 200	< 5.18
	Borderline	$200 - 239$	$5.18 - 6.19$
	High	≥ 240	≥ 6.22

*To convert results from mg/dl to mmol/L multiply mg/dl by 0.0259

2.5.4.2 **Determination of serum Triglyceride (T.G.)** (Fossati P, 1982), (McGowan MW, 1983):-

a- Principle of procedure:-

Triglycerides are enzymatically hydrolyzed by lipase to free fatty acids and glycerol. The glycerol is phosphorylated by adenosine triphosphate (ATP) with glycerol Kinase (GK) to produce glycerol-3-phosphate and adenosine diphosphate (ADP). Glycerol-3-phosphate is oxidized to dihydroxyacetone phosphate (DAP) by glycerol phosphate oxidase (GPO) producing hydrogen peroxide (H_2O_2). In a color reaction

catalyzed by peroxidase, the H_2O_2 reacts with 4-aminoantipyrine (4-AAP) and 4-chlorophenol (4-CP) to produce a red colored dye. The absorbance of this dye is proportional to the concentration of triglyceride present in the sample.

b- Reference range ((NCEP), 2001)**:-**

Table 2-8): Reference Range of Serum/ Plasma of Triglyceride

Diagnosis	Range (mg/dL)	Range (mmol/L)
Normal	< 150	< 1.70
Borderline High	150 to 199	1.70 to 2.25
High	200 to 499	2.26 to 5.64
Very High	≥ 500	≥ 5.65

2.5.4.3 **Determination of serum High Density Lipoprotein cholesterol (c-HDL)** (World Health Organization, 2004):-

a- Principle of procedure:-

The Ultra HDL assay is a homogeneous method for directly measuring HDL cholesterol concentration in serum or plasma without the need for off-line pretreatment or centrifugation steps.

The method uses a two-reagent format and depends on the properties of a unique detergent. This method is based on accelerating the reaction of cholesterol oxidase (CO) with non-HDL unesterified cholesterol and dissolving HDL cholesterol selectively using a specific detergent. In the first reagent, non-HDL unesterified cholesterol is subject to an enzyme reaction and the peroxide generated is consumed by a peroxidase reaction with DSBmT yielding a colorless product. The second reagent consists of a detergent (capable of solubilizing HDL

cholesterol), cholesterol esterase (CE), and chromogenic couple to develop color for the quantitative determination of HDL cholesterol.

b.Reference Range ((NCEP), 2001)**:-**

Table 2-9): Reference Range of Serum/Plasma High Density Lipoprotein cholesterol (c-HDL)

Diagnosis	Range (mg/dL)	Range (mmol/L)
Major risk factor for heart disease	< 40	< 1.04
Negative risk factor for heart disease	≥ 60	≥ 1.55

2.5.4.4 **Determination of serum Low and very Low Density Lipoprotein cholesterol (LDL-c) and (VLDL-c)** (World Health Organization, 2004)**:-**

Low and very Low Density Lipoprotein cholesterol are a risk factor of coronary heart diseases, and the estimation of there concentrations are most commonly by Friedewald's equation for triglyceride (TG) level less than 400 mg/dL. (Dansethakul, et al., 2015)

LDL cholesterol = total cholesterol - [triglycerides (TG)/5 +

high-density lipoprotein cholesterol]

VLDL-cholestero = triglycerides (TG)/5

2.5.5 **Determination of serum urea level (US Department of Health and Human Services., 2009):-**

  a- **Principle of procedure:-**

The urea nitrogen assay is a modification of a totally enzymatic procedure first described by Talke and Schubert. The test is performed as a kinetic assay in which the initial rate of the reaction is linear for a limited period of time. Urea in the sample is hydrolyzed by urease to

ammonia and carbon dioxide. The second reaction, catalyzed by glutamate dehydrogenase (GLD) converts ammonia and ɑ-ketoglutarate to glutamate and water with the concurrent oxidation of reduced nicotinamide adenine dinucleotide (NADH) to nicotinamide adenine dinucleotide (NAD). Two moles of NADH are oxidized for each mole of urea present. The initial rate of decrease in absorbance at 340 nm is proportional to the urea concentration in the sample.

b- Reference Range (Thomas L, 1998)**:-**

Table 2-10) Reference Range of serum urea

Age		Urea Nitrogen Range (mg/dL)*	Urea Range (mmol/L)**
Children	1 to 3 years	5.1 – 16.8	1.8 – 6.0
	4 to 13 years	7.0 – 16.8	2.5 – 6.0
	14 to 19 years	8.4 – 21.0	3.0 – 7.5
Adult, Male	< 50 years	8.9 – 20.6	3.2 – 7.4
	>50 years	8.4 – 25.7	3.0 – 9.2
Adult, Female	< 50 years	7.0 – 18.7	2.5 – 6.7
	>50 years	9.8 – 20.1	3.5 – 7.2

*Urea Nitrogen (mg/dL) × 2.14 =Urea (mg/dL).

**To convert results from mg/dl urea nitrogen to mmol/L urea, multiply by 0.357.

2.5.6 Determination of serum creatinine :-

a- Principle of procedure (Soldin S, 1978)**,** (Thomas L, 1998)**:-**

At an alkaline pH, creatinine in the sample reacts with picrate to form a creatinine-picrate complex. The rate of increase in absorbance at 500 nm due to the formation of this complex is directly proportional to the concentration of creatinine in the sample.

b- Reference Range (Krouwer JS, 2002)**:-**

Table 2-11): Reference Range of Serum/ Plasma creatinine

Gender	Range (mg/dL)	Range (µmol/L)
Adult, Male	0.72 – 1.25	63.6 – 110.5
Adult, Female	0.57 – 1.11	50.4 – 98.1

2.5.7 Determination of serum CA19-9 (Rittes R.E. et al., 1994), (Ikeda Y. et al., 1995), (Wild D., 2001)**:-**

a. Principle:-

The Human CA19-9 Ag ELISA intended for professional use is an ELISA for direct antigen detection including high affinity and specificity antibodies (enzyme labelled and biotinylated) with different and distinct epitope recognition. The assay makes use of the extremely high affinity of the system Biotin-Streptavidin. Streptavidin has been coated on the surface of microtiter wells. In the first incubation step, specimens, calibrators or controls and Conjugated B (monoclonal, biotinylated antibody, mouse) are mixed to form an antibody-antigen complex which is bound to the surface of the wells by the interaction of biotin with the immobilized Streptavidin.

After removing and washing out of unbound components Conjugate E (enzyme-labelled antibody, mouse) is added (step2). The enzyme conjugate binds to the immobilized antibody-antigen complex forming the final sandwich complex. After incubation excess conjugate is washed out and TMB Substrate added (step 3). A blue colour develops changing to yellow after stopping the reaction. The intensity of the colours is directly proportional to the CA19-9 concentration in the specimen.

The absorbance of calibrators and specimen is determined by using ELISA microplate reader or automated ELISA systems at 450nm. The concentrations for patient samples are obtained by means of a calibration curve which is established from the calibrators supplied with the kit.

b. Test procedure:-

➢ *Step one*

1. Twenty five microliters of each calibrator, control, sample was added (in duplicate) to the well.

2. One hundred microliters of CON-B was added to each well.

3. Each well was mixed and covered with Adhesive Strips then incubated 60 minutes at (20-25)°C

4. Each well washed 3 times (using 300 µl of washing solution in each time).

➢ *Step two*

1. One hundred microliters of CON-E was added to each well.

2. Each well was covered with Adhesive Strips and incubated 60 minutes at (20-25)°C.

3. Each well was washed 3 times (using 300 µl of washing solution in each time).

➢ *Step three*

1. One hundred microliters of substrate solution was added to each well.

2. Each well was incubated for 15 minutes at (20-25)°C.

3. Fifty microliters of stop solution was added to each well and mixed carefully.

➢ ***Finally the absorbance was measured for each well at 450 nm.***

2.5.8 **Determination of Antigen Affinity:-**

   a. **CA 19-9 Antibody:-**

Monoclonal antibody reacts specifically with Sialyl Lewisa containing glycolipids, without detectable reactivity with Lewisa, Lewisb or structurally related substances as determined by solid-phase binding assays against glycolipid antigens. It shows minimal cross-reactivity (<1%) with Sialyl Lacto-N-tetraosylceramide.

The source of this liquid CA 19-9 antibody is Mouse Ascites with concentration of 100µg/100µl and can be diluted by using phosphate buffer saline (0.01 M) with pH (7.4). It's suitable for enzyme linked immunosorbent assay ELISA (EIA), Immunoprecipitation (IP), ICH, Western Blot (WB).

Capture antibody in serological assays for the research of CA19-9. Immunohistochemistry on frozen or paraffin sections for the detection of Sialyl Lewisa. Immunochemical detection of Sialyl Lewisa determinant in mucins and glycolipids. Each laboratory should determine an optimum working titer for use in its particular application. Other applications have not been tested but use in such assays should not necessarily be excluded.

 b. **Procedure:-**

   ➢ *Step one*

  1. Phosphate buffer saline (PBS) pH (7.4): PBS buffer (0.01M, pH7.4). This buffer was prepared by dissolving 0.2995gm of monobasic sodium phosphate and 2.25gm of NaCl in deionized water. Then the pH was adjusted to 7.4 using NaOH (0.2M). The volume was made up to 250ml with deionized water.

2. The CA 19-9 Antibody vial was centrifuged briefly to dislodge any liquid in container's cap.

3. Nine hundred microliter of (0.01 M) Phosphate Buffer Saline (PBS) was added to CA19-9 Antibody vial.

4. The test in the well of ELISA plate was prepared as written in the following tables (in duplicate):-

Table 2-12): Standard CA19-9 (100 U/ml)

No.	Volume of CA 19-9 Antibody	Volume of (0.01M) PBS	Volume of standard CA 19-9 (100 U/ml)
1	25 μl		25 μl
2	20 μl	5 μl	25 μl
3	15 μl	10 μl	25 μl
4	10 μl	15 μl	25 μl
5	5 μl	20 μl	25 μl
6		25 μl	25 μl

Table (2-13): Patient have CA19-9 (99.568 U/ml)

No.	Volume of CA 19-9 Antibody	Volume of (0.01M) PBS	Volume of patient have CA 19-9 (99.568 U/ml)
1	25 μl		25 μl
2	20 μl	5 μl	25 μl
3	15 μl	10 μl	25 μl
4	10 μl	15 μl	25 μl
5	5 μl	20 μl	25 μl
6		25 μl	25 μl

Table (2-14): Healthy individual have CA 19-9 (23.494 U/ml)

No.	Volume of CA 19-9 Antibody	Volume of (0.01M) PBS	Volume of healthy individual have CA 19-9 (23.494 U/ml)
1	25 µl		25 µl
2	20 µl	5 µl	25 µl
3	15 µl	10 µl	25 µl
4	10 µl	15 µl	25 µl
5	5 µl	20 µl	25 µl
6		25 µl	25 µl

The wells were incubated at room temperature with shaking for one hour.

➢ *Step two*

1. One hundred microliters of CON-B was added to each well.

2. Each well was mixed and covered with Adhesive Strips then incubated for 60 minutes at (20-25)°C.

3. They are washed 3 times (using 300 µl of washing solution in each time).

➢ *Step three*

1. One hundred microliters of CON-E was added to each well.

2. Each well was covered with Adhesive Strips and incubated 60 minutes at (20-25)°C.

3. They are washed 3 times (used 300 µl of washing solution in each time).

➢ *Step four*

1. One hundred microliters of substrate solution was added to each well.

2. Each well incubated for 15 minutes at $(20\text{-}25)°C$.

3. Fifty microliters of stop solution was added to each well and mixed carefully.

➢ ***Finally the absorbance was measured for each well at 450 nm.***

2.5.9 Determination of protein concentration using Bradford method (Bradford, 1976), (Compton, 1985):-

Test procedure:-

a. Prepared the assay reagent by dissolved 100 mg of Coomasie Blue (G250) in 50 ml of 95% ethanol, then mixed the solution with 100 ml of phosphoric acid and made up to 1 L with distilled or deionized water.

