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Relationship between Leptin Levels, Lipid Profile and Oxidative Stress in Obese and Lean Libyan Diabetics (Type II)

العلاقة بين مستويات الليبتين وصورة الدهون والإجهاد التأكسدى فى النحفاء والبدناء من مرضى السكرى (الليبيين النوع الثانى)

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Abstract

This research was aimed to study the correlation between Leptin Levels, Lipid Profile and Oxidative Stress to severity and complication of diabetes mellitus type II.

Data were obtained from questionnaire interview, and biochemical analysis of blood samples of 57 type 2 diabetic patients and twenty-two non –diabetic person were included.

Degree of Oxidative Stress was measured in terms of malondialdehyde (MDA) along with lipid profile, serum glucose, urea, and creatinine in diabetes mellitus. It is categorized into non-insulin dependent diabetes mellitus (NIDDM) with complication.

ELISA techniques were used to investigate serum leptin and Malondialdehyde. In addition, urea, creatinine and lipid profile were measured by Enzymatic and colorimetric techniques.

From one hand, a significant effect (1% - 5%) BMI (kg/m^2); creatinine; Leptin (ng/mL) and MDA parameters for female and male in patients group was observed. On the other hand, a not sig. effect was found for the rest of parameters in both sexes in patients group. For the BMI (The value of 29.33 kg/ m^2 for males compared to 33.36 for females) ; creatinine (The value of 0.90 for males compared to 0.73 for females) ; Leptin (The value of 3.97 ng/mL for males compared to 14.71ng/dl for females) ; (The value of MDA 2.19 nmol/ml for males compared to 2.55 nmol/ml for females).

Oxidative stress is raised in type 2 DM patients. According to the result of final stepwise regression it can observed that the factors BMI , Gender, FBS , LDL , HDL , MDA , and AGE affected leptin hormone significantly at level 1% - 5% .

On the other hand, the effect of factors smoking, family history, HbA_{1C}, TG, cholesterol, creatinine, and urea were not significant on leptin hormone.

In addition, the correlation coefficients among studied traits for males and females were estimated.

The conclusion from this study that a positive relationship between Leptin levels and both Lipid Profile and Oxidative Stress in diabetes mellitus type II patients was observed.

Key words: Malondialdehyde, Lipid Peroxide, Diabetes Mellitus, Leptin, Lipid Profile and Oxidative Stress

الملخص

أجرى هذا البحث بهدف دراسة العلاقة بين مستويات هرمون اللبتين و الليبيدات والاجهاد التأكسدي للمرضى الذين يعانون بدرجة كبيرة من مرضى السكر النوع الثاني ومضعفاته. تم تجميع البيانات عن طريق مصدرين المصدر الأول استمارة استبيان والأخر عن طريق اجراء تحليل كيميائي لدم عدد57 مريض يعانون من مرض السكر النوع الثاني وعدد22شخص من الاصحاء وتم تقدير صفات اجهاد التأكسدي عن طريق قياس مالوني دي الدهيد بالإضافة الى قياس سكر جلوكوز في مصل الدم والسكر التراكمي والليبيدات واليوريا والكرياتين. تم استخدام تقنية الاليزا في قياس كل من هرمون اللبتين والمالونيل داى الدهيد. ومن ناحية أخرى تم استخدام تقنية القياس اللوني والانزيمى في تقدير الجلوكوز واليوريا والكرياتنين والليبيدات وقد وجد تأثير معنوي (5% او 1%) لتأثير الجنس على كل من دليل كتلة الجسم والكرياتينين واللبتين نانوجرام /مللى لتر والمالونيل داى الدهيد من ناهية أخرى لم يلاحظ هذا التأثير على باقي المقاييس. فيما يخص دليل كتلة الجسم كان المتوسط 29.33 للذكور مقارنة 33.36 فى الاناث، ومتوسط قيمة الكرياتينين للذكور كان 0.90 مقارنة بالإناث 0.73، وكان متوسط قيمة المالونيل داى الدهيد للذكور 2.19 مقارنة بالإناث 2.55، وبلغت قيمة المتوسط للبتين 3.97 مقارنة بالقيمة 14.7 للإناث. ووجد بتطبيق اجراء الانحدار المرحلى ان هناك تأثير معنوي (1% -5%) لعوامل كل من سكر الجلوكوز والجنس. والمالونيل داى الدهيد والعمر والكولسترول منخفض الكثافة والكولسترول عالى الكثافة على هرمون اللبتين. من ناحية أخرى لم يكن هناك تأثير معنوي للعوامل التالية التدخين والدهون الثلاثية والكولسترول واليوريا والكرياتينين والسكر التراكمى والعامل الوراثى على هرمون اللبتين. وأيضا تم حساب معاملات الارتباط بين جميع الصفات في كل من الذكور والاناث.

نستنتج من هذه الدراسة وجود علاقة موجبة ما بين مستويات كل من هرمون اللبتين واللبيدات والاجهاد التأكسدي للذين يعانون من مرض السكر النوع الثاني ومضعفاته.

Table of contents

List of abbreviations

HDL-C	High-density lipoprotein cholesterol
HbA1c	Glycated haemoglobin
LDL-C	Low-density lipoprotein cholesterol
TG	Triglycerides
DM	Diabetes mellitus
BMI	Body mass index
CVD	cardiovascular disease
IDF	International Diabetes Federation
LepR	leptin receptor
COX	cyclooxygenase
HHT	hydroxyl heptadecatrienoate
VLDL	very low-density lipoproteins
HRP	Horseradish Peroxidase
SST	serum separator tube
ELISA	enzyme-linked immune sorbent assay
SAS	statistical Analysis system
Glucagon- like peptide-1	GLP-1
Centers for Disease Control	CDC
Neuropeptide Y	NYP
BBB	blood-brain barrier

Malondialdehyde	MDA
Reactive oxygen and nitrogen species	ROS/RNS
CE	Cholesterol esterase
CHOD	Cholesterol oxidase
POD	Peroxidase
GLDH	glutamate dehydrogenase
Lower Limit of Detection	LLD
TMB	Tetra methyl benzidine
4-AAP	4-amino-antipyrine
BP	Blood pressure
HMGCoA	3-hydroxy-3-methylglutaryl coenzyme A

List of Tables

List of Figures

Introduction

1.1 Overview

In 2010, diabetes mellitus affects approximately 285 million people worldwide, the incidence is increasing in the developing countries, and this is estimated to reach 435 million by 2030. (Ravipati Sarath and B Rajkuma)

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action or both. Two major forms of diabetes were identified; type 1 and type 2. Lack of or severe reduction in insulin secretion due to autoimmune destructions of β cells is responsible for type 1 diabetes, which accounts for 5-10% of diabetic patients. The more prevalent form type 2 diabetes, accounts for more than 90% of cases (Olefsky *et al*., 2001). Type 2 diabetes usually begins as insulin resistance, a disorder in which the cells do not use insulin properly. As the need for insulin rises, the pancreas gradually loses its ability to reduce it. Lack of insulin action and/or secretion in type 2 diabetes induces hepatic glucose output by inhibiting glycogen synthesis and stimulating glycogenolysis and gluconeogenesis then increased rates of hepatic glucose production result in the development of overt hyperglycemia, especially fasting hyperglycemia (Defronzo *et al.,* 1992). In such conditions, lipolysis in adipose tissue is promoted leading to elevated circulating levels of free fatty acids. Ketones are produced, and are found in large quantities in ketosis, the liver converts fat into fatty acids and ketone bodies, which can be used by the body for energy (Botion *et a*l., 1999). In addition, excess fatty acids in serum of diabetics are converted into phospholipids and cholesterol in liver. These two substances along with excess triglycerides formed at the same time in liver may be secreted into blood in the form of lipoproteins (Jaworski *et al.*, 2007).

Leptin is a small peptide hormone (16-kDa protein) that is mainly, but not exclusively, produced in adipose tissue. The circulating leptin concentration therefore directly reflects the amount of body fat (Merabet et al., 1997) as a small peptide; principally the kidney (Chabova et al., 1999) clears leptin. Not surprisingly, serum leptin concentrations are increased in patients with diabetic nephropathy (El Meligi *et al.*, 2003) .

Leptin, a hormone known mainly for regulating appetite control and energy metabolism, plays a major role in islet cell growth and insulin secretion. This finding opens up new avenues for studying leptin and its role in islet cell biology, which may lead to new treatments for diabetes. (Sarath and Rajkumar, 2011) Leptin can curtail insulin release directly. Nevertheless, there is also a back-door route that researchers are still trying to piece together. Scientists knew that leptin nudges osteoblasts, which manufacture osteocalcin, a protein that stimulates insulin release. Aside from keeping blood sugar and insulin levels down, the rodents that received gene therapy also lived longer than obese rodents that did not. Currently we do not know if that is due to the correction of the diabetes or many of the diseases associated with diabetes. More specifically, interventional studies have demonstrated that several neuroendocrine, metabolic, or immune disturbances in these states could be restored by leptin administration. Extensive studies compounded with clinical trials will be needed to determine long-term safety and efficacy (Sarath and Rajkumar, 2011)

1.2 Objectives.

This study was designed to realize the following aims:

1. To assess serum leptin and other biochemical parameters such as serum glucose, urea, creatinine, cholesterol, triglycerides, high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) and malondialdehyde (MDA) in type 2 diabetic patients.

2. To study the effects of unchanged risk factors Age, mentioned member, Gender, BMI.

3. To investigate the relationships and indications between leptin and other biochemical parameters.

Chapter 3

Background.

2.1.1 Definition of diabetes mellitus

Diabetes mellitus is a chronic disease that affects the lives of millions around the world. It is metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both (International Diabetes Federation, IDF 2006).

2.1.2 Types of diabetes

The most common types of diabetes mellitus are:

2.1.2.1.Type 1 diabetes

It was previously called insulin-dependent diabetes mellitus (IDDM) or juvenile-onset diabetes. Type 1 diabetes develops when the body's immune system destroys pancreatic beta cells resulting in failure of insulin production. This form of diabetes usually strikes children and young adults, although disease onset can occur at any age. Type 1 diabetes accounts for 5-10% of all diagnosed cases of diabetes (olefsky *et al*., 2001).

2.1.2.2. Type 2 diabetes.

It was previously called non-insulin dependent diabetes or adult-onset diabetes Type 2 diabetes results from insulin resistance, a condition in which the body fails to properly use insulin, combined with relative insulin deficiency (Robbins *et al.,* 2004) This form of diabetes accounts for about 90-95% of all diagnosed cases of diabetes. Type 2 diabetes is associated with older age, obesity, history of gestational diabetes, impaired glucose metabolism, physical inactivity, and race/ ethnicity (olefsky *et al*., 2001).

2.1.3 Metabolism in type 2 diabetes.

Circulating glucose is derived from two sources, first intestinal absorption during the fed state in which the rates of gastric emptying determine how quickly glucose appears in the circulation during the fed state, and second Hepatic processes

including glycogenolysis and gluconeogenesis. Renal gluconeogenesis contributes substantially to the systemic glucose pool only during periods of extreme starvation Although most tissues have the ability to hydrolyze glycogen, only the liver and kidneys contain glucose-6-phosphatase, the enzyme necessary for the release of Glucose into the circulation .The rate of glucose entering the circulation balanced by the rate of removal from the circulation. The glucoregulatory hormones of the body are designed to maintain circulating glucose concentrations in a relatively narrow range Glucoregulatory hormone include insulin, glucagon, amylin, and glucagon-like peptide-1 (GLP-1), glucose-dependent insulin tropic peptide (GIP), epinephrine, cortisol, and growth hormone. Of these, insulin and amylin are derived from the β-cells, glucagon from the α-cells of the pancreas, and glucagon- like peptide-1and glucose-dependent insulin tropic peptide from the L-cells of the intestine. In the bi-hormonal model of glucose homeostasis, insulin is the key regulatory hormone of glucose disappearance, and glucagon is a major regulator of glucose appearance.

After reaching a post-meal peak, blood glucose slowly decreases during the next several hours, eventually returning to fasting levels. In the immediate post-feeding state, glucose moved to skeletal muscle and adipose tissue is driven mainly by insulin. At the same time, endogenous glucose production is suppressed by the direct action of insulin on the liver, and the paracrine effect or direct communication within the pancreas between the α- and β-cells, which results in glucagon suppression (Wallum *et al*., 1992).

Type 2 diabetes is a disorder characterized by lack of insulin action and/or secretion that induces hepatic glucose output by inhibiting glycogen synthesis and stimulating glycogenolysis and gluconeogenesis then increased rates of hepatic glucose production result in the development of overt hyperglycemia, especially fasting hyperglycemia (Defronzo *et al*., 1992) . In such conditions, lipolysis in adipose tissue is promoted leading to elevated circulating levels of free fatty acids.

ketone bodies, which can be used by the body for energy (Botion *et al.,* 1999). In addition, excess fatty acids in serum of diabetics are converted into phospholipids and cholesterol in liver. These two substances along with excess triglycerides formed at the same time in liver may be discharged into blood in the form of lipoproteins (Jaworski *et al*., 2007). Several studies showed that cholesterol, triglycerides and LDL-C are elevated in diabetic patients (Barrett-Connor *et al*., 1982). In contrast, other studies documented that HDL-C was decreased (Yassin *et al*., 2011).

2.1.4. Complications of type 2 diabetes mellitus.

Complications of type 2 diabetes include acute and chronic complications. The acute complications comprise diabetic ketoacidosis, hyperosmolar hyperglycemic non-ketotic coma, lactic acidosis and hypoglycemia (Bardin *et al.,* 1994). The chronic complications include cardiovascular disease, peripheral vascular disease, cerebrovascular disease, diabetic retinopathy and diabetic nephropathy (Savage *et al.,* 1996) Other complications include foot problems and leg amputations, skin disorders, decreased cognitive abilities and dementia, sexual dysfunction, pregnancy complications, some types of cancer, yeast infections, urinary tract infections, gingivitis, thrush, tuberculosis and other infections (Debra Manzella *et al.*, 2008)

Lactic acidosis

Lactic acidosis is a form of metabolic acidosis due to the inadequate clearance of lactic acid from the blood. Lactate is a byproduct of anaerobic respiration and is normally cleared from the blood by the liver, kidney and skeletal muscle. Lactic acidosis occurs when the body's buffering systems are overloaded and tends to cause a pH of ≤7.25 with plasma lactate ≥5 mmol/L. It is usually caused by a state of tissue hypoperfusion and/or hypoxia. This causes pyruvic acid to be preferentially converted to lactate during anaerobic respiration. Hyperlactataemia is defined as plasma lactate >2 mmol/L.

Classification

Cohen and Woods devised the following system in 1976 and it is still widely used:

Type A: lactic acidosis occurs with clinical evidence of tissue hypoperfusion or hypoxia.

Type B: lactic acidosis occurs without clinical evidence of tissue hypoperfusion or hypoxia. It is further subdivided into:

Type B1: due to underlying disease.

Type B2: due to effects of drugs or toxins.

Type B3: due to inborn or acquired errors of metabolism.

Causes of lactic acidosis

The list of causes is virtually endless, but the major causes are covered below. Lactic acidosis may occur in conjunction with a wide variety of underlying disease, in extremis, and indeed is a marker for severe progression and deterioration of the primary illness. Lactic acidosis is commonly found in cardiopulmonary failure, other causes of tissue ischaemia, or due to the effects of drugs/toxins/severe illness. A variety of acquired and congenital diseases may cause it, or contribute to its presence in ill patients. Lactic acidosis may occur as a consequence of vigorous or prolonged exercise but is usually of no consequence and self-correcting, unless other pathology such as hyperthermia is present.

Differential diagnosis

Any other cause of metabolic acidosis, particularly those due to diabetic ketoacidosis, other organic acidosis, chronic kidney disease, alcoholic ketoacidosis, hyperosmolar hyperglycaemic non-ketotic coma (HONK), poisoning or drug toxicity.

Complications

1. The major problem is the increasing myocardial suppression that occurs with decreasing blood pH. Avicious cycle of lactic acidosis, further hypoperfusion and multiorgan failure may lead to death.

2. There is an increased risk of a variety of cardiac arrhythmias (Kruse O, Grunnet N, Barfod C; 2011)

2.1.5 Risk factors and symptoms of type 2 diabetes

The most common risk factors for type 2 diabetes comprise obesity, poor diet, sedentary lifestyle, increased age; 21% of people over 60 years have diabetes with family history; diabetes tends to run in families.

Not everyone with type 2 diabetes has symptoms, particularly in the early stages of the disease. In fact, 5.7 million of the 23.6 million people are unaware that they even have disease. Of those, 90 to 95% are those with type 2 diabetes (Centers for Disease Control and Prevention, CDC, 2007). However, type diabetes symptoms may include one or more of the following:

Excessive thirst , Frequent urination , Extreme hunger , Unexplained weight loss , Fatigue, or a feeling of being "run down" and tired , Rapid breathing , Blurred vision , Dry, itchy skin ,Headache,Tingling or burning pain in the feet, legs, hands, or other parts of the body , High blood pressures , Mood swings ,Irritability, depression , Frequent or recurring infections, as urinary tract infections, yeast infections, and skin infections , Slow healing of cuts and bruises (Fujita *et al*., 2009).

2.2 Leptin

Leptin (from the Greek leptos meaning thin) is a protein hormone produced from fat tissue; it is critical in regulating appetite and metabolism**.**

Leptin is a peptide hormone secreted by adipose tissue. It is made up of 167 amino acids with an amino-terminal secretory signal sequence of 21 amino acids. (Glaum *et al.*, 1996).

