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# Obestatin level and some biochemical parameters in type 2 diabetic women attending Medical Relief Center in Gaza Governorate

**Submitted in Partial Fulfillment for the degree of Master of Science in Biological Sciences- Zoology**

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# Dedication

**To my parents who have always supporting me**

**To my husband Rashad who helped me to accomplish this thesis.**

**To my children, Renad and Kareem**

**To my brothers and sisters**

**To my university The Islamic university of Gaza which is continuously improving**
**the research**

**To all of them I dedicate this work**

**Mona M. Hamam**

## Declaration

I certify that this submission is my own research and that, to the best of my knowledge and belief, it contains material neither previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree of the university of other institute, except where due a acknowledgment has been made in the text.

| Signature | Name | Date |
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| Mona | Mona M. Hamam | September, 2012 |

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# Obestatin level and some biochemical parameters in type 2 diabetic women attending Mediacl Relief Center in Gaza


## Abstract

**Background:** Obestatin is a novel 23-amino acid peptide derived from the mammalian prepro-ghrelin polypeptide. Recent studies demonstrated that obestatin is a metabolic hormone associated with type 2 diabetes. Therefore, assessment the status of obestatin and clarifying such association in diabetes could constitute a promising therapy of the disease.

**Objective:** To asses obestatin level and some biochemical parameters among type 2 diabetic women attending Mediacl Relief Center in Gaza.

**Materials and Methods:** This case-control study comprised 64 type 2 diabetic women and 64 healthy non diabetic women. Questionnaire interview was applied. Serum obestatin was measured by ELISA. Serum glucose, cholesterol, triglycerides, high density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LDL-C), urea and creatinine were determined. Blood HbA1c was also measured. Data were computer analyzed using SPSS version 18.0.

**Results:** The mean ages of controls and cases were 52.7±8.9 and 52.9±8.3 years. Type 2 diabetes was found to be significantly associated with family history and diet. More than half of patients had diabetes since less than 5 years. The main self-reported complications among patients were neuropathy, retinopathy and cardiovascular disease. The longer the duration of diabetes, the higher the prevalence of self-reported complications. The mean level of serum obestatin was significantly lower in diabetic women compared to healthy non diabetic controls (3.4±0.8 ng/ml vs. 4.1±1.5 ng/ml, P=0.023).The mean HbA1c and glucose levels in cases were significantly higher than that in controls (6.4±0.9 and 190.7±86.7 vs 4.7±0.6 and 101.6±21.0) with P=0.000. The Pearson correlation test showed negative significant correlations between obestatin levels and HbA1c and glucose (r=-0.320, P=0.009 and r=-0.469, P=0.000 respectively). The average level of triglycerides was significantly increased in diabetic women (183.3±80.0mg/dl) compared to controls

(122.4±42.9 mg/dl) with P=0.000. On the other hand, HDL-C was significantly lower in cases (40.7±11.2vs. 47.2±12.0mg/dl, % difference=14.8 and t=2.228, P=0.030). The Pearson correlation test showed negative significant correlations between obestatin levels and triglycerides levels (r=-0.275, P=0.026).

**Conclusions:** Serum obestatin was significantly lower in diabetic women compared to healthy non diabetic women. There were negative significant associations between obestatin levels with HbA1c, glucose and triglycerides levels.

**Keywords:** Obestatin, diabetes, HbA1c, glucose, lipid profile, Gaza.

## مستوى الاوبستاتين وبعض المعايير الكيميائية لدى مرضى السكري من النوع الثاني من الاناث اللواتي يرتدن مركز الاغاثة الطبية في غزة

**المقدمة:** اكتشف هرمون الاوبستاتين حديثا وهو يتكون من 23 حمض اميني مشتق من عديد الببتيد بدائي الجريلين في الثدييات. وقد اظهرت الدراسات الحديثة ان الاوبستاتين هرمون متعلق بالأيض ويرتبط بمرض السكري من النوع الثاني ولذلك فان تقييم حالة الاوبستاتين وتوضيح هذا الارتباط في مرض السكري ممكن ان يشكل علاج واعد لهذا المرض.

**الهدف:** تهدف الدراسة الى قياس مستوى هرمون الاوبستاتين وبعض المعايير البيوكيميائية في مرضى السكري من النوع الثاني من الاناث اللواتي يرتدن مركز الاغاثة الطبية في غزة.

**الطرق والادوات:** منهج الدراسة (مجموعة مرضية-مجموعة ضابطة)، المجموعة المرضية تحتوي على 64 امرأة مصابة بالنوع الثاني من داء السكري والمجموعة الضابطة تحتوي على 64 امرأة غير مصابة بداء السكري. تم الحصول على البيانات المستخدمة في الدراسة من خلال المقابلة المباشرة مع المرضى. تم قياس مستوى كل من هرمون الاوبستاتين، السكر، الكوليسترول، الدهون الثلاثية، البروتين الدهني عالي الكثافة، البروتين الدهني منخفض الكثافة، البولينا و الكرياتينين وايضا تم قياس نسبة ارتباط السكر بالهيموجلوبين في الدم. تم تحليل البيانات والنتائج التي تم الحصول عليها باستخدام البرنامج الاحصائي المحوسب spss 18.0.