   Filtered the assay reagent through Whatman No.1 filter paper before storage in an amber bottle at room temperature. This solution is stable for several weeks, but slow precipitation of the dye will occur, so filtration of the stored reagent is necessary before each use.

b. Prepared protein standard by dissolved 1mg of Bovine serum albumin in 1ml of distilled or deionized water, used as a stock solution. (This should be stored frozen).

1. For calibration curve, pulled duplicate volumes of 10, 20, 40, 70 and 100µl of stock solution (Bovine serum albumin solution) into test tubes, and make each up to 100 µl with distilled or deionized water.

2. For the reagent blank pulled 100 µl with distilled or deionized water into a further tube.

3. For unknown protein concentration (in duplicate):-

a. 10 U/ml of standard CA19-9 Antigen.

b. 50 U/ml of standard CA19-9 Antigen.

c. 100 U/ml of standard CA19-9 Antigen.

d. Serum of five patients with a different concentration of CA19-9.

e. Serum of five healthy individuals with a different concentration of CA19-9.

4. Added 5 ml of the assay reagent for each tubes and mixed well by vortex instrument.

5. Measured the absorbance of the samples and standard against the reagent blank at 600nm.

2.5.10 Effect of some chemicals on the antigen affinity:-

➢ *Step one*

1. Tris 0.2M, buffer pH (7.4) was prepared by:-

   a. Dissolving 24.2gm of Tris hydroxymethyl aminomethane in 1L of distilled water.

   b. Then the pH was adjusted to 7.4 using HCl (0.1M). The volume was made up to 1000 ml with distilled water.

2. The stock solutions (0.15 M) (Martin I. Surks, 1989), (A. Paul Mould, 1995) of divalent cations and halides are prepared by dissolving each of the following amounts of salts in 25 ml Tris buffer pH (7.4): (0.639gm) $CuCl_2$, (0.416gm) $CaCl_2$, (0.511gm) $ZnCl_2$, (0.357gm) $MgCl_2$, (0.472gm) $MnCl_2$, (0.157gm) NaF, (0.219gm) NaCl and (0.562 gm) NaCl.

3. The stock solution 30% of Polyethyleneglycol PEG (6000) is prepared by dissolving 7.5gm of Polyethyleneglycol in 25ml Tris buffer pH (7.4).

4. The tests in the well of ELISA are prepared plate as written in the following tables(in duplicate):-

Table 2-15): Standard CA 19-9 (500 U/ml)

No.	Chemical solution	Volume of chemical solution	Volume of standard CA 19-9 (500 U/ml)
1	$CuCl_2$	25 µl	25 µl
2	$CaCl_2$	25 µl	25 µl
3	$ZnCl_2$	25 µl	25 µl
4	$MgCl_2$	25 µl	25 µl
5	$MnCl_2$	25 µl	25 µl
6	NaF	25 µl	25 µl
7	NaCl	25 µl	25 µl
8	NaI	25 µl	25 µl
9	Polyethyleneglycol 6000	25 µl	25 µl

Table 2-16): Patient have CA 19-9 (99.568 U/ml)

No.	Chemical solution	Volume of chemical solution	Volume of patient have CA 19-9 (99.568 U/ml)
1	$CuCl_2$	25 µl	25 µl
2	$CaCl_2$	25 µl	25 µl
3	$ZnCl_2$	25 µl	25 µl
4	$MgCl_2$	25 µl	25 µl
5	$MnCl_2$	25 µl	25 µl
6	NaF	25 µl	25 µl
7	NaCl	25 µl	25 µl
8	NaI	25 µl	25 µl
9	Polyethyleneglycol 6000	25 µl	25 µl

Table 2.17): Healthy individual have CA 19-9 (23.494 U/ml)

No.	Chemical solution	Volume of chemical solution	Volume of healthy individual have CA 19-9 (23.494 U/ml)
1	$CuCl_2$	25 µl	25 µl
2	$CaCl_2$	25 µl	25 µl
3	$ZnCl_2$	25 µl	25 µl
4	$MgCl_2$	25 µl	25 µl
5	$MnCl_2$	25 µl	25 µl
6	NaF	25 µl	25 µl
7	NaCl	25 µl	25 µl
8	NaI	25 µl	25 µl
9	Polyethyleneglycol 6000	25 µl	25 µl

5. One hundred microliters of CON-B was added to each well.

6. Each well was mixed and covered with Adhesive Strips then incubated 60 minutes at (20-25)°C

7. Each well washed 3 times (using 300 µl of washing solutionin each time).

➢ *Step two*

1. One hundred microliters of CON-E was added to each well.

2. Each well was covered with Adhesive Strips and incubated for 60 minutes at (20-25)°C.

3. Each well was washed 3 times (using 300 µl of washing solution in each time).

➢ *Step three*

1. One hundred microliters of substrate solution was added to each well.

2. Incubated for 15 minutes at (20-25)°C.

3. Fifty microliters of stop solution was added to each well and mixed carefully.

➤ *Finally the absorbance was measured for each well at 450 nm.*

2.6 Statistical analysis:-

- In this study the data obtained was analyzed by Microsoft excel 2013 and SPSS version 20.

- The numerical data expressed as mean ±SE.

- Student's t-test was used to calculate individual p-value, p-value < 0.05 was considered significant.

- For comparing mean of more than two groups the ANOVA (F-test) was used.

Results

During the period of the study a total sample of 80 patients were studied. Among this sample 40 were patients with type 2 diabetes mellitus and take medication and 40 patients newly diagnosis with type 2 diabetes mellitus and don't take medication in addition to 40 individuals represent the control group.

The Correlation of demographic characteristic of diabetes mellitus patient groups and control group are shown in table (3.1).

Table 3.1): The Correlation of demographic characteristic of diabetes mellitus patient group and control group

Parameters	Control (N=40)	With treatment (N=40)	Newly diagnosis (N=40)
Gender Male/Female	19/21	18/22	16/24
Age (year) (mean±SE)	44.75±1.454	48.25±1.723	44.98 ±1.348
BMI (kg/m^2) (mean±SE)	27.48 ±0.326	31.05±0.989	33.93± 1.082

3.1 Serum glucose:-

Fasting Blood Sugar (FBS) were measured for each person participated in this study (whether diabetic or control individual), Group I (with treatment group) showed a significant elevation of FBS level in comparison with the control group (p<0.0001), and there is a significant difference between group II (newly diagnosis group) and control group (p<0.0001). While there is no significant difference had been observed between the two diabetic groups (p = 0.593).

Furthermore, there is a significant difference between the two diabetic groups and control group showed by the p-value of F-test. Table (3.2)

3.2 Glycated hemoglobin (HbA1c):-

Glycated hemoglobin (HbA1c) has been measured for each person participated in this study (whether diabetic or control individual). Group I (with treatment group) showed a significant elevation of HbA1c level in comparison with the control group ($p<0.0001$), and there is a significant difference between group II (newly diagnosis group) and control group ($p<0.0001$). Also there is a significant difference had been observed between the two diabetic groups ($p = 0.029$)

Furthermore, a significant difference had been observed among all as it clear in table (3.2)

Table (3.2): Fasting blood sugar and Glycated hemoglobin.

Parameter	Control	With treatment	Newly diagnosis	p-value according to F-test
FBS (mg/dl)	88.68±0.98	223.08±16.266	171.65±9.913	<0.0001
HbA1c (%)	5.78±0.067	9.38±0.341	8.53±0.320	<0.0001

3.3 Serum lipid profile:-

Total serum cholesterol (TC), Triglyceride (TG), High density lipoprotein cholesterol (HDL-c), Low density lipoprotein cholesterol (LDL-c) and Very low density lipoprotein cholesterol (VLDL-c) were measured for each individual studied. The mean of serum lipid profile in control and the two diabetic groups is shown in table (3.3).

3.3.1 Total serum cholesterol:-

Total cholesterol (TC) has a significantly elevated in group Ⅰ (with treatment group) compared with control group (p=0.0177) and no significantly elevated in group Ⅱ (newly diagnosis group) compared with control group (p=0.5307). Also there is no significant elevation when compared between the values of two diabetic groups (p=0.158). While there is a significant difference among all groups according to the F-test as seen in table (3.3).

3.3.2 Serum triglyceride:-

There is a significant difference between the two diabetic groups (group Ⅰ and group Ⅱ) when compared with control group (p<0.0001), (p<0.0012) respectively.

Furthermore there is no significant elevation between the two diabetic groups (p=0.987). While there is a significant difference among all groups according to the F-test as shown in table (3.3).

3.3.3 Serum high density lipoprotein cholesterol (HDL-c):-

High density lipoprotein (HDL-c) was not significantly elevated in group Ⅰ (with treatment group) compared with control group (p=0.0847) while a significant difference is shown in group Ⅱ (newly diagnosis group) compared with control group (p=0.0412). Also there is no significant difference when compared between the values of two diabetic groups (p=0.997).While a significant difference among all groups according to the F-test as seen in table (3.3).

3.3.4 Serum low density lipoprotein cholesterol (LDL-c):-

There is no significant elevation between the two diabetic groups (group Ⅰ and group Ⅱ) when compared with control group (p=0.8926), (p=0.0863) respectively.

Furthermore there is no significant difference between the two diabetic groups (p= 0.375), also no significant difference among all groups according to the F-test as shown in table (3.3).

3.3.5 Serum very low density lipoprotein cholesterol (VLDL-c):-

Very low density lipoprotein (VLDL-c) were significantly elevated in group Ⅰ compared with control group (p< 0.0001) and group Ⅱ (p<0.0012) compared with control group. While there is no significant differences when comparing between the values of two diabetic groups (p=0.999) and.

Furthermore there is a significant difference among all groups according to the F-test as seen in table (3.3).

Table (3.3): Serum lipid profile in the two diabetic and control groups

Parameters	Control	With treatment	Newly diagnosis	p-value according to F-test
Total Cholesterol (mg/dl)	179.93±3.856	206.60±7.953	185.83±4.463	0.003
Triglyceride (mg/dl)	97.75±5.780	184.18±16.263	134.70±7.886	0.001
HDL-c (mg/dl)	37.60±1.625	41.05±1.348	43.75±2.079	0.042
LDL-c (mg/dl)	122.73±3.91	126.28±6.966	115.78±3.591	0.328
VLDL-c (mg/dl)	19.68±1.177	38.48±3.492	27.13±1.526	0.001

3.4 Serum urea level:-

serum urea was measured of each person participated in this study (whether diabetic or control individual) to exclude any patient has a high level of urea. The diabetic groups (with treatment & newly

diagnosis) showed no significant elevation of the urea serum in comparison with control group (p=0.358), (p= 0.166) respectively.

As a result there is no significant difference had been observed between the two diabetic groups (p=0.355).

Furthermore, there is no significant difference between the two diabetic groups and control group as showed by the p-value of F-test. Table (3.4).

3.5 Serum Creatinine level:-

serum urea was measured of each person participated in this study (whether diabetic or control individual) to exclude any patient has a high level of serum creatinine, the diabetic groups (with treatment & newly diagnosis) showed no significant elevation of the serum creatinine in comparison with control group (p=0.185), (p=0.358), As a result there is no significant difference had been observed between the two diabetic groups (p=0.3582).

Furthermore, there is no significant difference between the two diabetic groups and control group as showed by the p-value of F-test. Table (3.4).

Table (3.4): Serum Urea and Creatinine level.