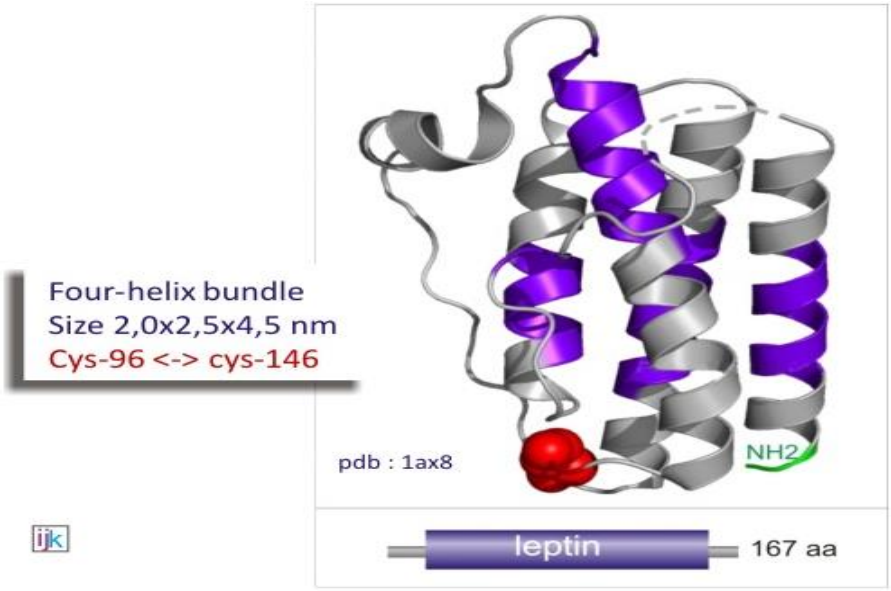
It is a globular protein with a tertiary structure similar to a haemopoitic cytokine. It regulates food intake, body adiposity and reproductive competence, and plays a role in fetal growth; gut derived satiety, immune or proinflammatory responses and angiogenesis and lipolysis (Mantzoro *et al.,* 1998).

2.2. 1 Structure of leptin

Hence, leptin is a globular protein with a tertiary structure (Figure 1) (Isse *et al.*, 1995).

Leptin is an adipocyte-derived hormone that acts as a major regulator for food intake and energy homeostasis. Leptin deficiency or resistance can

Figure 1



result in profound obesity, diabetes, and infertility in humans. Since the discovery of leptin, the breadth of biological actions has dramatically expanded and served to broaden the initial perspective, where this protein was viewed solely as an ant obesity hormone. Important biological activities have been discovered in peripheral tissues that demonstrate the pleiotropic effects of this molecule in such areas as hematopoiesis, angiogenesis, blood pressure, bone mass, lymphoid organ

homeostasis, and T lymphocyte function. Recent data indicate that leptin (116-130), an active fragment of the native molecule, exerts effects similar to those of the native peptide on body weight and food intake. (Isse *e al*., 1995)

2.2.2 Biosynthesis of leptin

Leptin is synthesized mainly in white adipose tissue and is the gene product of the ob gene (MacDougald *et al.*, 1995). White adipose tissue stores energy in the form of triglycerides and releases energy in the form of free fatty acids (Pankov *et al.*, 1996).

On the other hand, brown adipose tissue is responsible for the expenditure of fatty acids derived energy for maintenance of the organism`s thermal stability, dissipating energy as heat A direct correlation has been reported between adipocyte tissue mass and leptin levels. Leptin biosynthesis also occurs in other tissues of the body including: the placenta, fetal tissues and gastric mucosa. (Fielding *et al*., 1996).

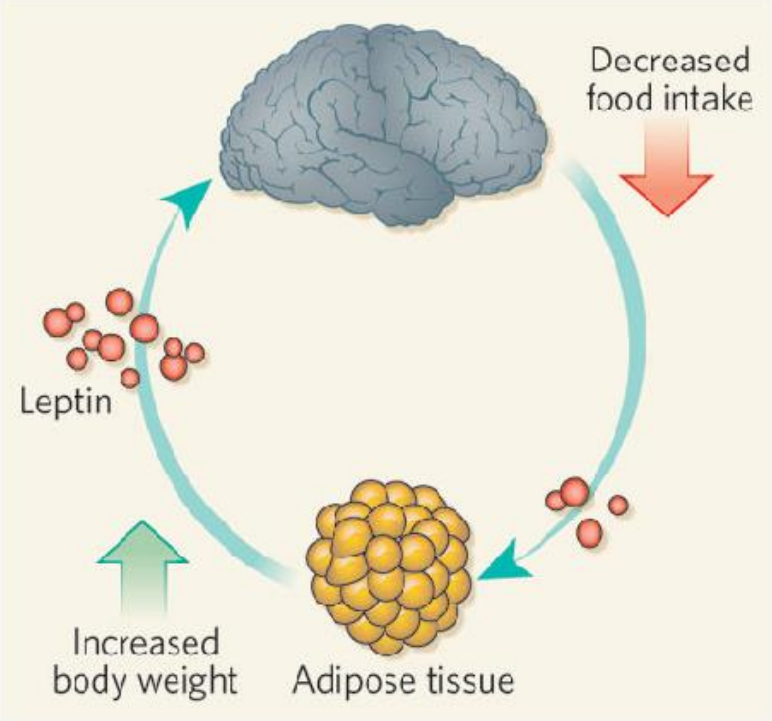


Figure (2-2)

2.2.3 Secretion of leptin

The secretion of leptin is affected by food intake, total body fat and serum levels of several hormones (Mantzoros *et al.,* 1998). Insulin, and to a lesser extent other peptide hormones such as pancreatic hormones including glucagon, amylin and pancreatic polypeptide which reduce food intake, have an influence on the secretion of leptin Insulin is the major regulator of leptin production by adipose tissue (Fried *et al.,* 2000). Infusions of insulin increases circulating leptin levels in humans (Flier *et al.,* 1998). Based on in vitro studies, the effect of insulin in stimulating leptin production appears to involve increased glucose metabolism (Mantzoros *et al.,* 1998). Blockade of glucose transport or glycolysis, inhibits leptin expression and secretion in isolated adipocytes (Mueller *et al.,* 1998). Alterations in insulin-mediated glucose metabolism in adipose tissue are likely to mediate the effects of energy restriction to decrease, and refeeding to increase, circulating leptin levels Changes in glucose metabolism may also explain the observation that high fat meals lower 24h circulating leptin levels relative to high carbohydrate meals in humans, suggesting a mechanism that may contribute to the effects that high fat diets have in promoting increased food intake, weight gain and obesity (Havel *et al*., 2000). The decreased circulating leptin levels observed during energy restriction is related to increased sensations of hunger in human (Mantzoros *et al.,* 1998). Thus, decrease in leptin during energy- restriction and weight-loss regiments might contribute to the strong propensity for weight gain (Flier *et al.,* 1998). Hence, leptin is secreted by adipocytes into the bloodstream and crosses to specific regions in the brain involved in regulating energy balance in the hypothalamus. To reach its target areas, leptin crosses the brain-blood barrier (Burguera *et al.,* 2000).

2.2.4 Transport of leptin

Leptin circulates in blood serum in both free and bound forms the free form is the biologically active form, while the bound form is bound to a carrier protein. The balance between free and bound leptin is a potential regulator of leptin bioavailability (Mantzoro et al., 1998).

2.2.5 Mode of action of leptin

The action of leptin is mediated by binding to various isoforms of the leptin receptor (LepR) that are expressed in a variety of tissues including the brain, ovaries and hemopoitic stem cells (Burguera *et al.*, 2000). After binding to its receptors, leptin alters the release of several neuropeptides, especially neuropeptide Y (NYP) from the hypothalamus (Baskin *et al*., 1999), and in the hypothalamus leptin inhibits the expression of NPY. Neuropeptide Y is a 36 amino acid protein, from the pancreatic peptide family, which acts as a transmitter in the nervous system. It is important due to its appetite stimulating effect. High hypothalamic concentrations of neuropeptide Y elicit food intake, whereas low concentrations have the opposite effect (Wang *et al*., 1997)

2.2.6 Leptin and leptin receptor in obesity

Leptin is the adipose tissue peptide hormone which plays an important role in the regulation of body fat and therefore it was called the obesity hormone (Clement *et al.*, 1998). Since leptin inhibits food intake by its action on Neuropeptide Y (NPY), initially it was believed that reduced leptin levels may be the cause of obesity (Davies *et al*., 1994) and indeed several individuals were identified with low leptin levels (Mantzoros *et al*., 1998).

However, several studies have shown that in most obese individuals, leptin levels are either normal or higher than in normal individuals (Oksanen *et al.*, 1998). These

results indicate that in obese individuals there may be a leptin resistance (Igel *et al.*, 1997), This resistance is believed to play a role in the development of obesity due to the fact that the excess amount of leptin cannot perform its role in controlling food intake, due to the presence of leptin receptor resistance (Oksanen *et al.*, 1998).The leptin resistance is believed to result from genetic defects in the leptin receptor gene, where the product of the gene is a truncated receptor which lacks most of the cytoplasmic region, Hence, individuals with this mutation, tend to eat more than needed and keep gaining weight.

Recent studies have shown that suppressor of cytokine signaling three is a leptin inducible inhibitor of leptin signaling and a potential mediator of leptin resistance in obesity. Leptin resistance can be caused by glucocorticoids, which interfere with the interaction of leptin with its receptor (Mantzoros *et al*., 1998). Abnormal leptin receptor, as well as abnormal leptin catabolism and a decreased blood-brain barrier (BBB) leptin transport have been indicated in obesity development Leptin crosses the BBB by a saturable transport system and elevated endogenous leptin levels during obesity cause an increased competition for binding to the transport system(Burguera *et al*., 2000).

2.2.7 Target tissues of leptin

The target tissues for leptin include the hypothalamus as well as peripheral tissues: skeletal muscle, reproductive organs like the placenta, testis and ovaries (Hoggard *et al.*, 2000). In addition, leptin also acts on the lung, stomach and in macrophages and platelets (O` Rourke *et al.*, 2001; Lu *et al.*, 2000).

2.2.8 Physiological role of leptin

The physiological roles of leptin include the following:

*control of food intake, energy expenditure and lipolysis.

* a role in reproductive competence .

*it plays a role in fetal growth.

*it has a role in immune and proinflammatory responses (Marti *et al.,* 1999).

2.2. 9 Leptin and the development of obesity:

Since body weight is regulated by complex mechanisms involving numerous metabolic and hormonal signals, lepton's mode of action and it`s interactions with other molecules regulating energy homeostasis have been the focus of several studies in order to provide the long needed answers to the pathogenesis of obesity. The result of a recent study was that initially low plasma leptin levels may predispose to future weight gain, due to the lack of control of food intake and energy expenditure by leptin (Mantzoros *et al.,* 1998). Other factors which have the potential of causing a positive energy balance which in turn lead to an increase in body weight include a high fat diet and a low level of physical activity, as well as a low resting metabolic rate (Marik *et al.*, 2000). Leptin`s effect on fat metabolism is that it reduces the synthesis of free fatty acids and triglycerides and increases lipolysis. This effect is brought about by an inhibitory effect on acetyl CoA carboxylase, which is the rate-limiting enzyme in fatty acid synthesis (Marik *et al*., 2000).

2.2.10 Leptin and its role in reproduction and fetal growth:

Leptin has a role in the onset of puberty and the sexual development (Hoggard *et al*., 2001). It was noticed that leptin stimulates the reproductive system in both sexes (Barash *et al.,* 1996), this is through an increased release of the pituitary leutinizing

hormone and the hypothalamic gonadotropin releasing hormone. Based on the findings it had been suggested that food availability is the most important factor influencing mammalian reproduction (Hileman *et al*., 2000). Leptin has a role in regulating growth and promoting hematopoisis in newborn infants, as well as regulating food intake (Mantzoros *et al*., 1999).

2.3. **Malondialdehyde (MDA)**

Malondialdehyde (MDA) is a naturally occurring product of lipid peroxidation; it can also be generated during prostaglandin biosynthesis in cells. MDA reacts with amino groups on proteins and other biomolecules to form a variety of adducts, including adducts with DNA bases that are mutagenic and possibly carcinogenic. Increased levels of lipid peroxidation products. by measurement of MDA, have been associated with various conditions and pathological states of diseases. Therefore, it is important to understand the characteristics and function of MDA as well as the effective techniques of measurement for using MDA as a tool in clinical monitoring of the diabetic patient. (lefevre et al., 1996). There was no significant relationship between malondialdehyde and HbA_1c type 2 diabetes. (Nayereh Parsaeyan and Bemaanali Jalali-Khanabadi, 2011). There is significant weak positive correlation between HbA_1c and plasma levels of malondialdehyde (Mohamed Elrasheed Ibrahim, 2011).

2.3.1 Chemical and biological properties

MDA is also called malonaldehyde or bis (dimethyl acetal). It has the molecular formula $C_7H_{16}O_4$ and molecular weight 164.2. The boiling point is $183^{o}C$, and the freezing point is $130^{o}C^{1}$. It was suggested that the bulk of MDA in human plasma

is bound to protein; this would explain the very low levels of MDA in plasma as measured under standard assay conditions (Lefevre *et al.*, 1996)

2.3 .2 Biological and health aspects

MDA can be found in most biological samples including foodstuffs, serum, plasma, tissues and urine, as a result of lipid peroxidation. The distribution of plasma lipid peroxides in men and women, determined by MDA, was found to be well approximated by a normal distribution. The median level increased by about 10% between 30 and 70 years of age in both sexes, which may be relevant to the increasing prevalence of atherosclerosis with age

(Rumley *et al.*, 2004)

2.4.1 Obesity and risk factors affected it.

Obesity results from interactions between multiple genes and the environment. It does not follow a clearcut pattern of inheritance, but concentrates in families. Extensive studies on twins have confirmed it`s genetic aetiology, where it has been shown that several genes (polygenic) contribute to susceptibility to development of obesity in presence of predisposing environmental factors (Bougneres *et al.,* 2002). Parents obesity is the most important risk factor for childhood obesity. Obesity is on the rise in children and in a study on Saudi children, 1-15 years old, 10.68 %boys and 12.7 % grills were overweight, while 5.9 % and 6.74 % respectively, were reported as obese (EL-Hazmi *et al*., 2001).

Some of the environmental factors which contribute to obesity development, include: eating habits and lack of physical activity, as well as some diseases and drugs that might cause obesity (Chagnon *at al*., 1999). Several genetic risk factors contributing to the development of obesity have been investigated in recent studies, and a number of genetic defects in development of obesity in humans have been identified. These include defects in leptin gene, pro-opiomelanocortin gene, insulin

gene and the gene which encodes for the leptin receptor (Arner et al., 2000).

2.4.2 Lipid levels in obesity

Obesity is a risk factor for cardiovascular disease (CVD) which has a high mortality rate in the world (Haffner *et al.*, 1998). The link between obesity and CVD are the elevated lipid levels in the serum (especially triglycerides & cholesterol) (Alexander *et al.*, 2003). The high levels of cholesterol predispose to several health problems including coronary heart disease, atherosclerosis and gall stones. The main step which triggers the development of atherosclerosis is the deposition of cholesterol-ester filled macrophage foam cells, which contribute to the formation of the atherosclerotic plaque (O`Rourke et al., 2001). Gallstones are formed as the bile of obese people is saturated with cholesterol and hence is liable to form stones (Mayes *et al.*, 1988). The cause of this saturation is the increased hepatic production of cholesterol as well as low HDL-C levels, which cause the accumulation of cholesterol in the bile of the affected individuals; these are accompanied by hypertriglyceridemia. In addition, the risk of cancer is increased possibly due to the increase in sex hormones (Machinal *et al*., 1999). Elevated plasma levels of triglycerides are seen in obesity and in type 2 non-insulin-dependent diabetes mellitus, which are conditions, associated with coronary heart disease development (Szapary *et al.*, 2002). The link between obesity, diabetes and triglycerides is a pancreatic β-cell dysfunction, which is caused by the excessive amounts of triglycerides A study has revealed the role of leptin in the depletion of triglycerides in all cells which express the leptin receptor at its surface via inducing an increase in free fatty acid (FFA) oxidation and a decrease in esterification. This action of leptin is absent in obesity due to leptin resistance (Shimabukuro *et al*., 1997).

2.4.3 Dietary lipids, lipid digestion and transport

An adult ingests about 60-150 g of lipids per day, of which more than 90% is triacylglycerols, the remainder is made up of cholesterol, cholesteryl esters, phospholipids and free fatty acids (Champe and Harvey, 1994).

The digestion of lipids begins in the stomach by an acid stable lipase and continues in the small intestine (duodenum) through emulsification by bile salts and degradation by pancreatic enzymes. Triacylglycerols are degraded by the action of an esterase which removes the fatty acids at carbons 1 and 3. Cholesteryl esters are hydrolyzed by pancreatic cholesteryl ester hydrolase.

2.5. Lipids related to obesity

The lipids that are related to obesity are mainly triglycerides, cholesterol and their transport forms HDL and LDL-cholesterol

2.5.1 Triglycerides

Triglycerides are the chemical form of lipids which exists in food as well as in the body. They are also present in blood plasma and in association with cholesterol from the plasma lipids. Triglycerides in plasma are derived from fats eaten in food (triglycerides are found in both plant and animal sources mainly in meat and fats like Crisco and puritan oil) or are made in the body from other energy sources like carbohydrates . Calories ingested in a meal and not used immediately by tissues are converted to triglycerides and transported to adipocytes to be stored (Mayes *et al., 1988*).