**النتائج:** كان متوسط العمر للمجموعة الضابطة هو 8.9±52.7سنة ومتوسط العمر للمجموعة المرضية هو 52.9 ±8.3 سنة. اوضحت النتائج ان مرض السكري يرتبط بالتاريخ العائلي والتغذية وان اكثر من نصف مرضى السكري يعانون من السكري منذ خمس سنوات او اقل. كما ان المضاعفات الرئيسية التي سجلت على لسان المرضى اوضحت وجود اعتلال الاعصاب واعتلال الشبكية وامراض القلب والاوعية الدموية وانه كلما زادت مدة الاصابة بالسكري كلما زادت نسبة المضاعفات بين المرضى. وقد أظهرت النتائج أن متوسط مستوي هرمون الأوبستاتين كان أقل في المجموعة المرضية مقارنة بالمجموعة الضابطة وقد كانت النتائج ذات دلالة إحصائية. كانت نتيجة متوسط كل من الجلوكوز ونسبة ارتباط السكر بالدم أعلى بدلالة احصائية في المجموعة المرضية منها في المجموعة الضابطة. وقد اظهرت النتائج وجود علاقة عكسية بين مستوى هرمون الاوبستاتين والجلوكوز ونسبة ارتباط السكر بالهيموجلوبين (مخزون السكر). كانت نتيجة متوسط مستوي الدهون الثلاثية أعلى بدلالة احصائية في المجموعة المرضية منها في المجموعة الضابطة. اما مستوى البروتين الدهني عالي الكثافة فقد كان اقل بدلالة احصائية في المجموعة المرضية منها في المجموعة الضابطة. و اظهرت النتائج وجود علاقة عكسية ذات دلالة احصائية بين الدهون الثلاثية و مستوى هرمون الأوبستاتين حيث كلما زادت الدهون الثلاثية كلما قل مستوى هرمون الأوبستاتين.

**الاستنتاجات:** تبين ان مستوي هرمون الأوبستاتين أقل بدلالة أحصائية في النساء المصابات بداء السكري من النوع الثاني مقارنة مع النساء الغير مصابات. كما انه توجد علاقة عكسية ذات دلالة احصائية بين هرمون الأوبستاتين و الجلوكوز ،نسبة ارتباط السكر بالهيموجلوبين والدهون الثلاثية.



**الكلمات المفتاحية:** الأوبستاتين، الجلوكوز، نسبة ارتباط السكر بالدم ، الدهون، السكري، غزة.

# Table of Contents

# Abbreviations

| | |
|---|---|
| Adenosine diphosphate | **ADP** |
| Adenosine triphosphate | **ATP** |
| Adenylyl cyclase | **AC** |
| Advanced glycosylated end-products | **AGEs** |
| Body Mass Index | **BMI** |
| Cardiovascular disease | **CVD** |
| Center for Disease Control and prevention | **CDC** |
| Cholesterol esterase | **CHE** |
| Cholesterol oxidase | **CHOD** |
| Cyclic Adenosine mono-phosphate | **cAMP** |
| Deoxyribonucleic acid | **DNA** |
| Diabetes mellitus | **DM** |
| Diastolic blood pressure | **DBP** |
| Enzyme immunoassay | **EIA** |
| Enzyme linked immunosorbant assay | **ELISA** |
| Ethylenediamine tetraacetic acid | **EDTA** |
| Extracellular signal-regulated kinase | **ERK** |
| Fasting plasma glucose | **FPG** |
| Gestational diabetes | **GD** |
| G protein-coupled receptor 39 | **GPR39** |
| Ghrelin-O-acyltransferase | **GOAT** |
| Glucagon-like peptide-1 | **GLP-1** |
| Glucagon-like peptide-1 receptor | **GLP-1R** |
| Glucose-dependent insulinotropic peptide | **GIP** |
| Glycerol kinase | **GK** |
| Glycerol phosphate oxidase | **GPO** |
| Growth hormone | **GH** |
| Growth hormone secretagogue receptor type 1a | **GHS-R1a** |
| Hemoglobin A1c | **HbA1c** |
| High density lipoprotein cholesterol | **HDL-C** |
| Horse radish peroxidase | **HRP** |