Parameter	Control	With treatment	Newly diagnosis	p-value according to F-test
Urea (mg/dl)	26.55±0.965	28.28±1.501	23.45±1.011	0.160
Creatinine (mg/dl)	0.678±0.017	0.736±0.019	0.697±0.016	0.757

3.6 Serum level of C - reactive protein (CRP):-

Serum level of C-reactive protein (CRP) was measured for each person participated in this study (whether diabetic or control individual), the negative value distribution of the three studied groups is shown in figure (3.1).
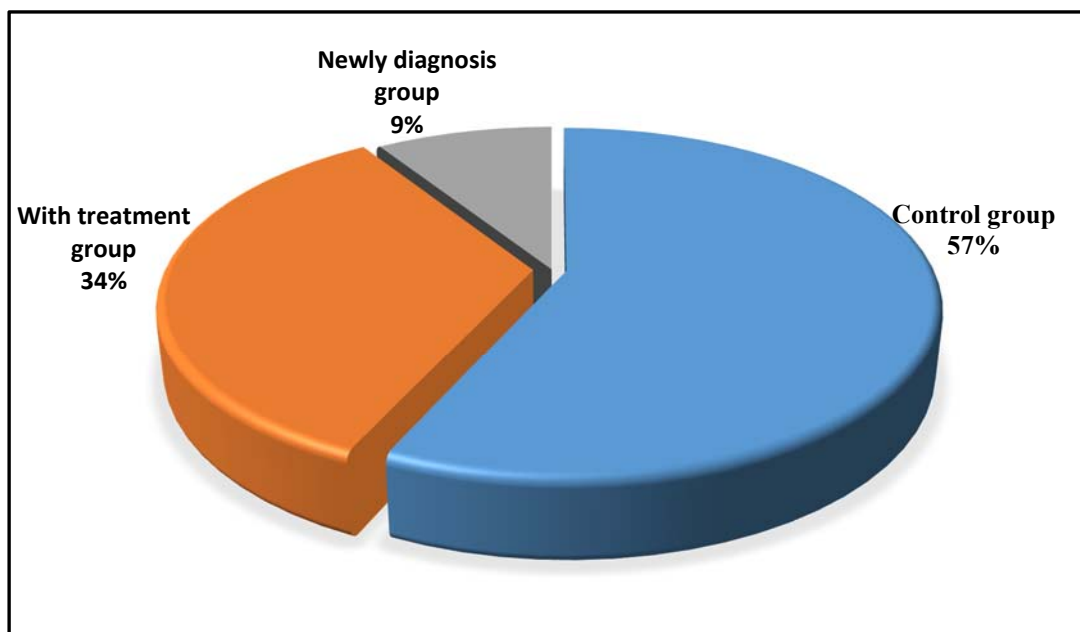

Figure (3-1): Distribution of negative CRP value.

3.7 **Determination of CA19-9 levels in sera of patients with type 2 diabetes mellitus by enzyme-linked immunosorbent assay (ELISA).**

Serum level of Carbohydrate antigen CA19-9 was measured for each person participated in this study (whether diabetic or control individual). The diabetic groups (with treatment & newly diagnosis) showed a significant elevation of serum CA19-9 level in comparison with control group ($p< 0.001$), while there is no significant difference observed between the two diabetic groups ($p=0.308$).as shown in tables (3.5), (3.6) and (3.7) and figure (3.2).

Table (3.5): Comparison of CA19-9 between control group and T2DM with treatment group.

Parameters	Control n=40 Median (Range)	With treatment n=40 Median (Range)	P-value
CA19-9 U/ml	1.68 (0.001-28.965)	22.03(10.142-99.568)	<0.001

Table (3.6): Comparison of CA19-9 between control group and T2DM newly diagnosis group.

Parameters	Control n=40 Median (Range)	Newly diagnosis n=40 Median (Range)	P-value
CA19-9 U/ml	1.68 (0.001-28.965)	30.19(10-63.667)	< 0.001

Table (3.7): Comparison of CA19-9 between T2DM with treatment group and newly diagnosis group.

Parameters	With treatment n=40 Median (Range)	Newly diagnosis n=40 Median (Range)	P-value
CA19-9 U/ml	22.03(10.142-99.568)	30.19(10-63.667)	0.308
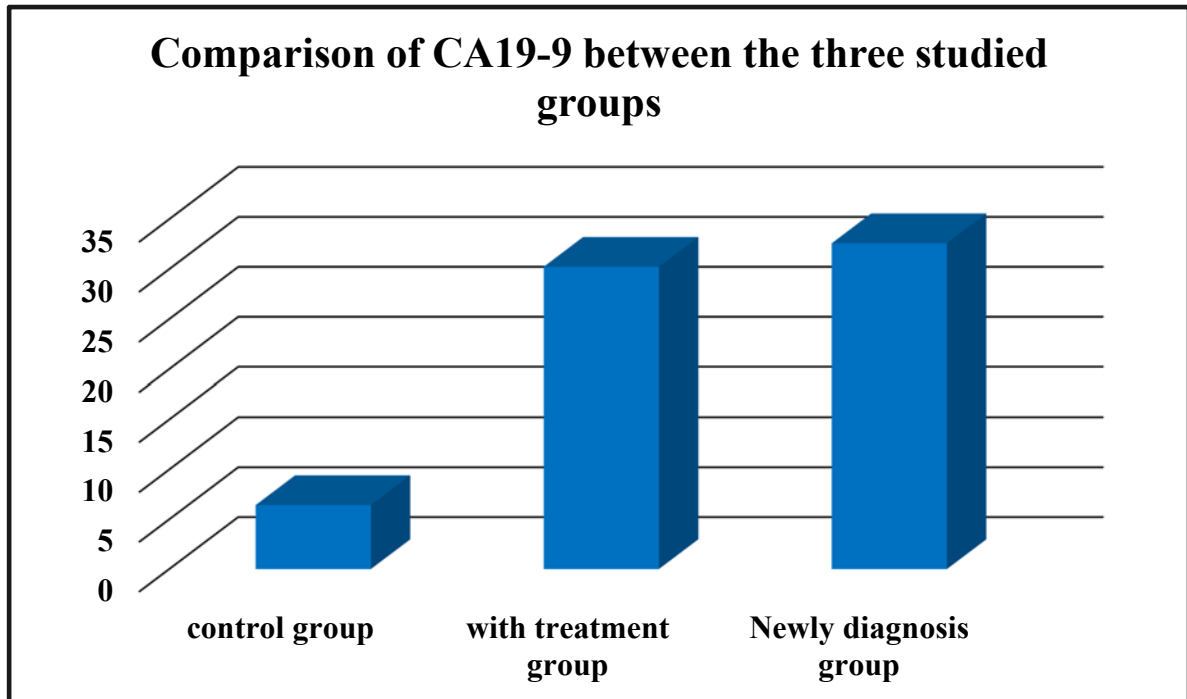
Figure 3-2):- Comparison of CA19-9 between the three studied groups

3.8 Define the normal range (cutoff value) of CA19-9 in type 2 diabetic patients:-

The Receiver Operator Characteristic (ROC) curve shows a significant discriminatory ability of increase serum CA19-9 levels for type 2 diabetes mellitus as in figure (3.3)
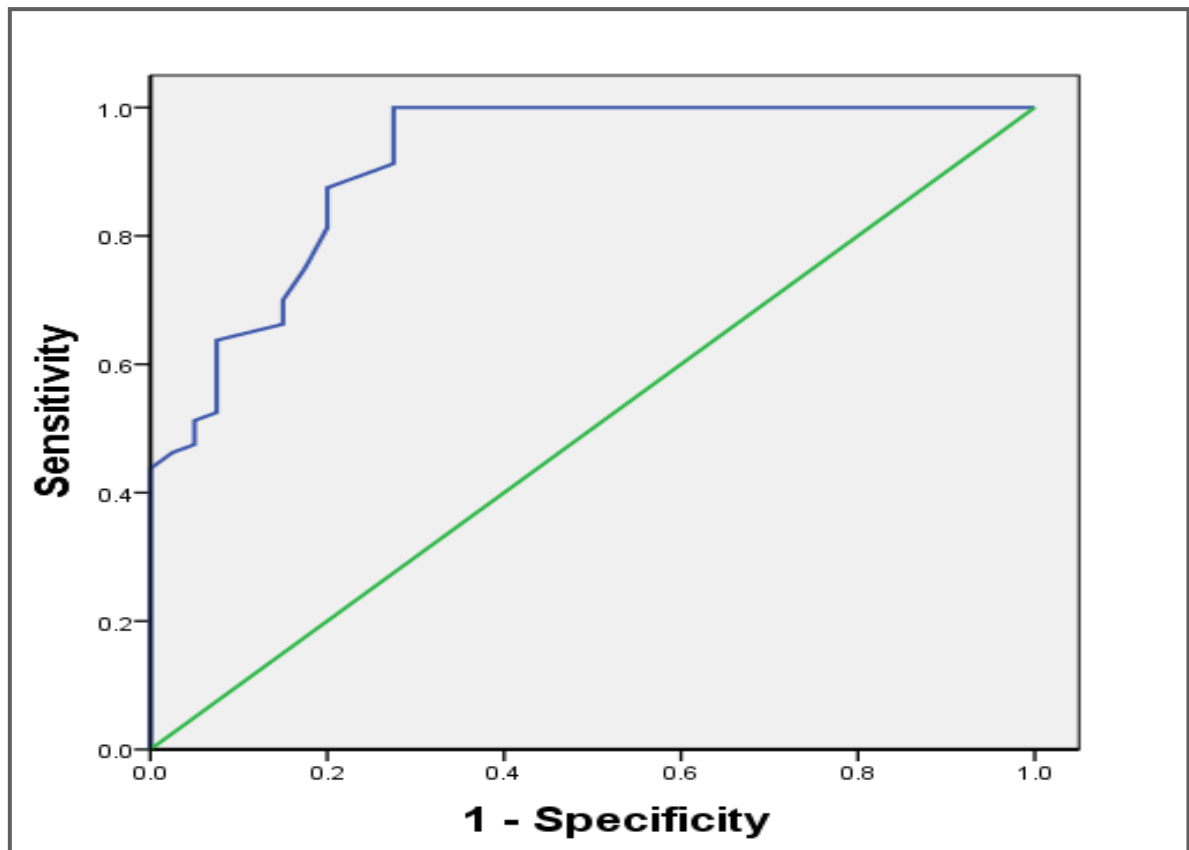
Figure (3-3): ROC for CA19-9 in control and diabetic groups.

When the serum CA19-9 concentration of 49.5 U/ml was used as cut off value for this marker in patients suffered from type 2 diabetes mellitus, the sensitivity is 88.8% and specificity is 77.5% as shown in Table (3.8)

Table (3.8): Sensitivity and specificity CA19-9 in control and patients groups

Cutoff value	Specificity	Sensitivity	Area under curve	P-value
49.50	77.5%	88.8%	0.914	< 0.001

3.9 **Investigate the possible relationships of CA19-9 with fasting blood sugar and HbA1c:-**

Serum level of Carbohydrate antigen CA19-9 was compared with FBS and HbA1c levels to investigate the possible relationships between them as shown in Table (3.9) and figure (3.4).

Table (3.9): Correlation of CA19-9 with fasting blood sugar and HbA1c.