2.5.2 Structure of triglycerides:

Triglycerides are also called neutral fats, and are esters of the alcohol, glycerol with fatty acids. They are nearly all mixed acylglycerols, with three different fatty acids. The proportion of triglycerol molecules containing the same fatty acid residue in all 3 ester positions is very small (Mayes *et al* 1998).

2.5.3 Regulation and processing of triglycerides:

There are two sources for the triglycerides: dietary and endogenous sources. The level of triglycerides is regulated by hormones, which regulate the synthesis and release of triglycerides from fat tissue so they meet the body`s needs for energy between meals. Dietary triglycerides mainly come from eating animal products and saturated fat. In the body, triglycerides are mainly synthezised in the liver by two pathways. The first is initiated by the synthesis of glycerol phosphate from glucose and in the second glycerol is converted to glycerol phosphate by glycerol kinase; the lipoproteins are released into the circulation, and are delivered to the cells of the body. (Champe and Harvey 1994). Triglycerides are released from the chylomicrons in skeletal muscle and adipose tissue and degraded to free fatty acids and glycerol by lipoprotein lipase. Cholesterol in the form of cholesteryl esters in chylomicrons is taken up by the liver and hydrolyzed to its component parts.

2 .6 Cholesterol

Cholesterol is a waxy- like compound that belongs to a class of molecules called steroids. It is widely distributed in all cells of the body, but particularly in nervous tissue. It is a major component of the plasma membrane and of plasma lipoproteins. Cholesterol has two sources: dietary and endogenous. It is present in animal products, like eggs, meat and cheese and is synthezised in the human body by all tissues mainly the liver,

intestine, adrenal cortex and reproductive tissues.

2. 6.1 Structure of cholesterol:

Cholesterol is a 27-carbon atom compound designated as 3-hydroxy-cholestene. It consists of four fused rings with the carbons numbered in sequence and an eight-membered branched hydrocarbon chain attached to the D ring (Mayes *et al* 1988).

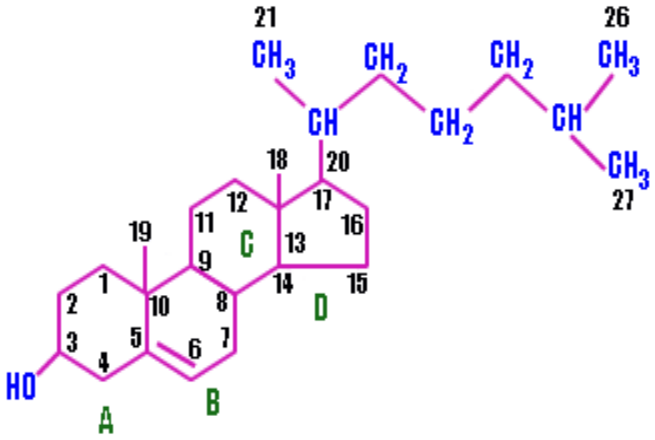


Figure **(2-3): Structure of cholesterol**

2.6.2.Functions of cholesterol:

Cholesterol is a lipid which has no role in providing energy unlike triglycerides However, it is essential for: - formation and maintenance of cell membranes (helps the cell to resist changes in temperature and protects and insulates nerve fibers). - formation of sex hormones (progesterone, testosterone, estradiol) (Champe and Harvey, 1994)

- production of bile salts, which help to digest food.- conversion into vitamin D in the skin when 7-dehydrocholesterol is exposed to sunlight. (Mayes *et al* 1988)

2.6.3. Synthesis of cholesterol:

Cholesterol is mainly synthsized in the liver (1000 mg/day), but also in cells lining the small intestine. The precursor of cholesterol is acetyl CoA and isconverted to 27 carbon cholesterol by a series of complex reactions involving the following steps:

Acetyl CoA (C2) → mevalonate (C6) → isopentenyl pyrophosphate (C5) → sqalene (C30) → Cholesterol (C27).

18Acetyl CoA + 18ATP + 16NADPH + $4O_2$ → Cholesterol + $9CO_2$ + 16NADP + 18ADP + 18P

The pathway can be described in four stage as the following:

The first stage: It is the formation of HMG CoA by the sequential condensation of three molecules of acetyl CoA. **The second stage:** This stage is the conversion of HMG CoA to two types of activated isoprenoids, is Isopentyl pyrophosphate and di methyl allyl pyrophosphate. The synthesis of each isoprenoid requires three molecules of ATP. These C5 compounds readily polymerize and are used as building blocks in several synthetic pathways.

The third stage: It is the condensation of six isoprenoids to form squalene, a process that involves C10 and C15 intermediates. **The fourth stage:** The last stage is the conversion of squalence a linear C30 compound, to cholesterol, a cyclic C27 compound. Lanosterol is the first intermediate with a steroid nucleus

2.6.4 Relation between cholesterol and obesity

Elevated cholesterol levels are a major risk factor for coronary heart disease. There are two kinds of cholesterol: good cholesterol which is carried in HDL, and bad cholesterol which is carried in LDL. Cholesterol levels and LDL –cholesterol

are increased in obesity due to the high fat intake of these individuals and their lack of movement, so they are at a high risk of heart disease. When LDL levels are too high, the LDL tends to stick to the lining of the blood vessels, leading to the stimulation of atherosclerosis or hardening of the arteries. Atherosclerosis plaques cause narrowing of the arteries and lead to heart attacks and strokes, whereas, increased levels of HDL cholesterol are associated with a lower risk of heart disease. Thus, HDL cholesterol appears to be a good protector against heart disease (Anderson *et al*., 1997). There is some evidence that the HDL molecule scours the walls of blood vessels, and cleans out excess cholesterol which is transported to the liver for further processing. Elevated cholesterol levels can be caused by several factors including:

(i) Hereditary, eg familial hypercholesteanemia.

(ii) A diet high in saturated fat and cholesterol.

(iii) Being overweight increases LDL cholesterol and decreases HDL cholesterol.

(iv) Being sedentary increases LDL cholesterol and decreases HDL cholesterol.

(v) Age: cholesterol levels increase with age, beginning at about 20 years

(vi) gender: females prior to menopause have cholesterol levels lower than men at the same age but when menopause occurs their LDL cholesterol levels increase as does the risk of heart disease.

There are also some secondary causes for elevated levels of cholesterol. I.e. some people have elevated cholesterol levels as a result of specific diseases or medical conditions. In these individuals, treating the underlying medical problem often results in an improvement in cholesterol levels, thus, any patient whose cholesterol levels are elevated should be screened for one of the causes of secondary lipid disorders. These causes are diabetes, hypothyroidism, obstructive liver disease, chronic renal failure and drugs (anabolic steroids, progesterone drugs and corticosteroids) (Mayes *et al*., 1988)

2.7. Relation of triglycerides and cholesterol with obesity

Obesity in most individuals is linked to an elevated level of both triglycerides and cholesterol. Since the elevated levels of cholesterol (hypercholesterolemia) may have a health threatening effect due to the fact that it is a component of atherosclerotic plaques and that it elevates the individuals risk of coronary heart disease. In addition, it also increases the risk of formation of gallstones.

Excess triglycerides in plasma is called hypertriglyceridemia. It is linked to the occurrence of coronary artery disease in some individuals. Elevated triglycerides may be a consequence of other diseases, such as untreated diabetes mellitus, by accumulating in pancreatic β-cells and causing it`s dysfunction (Shimabukuro *et al*., 1997). Like cholesterol, increases in triglyceride levels can be detected by plasma measurements. Very high triglycerides can cause pancreatitis, an enlarged liver and spleen, and fatty deposits in the skin called xanthomas. It was reported that the levels of HDL-cholesterol decreases consistently with increasing BMI, while the levels of LDL-cholesterol and triglycerides and the ratio of total cholesterol to HDL-cholesterol increased steadily (Reeder *et al* 1992).

2.8. OVERVIEW OF DIABETES (TYPE 1&2) AND OXIDATIVE STRESS

The human body is exposed to free radicals from outside the body (exogenous) and inside the body (endogenous). Some of the factors that lead to free radicals are smog, cigarette smoke, radiation, consumption of excessive amounts of alcohol, and even sunlight. Yet, some factors that led to free radicals come from within the body.

The cells necessitate oxygen to produce the energy they need to work properly. In the process known as mitochondrial respiration, the cells take in oxygen, burn it, and release energy. During the process, free radicals are produced. Oxidative stress occurs when free radical production exceeds the body's ability to neutralize them. This imbalance happens for one of two reasons: a) when the antioxidant production is decreased, or, b) when the free radicals are produced in excess. For instance diabetes, or the aging process itself, can direct to increased speed of the production of these endogenous free radicals In addition, reduced antioxidant resistance. Oxidative stress functions on both sides, meaning that it help the progression and the development of diabetes and its complications.

In the study of Ha et al., 2000 it was shown that oxidative stress is one of the important mediators of vascular complications in diabetes including nephropathy. High glucose produces reactive oxygen species as a result of glucose auto-oxidation, metabolism, and the development of advanced glycation end products. (Ha and Lee *et al.*, 2000).

2 .8.1 Oxidative stress and the onset of diabetes

Type 2 diabetes mellitus is characterized as having insulin resistance and hyperglycemias and treatment of insulin is not very effective in reducing the plasma glucose levels in type 2 diabetes patients. Experimental diabetes can be induced in rodents by feeding alloxan or streptozotocin. Evidence showed that alloxan and

strptoxotocin caused diabetes by generating reactive oxygen species (Grankvst et al., 1981)

2 .8.2 Oxidative stress and type 2 diabetes mellitus

A large genetic component also exists in type 2 diabetes mellitus, and the concordance rate in identical twins is around 90%. This suggests that genes essentially determine the disease in the appropriate environment. Insulin resistance is one of the major characteristics of type 2 diabetes mellitus. If the insulin resistance can result from oxidative damage, then a prediction would be that chronic oxidative stress would lead to hyperinsulinaemia if plasma glucose is clamped at normal level by infusing the required insulin. Following experiment supports this hypothesis. Fat-fed mice, infused with insulin and glucose, showed impaired glucose clearance. However, glucose clearance was slowed 2-3 fold further by prior feeding with low-dose of streptozotocin, which had little hyperglycaemic effect by itself and led to chronic oxidative stress.

The streptozotocin effect was presumably not on the β-cell with continuous supplement with external insulin, but seemed to have caused insulin resistance directly. Evidence showed that membrane proteins are early targets of oxidative stress. An early event in the induction of the multiple low dose of streptozotocin diabetes is the gradual loss of GLUT2 glucose transporter from islet cell membranes, which could cause insulin resistance (Wang *et al.*, 1998)

2.8.3 External Factors of Oxidative Stress

Free radicals occur naturally within the body, and for the most part, the body's natural antioxidants can manage their detoxification. But, there are certain external factors that can trigger the production of these damaging free radicals. These factors include:

• Excessive exposure to UV rays
• Pollution
• Smoking
• Eating an unhealthy diet
• Excessive exercise
• certain medications and/or treatments

2. 8.4 Oxidative Stress and the Central Nervous System

Oxidative stress takes place within the brain while the production of reactive oxygen species take precedence over the capability of the endogenous antioxidant structure to eliminate excess reactive oxygen species afterwards directing to cellular injury. Cellular characteristics of the brain indicate that this process is very delicate to oxidative stress. The brain, for instance, needs a very high amount of oxygen in order to work. It necessitates about 20% of oxygen for the brain located in the entire body. Furthermore, the brain tissue includes a large amount of unsaturated fatty acids, which are metabolized through oxygen free radicals and therefore, the brain includes high percentages of iron, which have been connected to free radical damage (Miyajima *et al.,* 2003).

Chapter 3

Materials and Methods

3.1 **Target population**

The target population was type 2 diabetic males and females aged 30- 70 years from a hospital in Diabetic and Endocrine Canter in Tripoli.

3.2 Sample size

The Sample size was 57 type 2 diabetic males and females 22 healthy males and females served as controls. Patients and controls were matched for age.

3.3 Sampling

A total of 57 blood samples were collected from type 2 diabetic patients, which were previously diagnosed according to the current WHO diagnostic criteria for diabetes (World Health Organization, 2006), from the a hospital in the Diabetic and Endocrine Canter in Tripoli. The 22 healthy person of blood samples were also collected from healthy persons who were served as controls in Tripoli .

3.4 Storage of Plasma

Samples were taken from patients diagnosed with type II diabetes in a hospital Diabetic and Endocrine Canter in Tripoli and then stored blood bank in Sabratha temperature of -20 ° C to measure the of serum leptin

3.5 Data collection

3.5.1 Questionnaire interview

A meeting interview was used for filling in the questionnaire, which designated for matching the study need. All interviews were conducted face to face by Dr. Abd-ulkarim Zwawi

3.5.2 Body mass index

Body mass index (BMI) was calculated as the ratio of body weight in Kg/height in meter square. The subjects were asked to remove shoes and heavy clothes before measurement of weight and height. Participant with BMI=18.5−24.9 kg/m² was considered to have normal weight, Participants with BMI=25.0−29.9 kg/m² was classified overweight, Participants with BMI≥30.0 kg/m² was considered obese (WHO, 2000).

3.6. Materials

The following chemicals and kits were used during the course of this study.

3.9.1 Chemicals and reagents

Chemicals and reagents used in this study are shown in the following Table:

Reagent	Supplier
Glucose	EKF Diagnostics,Biosen C-Line
HbA1c	COBAS INTEGRA® 400 plus
Urea	COBAS INTEGRA® 400 plus
Creatinine	COBAS INTEGRA® 400 plus
Cholesterol	COBAS INTEGRA® 400 plus
Triglycerides	COBAS INTEGRA® 400 plus
LDL	COBAS INTEGRA® 400 plus
HDL	COBAS INTEGRA® 400 plus
Leptin	Single humareader human company
MDA	Single humareader human company

3.6. The Roche Cobas Integra 400 plus:

The Roche Cobas Integra 400 plus chemistry analyzer is used for diagnostic clinical chemistry testing. Classic chemistry, electrolytes, specific proteins, therapeutic drug monitoring, drugs of abuse, and thyroid hormone testing are consolidated into one system with one reagent cassette design. The instrument carries out all test orders automatically and is equipped with measuring modules:

- FP photometer → Fluorescence polarimetry
- Absorbance photometer → Absorbance photometry

ISE (Ion-Selective Electrode) →module Ion selective potentiometry
Samples are automatically transferred from a sample tube or cup to the module where the measurements are made. All optical measurements use the same transparent plastic containers, called cuvettes.

3.6.1 Determination of serum Glucose

Principle

Enzymatic reference method with hexokinase. (bondar RJ and mead DC1974) Hexokinase (HK) catalyzes the phosphorylation of glucose by ATP to form glucose-6-phosphate and ADP. To follow the reaction, a second enzyme, glucose-6-phosphate dehydrogenase (G6PDH) is used to catalyze oxidation of glucose-6-phosphate by NAD+ to form NADH.

$$\text{D-glucose + ATP} \xrightarrow{HK} \text{D-glucose-6-phosphate + ADP}$$

$$\text{D-glucose-6-phosphate} \xrightarrow{G6PDH} \text{D-6-phosphogluconate + NADH + } H^{+}$$

The concentration of the NADH formed is directly proportional to the glucose concentration. It is determined by measuring the increase in absorbance at (340) nm.

Reagents

Concentrations are those in the final test mixture.

Reagents	Concentrations
B:	
MES buffer	0.5mmol/L
pH	6
Mg^{++}	24 mmol/L
ATP	≥ 4.5 mmol/L
NADP	≥ 7.0 mmol/L
C:	
HEPES buffer	200mmol/L
Mg^{++}	4 mmol/L
pH	8
HK (yeast)	≥ 300 µkat/L
G6PDH	≥ 300 µkat/L

3.6.2 Determination of HbA_1c:

Principle

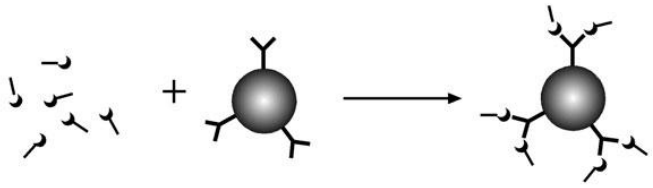
Total Hb and HbA_1c concentrations are determined after hemolysis of the anti-coagulated whole blood specimen. Total Hb is measured colorimetric ally. HbA1c is determined immunoturbidimetrically. The ratio of both concentrations yields the final percent HbA1c result [HbA_1c (%)].

The anti-coagulated whole blood specimen is hemolyzed automatically on COBAS INTEGRA systems with HbA_1c Hemolysis Reagent in the predilution cuvette. Erythrocytes are lysed by low osmotic pressure. The released Hb is proteolytically degraded by pepsin, to make the β-N-terminal structures more accessible for the immunoassay. Additionally, the heme portions are oxidized for the Hb assay.