| | |
|---|---|
| Immunoglobulin G | **IgG** |
| Impaired glucose regulation | **IGR** |
| Impaired glucose tolerance | **IGT** |
| Lipoproteinlipase | **LPL** |
| Low density lipoprotein cholesterol | **LDL-C** |
| MAP kinase kinase | **MEK** |
| Messenger ribonucleic acid | **mRNA** |
| Mitogen activated protein kinase | **MAPK** |
| Nicotinamide adenine dinucleotide | **NAD+** |
| Normal glucose tolerance | **NGT** |
| Palestinian clinical laboratory tests guide | **PCLTG** |
| Peroxidase | **POD** |
| Protein kinase A | **PKA** |
| Statistical Package of Social Sciences | **SPSS** |
| Streptavidin | **SA** |
| Systolic blood pressure | **SBP** |
| Tetramethylbenzidine | **TMB** |
| The homeostasis model assessment of insulin resistance | **HOMA-IR** |
| Triglyceride | **TG** |
| Type 2 diabetes mellitus | **T2DM** |
| United Kingdom | **UK** |
| United States | **US** |
| Very low density lipoprotein cholesterol | **VLDL-C** |
| Waist-to-hip ratio | **WHR** |
| World Health Organization | **WHO** |

# Chapter 1

# Introduction

## 1.1 Overview

Diabetes mellitus represents a group of diseases of heterogeneous etiology, characterized by chronic hyperglycemia and other metabolic abnormalities, which are due to deficiency of insulin action, insulin secretion 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 or viral 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, 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 produce it (Cohen, 2006).

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 and Simonson,1992 and Michael et al., 2000). In such conditions, lipolysis in adipose tissue is promoted leading to elevated circulating levels of free fatty acids. 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). In addition, disturbance in serum urea and creatinine was also reported in type 2 diabetes (Sharma et al., 2011 and Yassin et al., 2011).

Obestatin is one of the three ghrelin gene products. It is a novel 23-amino acid peptide derived from the mammalian prepro-ghrelin polypeptide (Zhang

et al., 2005). The expression of obestatin was reported in cells of the gastric mucosa, myenteric plexus and perinatal pancreas and in Leydig cells of the testis (Zhang et al., 2005; Chanoine et al., 2006 and Dun et al., 2006). Obestatin binds to an orphan G protein-coupled receptor, termed GPR39, to inhibit food intake (Zhang et al., 2005). On the other side, obestatin manifested various biological functions, such as improving memory performance, causing anxiolytic effects (Carlini et al., 2007), inhibiting thirst in rats (Samson et al., 2007), activating cortical neurons (Dun et al., 2006), stimulating proliferation of retinal pigment epithelial cells in vitro (Camiña et al., 2007), and profoundly influencing sleep (Szentirmai and Krueger, 2006b and Szentirmai et al., 2009).

There is great controversy about the effects of obestatin on food intake with some studies suggesting that it may inhibit food intake and suppress body weight under basal and ghrelin-stimulated conditions (Bresciani et al., 2006; Zizzari et al., 2007 and Hassouna et al., 2010) and others suggesting no effect (Seoane et al., 2006 and Gourcerol et al., 2007). In addition, articles linked obestatin and diabetes mellitus were very few and recent. Obestatin status in type 2 human diabetes remains controversial. One study showed decreasing concentration of obestatin with type 2 diabetes and impaired glucose regulation and insulin resistance (Qi et al., 2007). In another study, St-Pierre et al., (2010) showed that normal and diabetic subjects displayed similar levels of circulating obestatin in fasting condition. However, patients with type 2 diabetes looked refractory to the inhibitory effect of meal on obestatin secretion. Regarding lipid metabolism, it was reported that obestatin treatment led to decrease in plasma triglycerides (Nagaraj et al., 2008 and 2009 and Agnew et al., 2011).

Although the prevalence of diabetes in Gaza Strip is alarming, the available studies are limited and biochemical data are only restricted to traditional tests when the patient visits the clinic. However, a recent study investigated the status of leptin hormone in type 2 diabetic patients in Gaza Strip (Altawil, 2009). No previous study was conducted on the novel hormone obestatin in type 2 diabetes in Gaza Strip or even in the Arab World. Therefore, this study

will be the first study to assess obestatin status in type 2 diabetic females in Gaza Governorate, Gaza Strip.

## 1.2 General objective

The general objective of the present study is to assess obestatin level and some biochemical parameters in type 2 diabetic women attending Medical Relief Center in Gaza.

## 1.3 The specific objectives were:

1. To investigate obestatin level in type 2 diabetic women and compared them with healthy controls.

2. To determine HbA1c in blood and serum glucose, lipid profile including cholesterol, triglycerides, high density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LDL-C), urea and creatinine in diabetic patients and controls.

3. To study sociodemographic and clinical data in type 2 diabetic patients.

4.To verify the relationships of obestatin with the previous parameters.

## 1.4 Significance

1. Diabetes mellitus is prevalent globally as well as in the Gaza Strip, and leads to high rates of morbidity and mortality.