Parameter		Control	Newly diagnosis	With treatment
FBS (mg/dl)	**r**	0.076	0.281	0.104
	P	0.642	0.079	0.525
HbA1c (%)	**r**	0.243	0.197	0.013
	P	0.131	0.224	0.937
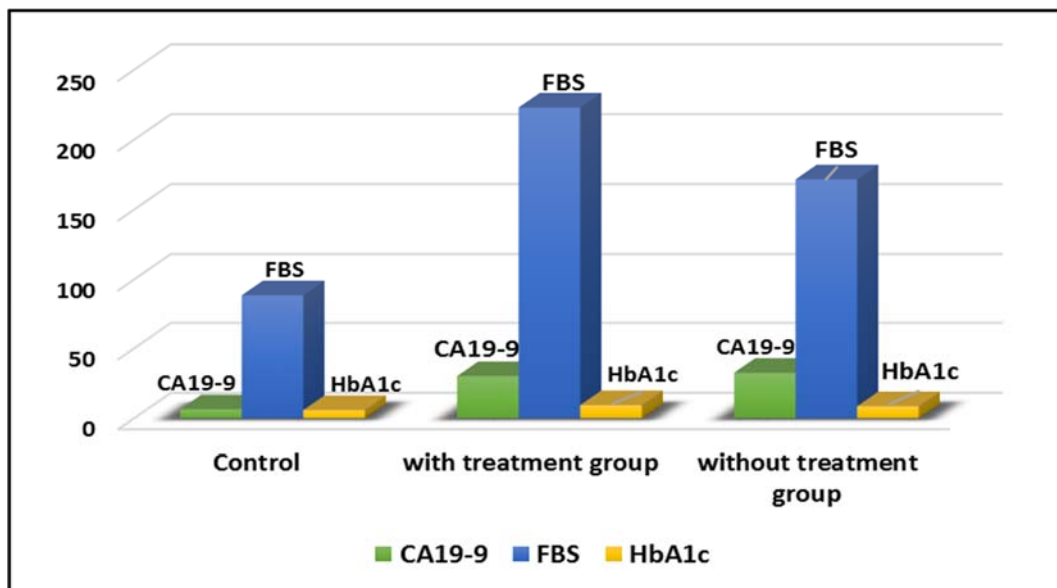
Figure (3-4): Correlation of CA19-9 with fasting blood sugar and HbA1c

3.10 Determination of protein concentration using Bradford method:-

Bradford method is used to determine the concentration of carbohydrate antigen CA19-9 in µg/ml instead of U/ml, the result is shown in figure (3.5):-
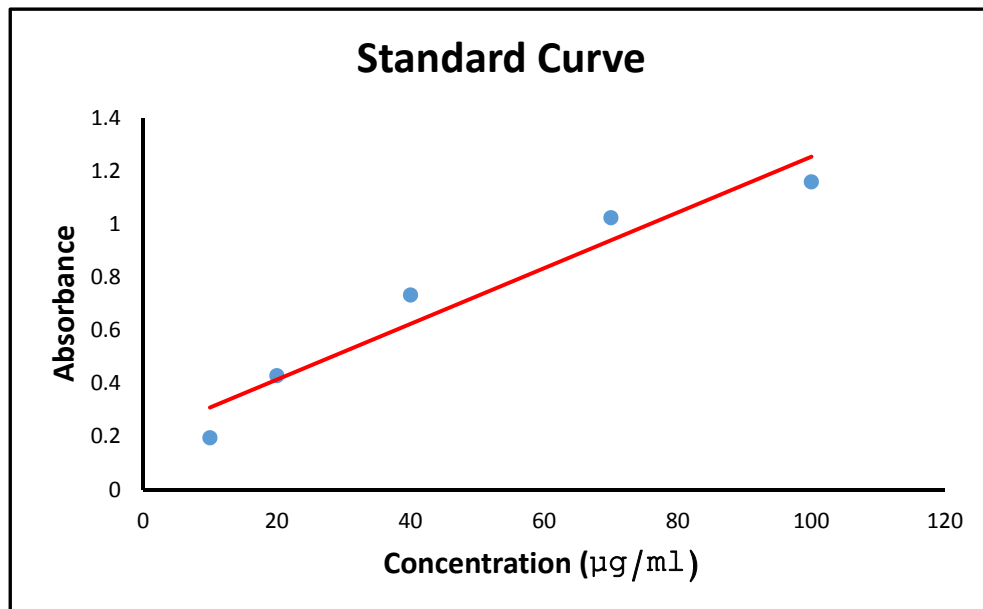
Figure (3-5): Standard Curve of Bradford method

Then the unknown concentration was extracted from its absorbance using standard curve, calculate the concentration in (µg/ml) by using the following equation:-

♦ The dilution factor is considered equal 2, as shown in table (3.10).

Concentration (µg/ml) = Diluted concentration* Dilution Factor

Table (3-10): The calculation of Bradford method.

| No.	Concentration (U/ml)	Diluted concentration (µg/ml)	Concentration (µg/ml)
1	10	5.05194	10.10388
2	50	25.2597	50.5194
3	100	50.5194	101.0388
4	99.568	50.3012	100.6023
5	56.849	28.7198	57.4395
6	63.667	32.1642	64. 3284
7	68.821	34.7679	69.5359
8	77.676	39.2414	78.4829
9	0.569	0.2875	0.5749
10	0.228	0.1152	0.2304
11	12.991	6.5629	13.1259
12	23.494	11.869	23.738
13	28.965	14.6329	29.2658

♦ So, the converter factor can be used to convert the concentration of carbohydrate antigen CA19-9 from (U/ml) to (µg/ml) is **1.010388**

3.11 Determination of a dissociation constant (Kd) of the binding CA19-9 to its antibody in type 2 diabetic patients using Scatchard plot through development of ELISA technique.

➢ To find the standard curve we used CA19-9 Antigen standard as below:-

(Each well containing 25 µl of Phosphate Buffer Saline PBS (0.01M)).

Table (3-11): concentration and Absorbance of CA19-9 standard

No.	Standard value (U/ml)	Volume (µl)	Absorbance	Concentration (U/ml)
1	0	25	0.094	0.001
2	10	25	0.220	10.00
3	50	25	0.551	50.001
4	100	25	0.904	100.00
5	250	25	1.595	249.999
6	500	25	2.336	499.993

The standard curve of CA19-9 by using Phosphate buffer saline is showed in figure (3.6)

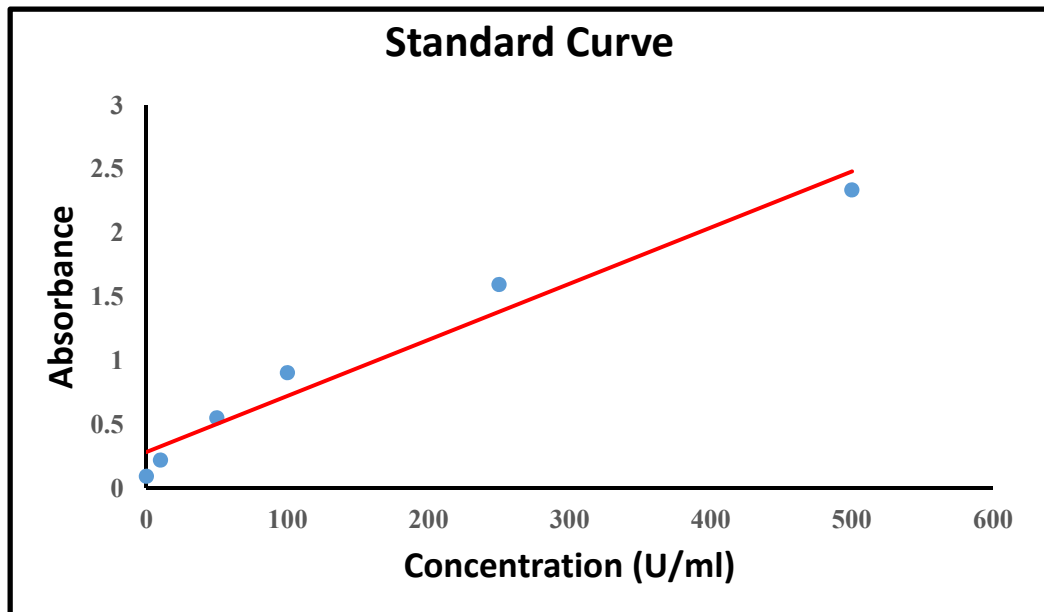**Figure (3-6):**Standard Curve

The original concentration of Monoclonal antibody solution was 100 µg /100 µl, as a result when made up the volume with 0.01M Phosphate Buffer Saline to 1 ml the concentration become 10 µg/ml = 0.01 mg/ml and used the last as a stock solution.

(Each well containing 25 µl of standard solution (100 U/ml))

Table (3-12):- Monoclonal Antibody dilution

No.	Dilution	Absorbance	Concentration (U/ml)
1	25 µl of Ab	0.135	0.020
2	20 µl of Ab + 5 µl of PBS	0.142	0.033
3	15 µl of Ab + 10 µl of PBS	0.169	0.232
4	10 µl of Ab + 15 µl of PBS	0.218	8.240
5	5 µl of Ab + 20 µl of PBS	0.379	21.634
6	25 µl of PBS	0.905	100.002

➢ To find the concentration of Ab in each well used the following equation:-

$$\text{Conc. Of Ab in the well} = \frac{\text{Volume of Ab in this well}}{\text{Total incubation volume}} \times \text{Conc. Of Ab (stock solution)}$$

Table 3-13): the concentration of Monoclonal Antibody in each well

No.	vol. of Ab (µl)	Total incubation volume (µl)	Conc. Of Ab (stock solution) (mg/ml)	Conc. Of Ab in the well (mg/ml)
1	25	50	0.01	0.005
2	20	50	0.01	0.004
3	15	50	0.01	0.003
4	10	50	0.01	0.002
5	5	50	0.01	0.001

➢ To find the concentration of Ag equivalent to the Ab concentration in each well applied the following equation:-

$$\text{The equivalent conc. of Ag} = \frac{\text{Conc. of Ab in this well}}{\text{Conc. of Ab in stock solution}} \times \text{Conc. of Ag}$$

Table 3-14): The equivalent concentration of Ag for CA19-9 standard (100U/ml)

No.	Conc. Of Ab in this well (mg/ml)	Conc. Of Ab in stock solution (mg/ml)	Conc. Of Ag (U/ml)	Equivalent conc. of Ag (U/ml)
1	0.005	0.01	100.002	50.001
2	0.004	0.01	100.002	40.0008
3	0.003	0.01	100.002	30.0006
4	0.002	0.01	100.002	20.0004
5	0.001	0.01	100.002	10.0002

➢ To calculate the x-axis values (Bound) applied the equation below:-

$$\text{Bound} = \frac{\text{Absorbance of Ab}}{\text{Absorbance of PBS}} \times \text{The equivalent conc. of Ag}$$

Table 3-15): Bound value (x-axis in Scatchard plot) for CA19-9 standard (100U/ml)

No.	Absorbance of Ab	Absorbance of PBS	Equivalent conc. of Ag (U/ml)	Bound (x-axis value) (U/ml)
1	0.135	0.905	50.001	7. 4587
2	0.142	0.905	40.0008	6. 2763
3	0.169	0.905	30.0006	5.6023
4	0.218	0.905	20.0004	4.8177
5	0.379	0.905	10.0002	4.1879

➢ To calculate the free values used the following equation:-

> Free value = (Total Concentration) – Conc. Of Ab (Bound value)

Table 3-16): Free and (Bound/free) values for CA19-9 standard (100U/ml).

No.	Conc. Of PBS (Total Concentration) (U/ml)	Conc. Of Ab (Bound value) (U/ml)	Free value (U/ml)	Bound/Free (B/F)(y-axis value)
1	100.002	0.020	99.982	0.0002
2	100.002	0.033	99.969	0.0003
3	100.002	0.232	99.77	0.0023
4	100.002	8.240	91.762	0.0897
5	100.002	21.634	78.368	0.276

Scatchard plot:-

Applied the following equation to find dissociation constant Kd:-

$$\frac{B}{F} = \frac{1}{K_d} \times (B_{max} - B)$$

Where:

B: The bound of CA19-9 to its immobilized antibody (B) that can be obtaining from ELISA curve.

F: the free concentration of CA19-9 which represent the first incubation in solution that can be deduced from the total binding (TB) of CA19-9.

Where F= TB-B

Kd: The dissociation constant.

B$_{max}$: The maximal binding capacity.