Total Hb is determined on COBAS INTEGRA systems in the hemolysate using a cyanide-free colorimetric method based on the formation of a brownish-green

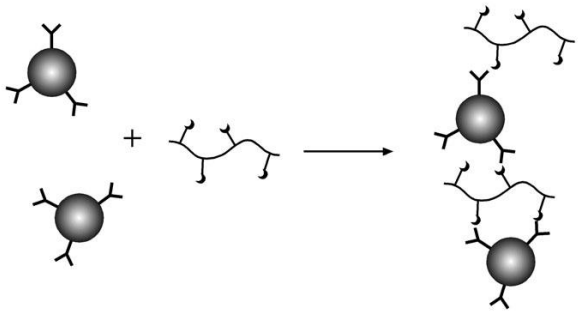
chromophore (alkaline hematin D-575) in alkaline detergent solution.(coleman *et al* 1978) The color intensity is proportional to the Hb

concentration in the sample and is determined by monitoring the increase in absorbance at 552 nm. The test result is calculated using a fixed factor determined from the primary calibrator chlorohemin.(world health organization, 2006). HbA_1c is measured on COBAS INTEGRA systems using monoclonal antibodies attached to latex particles. The antibodies bind the β-N-terminal fragments of HbA_1c.



Glycopeptides Antibody Latex Bound Glycopeptides

Remaining free antibodies are agglutinated with a synthetic polymer carrying multiple copies of the β-N-terminal structure of HbA_1c. The change in turbidity is inversely related to the amount of bound glycopeptides and is measured turbid metrically at 552 nm.



Antibody Latex Agglutinator Agglutinated Antibody Latex

Reagents

Concentrations are those in the final test mixture.

Reagents	Concentrations
B:	
MES buffer	0.025mmol/L
TRIP buffer	0.015mmol/L
pH	6.2
HbA1c antibody (ovine serum)	≥ 0.5mg/mL
C:	
MES buffer	0.025mmol/L
TRIP buffer	0015mmol/L
pH	6.2
HbA1c polyhapten	≥ 8µg/mL

3.6.3 Determination of serum cholesterol

Principle

Determination of cholesterol after enzymatic hydrolysis and oxidation. The colorimetric indicator is quinoneimine, which is generated from 4-aminoantipyrine and phenol by hydrogen peroxide under the catalytic action of peroxidase.

Cholesterol ester + H2O $\xrightarrow{CHE}$ cholesterol + fatty acid

Cholesterol + O2 $\xrightarrow{CHO}$ cholesterol-3-one + H2O2

H2O2 + 4- aminoantipyrine + Phenol $\xrightarrow{POD}$ Quinoneimine + 4 H2O

Reagents

Concentrations are those in the final test mixture.

Reagents	Concentrations
B:	
PIPES buffer	225 mmol/L
Mg^{2+}	10 mmol/L
Sodium cholate	0.6 mmol/L
4-amino-antipyrine	≥ 0.45 mmol/L
Phenol	≥ 12.6 mmol/L
Fatty alchol polyglycol ether	3%
CE Cholesterol esterase	≥ 25μkat/mL (microb)
CHOD Cholesterol oxidase	≥ 7.5 μkat/mL (E.coli)
POD Peroxidase	≥ 12.5 μkat/mL (horseradish)
C:Empty	

3.6.4 Determination of serum LDL

Principle

Homogeneous enzymatic colorimetric assay. This automated method for the direct determination of LDL-cholesterol takes advantage of the selective micellary solubilization of LDL-cholesterol by a nonionic detergent and the interaction of a sugar compound and lipoproteins (VLDL and chylomicrons). When a detergent is included in the enzymatic method for cholesterol determination (cholesterol esterase

cholesterol oxidase coupling reaction), the relative reactivities of cholesterol in the lipoprotein fractions increase in this order: HDL < chylomicrons < VLDL < LDL. In the presence of Mg++, a sugar compound markedly reduces the enzymatic reaction of the cholesterol measurement in VLDL and chylomicrons. The combination of a sugar compound with detergent enables the selective determination of LDL-cholesterol in serum. In the presence of oxygen, cholesterol is oxidized by cholesterol oxidase to Δ4-cholestenone and hydrogen peroxide.

This direct assay meets the 1995 NCEP goals of <4% Total CV, bias ≤4% versus reference method, and ≤12% total analytical error.

LDL-cholesterol ester + $H_2O \xrightarrow{detergent\,cholesterolesterase}$ cholesterol + free fatty acid (selective micellary solubilization)

LDL-cholesterol + $O_2 \xrightarrow{cholesteroloxidase}$ Δ4-cholestenone + H_2O_2

2 H_2O_2 + 4-aminoantipyrine + HSDA + H^+ + $H_2O \xrightarrow{peroxidase}$ purple blue pigment + 5 H_2O

Reagents

Concentrations are those in the final test mixture.

Reagents	Concentrations
B:	
MOPS	20.1mmol/L
pH	6.5
HSDA	0.958mmol/L
AOD(recomb)	≥ 50µkat/mL
POD(horseradish)	≥ 167µkat/mL
C:	
MOPS	20.1mmol/L
pH	6.5
POD(horseradish)	≥ 334µkat/mL
Mg_2SO_4. 7H2O	8.11mmol/L
4-aminoantipyrine	2,46mmol/L
CE(microb.)	≥ 50µkat/mL
CHOD(microb.)	≥ 33µkat/mL

3.6.5 Determination of serum HDL

Principle

Homogeneous enzymatic colorimetric assay.

In the presence of magnesium sulfate and dextran sulfate, water-soluble complexes with LDL, VLDL, and chylomicrons are formed which are resistant to PEG-modified enzymes. The cholesterol concentration of HDL-cholesterol is determined

enzymatically by cholesterol esterase and cholesterol oxidase coupled with PEG to the amino groups (approximately 40%). Cholesterol esters are broken down quantitatively into free cholesterol and fatty acids by cholesterol esterase. In the presence of oxygen, cholesterol is oxidized by cholesterol oxidase to Δ4-cholestenone and hydrogen peroxide. (Sugiuchi , et al 1995). This direct assay meets the 1995 NCEP goals of 13% total analytical error. (Kimberly M, 1999).

HDL-cholesterol esters + $H_2O \xrightarrow{PEG-cholesterol\ esterase}$ HDL-cholesterol + RCOOH

HDL-cholesterol + RCOOH+$O_2 \xrightarrow{PEG-cholesterol\ oxidase}$ Δ4-cholestenone + H_2O_2

2 H_2O_2 + 4-aminoantipyrine + HSDA +$H^+ \xrightarrow{peroxidase}$ purple blue pigment + 4 H_2O

The color intensity of the blue quinoneimine dye formed is directly proportional to the HDL-cholesterol concentration. It is determined by measuring the increase in absorbance at 583 nm.

Reagents

Concentrations are those in the final test mixture.

Reagents	Concentrations
B:	
MOPS	20.1mmol/L
pH	6.5
HSDA	0.958mmol/L
PEG	≥ 167µkat/mL
C:	
pH	6.5
4-aminoantipyrine	2,46mmol/L
CE (microb.)	≥ 50µkat/mL
CHOD (microb.)	≥ 33µkat/mL

3.6.6 Determination of serum triglycerides

Principle

Determination of triglycerides after enzymatic splitting with lipoprotein lipase Indicator is quinoneimine which is generated from 4-aminoantipyrine and 4-chlorophenol by hydrogen peroxide under the catalytic action of peroxidase.

Triglycerides $\xrightarrow{LPL}$ Glycerol + fatty acid

Glycerol + ATP $\xrightarrow{GK}$ Glycerol-3-phosphate + ADP

Glycerol-3-phosphate + O2 $\xrightarrow{GPO}$ Dihydroxyaceton phosphate + H2 O2

2H2O2 + 4-Aminophenazone + 4-Chlorophenol $\xrightarrow{POD}$ Quinoneimine + HCI + 4H2O

Reagents	Concentrations
B:	
Pipes	50mmol/L
pH	6.8
Na-cholate	0.20mmol/L
ATP	1.4mmol/L
4-aminophenazone	≥0.13mmol/L
4-Chlorophenol	4.7mmol/L
LPL(microb.)	≥83 µkat/L
GK(microb.)	≥3 µkat/L
GPO(E.coli)	≥41 µkat/L
POD (Horseradish)	1.6 µkat/L
C: empty	

3.6.7 Determination of serum creatinine

Principle

Creatinine forms a colored orange-red complex in an alkaline picrate solution. The difference in absorbance at fixed times during conversion is proportional to the concentration of creatinine in the sample.

Creatinine + Picric acid → creatinine picrate complex

Reagents

Concentrations are those in the final test mixture.

Reagents	Concentrations
B:	
Potassium hydroxide	900 mmol/L
Phosphate	135 mmol/L
pH	≥ 13.5
C:	
Picric acid	38 mmol/L
pH	6.5

3.6.8 Determination of serum Urea

Principle

Kinetic test with urease and glutamate dehydrogenase.

Urea is hydrolyzed by urease to form ammonium and carbonate. In the second reaction 2-oxoglutarate reacts with ammonium in the presence of glutamate dehydrogenase (GLDH) and the coenzyme NADH to produce L-glutamate. In this reaction two moles of NADH are oxidized to NAD for each mole of urea hydrolyzed.

$$\text{Urea} + 2\,H_2O \xrightarrow{urese} 2\,NH_4^+ + CO_3^{-2}$$

$$NH_4^+ + \text{2-oxoglutarate} + NADH \xrightarrow{GLDH} \text{L-glutamate} + NAD^+ + H_2O$$

The rate of decrease in the NADH concentration is directly proportional to the urea concentration in the specimen. It is determined by measuring the absorbance at 340 nm.

4.6.7 Determination of serum Malondialdehyde

Human Malondialdehyde (MDA) mybiosource ELISA Kit

For the quantitative determination of endogenic human malondialdehyde (MDA) concentrations in serum, urine, tissue homogenates. This package insert must be read in its entirety before using this product**.**

PRINCIPLE OF THE ASSAY

This assay employs the competitive inhibition enzyme immunoassay technique. Antibody specific for MDA has been pre-coated onto a micro plate. Standards and samples are pipetted into the wells with a Horseradish Peroxidase (HRP) conjugated MDA. A competitive inhibition reaction is launched between MDA (Standards or samples) and HRP-conjugated MDA with the pre-coated antibody specific for MDA. The more amount of MDA in samples, the less antibody bound by HRP-conjugated MDA. Following a wash to remove any unbound reagent, a substrate solution is added to the wells and color develops in opposite to the amount of MDA in the sample. The color development is stopped and the intensity of the color is measured.

DETECTION RANGE

0.1μg/ml-40μg/ml.

SENSITIVITY

The minimum detectable dose of human MDA is typically less than 0.04μg/ml. The sensitivity of this assay, or Lower Limit of Detection (LLD) was defined as the lowest human MDA concentration that could be differentiated from zero. It was determined the mean O.D value of 20 replicates of the zero standard added by their three standard deviations.

SPECIFICITY

This assay has high sensitivity and excellent specificity for detection of human MDA. No significant cross-reactivity or interference between human MDA and analogues was observed.

Note: Limited by current skills and knowledge, it is impossible for us to complete the cross-reactivity detection between human MDA and all the analogues, therefore, cross-reaction may still exist.

MATERIALS PROVIDED

Reagents	quantity
Assay plate	1(96 wells)
standard	5×0.5 ml
HRP -conjugate	1×6ml
Wash buffer (20 ×concentrate)	1×15ml
Substrate A	1×7ml
Substrate B	1×7ml
Stop solution	1× 7ml
Adhesive strip (for 96 wells)	4
Instruction manual	1

STANDARD CONCENTRATION

standard	**S1**	**S2**	**S3**	**S4**	**S5**
concentration(μg/ml**)**	**0.1**	**0.4**	**2**	**10**	**40**

STORAGE

Unopened kit	Store at 2 -8°C. Do not use the kit beyond the expiration date.
Opened kit	May be stored for up to 1 month at 2 -8° C.

***Provided this be within the expiration date of the kit.**

Other Supplies Required

*Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 600 nm -630 nm.

*An incubator which can provide stable incubation conditions up to 37°C±0.5°C.

*Squirt bottle، manifold dispenser، or automated micro plate washer.

*Absorbent paper for blotting the micro titer plate.

*100 mL and 500 mL graduated cylinders.

*Deionized or distilled water.

*Pipettes and pipette tips.

*Test tubes for dilution.

Precautions

The Stop Solution provided with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.

Sample collection and storage

Serum Use a serum separator tube (SST) and allow samples to clot for two hours at room temperature or overnight at 4°C before centrifugation for 15 minutes at 1000 ×g. Remove serum and assay immediately or aliquot and store samples at -20°C or -80°C. Avoid repeated freeze-thaw cycles.

Note:

1. is only responsible for the kit itself, but not for the samples consumed during the assay. The user should calculate the possible amount of the samples used in the whole test. Please reserve sufficient samples in advance.

2. Samples to be used within 5 days may be stored at 2-8°C, otherwise samples must be stored at -20°C (≤1month) or -80°C (≤2month) to avoid loss of bioactivity and contamination.

3. Grossly hemolyzed samples are not suitable for use in this assay.

4. If the samples are not indicated in the manual, a preliminary experiment to determine the validity of the kit is necessary.

5. Please predict the concentration before assaying. If values for these are not within the range of the standard curve, users must determine the optimal sample dilutions for their particular experiments.

6. Tissue or cell extraction samples prepared by chemical lysis buffer may cause unexpected ELISA results due to the impacts of certain chemicals.

7. Owing to the possibility of mismatching between antigen from other resource and antibody used in our kits (e.g., antibody targets conformational epitope rather than linear epitope), some native or recombinant proteins from other manufacturers may not be recognized by our products.

8. Fresh samples without long time storage are recommended for the test. Otherwise, protein degradation and denaturalization may occur in those samples and finally lead to wrong results.

Reagent preparation

Note:

*Kindly use graduated containers to prepare the reagent.

*Bring all reagents to room temperature (18-25°C) before use for 30min.

*Distilled water is recommended to be used to make the preparation for reagents or samples. Contaminated water or container for reagent preparation will influence the detection result.

Wash Buffer(1x)- If crystals have formed in the concentrate, warm up to room temperature and mix gently until the crystals have completely dissolved. Dilute 15 ml of Wash Buffer Concentrate (20 x) into deionized or distilled water to prepare 300 ml of Wash Buffer (1 x).

Assay procedure

Bring all reagents and samples to room temperature before use. Centrifuge the sample again after thawing before the assay. It is recommended that all samples and standards be assayed in duplicate.

1. Prepare all reagents and samples as directed in the previous sections.

2. Determine the number of wells to be used and put any remaining wells and the desiccant back into the pouch and seal the Ziploc, store unused wells at 4°C.

3. Set a **Blank** well without any solution.

4. Add 50µl of **Standard** or **Sample** per well. Standard need test in duplicate.

5. Add 50µl of **HRP-conjugate** to each well (Not to **blank** well). Mix well and then incubate for 1 hour at 37°C.

6. Aspirate each well and wash, repeating the process two times for a total of three washes. Wash by filling each well with **Wash Buffer** (200µl) using a squirt bottle, multi-channel pipette, manifold dispenser, or autowasher, and let it stand for 10 seconds, complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating ordecanting. Invert the plate and blot it against clean paper towels.

7. Add 50µl of **Substrate A** and 50µl of **Substrate B** to each well, mix well. Incubate for 15 minutes at 37°C. Keeping the plate away from drafts and other temperature fluctuations in the dark.

8. Add 50µl of **Stop Solution** to each well, gently tap the plate to ensure thorough mixing.

9. Determine the optical density of each well within 10 minutes, using a microplate reader set to 450 nm.

Note:

1. The final experimental results will be closely related to validity of the products, operation skills of the end users and the experimental environments.

2. Samples or reagents addition: Please carefully add samples to wells and mix gently to avoid foaming. Do not touch the well wall as possible. For each step in the procedure, total dispensing time for addition of reagents or samples to the assay plate should not exceed 10 minutes. This will ensure equal elapsed time for each pipetting step, without interruption. Duplication of all standards and specimens, although not required, but it recommended. To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.

3. Incubation: To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary. Do not allow wells to sit uncovered for extended periods between incubation steps. Once reagents have been added to the well strips, DO NOT let the strips DRY at any time during the assay. Incubation time and temperature must be observed.

4. Washing: The wash procedure is critical. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Solution by aspirating or decanting, remove any drop of water, and fingerprint on the bottom of the plate. Insufficient washing will result in poor precision and falsely elevated absorbance reading. When using an automated plate washer, adding a 10-second soak period following the addition of wash buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.

5. Controlling of reaction time: Observe the change of color after adding Substrates (e.g. observation once every 10 minutes). Substrates should change from colorless or light blue to gradations of blue. If the color is too deep, add Stop Solution in advance to avoid excessively strong reaction, which will result in inaccurate absorbance reading.

6. Substrates are easily contaminated. Substrates should remain colorless or light blue until added to the plate. Please protect it from light.

7. Stop Solution should be added to the plate in the same order as the Substrates. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrates.

3.6.8 Determination of serum Leptin

3. 1 Human Leptin Kit

Intended Use

The DRG Leptin ELISA is an enzyme immune assay for tile quantitative in vitro diagnostic measurement of Leptin in serum and plasma.

PRINCIPLE OF THE TEST

The DRG Leptin ELISA Kit is a solid phase enzyme-linked immune sorbent assay (ELISA) based on the sandwich principle.

The microliter wells are coated with a monoclonal antibody directed towards a unique antigenic site on a Leptin molecule.