2. Globally, there are few studies linked obestatin with type 2 diabetes. Of course, this will be the first study to describe obestatin levels in type 2 diabetic patients in Gaza Strip or even in the Arab world.

3. Understanding the role of obestatin in diabetes mellitus may make it a promising target for the developing of new drugs for the treatment of the disease.

# Chapter 2

# Literature Review

## 2.1 Definition of diabetes mellitus

It is a metabolic disorder characterized by chronic hyperglycemia due to disturbances of carbohydrate, fat and protein metabolism that are associated with absolute or relative deficiencies in insulin secretion, insulin action or both. Diabetes poses a major and growing health and socio-economic burden on society that affects over 177 million people worldwide and this figure is likely to be more than double by the year 2030 (WHO, 2003).

## 2.2 Types of diabetes

The most common types of diabetes mellitus are:

### 2.2.1 Type 1 diabetes

Type 1 diabetes indicates the processes of beta-cell destruction that may ultimately lead to diabetes mellitus in which insulin is required for survival to prevent the development of ketoacidosis, coma and death. It is characterized by the presence of islet cell or insulin antibodies which lead to beta cell destruction. Type 1 diabetes accounts for 5-10% of all diagnosed cases of diabetes (Olefsky, 2001). Despite 30 years of research, the cause of type-1 diabetes remains unknown (Wilkin, 2006).

### 2.2.2 Type 2 diabetes

Type 2 diabetes mellitus is a heterogeneous syndrome which accounts for about 90-95% of all diagnosed cases of diabetes. The causes of type 2 diabetes are multi-factorial and caused by a group of both genetic and environmental elements that affect beta-cell function and tissue (muscle, liver, adipose tissue, pancreas) insulin sensitivity (Scheen, 2003). Type 2 diabetes develops in individuals who fail to compensate for insulin resistance by

increasing pancreatic insulin secretion. This insulin deficiency results from pancreatic beta-cell dysfunction and death (Cnop, 2008).

### 2.2.3 Gestational diabetes

Gestational Diabetes (GD) is defined as any degree of glucose intolerance which begins or is first recognized during pregnancy. The risk for developing type 2 diabetes within the first decade following pregnancy in GD cases ranges between 35% and 60% (Wroblewska-Seniuk et al., 2009). Similarly, children of women with GD are known to be at risk for obesity and diabetes mellitus in their later life (Bánhidy et al., 2011).

## 2.3 Prevalence and mortality rate of diabetes mellitus in Palestine

The prevalence of diabetes mellitus in Palestine was examined in a study conducted in 2000 in cooperation with Al-Quds University and Ministry of Health. The results indicated that the prevalence was about 9% (Ministry of Health, 2002). It is around the reported prevalence rate in Egypt and Tunisia (9%) and less than in Saudi Arabia (12%) and Oman (13%). However, in Palestine, there is under-diagnosis and under-reporting of the disease. This is due to lack of proper hospital and clinic recording system (Ministry of Health, 2005). The mortality rate of diabetes mellitus among Palestinians constituted 5.9 per 100,000 population in the year 2009 (Ministry of Health 2009), and this figure raised to 8.5 per 100,000 population in the year 2010 (Ministry of Health 2010).

## 2.4 Type 2 diabetes

### 2.4.1 Metabolism in type 2 diabetes

Circulating glucose is derived from 1) 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 from 2) 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 glucose removal from the circulation. The glucoregulatory hormones of the body are designed to maintain circulating glucose concentrations in a relatively narrow range. Glucoregulatory hormones include insulin, glucagon, amylin, glucagon-like peptide-1 (GLP-1), glucose-dependent insulinotropic 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 GLP-1 and GIP 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 removal into skeletal muscle and adipose tissue is driven mainly by insulin. At the same time, endogenous glucose production is suppressed by 1) the direct action of insulin on the liver, and 2) 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 and Simonson,1992 and Michael et al., 2000).

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 and Green, 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). In addition, disturbance in serum urea and creatinine was also reported in type 2 diabetes (Sharma et al., 2011).

### 2.4.2 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, C.W. 1994 and Harris et al., 1995). The chronic complications include cardiovascular disease, peripheral vascular disease, cerebrovascular disease, diabetic retinopathy and diabetic nephropathy (Savage, 1996; Dyck et al., 2002; Bate and Jerums, 2003; Susztak et al., 2003 and The National Eye Institute, 2006). 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, 2008).