The plot of **B/F** ratios vs. the **B** values gives a linear relationship. The value of the dissociation constant of the binding Kd can be calculated from the slope of the straight line.
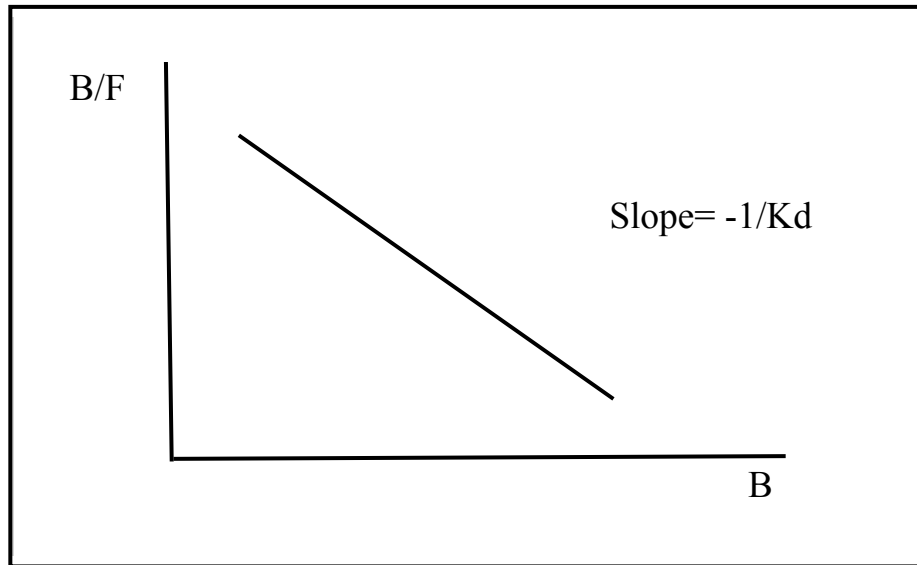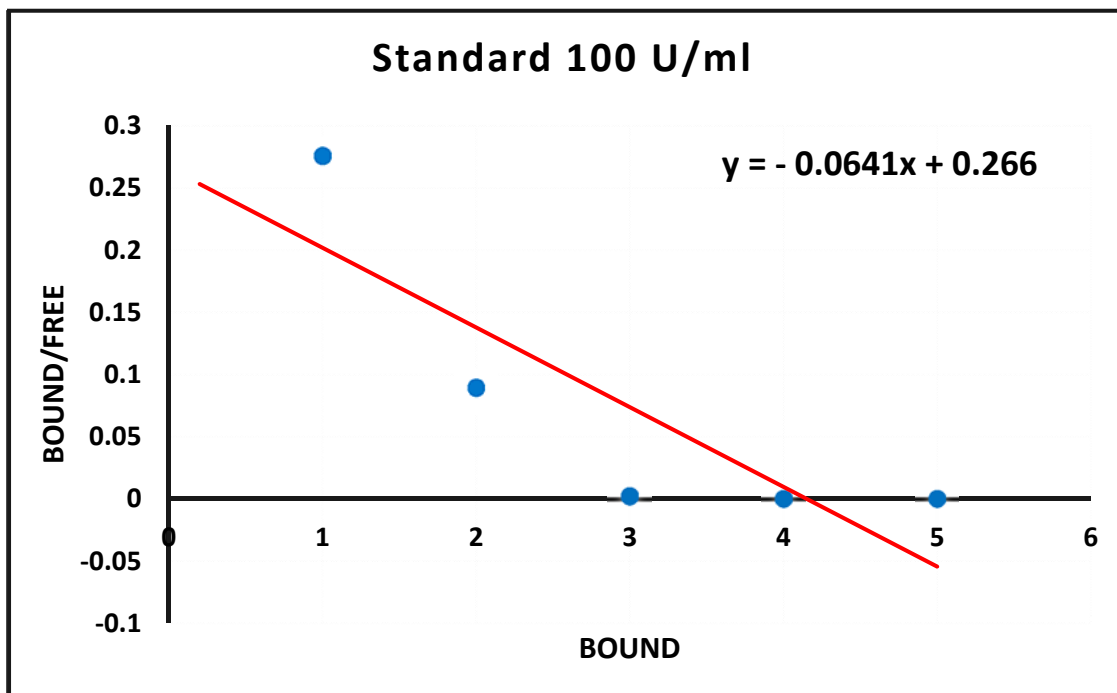

Figure 3-7: Scatchard plot

Figure (3-8): Scatchard plots of standard CA19-9 (100U/ml)

♦ Then the dissociation constant (Kd) value equal 15.6006 U/ml.

The same previous equations were applied for patient has a CA19-9 level (99.568U/ml) and for healthy individual has a CA19-9 level (23U/ml), which were gave the following results:-
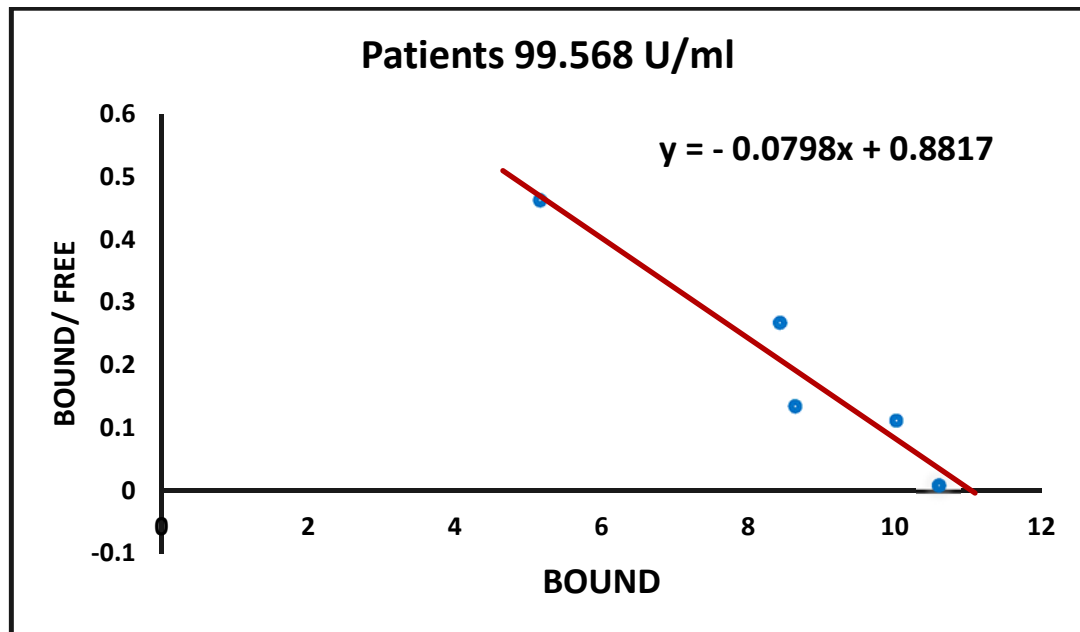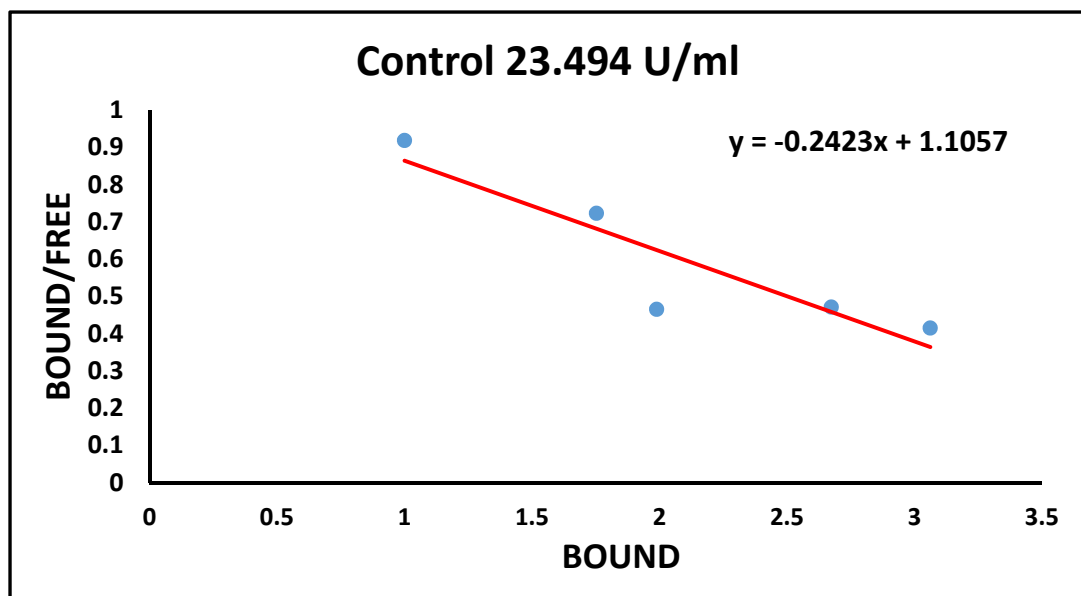

Figure (3-9): Scatchard plots of patient has a CA19-9 level (99.568U/ml)

♦ Then the dissociation constant (Kd) value equal 12.5313 U/ml

Figure(3-10): Scatchard plot for Healthy individual has a CA19-9 level (23.494 U/ml).

♦ Then the dissociation constant (Kd) value equal 4.1271 U/ml

The affinity constant and maximal binding capacity were determined according to Scatchard equation (Laskowski, 1960).

$$\frac{B}{F} = \frac{1}{K_d} \times (B_{max} - B)$$

$$K_a = \frac{1}{K_d}$$

Where:

K_a = The affinity constant.

K_d = The dissociation constant.

B_{max}= The maximal binding capacity.

The plot of B/F ratios vs. the B values gives a linear relationship, the value of the dissociation constant of the binding k_d can be calculated from the slope of the straight line, as a result the value of the affinity constant of the binding ka can be calculated from the dissociation constant value, while the value of the total concentration of CA19-9 (B_{max}) was calculated from the intercept with the x-axis the results referred in table.

Table (3-17): Summary of Scatchard plot

Cases	Slope	K_d (U/ml)	K_d (µg/ml) *	K_a (µg/ml)	B_{max} (µg/ml) *
Standard (100 U/ml)	- 0.0641	15.6006	15.76266	0.06344	0.26876
Patient (99.568 U/ml)	- 0.0798	12.5313	12.6615	0.07897	0.89085
Control (23.494 U/ml)	- 0.2423	4.1271	4.16997	0.2398	1.11718

* To convert the unit of the dissociation constant from (U/ml) to (µg/ml) multiply by the converter factor (**1.010388**) (which obtained from Bradford method).
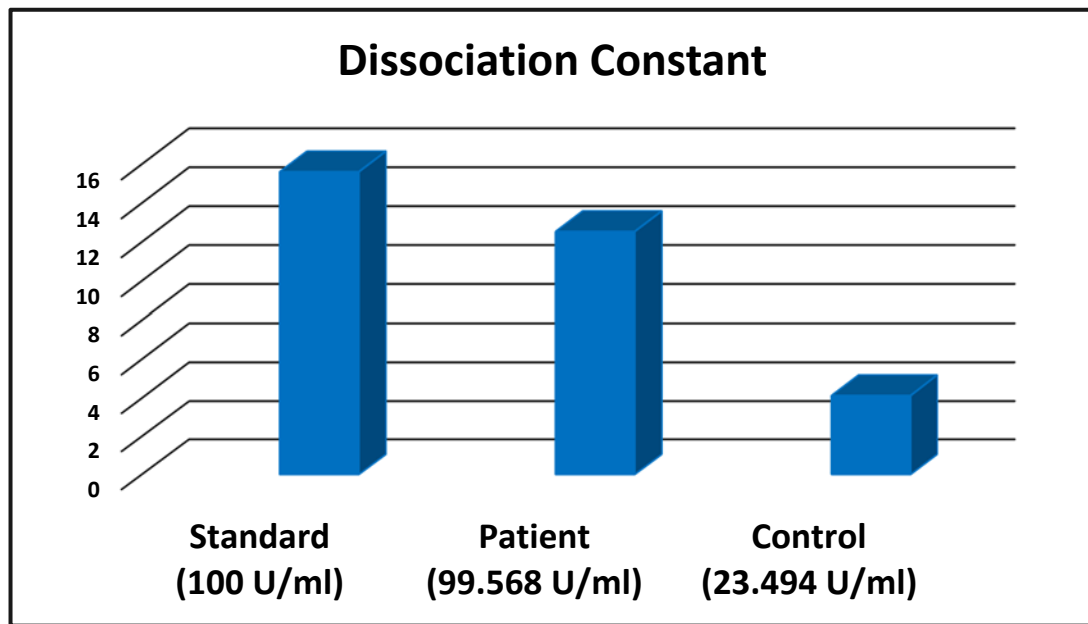
Figure (3-11):- Comparison between the dissociation constant values