An aliquot of patient sample containing endogenous Leptin is incubated in the coated well with a specific biotinylated monoclonal anti Leptin antibody. A sandwich complex is fonmed. After incubation the unbound material is washed off and a Streptavidin Peroxidase Enzyme Complex is added for detection of the bound Leptin.

Having added the substrate solution, the intensity of colour developed is proportional to the concentration of Leptin in the patient sample.

REAGENTS

Reagents provided

1 .Microliter wells, 12x8 (break apart) strips, 96 wells' Wells coated with human anti-Leptin antibody (monodonal).

2. Standard (Standard 0-5), 6 vials, (lyophilized), 0.5

Concentrations: 0 - 2- 5- 25 - 50 - 100 ng/ml Contain non-mercury preservative.

3. Control (Low & High), 2 vials, (lyophilized), 0.5 ml; for control values and ranges please refer to vial label or QC-Oatasheet, Contain non-mercury preservative.

4. Assay Buffer, 1 vial, 11 ml, ready to use, Contain non-mercury preservative.

5. Antiserum, 1 vial, 11 ml, ready to use, monoclonal biotinylated human anti-Leptin antibody; contain non-mercury preservative. 6. Enzyme Complex, 1 vial, 11 ml, ready to use, Streptavidin conjugated to horseradish Peroxidase; Contain non-mercury preservative.

7. Substrate Solution, 1 vial, 14 ml, ready to use,3,35,5 Tetra methyl benzidine (TMB)in buffer solution

8. Stop Solution, 1 vial, 14 ml, ready to use, contains 0.5 M H_2S0_4,

Avoid contact with the stop solution. It may cause skin irritations and burns.

9. Wash Solution, 1 vial, 30 ml (40X Concentra ted), see, Preparation of Reagents".

Note: Additional Standard zero for sample dilution is available on request.

Materials required but not provided

- A microliter plate calibrated reader (450 ± 10 nm) (e.g. the DRG Instruments Microliter Plate Reader).
- Calibrated variable precision micropipettes.
- Absorbent paper.
- Distilled or deionized water
- Timer
- Semi logarithmic graph paper or software for data reduction

Storage Conditions

When stored at 2 °C - 8 °C unopened reagents will retain reactivity until expiration date. Do not use reagents beyond this date.

Opened reagents must be stored at 2 °C - 8 °C. Microliter wells must be stored at 2 °C - 8 °C. Once the foil bag has been opened, care should be taken to close it tightly again

Reagent Preparation

Allow all reagents and required number of strips to reach room temperature prior to use.

Standards

Reconstitute the lyophilized contents of the standard vials with 0.5 ml Aqua dest and let stand for 10 minutes in minimum. Mix the vials several times before use.

Note: The reconstituted standards are stable for at least 6 weeks at 2 °C -8 °C . For longer storage freeze at -20°C.

Control

Reconstitute the lyophilized content of each vial with 0.5 ml Aqua de st. and Jet stand for 10 minutes in minimum. Mix the control several times before use.

Note: The reconstituted control is stable for at least 6 weeks at 2 °C -8 °C. For longer storage freeze at -20°C.

Wash Solution

Add deionized water to the 40X concentrated Wash Solution.

Dilute 30 ml of concentrated Wash Solution with 1170 ml deionized water to a final volume of 1200 ml.

The diluted Wash Solution is stable for 2 weeks at room temperature

Disposal of the Kit

The disposal of the kit must be made according to the national regulations. Special information for this product is given in the Material Safety Data Sheets (see chapter 13).

Damaged Test Kits

In case of any severe damage to the test kit or components, DRG has to be informed in writing, at the latest, one week after receiving the kit. Severely damaged single components should not be used for a test run.

They have to be stored until a final solution has been found. After this, they should be disposed according to the official regulations.

Specimen Collection and Preparation

Serum or plasma can be used in this assay.

Do not use hemolytic, icteric or lipaemic specimens.

Please note: Samples containing sodium azide should o be used in the assay.

Specimen Collection Serum:

Collect blood by venipuncture (e.g. Sarstedt Monovette for serum), allow to clot, and separate serum by centrifugation at room temperature. Do not centrifuge before complete clotting has occurred.

Patients receiving anticoagulant therapy may require increased clotting time.

Plasma: Whole blood should be collected into centrifuge tubes containing anti-coagulant (e.g. Sarstedt Monovette with the appropriate plasma preparation) and centrifuged immediately after collection.

Specimen Storage and Preparation

Specimens should be capped and may be stored for up to 24 hours at 2 °C - 8 °C prior to assaying.

Specimens held for a longer time should be frozen only once at -20 •c prior to assay. Thawed samples should be inverted several times prior to testing.

Specimen Dilution

If in an initial assay, a specimen is found to contain more than the highest standard, the specimens can be diluted with Standard 0 and reassayed as described in Assay Procedure. For the calculation of the concentrations, this dilution factor has to be taken into account.

Example:

a) Dilution 1:10: 10µL Serum+ 90 µL Standard 0 (mix thoroughly)

b) Dilution 1:100: 10-µL dilution a) 1:10 + 90 µL Standard 0 (mix thoroughly).

Assay procedure

General Remarks

All reagents and specimens must be allowed to come to room temperature before use. All reagents must be mixed without foaming.

- Once the test has been started, all steps should be completed without interruption.
- Use new disposal plastic pipette tips for each standard, control or sample in order to avoid cross contamination
- Absorbance is a function of the incubation time and temperature. Before starting the assay, it is recommended that all reagents are ready, caps removed, all needed wells secured in holder, etc. This will ensure equal elapsed time for each pipetting step without interruption.
- As a general rule the enzymatic reaction is linearly proportional to time and temperature.

Test Procedure

Each run must include a standard curve.

1. Secure the desired number of Microliter wells in the holder.
2. Dispense 15µL of each Standard, controls and samples with new disposable tips into appropriate wells.
3. Dispense 100 µL Assay Buffer into each well.

Thoroughly mix for 10 seconds. It is important to have a complete mixing in this step.

4. Incubate for 120 minutes at room temperature (without covering the plate).

5. Briskly shake out the contents of the wells. Rinse the wells 3 times with diluted Wash Solution (300 µL per well). Strike the wells sharply on absorbent paper to remove residual droplets.

Important note:

The sensitivity and precision of this assay is markedly influenced by the correct performance of the washing procedure!

6. Add 100 µL Antiserum to each well.

7. Incubate for 30 minutes at room temperature.

8. Briskly shake out the contents of the wells. Rinse the wells 3 times with diluted Wash Solution (300 µL per well). Strike the wells sharply on absorbent paper to remove residual droplets.

9. Dispense 100 µL Enzyme Complex into each well

10. Incubate for 30 minutes at room temperature.

11. Briskly shake out the contents of the wells.

Rinse the wells 3 times with diluted Wash Solution (300 µL per well). Strike the wells sharply on absorbent paper to remove residual droplets.

12. Add 100 µL of Substrate Solution to each well.

13. Incubate for 15 minutes at room temperature.

14. Stop the enzymatic reaction by adding 50 µL of Stop Solution to each well.

15. Determine the absorbance (OD) of each well at 450 ± 10 nm with a microliter plate reader.

It is recommended that the wells be read within 10 minutes after adding the Stop Solution.

Calculation of Results

1**.** Calculate the average absorbance values for each set of standards, controls and patient samples.

2. Construct a standard curve by plotting the mean absorbance obtained from each standard against its concentration with absorbance value on the vertical(Y) axis and concentration on the horizontal (X) axis.

3. using the mean absorbance value for each sample determine the corresponding concentration from the standard curve.

4. Automated method: The results in the IFU have been calculated automatically using a 4PL, (4 Parameter Logistics) curve fit. 4 Parameter Logistics is the preferred method. Other data reduction functions may give slightly different results.

5. The concentration of the samples can be read directly from this standard curve. Samples with concentrations higher than that of the highest standard have to be further diluted. For the calculation of the concentrations this dilution factor has to be taken into account.

Expected normal values

It is strongly recommended that each laboratory should determine its own normal and abnormal values.

In a study conducted with apparently normal healthy adults, using the DRG Leptin ELISA the following

values are observed:

Population ng/mL

Males 3.84 ± 1.79

Females7.36 ± 3.73

The results alone should not be the only reason for any therapeutic consequences. The results should be correlated to other clinical observations and diagnostic tests.

Performance characteristics

Assay Dynamic Range

The range of the assay is between 1.0- 100 ng/ml.

Sensitivity

The analytical sensitivity of the DRG ELISA was calculated by adding 2 standard deviations to the mean of 20 replicate analyses of the Standard 0 and was found to be 1.0 ng/ml.

Limitations of use

Reliable and reproducible results will be obtained when the assay procedure is performed with a complete understanding of the package insert instruction and with adherence to good laboratory practice. Any improper handling of samples or modification of this test might influence the results.

Drug Interferences

Until today no substances (drugs) are known to us, which have an influence to the measurement of Leptin in a sample.

Statistical Analysis

Data were analyzed using Analysis of variance (ANOVA), Correlation (r) and stepwise Regression procedures by statistical Analysis system (SAS, 2006).

1 **- Analysis of variance (ANOVA)**: A statistical procedure that determines whether any differences exist among two or more groups of subjects on one or more factors. The F test is used in ANOVA. Duncan A multiple –comparison method for comparing means following a significant F test in analysis of variance .It is a method highly recommended by statisticians.

2 – **Correlation coefficient (r)**: A measure of the linear relationship between two numerical measurements made on the same set of subjects. It ranges from (-1) to (+1), with zero indicating no relationship. Also called the Pearson product moment.

4- The stepwise regression: Tend to have a better chance of finding more nearly optimum model, the method depend on test all factors affecting the variable and chose only the significant factors and omitted the other factors from the model. Beside, it is important to see the R^2 improvement with every step in this method because the chose operation will stop when the R^2 decrease .

Results

The differences between blood parameters for female in two groups are shown in Table (4-1).

From one hand , A significant effect (5% or 1%) was observed for FBS , HbA_1C , triglyceride and MDA parameters for female in the two groups (Normal (G1) and Patient (G2), resp.). On the other hand, these is no significant effect for female in two groups was found in the rest of the parameters. FBS (The value of 90.9±5,89 for G1 compared to 199.86±13.40 for G2) ; HbA_1C (the value of 5.46±0.14 for G1 compared to 9.70±0.36 for G2) ;TG (The value of 112.40±12.26 for G1 compared to 179.67±19.22 mg/dl for G2) ; (The value of MDA 1.28±0.07nmol/ml for G1 compared to 2.55±0.12 nmol/ml for G2) ;

Table (4-1): Comparison between females in two groups (Normal & patient) for studied parameters

Parameters	Female		P
	Normal (G1) Means ±SE	Patient (G2) Means ±SE	
Age (years)	49.9±5.179	51.21±2.44	**NS**
BMI(kg/m2)	29.47±1.29	33.36±1.54	**NS**
FBS(mg/dl)	90.9±5.89	199.86±13.40	**
HbA_1C	5.46±0.14	9.70±0.36	**
Cholesterol (mg/dL	170.40±11.25	191.64±7.29	**NS**
HDL-C (mg/dL)	32.16±3.11	33.08±2.35	**NS**
LDL-C (mg/dL)	108.98±10.37	119.30±6.05	**NS**
Triglyceride (mg/dL)	112.40±12.26	179.67±19.22	*
Crea (mg/dL)	0.831±0.07	0.73±0.02	**NS**
Urea (mg/dL)	23.00±3.92	26.17±1.61	**NS**
MDA (nmol/mL)	1.28±0.07	2.55±0.12	**

Leptin (ng/mL)	18.40±2.02	14.71±2.03	**NS**

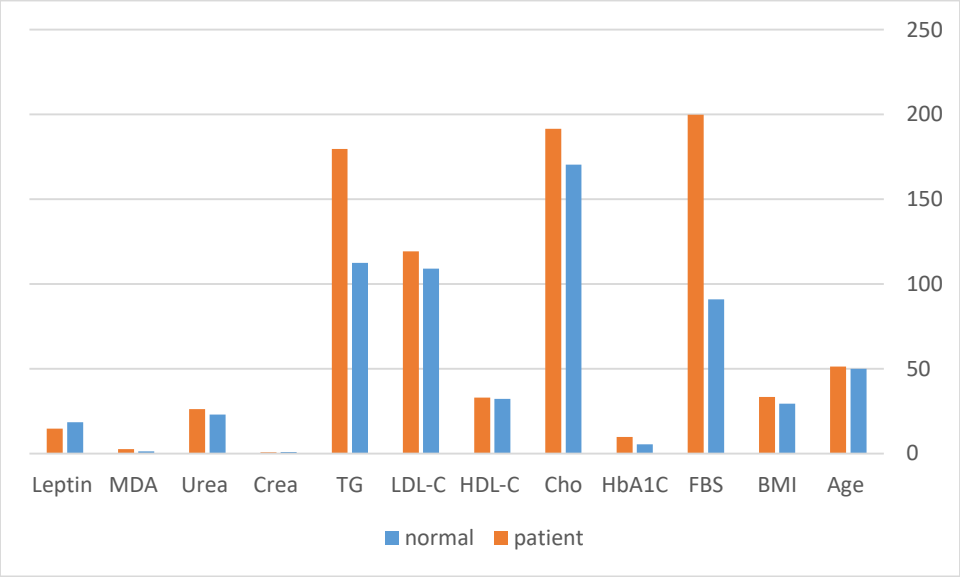


Fig (4-1): Comparison of female parameters in two groups (Normal & patient).

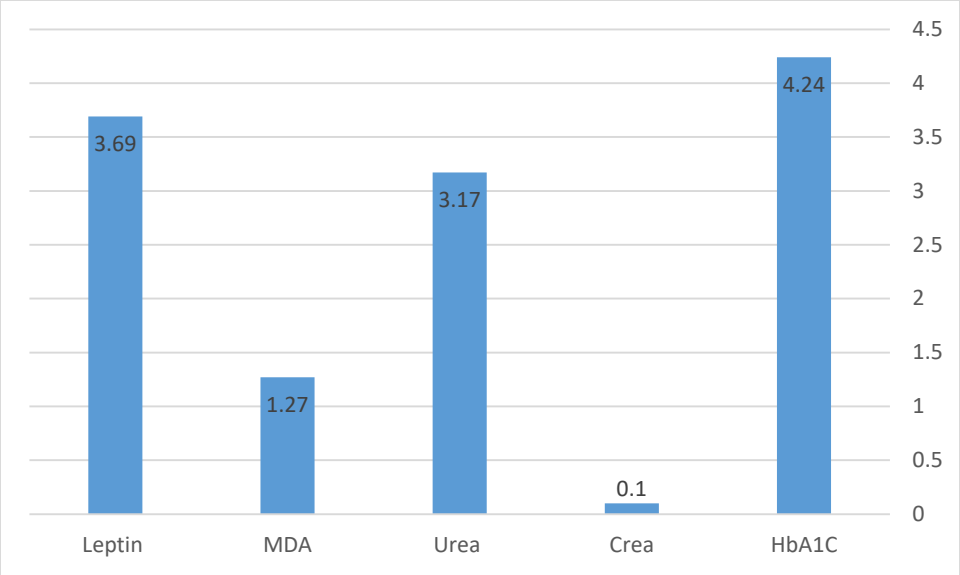


Fig (4-2): Comparison different between of female parameters in two groups (Normal & patient).

The differences between parameters for males in two groups are shown in Table (4-2).

From one hand , A significant effect (5% or 1%) for FBS , HbA_1C , HDL-C ,CREA , triglyceride and MDA parameters for female in two groups. On the other hand, a not sig. effect for female in two groups for the rest of parameters. Concern the FBS (The value of 108.60±15.73 mg/dl for G1 compared to 213.75±14.19 mg/dl for G2) ; HbA_1C (the value of 5.62±0.26 for G1 compared to 9.68±0.42 for G2) ; HDL-C (The value of 23±1.72mg/dl for G1 compared to 31.66±3.75 mg/dl for G2) ; (The value of MDA 1.38±0.04nmol/ml for G1 compared to 2.19±0.09 nmol/ml for G2) and CREA (The value of 1.040.05mg/dl for G1 compared to 0.90±0.3 mg/dl for G2).

Table (4-2): Comparison between males in two groups (Normal & patient) for studied parameters.