#### 2.4.2.1 Retinopathy

The risk of developing diabetic retinopathy or other microvascular complications of diabetes depends on both the duration and the severity of hyperglycemia. Retinopathy may begin to develop as early as 7 years before the diagnosis of diabetes in patients with type 2 diabetes. Retinopathy involves changes in the retina. These changes happen because of damage or growth problems in the small blood vessels of the retina. Usually, changes in the retinal blood vessels don't appear before a person has reached puberty. One reason why diabetes needs to have regular yearly eye exams is because people with retinopathy may not have any problems seeing at first. But if the condition gets worse, they can become blind. A person with diabetes may be able to slow or reverse the damage caused by retinopathy by improving blood sugar control. If retinopathy becomes more advanced, laser treatment may be needed to help prevent vision loss (The National Eye Institute, 2006).

#### 2.4.2.2 Diabetic neuropathy

Diabetic neuropathy can affect nerves in many different parts of the body. The most common early symptoms of the condition are numbness, tingling, or sharp pains in the feet or lower legs. An estimated 50% of those with diabetes have some form of neuropathy, but not all with neuropathy have symptoms. The highest rates of neuropathy are among people who have had the disease for at least 25 years. Diabetic neuropathy also appears to be more common in people who have had problems controlling their blood glucose levels, in those with high levels of blood fat and blood pressure, overweight people, and people over the age of 40 (Dyck et al., 2002). If it's not treated, nerve damage can cause a number of problems. For example, because of the numbness, people with nerve damage might not realize that they have a cut, and it could become seriously infected before they discover it (Debra Manzella, 2006).

#### 2.4.2.3 Cardiovascular disease

Cardiovascular disease is the number one killer of people with type 2 diabetes, people with diabetes developing certain problems with the heart and blood vessels. Some of these problems are Heart attack, stroke and blockage

of blood vessels in the legs and feet, which can lead to foot ulcers, infections, and even loss of a toe, foot, or lower leg (Marshall, 2006). Myocardial ischemia due to coronary atherosclerosis commonly occurs without symptoms in patients with diabetes. As a result, multivessel atherosclerosis often is present before ischemic symptoms occur and before treatment is instituted. A delayed recognition of various forms of coronary heart disease undoubtedly worsens the prognosis for survival for many diabetic patients. One reason for the poor prognosis in patients with both diabetes and ischemic heart disease seems to be an enhanced myocardial dysfunction leading to accelerate heart failure. Several factors probably underlie diabetic cardiomyopathy: severs coronary atherosclerosis, prolonged hypertension, chronic hyperglycemia, microvascular disease, glycosylation of myocardial proteins, and autonomic neuropathy (Savage, 2005).

## 2.5 Obestatin

### 2.5.1 Definition and site of secretion

Obestatin is a novel 23-amino acid peptide derived from the mammalian prepro-ghrelin polypeptide, with molecular weight 2546.8 (Zhang et al., 2005). The expression of obestatin was reported in cells of the gastric mucosa, myenteric plexus and perinatal pancreas, mammary gland, breast milk, Leydig cells of the testis, spleen and in plasma and saliva (Zhang et al., 2005; Chanoine et al., 2006; Dun et al., 2006; Aydin et al., 2008; Grönberg et al., 2008 and Ozbay et al., 2008).

### 2.5.2 Biosynthesis

Obestatin is one of the three ghrelin gene products. The sequential steps of the production of three ghrelin genes products and their relationship with ghrelin *O*-acyltransferase (GOAT) is illustrated in Figure 2.1 (Chen et al., 2009).The described wild-type human ghrelin mRNA codes for a 117 amino acid long peptide, preproghrelin. Preproghrelin contains a 23 amino-acid signal peptide and a 94 amino-acid called pro-ghrelin (1–94). This includes the 28 amino acid mature ghrelin (1–28) and a 66 amino acid tail (29–94)

(Jeffery et al., 2005). Recent evidence suggests that the 66 amino acid tail of wild type proghrelin (29–94) can circulate as a full-length peptide (C ghrelin) or be processed to smaller peptides, mainly obestatin. There may be two types of C-ghrelin: one derived from the wild type proghrelin and one derived from the exon 3 (or exon 4)-deleted proghrelin (Soares and Leite-Moreira, 2008).