3.12 The effect of different chemicals on the binding of CA19-9 to its antibody in type 2 diabetic patients:-

3.12.1 Effect of divalent cations:-

The current study proves that there is an effect for divalent cation at concentration (0.15 M) on the binding of CA19-9 to its antibody in type 2 diabetic patients, as shown in figure (3.12).
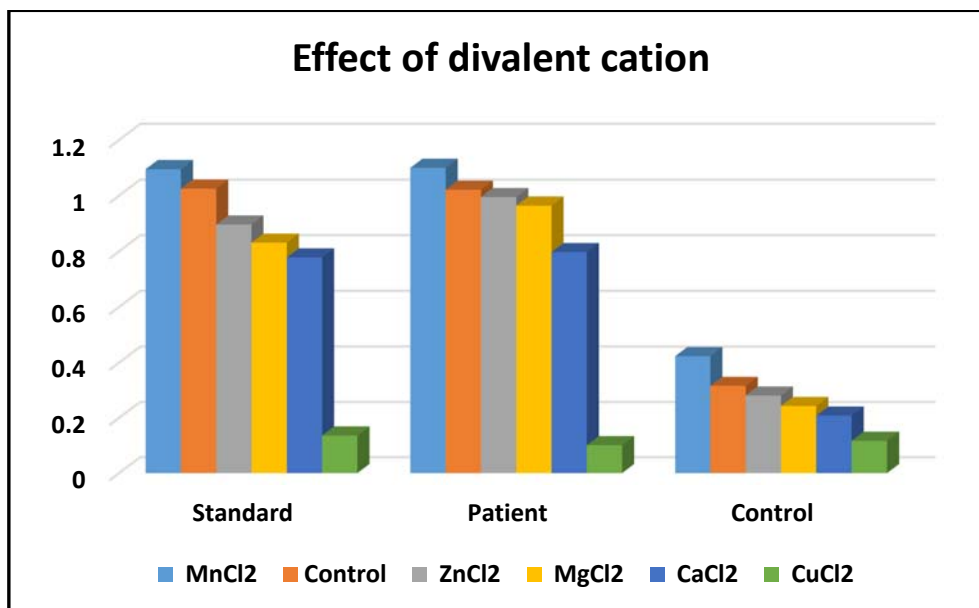
Figure (3-12): Effect of divalent cation

3.12.2 Effect of different halides:-

The current study proves there is an effect for different halides at concentration (0.15 M) on the binding of CA19-9 to its antibody in type 2 diabetic patients, as seen in figure (3.13).

Figure 3.13):- Effect of different halides

3.12.3 Effect of Polyethylene (PEG-6000):-

The current study proves there is a negative effect for Polyethylene glycol (PEG-6000) at concentration (30%) on the binding of CA19-9 to its antibody in type 2 diabetic patients, as seen in figure (3.14).

Figure (3-14): Effect of Polyethylene (PEG-6000)
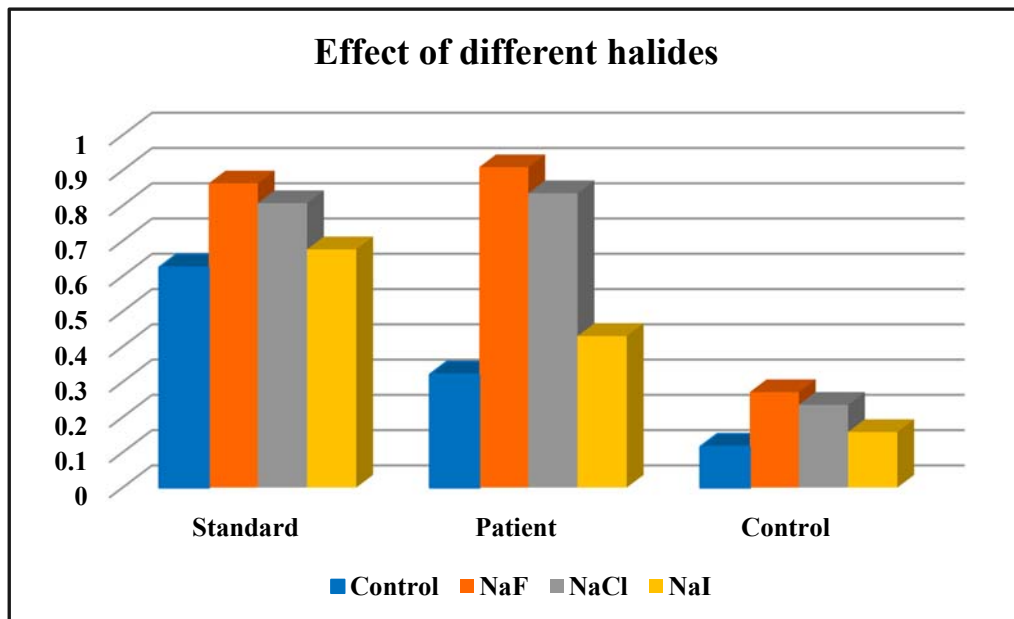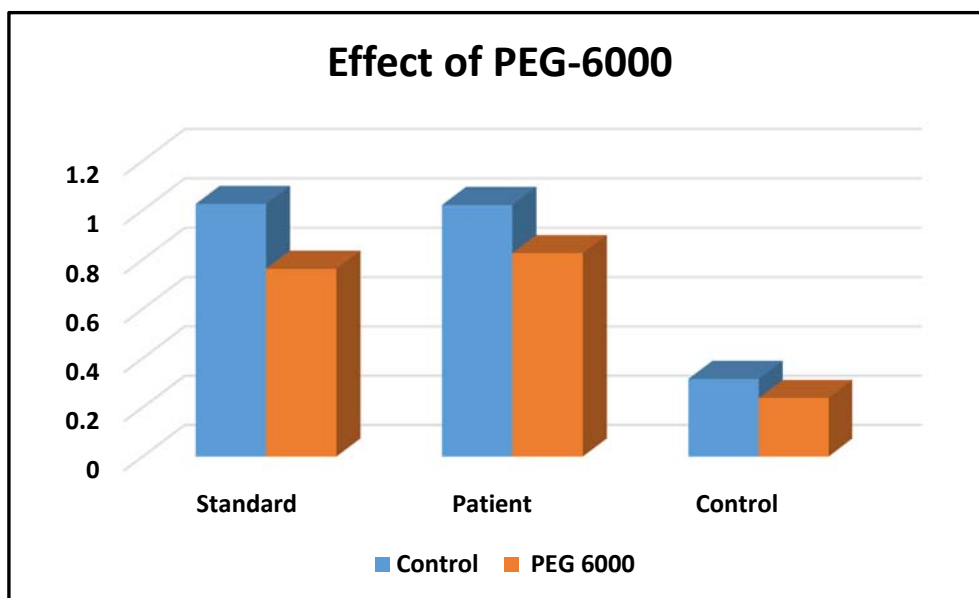
Discussion

Diabetes mellitus is a chronic inflammatory disease of the pancreas and the mechanism of glucose intolerance comprises insulin resistance and devastation of beta cells. (Mohan, et al., 2003).

The result of the present study showed that 57.5% of diabetic patients were females, this percentage was in agreement with other studies by Liu Zhaolan et al. and Ozoh et al. in China, in which the rates were (58.2%) and (53.5%) respectively. (Zhaolan, et al., 2010) (Ozoh, et al., 2010), but lower than that in other study in Basrah were the percentage was (80.5%) (Abass, et al., 2007)

The standard error of mean age for patients participated in the current study was (46.563 ± 1.105)years , their age ranged 35-65 years, and this comparable with other study by Anas H. Sadek et al. (2013) in Iraq, Heidari (2013) in Iran and Raj and Rajan (2013) in India in which the standard error of mean age (52.14±1.01), (49.26 ± 6.39) and (54.3 ± 9.2) years respectively (Anas Hashim Sadek, et al., 2013), (Heidari, 2013), (Raj, et al., 2013). The high distribution of diabetes mellitus in this age group mean that majority of diabetes victims were suffering from diabetes in the most productive years of life. The risk of diabetes developing also increases with age (Hartz, et al., 1983), Another study in USA showed (54.15%) of diabetic patients were above 50 years (Daly, et al., 2009) and this is similar for the results which reported by WHO 1993 in Iraq, that the age group (50-69) years is the majority of type 2 diabetic patients (World health organization, 1993) therefore type 2 diabetes is known as the main disease of older age group (Kahn, et al., 2006).

The mean of body mass index (BMI) in the current study of T2DM patient group 1 and 2 were (31.051 ± 0.989 kg/m2) and (33.915±

1.053 kg/m^2) respectively and these values in agreement with other study by Lewis et al. in New Zealand, in which mean BMI was 32.64 ± 4.52 kg/m^2 (Lewis, et al., 2008).

Al-Auqbi and Mustafa in Iraq found just 28.8% of the patients were non-obese, BMI<25 kg/m^2 while 71.2% of type 2 diabetic patients had BMI>25 kg/m^2 who categorized their patients as follows:

- Overweight, BMI 25-29.9 kg/m^2 included 832 patients.
- Obesity, BMI 30-39.9 kg/m^2 included 661 patients.
- Morbid obesity, BMI >40 kg/m^2 included 64 patients.(Al-auqbi, et al., 2005)

Fasting blood sugar was significant elevation in patient groups in comparable with control group (p<0.0001), while there is no significant difference had been observed between the two diabetic groups (p=0.593), also F-test shows a significant difference between all studied groups. These results were consent with other studies reported previously by Sindelka et al. in Prague and Kocak et al. in Turkey.

Sindelka et al. described that the serum blood glucose levels elevated in 18 obese diabetic patients (standard error of mean blood glucose 190.8 ± 70.2 mg/dl) compared with 14 controls have a normal weight (standard error of mean blood glucose 81.0 ± 12.6 mg/dl) p < 0.01 (Sindelka, et al., 2002).

Kocak et al. showed that serum glucose levels were significant elevation (p<0.001) for 28 diabetic obese patients (mean BMI = 34.3 ± 2.6 kg/m^2, mean blood glucose = 187.4 ± 78.8 mg/dl) compared with 14 healthy controls (mean BMI = 24.0 ± 1.2, mean blood glucose = 85 ± 7 mg/dl) (Kocak, et al., 2007)

The elevation of glucose level in diabetic patient may be explicated by that the glucose transporter (GLUT4) facilitates the transporting of glucose across plasma membrane into adipocytes and into skeletal muscle cells. This process is subject to stimulation by insulin and it is also considered the rate-limiting step in glucose uptake into those tissue. In pathogenic conditions of metabolic syndrome, obesity, and type 2 (noninsulin-dependent) diabetes, Insulin resistance occurs. The glucose transporter GLUT4 is then down regulated in adipose tissue, resulting in impaired glucose uptake. (Wolf, 2007)

In a normal glycemic individual, in response to raise the blood glucose level, the β −cells of the islets of Langerhans which found within the pancreas, will synthesize and secrete insulin into the blood in a biphasic pattern (Moreira, et al., 2010)

This study showed that glycated hemoglobin HbA1c was significant elevation in group I compared with Control group (p<0.001) also found a significant difference between group II and control group (p <0.001), while a significant difference between the two diabetic groups (p < 0.029). This is consistent with Murugan et al study, they stated in their study the obese diabetic patients have a high HbA1c level compared with the normal control group (p<0.05) and the statistical analysis using one way ANOVA detect that there was significant difference between the two groups p<0.05 (Murugan, et al., 2010). These data mean poor glycemic control and this differs from the results obtained by Chavez et al. they showed that good glycemic control in 39 obese T2DM, HbA1C was 6.5 ± 0.2 % indicating a reasonable level (Chavez, et al., 2009).