Parameters	Males		P
	male Normal	male patient	
Age (years)	40.15±1.80	49.85±3.30	NS
BMI(kg/m2)	28.35±1.14	29.33±1.03	NS
FBS(mg/dl)	108.60±15.73	213.75±14.19	**
HbA_1C	5.62±0.26	9.68±0.42	**
Cholesterol (mg/dL	166.22±11.90	180.20±7.43	NS
HDL-C (mg/dL)	23±1.72	31.66±3.75	*
LDL-C (mg/dL)	120.73±5.65	188.99±7.90	NS
Triglyceride (mg/dL)	151.75±19.27	168.95±21.33	NS
Crea (mg/dL)	1.04 ±0.05	0.90±0.03	*
Urea (mg/dL)	23.75±1.86	25.72±1.84	NS
MDA (nmol/mL)	1.38±0.04	2.19±0.09	**
Leptin (ng/mL)	5.34±0.72	3.97±0.63	NS

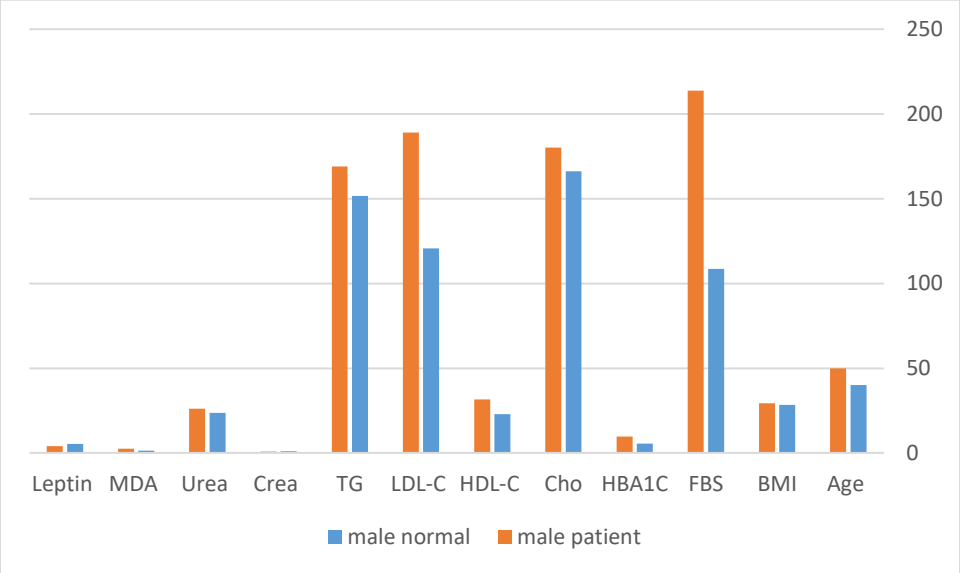


Fig (4-3): Comparison between males in two groups (Normal & patient) for studied parameters.

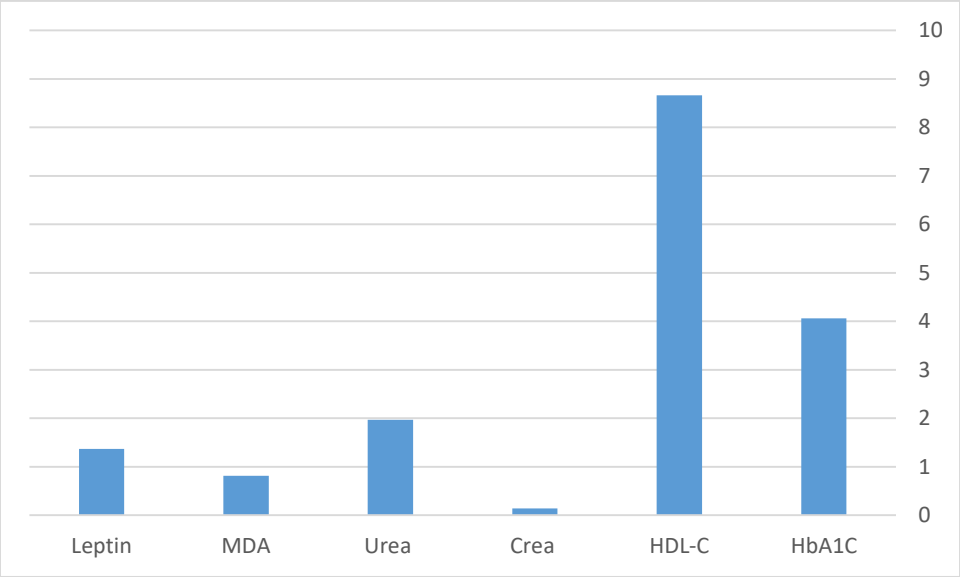


Fig (4-4): Comparison different between males in two groups (Normal & patient) for studied parameters.

The differences between parameters for males and females in control group are showon in Table (4-3).

From one hand, A significant effect (5% or 1%) for, leptin and Creatinine. parameters for males and female in two groups, On the other hand , a not significant effect for males and females in two groups for the rest of parameters. For the HDL-C (23±1.72 mg/dL for males compared to 32.16±3.11mg/dL mg/dL); Leptin (The value of 5.34±0.72 ng/dl for males compared to 18.40±2.02 ng/dl for females) and Creatinine (The value of 1.04±0.05mg/dl for males compared to 0.83±0.07 mg/dl for females).

Table (4-3): Comparison between males and females in control group for studied parameters.

Parameters	Control		P
	Male Means±SE	Female Means±SE	
Age (years)	40.15±1.80	49.9±5.179	NS
BMI(kg/m2)	28.35±1.14	29.47±1.29	NS
FBS(mg/dl)	108.60±15.73	90.9±5.89	NS
HBA_1C	5.62±0.26	5.46±0.14	NS
Cholesterol (mg/dL	166.22±11.90	170.40±11.25	NS
HDL-C (mg/dL)	23±1.72	32.16±3.11	**
LDL-C (mg/dL)	120.73±5.65	108.98±10.37	NS
Triglyceride (mg/dL)	151.75±19.27	112.40±12.26	NS
Crea. (mg/dL)	1.04 ±0.05	0.831±0.07	*
Urea (mg/dL)	23.75±1.86	23.00±3.92+	NS
MDA (nmol/mL)	1.38±0.04	1.28±0.07	NS
Leptin (ng/mL)	5.34±0.72	18.40±2.02	**

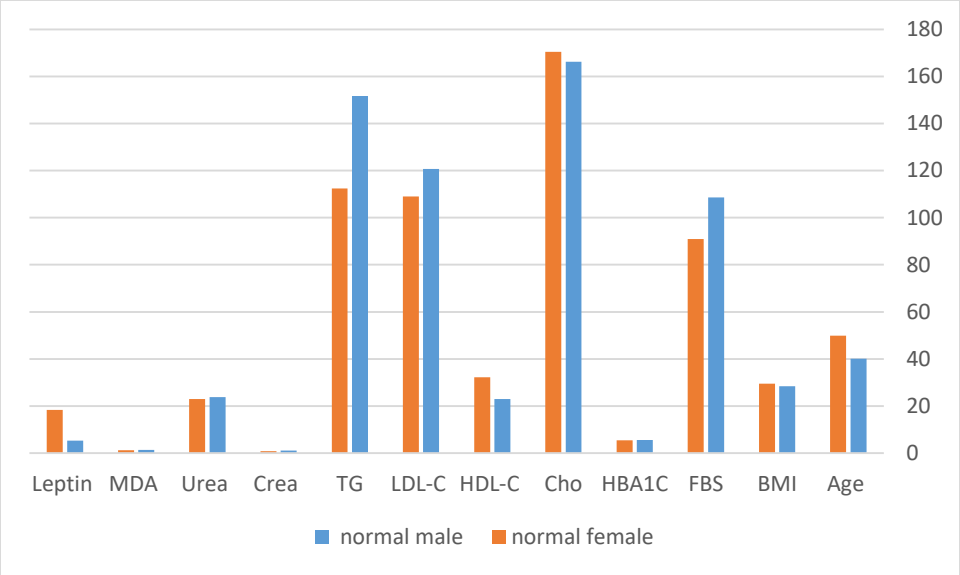


Fig (4-5): Comparison between males and females in control group for studied parameters.

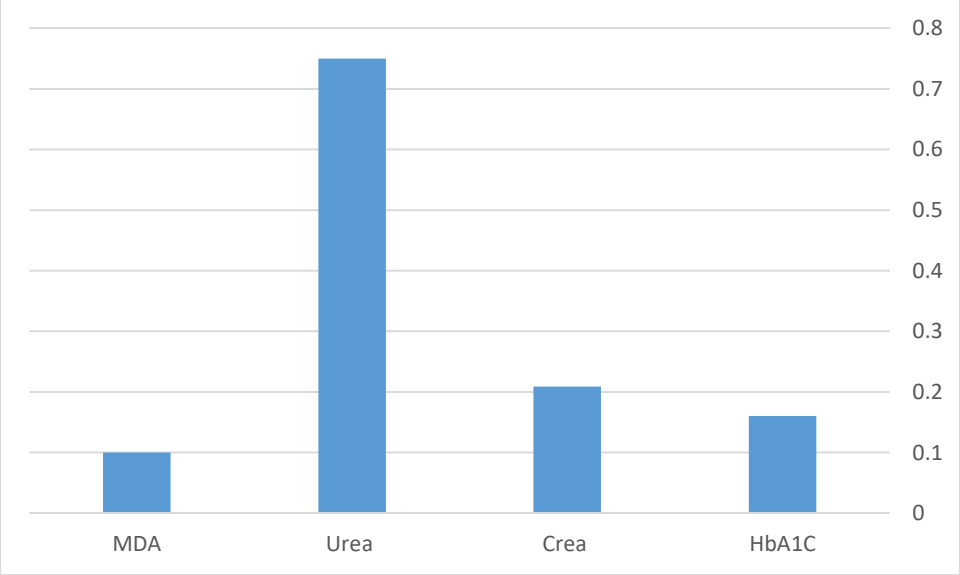


Fig (4-6): Comparison different between males and females in control group for studied parameters.

The differences between parameters for female and male in patients group are showon in Table (4-4)

From one hand, a significant effect (5% or 1%) for BMI (kg/m²); *Creatinine; Leptin (ng/mL)* and MDA parameters for female and male in patients group. On the other hand, a not sig. effect for female and male in patients group for the rest of parameters. For the BMI (The value of 29.33±1.03 kg/m² for males compared to 33.36±1.54 for females); Creatinine (The value of 0.90±0.03 for males compared to 0.73±0.02 for females); *Leptin* (The value of 3.97±0.63 *ng/mL* for males compared to 14.71±2.03ng/dl for females); (The value of MDA 2.19±0.09 nmol/ml for males compared to 2.55±0.12 nmol/ml for females).

Table (4-4) : Comparison between males and females in patients group for studied parameters.

Parameters	Patients group		p
	males	Females	
Age (years)	49.85±3.30	51.21±2.44	**NS**
BMI(kg/m2)	29.33±1.03	33.36±1.54	*
FBS(mg/dl)	213.75±14.19	199.86±13.40	**NS**
HBA1C	9.68±0.42	9.70±0.36	**NS**
Cholesterol (mg/dL	180.20±7.43	191.64±7.29	**NS**
HDL-C (mg/dL)	31.66±3.75	33.08±2.35	**NS**
LDL-C (mg/dL)	188.99±7.90	119.30±6.05	**NS**
Triglyceride (mg/dL)	168.95±21.33	179.67±19.22	**NS**
Crea(mg/dL)	0.90±0.03	0.73±0.02	**
Urea(mg/dL)	25.72±1.84	26.17±1.61	**NS**
MDA (nmol/mL)	2.19±0.09	2.55±0.12	*
Leptin (ng/mL)	3.97±0.63	14.71±2.03	**

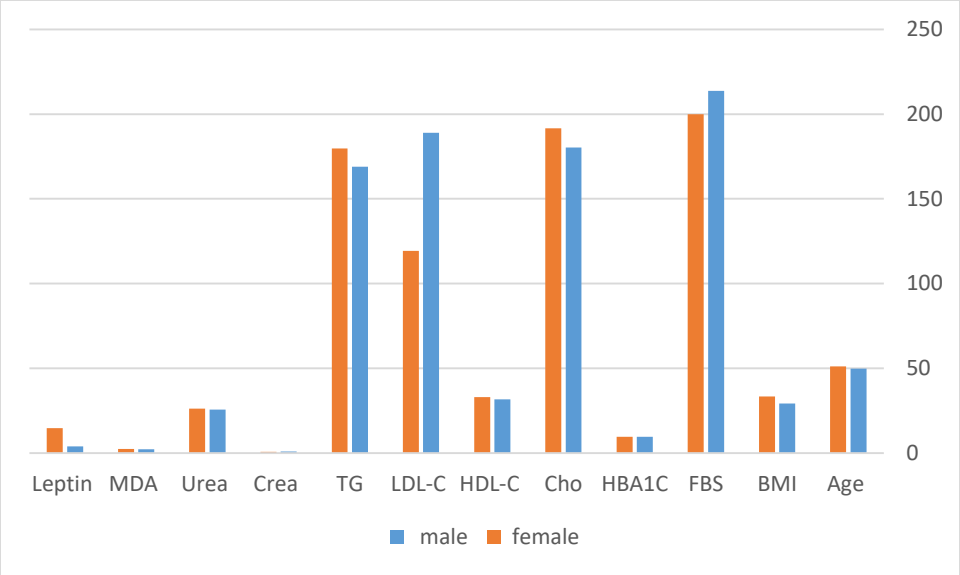


Fig.(4-7): Comparison between males and females in patients group for studied parameters.

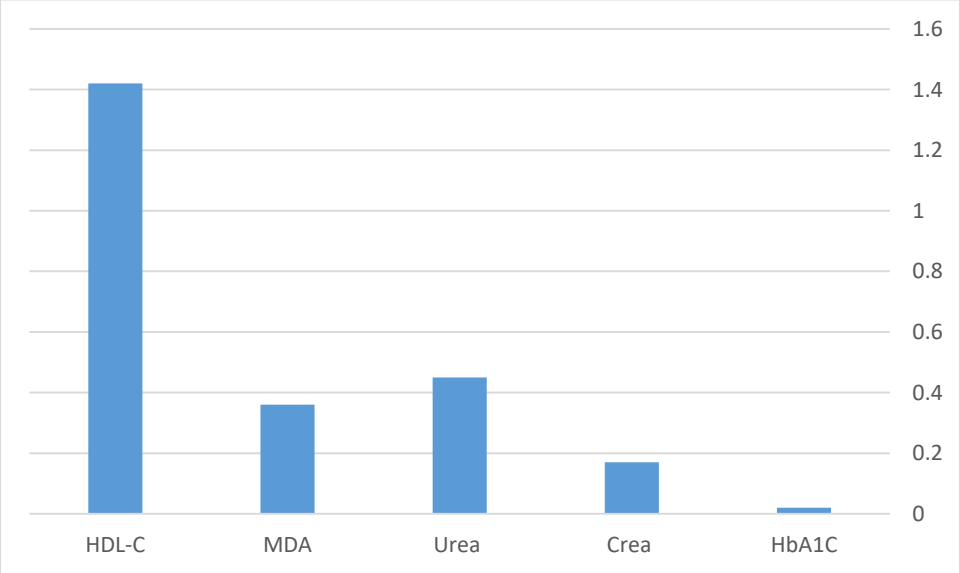


Fig.(4-8): Comparison different between males and females in patients group for studied parameters

Table (4-5) : The correlation coefficients among studied traits for males in control group.

	MDA	LDL	TG	Cho	Age	smok	BMI	Fmly	Crea	urea	FBS	HbA_1C	leptin
HDL	0.226	0.102	0.387	0.472*	-0.041	0.213	-0.297	0.209	0.082	-0.207	-0.266	-0.298	-0.123
MDA		- 0.007	0.043	0.140	-0.175	0.285	-0.421	0.074	-0.181	-0.196	-0.333	-0.395	-0.134
LDL			0.238	0.577**	0.023	0.313	0.043	-0.197	-0.119	-0.023	-0.309	-0.037	0.160
TG				0.544*	0.267	-0.159	0.306	-0.055	-0.022	-0.279	0.175	0.264	0.397
Cho					0.229	0.093	0.151	-0.156	0.037	-0.303	-0.088	0.057	0.299
Age						0.091	0.155	0.057	0.287	-0.112	-0.023	0.009	0.373
SMOK							-0.717**	0.548*	0.135	0.105	-0.988**	-0.897**	-0.347
BMI								-0.407	-0.139	-0.293	0.758**	0.792**	0.693**
FMLY									0.111	0.138	0.420	-0.439*	-0.458*
CREA										0.722**	-0.148	-0.241	0.090
UREA											-0.127	-0.178	-0.302
FBS												0.928**	0.347
HbA_1C													0.400

The correlation coefficients among studied traits for males in control group are shown in Table (4-5).

The results noticed that there was a strong positive correlation between cholesterol and the following traits HDL –C (r=0.472) ; LDL – C (r = 0.577) and Triglyceride (r= 0.544) . Also , correlation between BMI and the following traits FBS (r = 0,758) ; HbA_1C (r= 0.792) and leptin (0.693) and between FBS and HbA_{1C}(r = 0.928) . Likewise , positive correlation between leptin and the following traits Triglyceride (r = 0.397) ; Age (r = 0.373) ; FBS (r = 0.347) ; and between HbA_1C (R = 0.400) ;and BMI (r = 0.693) .

On the other hand , a negative correlation between MDA and BMI (r = - 0.421) ; FBS (r = -0.333) and HbA_1C (r = -0.95) similarly , the correlation between LDL – C and FBS (r = -0.309) ; smoking habit and the following traits BMI (r= -0.717) ; FBS (r = -0.980) ; HbA_1C (r = -0.897) ; and leptin (-0.347). In accordance, the correlation BMI and family history of diabetes (r = -0.407), urea (r = -0.293).

A strong negative correlation between family history and HbA_{1C} (r= -0.439); leptin (r = -0.458). Besides, negative correlation coefficients between creatinine and HbA_{1C} (r = -0.241) & urea and leptin (r =-0.302).