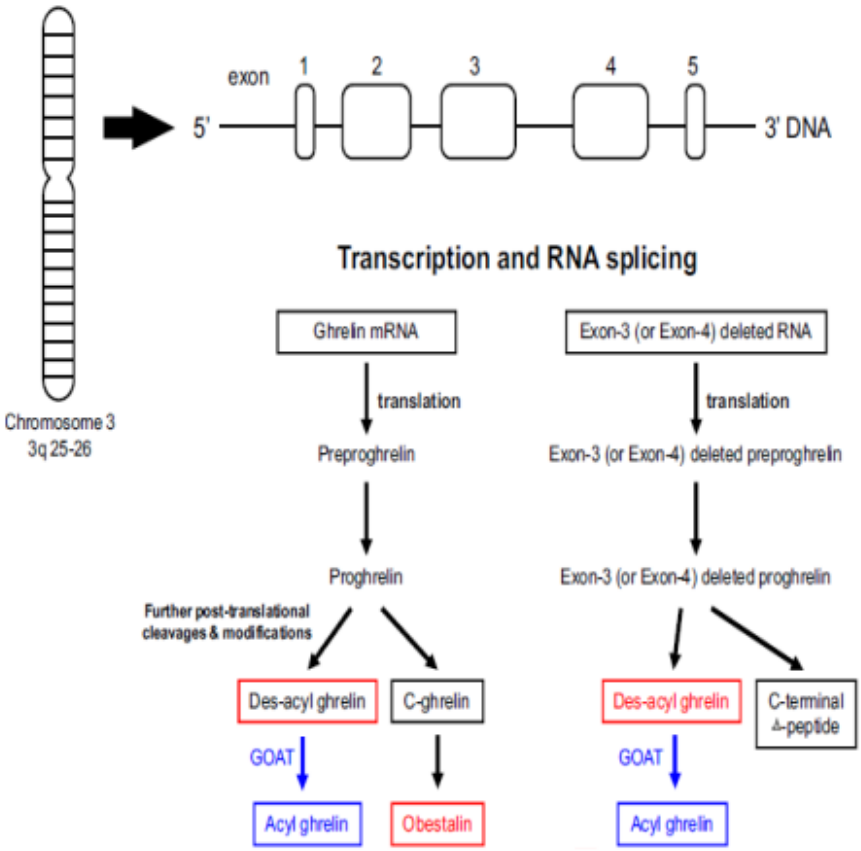



Figure 2.1. A diagram illustrating sequential steps of the production of three ghrelin genes products and their relationship with ghrelin *O*-acyltransferase, GOAT(Chen et al., 2009).

### 2.5.3 Obestatin in plasma and its degradation

Similar to ghrelin and growth hormone, obestatin is secreted in a pulsatile manner. Obestatin and ghrelin amounts in human plasma were found to be 6.9±0.28 and 132.4±13.1 fmol/ml, respectively with ratio of obestatin to ghrelin (%) 5.21 (Mondal et al. 2008). Plasma obestatin was below the detectable

value (less than 0.2 fmol) in rats. Differences in the metabolic degradation kinetics of human obestatin and mouse obestatin were found in plasma, liver and kidney, with half- lives ranging between 12.6 and 138.0 min (Vergote et al., 2008).

## 2.5.4 Biological effects of obestatin

It was originally projected that obestatin binds to an orphan G protein-coupled receptor, termed GPR39 (Zhang et al., 2005). High levels of GPR39 mRNA were found abundantly in the amygdala, the hippocampus, and the auditory cortex but not in the hypothalamus (Jackson et al., 2006). However, Lauwers et al., (2006) indicated that obestatin is not the endogenous cognate ligand for GPR39, whereas a more recent study demonstrated that obestatin was a metabolic hormone capable of binding to GPR39 and, in turn, of regulating the diverse biological functions of gastrointestinal and adipose tissues (Zhang et al., 2008). Furthermore, Granata et al. (2008) reported that obestatin promotes beta cell and human islet survival by binding to glucagon-likepeptide-1 receptor (GLP-1R), the receptor through which incretins act. Similarly, incretins also reduce gastrointestinal motility with a paracrine peripheral action and induce satiety and reduce food intake in the hindbrain specifically in the postrema and nucleus of the solitary tract (Yamamoto et al., 2003 and Holst, 2007). To date, the receptor for obestatin remains unknown and further studies are required to reveal the exact relationship between obestatin, GPR39 and GLP-1R.

### 2.5.4.1 Appetite and body weight

There is great controversy about the effects of obestatin on food intake with some studies suggesting that it may inhibit food intake and suppress body weight under basal and ghrelin-stimulated conditions (Zhang et al., 2005; Bresciani et al., 2006 and Zizzari et al. 2007), and others suggesting no effect (Seoane et al., 2006 and Gourcerol et al., 2007). Recent studies have partially clarified this issue. In one of these studies, intraperitoneal obestatin suppressed food intake and body weight gain in rodent in a U-shaped dose–response relationship. This relationship may explain the difficulties in

reproducing the effects of obestatin on feeding reported by some groups (Lagaud et al., 2007). In the other study, intracerebroventricular administration of obestatin inhibited water drinking in ad libitum-fed and - watered rats, and in food- and water-deprived animals (Samson et al., 2007).The effects on water drinking preceded and were more pronounced than any effect on food intake, and did not appear to be the result of altered locomotor/behavioral activity. Thus, it appears that obestatin possess biologic activity but that the effect on food intake may be secondary to an initial action in inhibiting thirst phenomenon referred to by some authors as dehydration anorexia (Samson et al., 2007).