Total cholesterol (TC) showed an elevation in diabetic patient groups compared with the control group table (3.3). The current findings

are not consistent with other study that showed no difference in cholesterol levels between 39 obese diabetes group (standard error of mean was 180 ± 6 mg/dl), 17 obese control (standard error of mean was 179 ± 7 mg/dl) and 16 normal control (standard error of mean was 183 ± 7 mg/dl) in Texas (Chavez et al., 2009).

In the current work diabetic groups had a significant differences in serum triglyceride level when compared with normal control group as showed in a paragraph 3.3.2 Furthermore, there was not significant elevation between the two diabetic groups, whereas p<0.001 among all groups according to the F-test, (table 3.3). This results along with other study by Riza et al. that triglyceride levels was highly significant elevated in diabetic obese (standard error of mean BMI was 33.56 ± 0.44 kg/m, standard error of mean TG was 250.26 ± 19.4 mg/dl) compared to normal control (standard error of mean BMI was 24.6 ± 0.61 kg/m2, standard error of mean TG was 108.64 ± 13.91 mg/dl) p<0.05 (Riza, et al., 2009).

Yerlikaya et al found that serum triglyceride of 41 patients was (2.79 ± 1.4 mmol/l) significant elevated (p<0.05) when compared with a 50 normal control group triglyceride level (0.8 ± 0.4 mmol/l) (Yerlikaya, et al., 2013).

High density lipoprotein HDL-c showed an difference in diabetic patient groups compared with the control group and there was no significant difference between the two diabetic groups table (3.3).

These results consent with Wiegand et al. and Muscelli et al. studies who compared on HDL-c levels between normal glucose tolerance of obese control with obese type 2 diabetes (Both groups have a mean of BMI > 30 kg/m^2).

Also they found that there was no significant difference between control serum LDL-c in compared with diabetic group (p>0.05) (Wiegand, et al., 2004), (Muscelli, et al., 2008).

The United Kingdom Prospective Diabetes Study found the beta cell functions were already reduced by fifty percent at diagnosis in addition to a subsequent deterioration appears regardless of therapy. (Group, 1995) So, diabetes mellitus can be qualified as the last stage of chronic pancreatitis (Göke, et al., 2005), on the other hand chronic pancreatitis considered as a risk factor for pancreatic cancer (Lowenfels, et al., 2005)

Carbohydrate antigen CA 19-9 is used mainly in the pancreatic cancer diagnosis but is also utilized as a marker of pancreatic tissue damage that might be caused by diabetes. So, the elevation degree in CA 19-9 level may be helpful in differentiating the pancreatic inflammatory conditions from pancreatic adenocarcinoma. Therefore The evaluation of CA19-9 level in patients with diabetes mellitus makes it imperative to find normal range for CA 19-9 level for these patient to eliminate the additional interventional approaches. (Uygur-Bayramiçli, et al., 2007), (United Healthcare, 2015)

The result of the present study found that patients suffering from type 2 diabetes mellitus have a serum CA19-9 level significantly higher than that in control group. Regression analysis was showed a positive correlation between CA 19-9 and diabetes independent from gender and age, and a positive relationship between CA19-9 level and fasting blood sugar and HbA1c.

Furthermore there is no obvious difference between the patients who are undergoing treatment (have a standard error of mean duration

(mean ± SE) 6.6± 0.9421 years)) and those who did not take the treatment (newly diagnosis patients) which is similar with the study of Benhamou et al. They concluded that CA 19-9 level in diabetic patients is elevated in acute metabolic situations, that correlated very well with the concentration of blood glucose (Benhamou, et al., 1991)

In contrast Banfi et al proved no correlation between biochemical markers of metabolic compensations in diabetes and CA 19-9 level (Banfi, et al., 1996), this study had only 28 sample with non-insulin dependent diabetes and may be this cause makes their result vulnerable to bias.

The present study also agree with Oya Uygur-Bayramiçli et al. study which found that CA 19-9 level was higher in diabetic patients when compared with control individuals and a positive correlation between CA 19-9 and diabetes independent from gender, age, HbA1c level and glucose level. (Uygur-Bayramiçli, et al., 2007)

While M. M. S. Bedi concluded that CA 19-9 level in excess of 300 U/mL in mass lesions in chronic pancreatitis was always indicative of malignancy (Bedi, et al., 2009), chronic pancreatitis related with diabetes and pancreatic cancer are reported to be accompanied by elevated CA19-9 levels (Guo, et al., 2010)

The current study concluded that the cut off value of CA19-9 level in diabetic patients is 49.5 U/ml while Alireza Esteghamati et al. suggest that CA19-9 level more than 10.83 U/mL in the absence of other pathologies are in favor of glycemic impairments. CA19-9 values above 34.30 U/mL may accompany an 84% of diabetic subjects .while CA19-9 values of less than 6.46 U/mL are likely to rule out the presence of diabetes. (Esteghamati, et al., 2014)

In 99.6 % of healthy adult, serum level of CA 19-9 is lower than 37 U per ml. while if the serum level is less than 100 U/ml it is considered as value in benign and malignant diseases which may overlap. In malignant tumors the value of CA 19-9 may be higher than 100,000 U/ml. CA19-9 is neither organ specific nor tumor specific. (Magnani, et al., 1983), (Harmenberg, et al., 1988), (Malati, et al., 1996), , (Duffy, et al., 2000), (Shukla, et al., 2006), (Sturgeon, et al., 2009), (Rückert, et al., 2010).

Enzyme-linked immunosorbent assay (ELISA) is one of immunoassay methods using antibody to capture an antigen (target antigen) then using an enzyme labeled antibody for estimate the antigen amount (Wakabayashi, 2010)

It is widely used as a clinical diagnostic tool to detect a vast range of diseases from infection diseases to cancer biomarkers. ELISA instrument is described as a versatile, precise, quantifiable and sensitive diagnostic method (Crowther, 2001), (Thiha, et al., 2015)

The specific reaction between an antibody (Ab) and an antigen (Ag) is usually driven by two main interacting energy component, electrostatic forces between oppositely charged amino acids and hydrophobic interactions, which are best understood by a study of the thermodynamic parameters of the interaction (Wiseman T., 1989). The equilibrium reaction, termed "biospecific interaction", is characterized by the affinity of reactants to form Ab-Ag complex (Rosier, 2000).

Kinetic studies supplement the information for differences between the initial, final states of each reactant and an intermediate activated complex, (i.e., the pathway taken by the reactants reach the final product) (Seeley, et al., 1980).On the other hand, thermodynamics of the

binding describes the system in its initial, final states. Using kinetic and equilibrium data also determined thermodynamic formation constant.

Several methods are known for calculating the dissociation constant, most of which are based on linearization procedures, such as the Scatchard (1949) plot (Scatchard, 1949), Lineweaver and Burk (1934) plot (Lineweaver, et al., 1934), etc. The main advantage of linearization methods is their simplicity. Previously, a simple linearization procedure was developed to determine the Kd of antigen –antibody (Ag – Ab) interactions (Liliom, et al., 1991), (Orosz, et al., 2002). This linearization does not require direct ligand labelling or the absolute concentration of the complex and is, therefore, especially suitable for Kd determination from an enzyme-linked. immunosorbent assay (ELISA).

Sang-Han Lee et al., 1996 obtained the dissociation constant (Kd) of antigen-antibody complex using Scatchard equation through indirect ELISA (Lee, et al., 1996).

The present study suggest a simple linearization procedure developed to determine the obvious dissociation constant (Kd) of the interaction between antibody and antigen from the data of competitive sandwich enzyme linked immunosorbent assays (ELISA) by Scatchard plot. This is proven by the results referred in paragraph (3.11).

The affinity constant and maximal binding capacity were determined according to Scatchard equation (Laskowski, 1960).

Furthermore when the affinity constant value was available the calculation of enthalpy and entropy be easier using van't Hoff Equation:-

$$\ln Ka = -\frac{\Delta H°}{RT} + \frac{\Delta S°}{R}$$

Where

K_a : Affinity constant.

$\Delta H°$: Standard Enthalpy changes.

R: Gas constant 8.314 JK^{-1} mol^{-1} .

T: Temperature (in kelvin).

$\Delta S°$: Standard entropy change.

The plot of lnK_a ratios vs. the 1/T values gives a linear relationship, the value of enthalpy can be calculated from the slope of the straight line, while the value of the entropy can be calculated from the intercept with the x-axis (WALTER, 1963), (Fadi Bou-Abdallah, 2011).

The Gibb's Energy is a measure of the maximum amount of useful work available from a reaction, when the affinity constant value was available can be calculate standard Gibb's Energy from the equation:-

$$\Delta G° = -RT \ln K_a$$

Where:

$\Delta G°$:- Standard Gibb's Energy

K_a : Affinity constant.

R: Gas constant 8.314 JK^{-1} mol^{-1} .

T: Temperature (in kelvin) (WALTER, 1963).

Ferenc Orosz and Judit Ova´di concluded no linearization procedure has been described for determination of dissociation constant (K_d) from displacement ELISAs (Orosz, et al., 2002).

Liliom, K et al. described a linearization procedure for determination of dissociation constants (K_d) of antigen–antibody

interaction using data from the enzyme-linked immunosorbent assays (ELISA) (Liliom, et al., 1991).

Katsumi discussed the impact of dissociation constant on the standard curve from the below equation of antigen-antibody interaction,

$$\mathbf{Kd = (H - b)x(R - b)/b}$$

$$\mathbf{2b - (Kd + H + R)b + RH = 0}$$

$$b = \frac{\mathbf{Kd + H + R} - \sqrt{(Kd + H + R)^2 - 4HR}}{\mathbf{2}}$$

Where antibody concentration: R, bound antigen concentration: b, initial antigen concentration: H, dissociation constant: K_d (unit:M)

And conclude that the standard curve shift to right depending on the value of dissociation constant (K_d), which indicates the sensitivity of assay is related to K_d. Therefore if K_d is small (the affinity constant is large), the sensitivity becomes excellent.

The effect of divalent cations ($CuCl_2$, $CaCl_2$, $ZnCl_2$, $MgCl_2$ and $MnCl_2$) on CA19-9 Antigen-Antibody binding with type two diabetes mellitus is shown in figure (3.12) which showed that the binding process is sensitive for the cations existence, it is found that Mn (II) at concentration 0.15M increases the binding more than the other divalent cations. While Cu (Ⅱ) in the same concentration increased the binding less than other divalent cations.

Study the effect of divalent cations and different halides on the binding of CA19-9 to its antibody is useful in the future work to obtain the maximum bind and create an optimum conditions for this binding.

One hypothesis presumes that salts may modify the nature of hydrophobic forces controlling the stabilization of the complex formed.