Table (4-6): The correlation coefficients among studied traits for females in control group.													
	MDA	LDL	TG	Cho	Age	smok	BMI	fmly	crea	urea	FBS	HbA1C	leptin
HDL	0.416	0.178	0.470	0.254	0.029	0.088	0.048	0.081	-0.242	0.069	0.310	-0.093	0.209
MDA		0.390	0.541	0.642*	0.534	-0.221	0.223	0.413	-0.567	-0.238	0.400	0.135	0.505
LDL			0.000	0.889**	0.248	0.490	-0.090	0.187	-0.560	0.091	0.332	0.129	0.622*
TG				0.140	0.381	-0.270	0.730	0.273	-0.041	-0.140	0.430	0.041	0.376
Cho					0.451	0.268	0.069	0.382	-0.546	0.054	0.628*	0.442	0.688*
Age						0.421	0.342	0.248	0.154	0.142	0.426	0.536	0.537
SMOK							-0.209	-0.218	0.137	0.233	-0.025	0.131	0.259
BMI								-0.006	0.297	0.027	0.550	0.302	0.548
FMLY									-0.313	-0.222	0.378	0.281	0.118
CREA										0.620*	-0.088	0.234	0.067
UREA											-0.007	0.206	0.300
FBS												0.802**	0.501

HbA1C												**0.245**

The correlation coefficients among studied traits for females in control group are observed in Table (4-6).

The results noticed that there was a strong positive correlation between cholesterol and the following traits MDA (r=0.642) ; LDL – C (r = 0.889) ; FBS (r= 0.628) ; and leptin (0.688) . In addition , correlation between leptin and LDL (r= 0.622) and between FBS and HbA_1C(r = 0.802) and between urea and creatinine (r= 0.620) . Further , positive correlation between leptin and the following traits Triglyceride (r = 0.376) ; Age (r = 0.537) ; FBS (r = 0.501) MDA (r = 0.505) ; urea (0.300) ; BMI (r = 0.548) . Furthermore , correlation between FBS and the following traits HDL-C (r = 0.310) ; MDA (r= 0.400); LDL (r= 0.332) ; TG (r= 0.430) ; age (r= 0.426) ; BMI (r= 0.550) ; family (r = 0.378) . Likewise, correlation between family and the following traits MDA (r= 0.413) ; cholesterol (r= 0.382) . Beside correlation between BMI and the following traits TG (r= 0.730); age (r = 0.342). Also , correlation between smoking and the following traits LDL (r=0.490) ; Age (r= 0.421) . In similar , correlation between Age and the following traits MDA(r= 0.534); TG (r= 0.381) ; cholesterol (r= 0.451). In addition , correlation between TG and the following traits HDL-C (r= 0.470) ; MDA (r= 0.390) and between LDL-C and MDA (r= 0.390) and between MDA and HDL-C (r= 0.416).

On the other hand , a strong negative correlation between creatinine and the following traits HDL-C (r = - 0.242) ; MDA (r = -0.567) ; LDL (r= -0.560) ' cholesterol (r = -0.546) ; family history (r= -0.313); Besides , the correlation between MDA and urea (r = -0.238) ; smoking habit and TG (r= -0.270) .

Table (4-7): The correlation coefficients among studied traits for males in patients.													
	MDA	LDL	TG	Cho	Age	smok	BMI	fmly	crea	urea	FBS	HbA_1C	Leptin
HDL	0.020	-0.120	-0.357	0.073	0.039	-0.040	-0.330	0.112	-0.072	0.310	0.029	0.082	-0.422
MDA		0.251	0.172	0.334	-0.210	-0.260	-0.018	-0.390	0.521*	0.238	0.107	0.117	-0.156
LDL			0.021	0.806**	-0.443	-0.029	0.286	0.005	-0.049	0.036	0.211	0.233	0.421
TG				0.238	-0.120	0.136	0.007	-0.125	0.226	0.410	0.010	-0.134	-0.135
Cho					-0.224	-0.197	0.004	-0.124	0.073	0.327	0.194	0.191	0.105
Age						0.274	-0.585**	0.455*	0.098	0.353	-0.172	-0.125	-0.287
SMOK							0.128	0.697**	-0.338	0.156	-0.191	-0.018	0.185
BMI								-0.377	-0.121	-0.427	-0.043	0.268	0.624**
FMLY									-0.281	0.117	-0.143	-0.275	0.153
CREA										0.239	-0.066	-0.169	-0.068

UREA											**0.237**	**0.139**	**-0.542***
FBS												**0.767****	**-0.099**
HbA_1C													**-0.001**

The correlation coefficients among studied traits for males in patient group are observed in Table (4-7).

The results noticed that there was a strong positive correlation between cholesterol and LDL – C (r = 0.806) . Also correlation between family history and the following traits Age (r = 0,455) ; Smok (r= 0.697) and correlation between creatinine and MDA (r = 0.521) and between FBS and HbA_1C(r = 0.767)and between leptin and BMI (r = 0.624) . Besides , positive correlation between urea and the following traits Triglyceride (r = 0.410) ; Age (r = 0.353) ; FBS (r = 0.237) HDL=C (r = 0.310) ; MDA (r= 0.238) ; Cho (r = 0.327) ; creatinine (r = 0.239) , and between smoke and Age (r= 0.274) . In similar correlation between LDL-C and the following traits MDA (r = 0.251); BMI (r= 0.286) ; FBS (r=0.211) ; HbA_1C (r= 0.233) ; and leptin (r= 0.421) . As well as, correlation between BMI and HbA_1C (r= 0.268).

On the other hand , a strong negative correlation between Age and BMI (r = - 0.585) .In accordance , the correlation between leptin an urea (r = -0.542) .In addition , negative correlation coefficients between HDL=C and the following traits Triglyceride(r = - 0.357); BMI (r=- 0.330)leptin (r=- 0.422).Further-more , negative correlation coefficients between MDA and the following traits Age (r = -0.210) ;smoke (r= -0.260) ; family history (r = -0.390) & LDL-C and Age (r =-0.443); and between Cho and Age (r= -0.224) and correlation between Age and leptin (r = -0.287) and between smoke and creatinine (r= -0.338) . Besides, negative correlation coefficients between family history and the following traits creatinine (r = -0.281); HbA_1C (r = -0.275).

Table (4-8): The correlation coefficients among studied traits for females in patients group.													
	MDA	LDL	TG	Cho	Age	smok	BMI	fmly	crea	urea	FBS	HbA_1C	Leptin
HDL	-0.265	-0.140	-0.433*	-0.186	-0.043	0.158	0.100	-0.127	-0.286	0.091	-0.028	0.177	0.323
MDA		-0.164	-0.132	-0.258	0.183	0.163	-0.117	0.026	-0.209	-0.098	-0.012	0.128	-0.276
LDL			0.365*	0.793**	0.143	0.057	-0.128	0.186	0.314	0.038	0.070	0.026	0.116
TG				0.600**	0.150	0.146	0.195	-0.151	0.104	0.230	0.238	0.014	-0.022
Cho					0.180	0.119	0.047	0.005	0.317	0.115	0.031	-0.093	0.225
Age						-0.077	0.028	0.253	0.133	0.502**	-0.288	-0.234	0.120
SMOK							0.171	0.249	-0.061	0.106	-0.046	-0.017	0.094
BMI								0.054	0.032	0.348	-0.390*	-0.343	0.714**
FMLY									0.277	0.337	-0.060	-0.016	0.002
CREA										0.282	0.038	-0.137	0.027
UREA											-0.253	-0.346	0.373*
FBS												0.733**	-0.505**
HbA_1C													-0.407*

The correlation coefficients among studied traits for females in patients group are shown in Table (4-8).

It can noticed that there was a strong positive correlation between LDL – cholesterol and the following traits cholesterol (r=0.793); and Triglyceride (r= 0.365). Besides correlation between Triglyceride and cholesterol (r = 0.600) ; and between Age and urea (r = 0.502)and between BMI and Leptin (r= 0.714)and between urea and Leptin (r= 0.373) & FBS and HbA1C (r =-0.733).

Furthermore , positive correlation between urea and the following traits Triglyceride (r = 0.230) ; BMI (r = 0.348) ; Family history (r = 0.337);creatinine (r = 0.282) .In addition positive correlation between creatinine and the following traits cholesterol (r = 0.317) ;and family history (r = 0.277) ; LDL-C (r – 0,314) .and between FBS and Triglyceride (r= 0.238) as well as positive correlation between family history and the following traits Age (r = 0.253) ; smoke (r = 0.249) . In accordance, positive correlation between leptin and the following traits HDL-C (r = 0.323); cholesterol (r = 0.225). On the other hand , a strong negative correlation between HDL-C and TG (r = - 0.433) ; In similar , the correlation between FBS and the following traits BMI (r = -0.390) ; Leptin (r = 0.505).

On the other hand , negative the correlation between MDA and the following traits HDL-C (r =- 0.265) ; cholesterol (r = -0.258); creatinine (r = -0.209) ; leptin (r = -0.276) . Besides , negative the correlation between Age and the following traits FBS (r= -0.288) ; HbA1C (r = -0.234) . Moreover, between BMI and HbA1C (r = -0.343). Also, negative the correlation between urea and the following traits FBS (r= -0.253); HbA1C (r = -0.346).

Factors affecting leptin according to stepwise regression are found in Table (4-9).

According to the result of final stepwise regression it can observed from the previous table that the factors BMI , Gender , FBS , LDL , HDL , MDA , and AGE affected leptin hormone significantly at level 5% or 1% . On the other hand, the effect of factors smoking, family history, HbA1C , TG , cholesterol , creatinine , and urea are not significant on leptin hormone

Table (4-9): Factors affecting leptin according to stepwise Regression

step	Variable entered	Model R^2	C (P)	F	Level of significance
1	BMI	0.392	96.71	49.12	**
2	SEX	0.602	39.54	39.54	**
3	FBS	0.678	20.61	17.29	**
4	LDL	0.702	15.85	5.88	**
5	HDL	0.722	11.96	5.45	*
6	MDA	0.742	8.51	5.34	*
7	AGE	0.760	5.49	5.20	*

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Discussions

Discussions

Results in Tables (1 and 2) are shown that higher values for all studied parameters in patients group compared to control group, except Creatinine and leptin in both females and males. When it compared the parameters between both sex in Tables (3 and 4), it can observed that higher values for FBS, HBA1C, LDL-C, Triglyceride, Creatinine , Urea and MDA traits in males compared to females in control and patients groups. On the other hand, the BMI, Cholesterol, HDL-C, and Leptin traits in females are higher compared to males in control and patients groups.

Type 2 diabetes (non-insulin-dependent diabetes) is a multi-causal disease which develops slowly and in a stepwise order. Type 2 diabetics produce insulin, but the cells simply don't react well to it anymore. This type is very curable, usually in a year or less. Here, the pancreas not only produces insulin, but usually overproduces it, since the effectiveness is so reduced. **(Buguslaw, 2001; Stumvoliet *al.*, 2005; Waeber and Vollenweider, 2007).** Initially it commences with insulin resistance, which progress gradually with time until the body fails to maintain glucose haemostasis resulting in glucose intolerance. Systemically these perturbations are accompanied with changes in a variety of biochemical processes such as obesity, an altered lipid profile and lipid peroxidation

(Maharjan*et al.*, 2008).

Oxidative stress induced by reactive oxygen species (ROS), which is generated by hyperglycaemia. Diabetes mellitus is characterized by hyperglycaemia together with biochemical alterations of glucose and lipid peroxidation. It has been demonstrated by high levels of serum TC, triglycerides, LDL, VLDL, low concentration of HDL **(Milzani,2006).**

Several studies have evaluated free radical induced lipid peroxidation and the antioxidants in diabetic patients **(Maritim, *et al.*, 2003 and Stephens et al., 2006)**.

Some complications of diabetes mellitus are associated with increased activity of free radical-induced lipid peroxidation and accumulation of lipid peroxidation products **(Cannon, 2010).** Abnormally high levels of peroxidation and the simultaneous decline of antioxidant defense mechanisms can lead to damage of cellular organelles and lead to oxidative stress **(American Diabetic Association Guidelines, 2005)**. Under normal physiological conditions, there is a critical balance in the generation of oxygen free radicals and antioxidant defense systems used by organisms to deactivate and protect themselves against free radical toxicity **(Guillermo,2006)**.

Impairment in the oxidant/antioxidant equilibrium creates a condition known as oxidative stress. Oxidative stress is known to be a component of molecular and cellular tissue damage mechanisms in a wide spectrum of human diseases **(Wolff, 1993).** Diabetes is associated with a number of metabolic alterations and principal among these is hyperglycemia. Increased extra cellular matrix production and vascular dysfunction have all been implicated in the pathogenesis of vascular disease in type 1 and type 2 diabetes **(Brownle ,2001).**

A variety of natural antioxidants exist to scavenge oxygen free radicals and prevent oxidative damage to biological membranes. One group of these antioxidants is enzymatic (intracellular), which include super oxide dismutase, glutathione peroxidase and catalase. In addition to enzymatic antioxidants, the major natural antioxidants, most of them derived from natural sources by dietary intake are vitamin A, vitamin C and vitamin E and carotenoids. Also, numerous small molecules are synthesized or produced within the body that has antioxidant capacity (e.g. glutathione and uric acid) **[Wieruszwysocka*et al.*, 1995 and Heistad, 2005].**

Oxidative damage to unsaturated lipids is a well-established general mechanism for oxidative stress-mediated cellular injury **(Yagi K, 1994),** in addition to increased lipid peroxidation **(Syryawansh*et al.*, 2006).** The occurrence of free-radical-induced lipid peroxidation causes considerable changes in the cell membrane **(Agrawal*et al.*, 1985).**

Peroxidation of the lipid membrane has been related to the pathogenesis of many degenerative diseases, such as atherosclerosis, aging, carcinogenesis and diabetes mellitus **(Nair*et al.*, 2007).** Evidence suggests that oxidative stress is increased in diabetes, because of excessive production of reactive oxygen species (ROS) and an impaired antioxidant defense mechanism **(West, 2000 and Antoine*et al.*,2002).**

It has been suggested that ROS induce membrane lipid peroxidation and that the toxicity of the generated fatty acids peroxides are important causes of cell malfunction **(Sanocka and Kurpisz, 2004)**. The most widely used assay for lipid peroxidation involves the measurement of malondialdehyde (MDA) due to its simplicity. Thus, the lipid peroxide in the blood provides useful information for the prognosis of diabetes in which secondary disorders are often fatal **(Tappel, 1973).** Antioxidants can be defined as substances whose presence in relatively high concentration significantly inhibits the rate of oxidation of lipids, proteins, carbohydrates and DNA. Antioxidants such as uric acid (UA), superoxide dismutase (SOD) and glutathione (GSH) act as potent electron donors; they donate hydrogen atoms to pair up with unpaired electrons on free radicals.

The relationship between leptin and other parameters are shown in Tables (4-8), It found strong positive correlation between leptin and BMI; sex and FBS in both control and patient groups. This agrees with RahmaniNia *et al.*, (2009).

A significant effect (1% or 5%) for MDA, HDL-C, LDL-C, Age, Sex, BMI and FBS variables on Leptin are noticed by Stepwise regression procedure Table(4-9).

Leptin (Greek leptos meaning thin) is a 16-kDa protein (a small peptide) hormone; it plays a key role in regulating energy intake and energy expenditure, including appetite and metabolism. Leptin is produced mainly, but not exclusively by adipocytes. In the general population, it is believed to be an "appetite inhibitor". It is partly cleared by the kidney and is increased in the patients of end-stage renal disease, who are undergoing hemodialysis.

There are conflicting reports in the literature about the relationship of serum leptin with the nutritional marker, body mass index (BMI). Leptin secretion from the adipose tissue, its role has been more clear in the endocrinology area. It is one of the most important adipose derived hormones. It has been found high in obesity. This can be explained by the statement that ''obesity may be the consequence of leptin resistance'' **(Wei *et al.*, 1997&Brennan and Mantzoros, 2006).**

Ibrahim *et al.*,.(2011) found that the levels of leptin were significantly elevated in female patients group, in male patients group and in total patients group, cholesterol and LDL-C were significantly elevated, while HDL-C was significantly lower, there was positive correlation between leptin , cholesterol LDL-C and triglyceride. On the other hand, serum LDL-C and total cholesterol had no significant association with leptin. Thus, fasting glucose level remains as the clinical parameter that might have influence on leptinemic status though high blood glucose level is a presentation of diabetes (**Mahler and Adler, 1999).**

Decreased levels of leptin concentrations during food deprivation leading to impaired immune function gave rise to spending more time to understand role of this molecule in chronic inflammation and autoimmunity together with therapeutic implications of its modulators **(La Cava *et al.*, 2004).**

Leptin has the ability of regulation of insulin secretion from the pancreatic islet cells **(Ceddia *et al.*, 1999 &Muoio and Lynis ,2002).**

After leptin was given to mice who had leptin deficiency, it has been demonstrated that there had been a decrease in hyperglycemia and hyper insulinemia, inhibition in hepatic gluconeogenesis and insulin secretion via direct effects on beta cells **(Pelleymounter*et al.*, 1995 and Rossetti, *et al.*, 1997).**

Fat cells are the cells that primarily compose adipose tissue, specialized in storing energy as fat **(Hong *et al.*, 2008)**. It is technically composed of roughly only 80% fat; fat in its solitary state exists in the liver and muscles. Its main role is to store energy in the form of fat, although it also cushions and insulates the body. Adipose tissue also serves as an important endocrine organ by producing hormones such as leptin, resistin, and the cytokine **(Cannon and Nedergaard, 2008)**.