#### 2.5.4.2 Metabolism

Data regarding effects of obestatin on insulin secretion are limited and controversial as stimulation (Granata et al., 2008), inhibition (Qader et al., 2008), and lack of effect (Green et al., 2007) have been reported. Further studies are necessary to clarify this issue and the effects of obestatin on glucose-sensing neurones, insulin action and hepatic glucose production. In addition, previous studies suggest that obestatin is involved in lipid metabolism. Nagaraj et al. (2008 and 2009) reported that 8-day obestatin treatment led to 22-32% decrease of plasma triglyceride levels. Agnew et al. (2011) did not observe any changes in fasting cholesterol levels following 14-day continuous infusion with obestatin, however obestatin led to a significant 40% reduction in plasma triglyceride levels.

#### 2.5.4.3 Growth hormone secretion

Most studies showed that neither intravenous nor intracerebroventricular administration of obestatin affects the secretion of growth hormone (GH) in rats (Bresciani et al., 2006 and Yamamoto et al., 2007). Nevertheless, one study showed that obestatin, in some conditions, can inhibit exogenous ghrelin action on GH release (Zizzari et al., 2007). It remains to be determined whether obestatin modulates endogenous ghrelin actions.

#### 2.5.4.4 Reproduction

Data regarding obestatin effects on reproductive function are very limited. A recent study demonstrated that obestatin may directly control porcine ovarian granulosa cells functions, stimulating proliferation and apoptosis of these cells and the secretion of progesterone (Mészárosová et al., 2008).

#### 2.5.4.5 Gastrointestinal function

Recently, obestatin was demonstrated to stimulate the secretion of pancreatic juice enzymes through a vagal pathway in anaesthetized rats (Kapica et al., 2007). As with the effects of obestatin on regulation of food intake, its effects on gastrointestinal motility are involved in great controversy with most studies reporting no effect (Bassil et al., 2007; De Smet et al., 2007; Gourcerol et al., 2007; Depoortere et al., 2008 and Chen et al., 2010), and others reporting a negative effect (in gastric and jejunal motility) under basal and ghrelin-stimulated conditions (Zhang et al., 2005). Further studies should clarify this issue and its effects on gastric exocrine secretion and epithelial protection.

#### 2.5.4.6 Cellular proliferation

Obestatin may also be able to influence cell proliferation and apoptosis. It was demonstrated that obestatin induces cell proliferation in primary cultures of human retinal epithelial cells by MEK/ERK 1/2 phosphorylation (Camiña et al., 2007) and promotes human islets cells survival through cAMP increase and involvement of AC/cAMP/PKA signaling (Granata et al., 2008).

#### 2.5.4.7 Bone physiology

Data regarding effects of des-acyl ghrelin and obestatin on bone physiology are very limited. A recent study demonstrated that des-acyl ghrelin stimulates human osteoblasts proliferation in the absence of GHS-R1a (Delhanty et al., 2006). Unlike ghrelin, obestatin does not exert any relevant activity in chondrocytes (Lago et al., 2007).

#### 2.5.4.8 Other effects

Neither intravenous nor intracerebroventricular administration of obestatin affects the secretion of prolactin, thyroid stimulating hormones and adrenocorticotropic hormone in rats (Bresciani et al., 2006 and Nogueiras et al., 2007). On the other hand, a recent report provides evidence indicating that obestatin effects are functionally opposite on anxiety to that of ghrelin, while both these related peptides increase memory retention (Carlini et al., 2007). Recently, it was demonstrated that obestatin has also a sleep-promoting effect (Szentirmai and Krueger, 2006b and Szentirmai et al., 2009).

## 2.6 Related studies

Catalán et al. (2007) evaluated the actual presence of obestatin receptor (GPR39) mRNA in human adipose tissue and whether GPR39 expression levels are altered in obesity and obesity-associated type 2 diabetes. Omental adipose tissue biopsies obtained from 15 women were used in the study. Patients were classified as lean (BMI=20.8±1.0 kg/m$^2$), obese normoglycaemic (BMI=48.4±2.1 kg/m$^2$) and obese type 2 diabetic patients (BMI=52.6±4.9 kg/m$^2$). Obese type 2 diabetic patients exhibited significantly lower GPR39 expression levels compared to lean (P=0.016) and obese normoglycaemic subjects (P=0.008), while no differences between lean and obese normoglycaemic patients were observed. The mRNA expression levels of GPR39 were negatively correlated to fasting glucose concentrations (r=-0.581, P=0.023), while exhibiting a positive correlation to adiponectin mRNA expression levels (r=0.674, P=0.006). The authors concluded that the reduced expression levels of GPR39 in omental adipose tissue observed in obese type 2 diabetic patients suggest an involvement of obestatin signalling in glucose homeostasis and type 2 diabetes development.