These differences depend on the nature of the interacting groups. (Paul, 2006)

The inhibitory influence of divalent cations is stronger in a medium which has a low concentration of Potassium than in presence of Potassium chloride (20-50 mM). (Demmig, et al., 2014)

The effect of different halides (NaI, NaCl and NaF) on CA19-9 Antigen-Antibody binding with type two diabetes mellitus is shown in figure (3.13). The incubation mixture that contain sodium halides which causes activation of the percent of binding as the following sequence:

<div align="center">

NaF > NaCl > NaI

</div>

Melander and Horvath declared that the influence of halide salt kind on hydrophobic interaction is quantified by the molal surface tension increment (MSTI) that is a measure for the rise in surface tension, by salt. They found too that this parameters increase as the following series:

<div align="center">

NaF > NaCl > NaI

</div>

Also it is found that halides with a high MSTI value will strengthen the hydrophobic interaction while halides with a low MSTI value reverse this effect (Melander, et al., 1977)

On the other hand Edigington reported that chaotropic ions (I^-, Cl^-, F^-, Br^- and SCN^-) distort, especially three-dimensional structure leading to the disruption of antibody-antigen interaction surface. This may occur with high concentrations. (Edigington, 1971)

The strength of halide binding decreased with the size decreases of the anion: F>Cl>Br>I (Nepal, et al., 2015)

The effect of Polyethylene glycol (PEG-6000) on CA19-9 Antigen-Antibody binding with type two diabetes mellitus is shown in figure (3.14)

The effect of Polyethylene glycol on the protein- protein interactions can be interpreted according to the mechanism of steric exclusion which suggested by Laurent, assuming a total fixed volume **(VT)** of solvent occupied by both protein and polymer, and supposing that **(V′)** is the volume occupied by protein while **(VE)** is the volume occupied by polymer (excluded volume i.e., volume not accessible to proteins) The relation **(VT = V′ + VE)** means that any elevation in VE, due to rise in size or number of polymer molecules, forces a reduction in (V′) and an effective raise in the protein molecules concentration. Therefore, the effective protein concentration is increased, VE increases, as well as clash and particles association to form product. The binding continue to decrease. After ten percent concentration when the concentration rise due to enhancing the precipitation of protein because of formation of large insoluble aggregates. (Laurent, 1963), (Deutscher, 1990).

Conclusions :-

1. A higher incidence of serum CA19-9 was found in patients with type 2 diabetes mellitus. It is known that CA19-9 used as a good marker in the diagnosis of pancreatic cancer. This may be indicating that the diabetes has been claimed to be a risk factor for pancreatic cancer, which is increasing its incidence.

2. The serum CA19-9 concentration of 49.5 U/ml was used as cut off value for differentiate between type 2 diabetes mellitus and pancreatic cancer.

   It proposed that a higher cutoff value of CA 19-9 should be used in diabetics to differentiate benign and malignant pancreatic disease, and subtle elevations of CA 19-9 in diabetics should be considered as the indication of exocrine pancreatic dysfunction. CA 19-9 is used in the diagnosis of pancreatic cancer but is also a marker of pancreatic tissue damage that might be caused by diabetes.

3. Regression analysis was showed a positive correlation between CA 19-9 and diabetes independent from gender and age, and a positive relationship between CA19-9 level, fasting blood sugar and HbA1c.

4. The dissociation constant of the interaction between antigen and antibody can be calculated using a new modification for the data of competitive sandwich enzyme linked immunosorbent assay (ELISA) using Scatchard plot by a simple linearization procedure.

5. **a.** The binding process is sensitive for the cations existence, it is found that Mn (II) at concentration 0.15M increases the binding more than the other divalent cations. While Cu (Ⅱ) in the same concentration increased the binding less than other divalent cations.

b. The incubation mixture that contain sodium halides which causes activation of the percent of binding as the following sequence:

<div align="center">

NaF > NaCl > NaI

</div>

c. Polyethylene glycol (PEG-6000) have a negative effect on CA19-9 Antigen-Antibody binding with type two diabetes mellitus.

<u>*Recommendations:-*</u>

It was recommended to:

1. Measure the CA19-9 level for patients with type 2 diabetes mellitus, take medication and have FBS and HbA1c within normal range.
2. Conduct a study aimed to the comparison between the CA19-9 level in patients with type 1 and type 2 diabetes mellitus.
3. Study the effect of the concentration of cations and halides in blood on the CA19-9 Antigen-Antibody binding because that might give false positive or false negative results.
4. The development of enzyme-linked immunosorbent assay through insert external antibody may open up new prospects as a tool research through which study the characterization of antibody- antigen interactions in tumor tissues as well as in serum.

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Questionnaire Information

Code no: **Date:**

Name: **Sex:**

Age:

Height: **Weight:**

BMI:

Blood pressure:

Family history:

Duration of diabetes mellitus:

Diabetes medication:

Other drugs:

Complications:

الــخـــلاصــة

خلفية الدراسة:ـ

يستخدم المستضد الكاربوهيدراتي CA19-9 بشكل واسع في تشخيص انواع مختلفة من الأمراض السرطانية ومن أهم هذه الانواع سرطان البنكرياس و سرطان الجهاز الهضمي العلوي بالإضافة إلى ذلك فإنه يستخدم أيضا كمؤشر لتلف أنسجة البنكرياس التي قد تنجم عن مرض السكري.

مقايسة الممتز المناعي المرتبط بالانزيم (الإليزا) هي إحدى الطرق المستخدمة في قياس مستوى المستضد الكاربوهيدراتي CA19-9 في مصل دم المرضى المصابين بداء السكري من النوع الثاني والتي تم تطويرها بإستخدام ضد خارجي للمستضد الكاربوهيدراتي CA 19-9 (تقنية الإليزا الغير مباشرة التنافسية) وهذه الطريقة وجدت لتكون مناسبة لتعيين ثابت التفكك (K_d) لربط مستضد CA19-9 مع ضده في مرضى السكري من النوع الثاني بإستخدام Scatchard plot.

هدف الدراسة:ـ

تهدف هذه الدراسة لتقييم مستويات مستضد الكاربوهيدرات CA19-9 مصل دم المرضى المصابين بداء السكري من النوع الثاني, بواسطة المقايسة الممتز المناعي المرتبطة بالانزيم (الإليزا) وتحديد المعدل الطبيعي لنفس المستضد في هؤلاء المرضى بالاضافة الى التحقق من إمكانية وجود علاقات محتملة بين CA19-9 من جهة ومستوى السكر في الدم بعد الصيام و نسبة الهيموجلوبين السكري في الدم من جهة أخرى.

هذه الدراسة تقدم طريقة جديدة لتعيين ثابت التفكك (K_d) لربط مستضد CA19-9 مع ضده في مرضى السكري من النوع الثاني بإستخدام Scatchard plot من خلال تطوير تقنية الإليزا (تقنية الإليزا الغير مباشرة التنافسية).

كذلك تهدف الى دراسة تأثير مواد كيميائية مختلفة (الأيونات الموجبة ثنائية التكافؤ و الهاليدات المختلفة و والبولي إثيلين (PEG-6000)) على ربط هذا الربط.

طريقة العمل:ـ

أجريت دراسة مقارنة بين مجموعتي مرضى ومجموعة سيطرة خلال الفترة من شباط 2015 الى آيار 2015, إستندت الدراسة على متابعة 80 مريض (معدل العمر± الخطأ القياسي؛ 46.5625±

1.1054 سنة) قُسموا الى مجموعتين : 40 شخص مصاب بداء السكري من النوع الثاني و يخضعون للعلاج أما المجموعة الثانية فهي عبارة عن 40 شخص مصابين حديثاً بنفس المرض و لم يخضعوا للعلاج بعد, بالإضافة الى 40 شخص سليم (مجموعة السيطرة).

تتضمن الدراسة الحالية قياس مستوى كل من السكر في الدم و الدهون بعد الصيام و مستوى اليوريا والكرياتينين وكذلك مستوى بروتين c التفاعلي في مصل الدم بالإضافة الى نسبة الهيموجلوبين السكري في الدم بإستخدام جهاز المحلل الذاتي و NycoCard reader .

كما تم قياس مستوى مستضد الكاربوهيدرات CA19-9 وتحديد ثابت التفكك لنفس المستضد بطريقة مقايسة الممتز المناعي المرتبط بالانزيم (الإليزا) وقياس تركيز البروتين (بإستخدام طريقة Bradford).

وتم مقارنة النتائج مع نتائج مجموعة السيطرة المؤلفة من 40 شخص سليم كان معدل أعمارهم (44.7±1.4539).

النتائج:-

أظهرت نتائج الدراسة أن المرض المصابين بالسكري من النوع الثاني في كلا المجموعتين (الخاضعين للعلاج و المشخصين حديثاً بداء السكري) يعانون من إرتفاع ملموس في CA19-9 في مصل الدم بالمقارنة مع مجموعة السيطرة، بينما لا يوجد إختلاف ملحوظ بين مجموعتي السكري.

تم إستخدام تركيز **49.5 وحدة \ مل** كمعدل طبيعي للمستضد الكاربوهيدراتي CA19-9 لتشخيص داء السكري من النوع الثاني بحساسية وخصوصية تقدر **88.8%** و**77.5%** على التوالي.

عامل تحويل **1.010388** يمكن استخدامه لتحويل تركيز المستضد الكربوهيدرات CA19-9 من (وحدة \ مل) إلى (ميكروغرام \ مل) والذي تم الحصول عليه بإستخدام طريقة Bradford.

قيمة ثابت التفكك (K_d) للمستضد الكاربوهيدراتي القياسي **100 وحدة \مل** و مريض **99.568 وحدة \مل** يساوي **15.6006 وحدة \مل** و **12.5313 وحدة \مل** على التوالي، في حين تكون قيمته لدى شخص سليم **23.494 وحدة \مل** (من مجموعة السيطرة) هي **4.1271 وحدة \مل**.

الدراسة الحالية تُثبت وجود تأثير للكاتيونات ثنائية التكافؤ إذ أن عملية الربط حساسة لوجود مثل هذه الكاتيونات، فقد تبين أن المنغنيز (II) بتركيز (**0.15 M**) يزيد من عملية الربط أكثر من الكاتيونات

ثنائي التكافؤ الأخرى. بينما النحاس (II) في نفس تركيز يزيد من عملية الربط بنسبة أقل من الكاتيونات ثنائي التكافؤ الأخرى.

كذلك إن للهاليدات المختلفة تأثير على عملية ربط المستضد الكاربوهيدراتي CA19-9 مع ضده، إذ إن الخليط الحاضن الذي يحوي على هاليدات الصوديوم يحدث فيه تنشيط لعملية الربط تبعاً للتسلسل التالي:-

NaF > NaCl > NaI

إن دراسة تأثير الكاتيونات ثنائية التكافؤ و الهاليدات المختلفة على عملية ربط المستضد الكاربوهيدراتي CA19-9 مع ضده يمكن الاستفادة منها في المستقبل للحصول على أقصى حالة إرتباط وكذلك تهيئة ظروف مثلى لهذا الإرتباط.

أيضا البولي ايثيلين (PEG-6000) تؤثر سلبا على إرتباط مستضد CA19-9 مع ضده.

الاستنتاج:-

مستوى CA19-9 لا يقتصر في إرتفاعه على وجود الانسجة السرطانية فحسب بل انه يرتفع في الحالات غير السرطانية أيضاً مثل داء السكري من النوع الثاني نتيجة تلف أنسجة البنكرياس التالفة التي قد تنجم عن مرض السكري.

وثابت تفكك التفاعل بين الأجسام المضادة والمستضد لنفس علامة الورم فيمكن حسابه من بيانات مباشرة، بطريقة المقايسة المناعية المرتبطة بالانزيم (الإليزا) من خلال Scatchard plot.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين \ كلية الطب
فرع الكيمياء والكيمياء الحياتيه

دراسة كيميائية حياتية لإرتباط المستضد الكاربوهيدراتي CA19-9 بضاده الحيوي في مرضى داء السكري من النوع الثاني بإستخدام مقايسة الممتز المناعي المرتبط بالأنزيم مع بعض التحويرات

مقدمة الى مجلس كلية الطب ـ جامعة النهرين كجزء من متطلبات نيل درجة

الماجستير في الكيمياء الطبية

من قبل
رسل رياض عباس
بكالوريوس علوم كيمياء ـ جامعة بغداد 2012

<table>
<tr><td>الإستشاري</td><td>بأشراف</td></tr>
<tr><td>د.محمود شاكر خضير</td><td>د.حسن حسين طه السعيد</td></tr>
<tr><td>2016م</td><td>1437هـ</td></tr>
</table>

CHAPTER ONE

CHAPTER TWO

CHAPTER

THREE

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FOUR

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FIVE

APPENDIX

REFERENCES