Several traditional cardiovascular risk factors track with inflammatory biomarkers, in particular central obesity and body mass index. Serum leptin levels are found higher in women than in men **(Wei*et al.*, 1997; Vettor*et al.*, 1997 and Donahue, *et al.*, 1999)** and this is probably owing to adipose tissue in women being higher than in the opposite sex, the existence of negative correlation between leptin and testosterone levels (**Vettor*et al.*, 1997 andJockenhovel*et al.*, 1997)** and the stimulation of mRNA production by 17b-estradiol, which is one of the female sexuality hormones **(Sweeney, 2002)**. All the subjects enrolled in our study were female obese subjects and we found no significant correlation between the age and serum leptin levels. Many investigators demonstrated that leptin had a major

correlation with BMI **(Kieffer *et al.*, 1997; Bertin, *et al*., 1998; Haluzik *et al.*, 2001 and Gomez-Ambrosi *et al.*, 2002)**

In addition, In current study, leptin had a correlation with BMI both for subjects with diabetes and subjects who did not have diabetes as in Tables (7 and 8). The BMI seems to be applied easily at outpatient departments; however, obesity itself cannot be defined simply by this method only. Therefore, waist and hip circumferences and the waist-to-hip ratio have all been performed. As a result, we found no relationship with leptin and waist or waist-to-hip ratio measurements in diabetic and non-diabetic groups.

Obese individuals have markedly increased leptin production probably as a consequence of resistance to its function, and this agrees with our control groups, we noticed that leptin elevated markedly in obese individual in control groups and in patients group and this elevation may be due to high insulin level in patients because leptin have positive correlation with insulin in addition elevated value of BMI.

Leptin correlated positively with HDL; cholesterol and weak correlated with triglycerides in the diabetic group, as in the study where serum leptin concentrations were investigated in a group of patients with moderate and severe obesity (**Liuzzi*et al.*, 1999**).

In our study, Creatinine was found to have a difference between both sexes and groups. It can noticed that the values are higher in males than females. This was matching with the findings of another investigation (**Bedir*et al.*, 2003)**. Leptin is known to accumulate in a uremic state; however, leptin levels and renal creatinine clearance showed no significant correlation in peritoneal dialysis patients (**Aguilera *et al.*, .,2002).**

Some investigators have found that in more obese patients with type 2 diabetes mellitus, the leptin levels were less in patients with not well controlled diabetes than

in well-controlled diabetic subjects **(Clement *et al.*,1997)**. This was related to the insulin deficiency. Leptin levels were significantly lower in patients who have HbA1c above 8.5%. It has been found that untreated diabetes gave rise to a reduction in leptin levels owing to an ineffective release of insulin by the monodrug therapy **(Sivitz *et al.*, 2003)**.

An absence of leptin receptor expression in human diabetic foot ulcer, which is the consequence of poor glycemic control for many years, was well documented while investigating the role of leptin for the inflammatory response in diabetes-impaired skin repair .This may show that leptin itself might function as a regulatory link between endocrine and immune system in the context of skin repair (**Goren*et al.*, 2003).** However, contrarily to these findings, no significant relation between leptin levels and the degree of the severity of diabetes was observed in another study **(Tasaka*et al.*, 1997).**

Abdominal fat tissue that can be detected by densitometry, hydrometry, dual-energy X-ray absorptiometry, a chemical multi- compartment model, bioelectrical impedance, computerized tomography and magnetic resonance imaging is also well correlated with leptin levels (**Johannsson *et al.*, 1998)**.

Leptin's relationship with total body fat and insulin resistance was independent of age and gender (**De Courten *et al.*, 1997;Liuzzi ,*et al.*, 1999 and Donahue*et al.*,1999).**

In the present study, we did not investigate the abdominal fat tissue percentage in the participants with the aforementioned instruments and

imaging techniques. We speculate that as a consequence of insulin deficiency and the different distribution of fat tissue throughout the body, we have found low leptin levels in diabetic subjects and even lower levels in subjects who have poorly controlled diabetes.

The U.S. Department of Health and Human Services found that losing a mere 7% of body weight resulted in more than a 50% reduction in incidence of adult onset diabetes. For a 200 pound person this is a mere 14 pounds. Obesity is clinically associated with high insulin, high glucose, hypertension, high cholesterol, high triglycerides, increased insulin resistance, high CRP levels, high homo cysteine levels, high uric acid, high leptin, The American Diabetes Association, the North American Association for the Study of Obesity, and the American Society for Clinical Nutrition recently issued a statement **(American Journal of Clinical Nutrition v 80, 2004)** Weight Management Through Lifestyle Modification for the Prevention and Management of Type 2Diabetes. "Overweight and obesity are important risk factors for type 2 diabetes **(Mason, 2012)**.

The marked increase in the prevalence of overweight and obesity is presumably responsible for the recent increase in type 2 diabetes. Lifestyle modification aimed at reducing energy intake and increasing physical activity is the principal therapy for overweight and obese patients with type 2diabetes. The prevalence of diabetes in the U.S. continues to rise by epidemic proportions. This increase parallels the rising rates of obesity and overweight observed over the last decade. Indeed, as BMI increases, the risk of developing type 2 diabetes increases in adose-dependent manner.

Obesity is one of the cornerstone factor. What specific diagnostic tests can you get? When you get your standard, basic blood analysis profile you'll test glucose, uric acid, white blood cell count, total cholesterol, HDL (high density or "good"), LDL (low density or "bad"), and triglycerides. Total cholesterol should be about 150 ideally, and very definitely well under 200. Triglycerides are very crucial here, and should be under100. Uric acid should be under 5 mg/dl for men and under 4 mg/dl for women. The white blood cell count should be in normal range.

The liver is very important here for a lot of reasons. Get both SGOT and SGPT tests. Surprisingly, you do not need to get your insulin tested. You should also have your homo cysteine (Hcy) tested and look for 10mmol or less. This is a also good predictor of coronary heart disease (CHD) in general. Currently we do not have practical, inexpensive tests to determine total oxidative stress or general free radical levels. It is costly and unnecessary to test the basic status of antioxidants such as SOD, glutathione, vitamin C, beta-carotene, and vitamin E **(Mason, 2012).**

The prevalence of type 2 diabetes in obese adults is 3-7 times that in normal weight adults. Those with a BMI greater than 35 are twenty times as likely to develop diabetes as those with a BMI between 18.5 and 24.9. In addition, weight gain during adulthood is directly correlated with an increased risk of type 2 diabetes. Obesity also complicates the management of type 2 diabetes by increasing insulin resistance and blood glucose concentrations. Obesity is an independent risk factor for dyslipidemia, hypertension, and CHD, and thus increases the risk of cardiovascular complications and cardiovascular mortality in patients with type 2 diabetes. Weight loss is an important goal for overweight and obese persons, particularly those with type 2 diabetes because it improves glycemic control. Moderate weight loss (5%of body weight) can improve insulin action, decrease fasting blood glucose concentrations, and reduce the need for diabetes medications **(Mason, 2012)**. Moreover, improvements in fasting blood glucose are directly related to the relative amount of weight loss". Want clinical proof from Cornell University **(American Journal of Clinical Nutrition, 1987)** that the person can literally eat all he want, lose weight and never be hungry? Women were allowed to eat all the whole natural foods they wanted, as long as they had 20% or less fat calories. They could eat 24 hours a day! In only 30 days, they lost considerable weight by just eating foods lower in fat. The ones who ate the 30% fat diet lost no weight.

Your fat calorie intake must be under 20%. 15% is better. Again, the average American eats about 42% fat calories, and most of these are saturated animal fats. There are many more similar published clinical studies showing the very same results.

Diabetes is also associated with elevated blood levels of low-density lipoproteins. Low-density lipoproteins are carriers of “bad” holesterol. Resveratrol inhibits copper mediated low-density lipoprotein oxidation **(Belguendouz *et al.*, 1997).**

In diabetes mellitus, abnormally increased levels of lipids, lipoproteins and lipid peroxides in plasma may be due to the abnormal lipid metabolism. Patients with type 2 diabetes frequently have an abnormal blood lipid profile consisting of moderately elevated LDL-C, moderately decreased HDL-C, and high TC and triglycerides. Thus, inadequate levels of HDL-C, in conjunction with more atherogenic forms of LDL-C may contribute to atherogenesis **(Libby, 2004).**

The best way to understand the dysfunction of insulin and blood sugar is the theory of oxidative stress. Here free radicals run rampant through the body, and use up our antioxidants– glutathione,SOD (superoxide dismutase), beta carotene, vitamin E, vitamin C, melatonin, lipoic acid and others. This is why it is so important to; first, lower the oxidative stress with better diet and exercise. Secondly, we need to take all the known antioxidant supplements to neutralize the excess free radicals.

The high rates of alcohol and nicotine use add to oxidative stress. Coffee (or any form of caffeine) raises blood sugar, and has other very serious health effects. The scientists of the world are in basic agreement that free radical oxidative stress is central to blood sugar conditions.

It can observed the diagnostic indications of blood sugar dysmetabolism ,If we look for any combination of obesity, hypertension, insulin resistance, low HDL cholesterol, high LDL cholesterol, high triglycerides, hypertension, high homo

cysteine, elevated uric acid, increased C-reactive protein, hyper coagula ability of the blood, fasting blood glucose over 85 mg/dL (European mmol/L is 4.7),HbA1c over 4.7%, low white blood cell count, high creatinine, and proteinuria (albumin in the urine). Age is critical here. The older we are, the more blood sugar problems you can expect to have. Diabetes rates go up dramatically after the age of 50. Genetics is obviously important, and any family history of such problems increases your chances.

The most accurate test you can get is a glucose tolerance test (GTT). This is more predictive than testing your insulin level per se, as it shows the sensitivity of your insulin. If our blood sugar level is over 85 mg/dl you need to do this. A GTT is the "gold standard", accurate, well known, inexpensive, but very under-utilized. It is just not commonly done due to lack of knowledge in the medical profession. Simply get a one hour, one blood draw test (we should already know our fasting blood glucose level so our won't need a draw before our drink the glucose solution).

An HbA_{1c} test is very accurate. This tests long-term glycation (sugar molecules attached to hemoglobin), and the result should be 4.7% (equals 85 mg/dL blood sugar) or less, and not the 5.6% (100 mg/dL) the doctors will tell you. 4.7% or less. There are exotic tests we can get such as leptin, malondialdehyde, thrombomodulin, tumor necrosis factor-alpha (TNF), adoponectin, plasminogen, fibrinogen, and others. These are simply not practical or necessary; plus they can be expensive and hard to obtain. It is very difficult to test the amount of oxidative stress you suffer from, or your antioxidant status in general. There just is not any reason to Spend the time and money on these tests. Your attention needs to be on curing yourself and changing your diet and lifestyle, rather than getting exotic diagnostic tests you do not need.

Generally, people can simply get their total cholesterol (TC) and triglycerides (TG) tested. Here you can also test your high density ("good") HDL and your low-density ("bad") LDL levels.

Our TC should ideally be about 150 mg/dl. The media and medical profession will tell you that a TC 200 level or less is good,but that's just not the case at all. Even if you have genetically, high cholesterol you can still keep your level well under 200 with diet, supplements, hormones, and exercise. Please read my book Lower Cholesterol without Drugs. The TG level is the most important blood lipid marker of all for blood sugar problems. Even vegans and ethical vegetarians can (and usually do) have high levels due to an inordinate intake of sweets. Our TG means more than your TC, LDL, or HDL. We should keep our triglyceride level below100. We can do this with the same means as for total cholesterol.

People with blood sugar problems usually have low HDL and high LDL levels. People with low cholesterol (e.g. about150) will naturally have lower HDL levels. You can raise your HDL and lower your LDL the same way with diet and lifestyle.

Fasting blood plasma glucose is part of our basic routine blood analysis. Again, our level should be at 85 mg/dl or less (European 4.7 mmol/L). Levels of 100 and higher are clearly prediabetic. We need to have low blood sugar, and the usual accepted level of 100 and less just is not good enough. Doctors will tell us that any reading under 100 is "normal", but this just is not true. This was proven at the Riks-hospitalet Hospital, in Norway, with a 22-year follow-up study of 1,998 healthy men. Those with glucose levels of 85 or less lived the longest, and had the least cardiovascular disease - the biggest killer of people in the world by far **(Mason, 2012).**

Serum creatinine is another. Eating a low protein diet is vital. Eat no more than 10% seafood if you like. Americans eat twice the protein they need, which

causes many health problems. Again, don't get carried away with diagnostic tests. Put your time and energy into curing ourself.

Cholesterol is a soft, waxy substance found in all areas of the body, including the heart, liver, intestines, muscle, skin, and nervous system. It is produced by the liver and derived from animal-based foods (like meat, eggs, and butter) in the diet. Its job is to help form cell membranes, some hormones, and vitamin D. There are two types of cholesterol:"good" and "bad." "Bad" cholesterol (LDL) flows from the liver to the rest of the body. When there is too much LDL cholesterol in the bloodstream, some of it can stick to the inside of the arteries, causing a buildup of plaque and hampering the flow of blood. This can lead to stroke or heart attacks. It is recommended that people maintain blood levels that are low in "bad" cholesterol and relatively high in "good" cholesterol."Good" cholesterol (HDL) flows back in the blood from the rest of the body to the liver. It helps blood vessels and the liver to clean up and eliminate excess cholesterol.

The healthiest levels of cholesterol are total cholesterol: Less than 200; LDL: Less than 100and HDL: Greater than 40. There are four different types of fats in our diet. The first two are unsaturated fats (mono unsaturated fats and poly unsaturated fats) and are found in olives and olive oil, most nuts, avocados, fish, and most liquid cooking oils, such as corn and soybean. These types of fats give us a good balance of cholesterol in our bloodstream. The fats that increase our bad cholesterol levels are saturated fats and trans fatty acids. Saturated fats are found in whole milk and other dairy products, Nevertheless, we still do not know whether metabolic age-dependent remodelling is an adaptive process affecting only one parameter such as insulin action or whether it is also due toa more general process encompassing the main causes of age-related metabolic derangement.

Leptin is the product of the LEP gene and has been shown to regulate body weight and food intake. Hyper-leptinemia is the result of an imbalance between

leptin overproduction (by the adipose tissue) and peripheral leptin resistance at the level of the hypothalamus and it is associated with raised energy intake and obesity. Since healthy centenarians eat less than subjects >75years of age **(Paolisso *et al.*, 1995)**

and have a more favorable body fat content and distribution, one could hypothesize that differences in plasma leptin concentrations may play a role in explaining longevity.

Indeed, plasma leptin concentrations in healthy centenarians were found to be between those of subjects <50 years old and those of subjects

>75 years old and were positively correlated with BMI and body fat content **(Paolisso *et al.*, 1997)**. The healthy centenarians have lower leptin production and/or better hypothalamic sensitivity in comparison with elderly subjects. Both phenomena may contribute to controlling energy intake and avoiding a rise in body fat content in healthy centenarians.

Healthy centenarians have been found to have a lower degree of oxidative stress. Analysis of the causes of this striking difference revealed that healthy centenarians had greater plasma antioxidant defences (mainly vitamin E) in comparison with subjects old aged ≥ 70 years. It could be hypothesized that elevated plasma vitamin E helps to preserve insulin action in healthy centenarians. Nevertheless, due to the viciouscircle between insulin action and oxidative stress, one cannot rule out that healthy centenarians have lower oxidative stress in comparison with elderly subjects due to preserved insulin action, and vice versa**.(Paolisso *et al.*,1998).** observed that although the source of this oxidative stress remains unclear, it has been suggested that the chronic hyperglycemia in diabetes enhances the production of ROS from glucose autoxidation, protein glycation and glycoxidation, which leads to tissue damage **(Brownlee *et al.*, 2000**). Reported that many studies have shown that increased lipid peroxides and/ or oxidative stress are present in diabetic subjects

(Bonnefont *et al*., 2000). Noticed that Oxidative stress could be increased before clinical signs of diabetic complications. However, the role of oxidative stress in the initiation and progression of diabetes remains uncertain. **(West *et al*., 2000**). It is debatable whether oxidative stress precedes the appearance of diabetic complications or whether it merely reflects the presence of complications or consequence of complications. In diabetes, oxidative stress seems caused by both increased production of ROS, sharp reduction in antioxidant defenses and altered cellular redox status. Increasing evidence in both experimental and clinical studies suggests that there is a close link between hyperglycemia, oxidative stress and diabetic complications. High blood glucose level determines overproduction of reactive oxygen species (ROS) by the mitochondria electron transport chain. High reactivity of ROS determines chemical changes in virtually all cellular components, leading to DNA and protein modification and lipid peroxidation. Measurement of biomarkers such 8-hydroxy-2′deoxyguanosine (8-OHdG), isoprostanes, malondialdehyde (MDA) and nitrotyrosine is a useful tool to assess the oxidative stress of the organism. Knowledge of the mechanisms of ROS damage of is the first step for development of new therapeutic molecules and for rationalizing the use of existing drugs.

Chapter 5:

Conclusion and Recommendations

Conclusion and Recommendations

Concludo

It can conclude from this study that a positive relationship between Leptin levels and both Lipid Profile and Oxidative Stress of diabetes mellitus type II patients was observed.

Recommendations:

*****Further large-scale studies which should investigate the physiopathologic mechanisms are required to make clear the issue for lower leptin levels, whether it is a reason or an outcome.

*The interactions between genetics and environmental factors require further elucidation. However, the current state of knowledge provides convincing evidence that the major determinants of differences in metabolic risk factors within and across populations primarily are due to behavioral and lifestyle factors (diet, physical activity, obesity, smoking, alcohol use), rather than genetic differences.

Chapter 7:

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