Qi et al. (2007) investigated whether plasma obestatin level is different in patients with impaired glucose regulation (IGR) and type 2 diabetes mellitus (T2DM). Forty-seven patients with T2DM, 30 subjects with IGR, and 38 sex- and age-matched normal controls participated in the study. Plasma obestatin levels were lower in patients with T2DM and IGR than in controls (37.5±9.2 ng/l and 39.2±9.7 ng/l vs. 43.8±8.0 ng/l, P=0.002 and P=0.039, respectively). Decreasing concentrations of obestatin were independently and significantly associated with IGR and T2DM. Multiple logistic regression analysis revealed obestatin to be independently associated with IGR and T2DM. In a multiple linear regression analysis, only waist-to-hip ratio and homeostasis model assessment of insulin resistance were independently associated with plasma obestatin level.

Basal plasma obestatin levels were investigated in 321 normal weight and obese subjects in relation to BMI, gender, age, insulin concentrations, and type 2 diabetes (Lippl et al., 2008). Additionally, postprandial obestatin levels were determined in 20 normal weight subjects. Basal obestatin levels in females were higher compared to males (193.6±5.8 vs. 140.6±5.1 pg/ml). Obestatin levels correlated inversely and significantly with BMI (f: r=-0.632, p<0.001; m: r=-0.487, p<0.001) and basal insulin levels (f: r=-0.536, p<0.001; m: r=-0.320, p=0.008) in females and males. However, in a multiple regression analysis as well as in a matched comparison of a low and high insulin group no significant relationship between insulin and obestatin levels was observed in nondiabetics. On the other hand, inclusion of type 2 diabetics with higher insulin levels resulted in a significant inverse correlation. Obestatin levels were independent of age in both sexes. In patients with type 2 diabetes basal obestatin levels were not different compared to nondiabetic subjects when matched for gender, body mass index, and insulin. In normal weight subjects, postprandial obestatin levels showed a significant decrease between 60 and 90 minutes rising to basal levels thereafter.

St-Pierre et al. (2010) clarified obestatin secretion in normal subjects and in patients with type 2 diabetes in basal conditions and after a standardized meal. Five normal subjects and 5 type 2 diabetic patients were studied during infusion of saline (iv for over 5 h from -120 to +180 min). A standardized lunch was served at 0 min. Obestatin, glucose, and insulin levels were assayed at -120, -90, -60, -45, -30, -15, 0, 15, 30, 45, 60, 90, 120, 150, and 180 min. From -120 to 0 min, obestatin levels in normal and type 2 diabetic patients were similar (area under the curve: 32.3±5.6 pg/ml/min vs 31.1±1.0 pg/ml/min). After the meal, circulating obestatin levels underwent a clear decrease in normal subjects (0 min: 300.6±34.7 pg/ml vs nadir at 60 min: 161.8±29.4 pg/ml; p=0.002) but not in diabetic patients (0 min: 267.2±16.5 pg/ml vs nadir at 180 min: 226.0±10.5 pg/ml).

Jack, (2012) measured fasting plasma obestatin concentrations of the subjects devided in type 2 diabetes (T2DM), impaired glucose tolerance (IGT) and normal glucose tolerance (NGT) to investigate the relationship between

obestatin and fasting plasma glucose (FPG), 2 hour plasma glucose (2hPG), fasting plasma insulin (FIns), insulin resistance (IR) and plasma lipid as well as other factors in population with different glucose tolerances. Plasma obestatin levels were measured by radioimmunoassay. The relationship between plasma Obestatin levels and BMI, the homeostasis model assessment of insulin resistance (HOMA-IR), plasma glucose, insulin, blood lipids levels were also analyzed by bivariate, and multivariate regression analysis. Plasma obestatin levels in patients with IGT and T2DM were significantly reduced compared with controls (2.41±0.78 ng/ ml) and (2.95±0.56 ng/L) vs (3.41±0.61 ng/ ml), P= 0.01. Plasma obestatin levels were also found to be markedly decreased in obese subjects in three groups when compared to subject with BMI 25 kg/m². Plasma obestatin levels correlated negatively with systolic blood pressure (SBP), diastolic blood pressure (DBP), BMI, waist to hip ratio (WHR), FPG, 2hPG, Fins, HOMA-IR, triglycerides, LDL-C, C-reactive protein ($P<0.01$ and $P<0.05$) in simple regression analysis. But in a multiple regression analysis, only BMI and FPG were independently associated with plasma obestatin levels ($P<0.01$). In non-obese groups only HOMA-IR was independently associated with plasma obestatin levels ($P<0.01$).

# Chapter 3

# Materials and Methods

## 3.1 Study design

Case control study design.

## 3.2 Study population

The target population was type 2 diabetic women aged 40-65 years attending Medical Relief Center in Gaza Governorate. Healthy non diabetic women were served as controls.

## 3.3 Sampling and sample size

Non probability accidental sample of type 2 diabetic women, previously diagnosed according to the WHO diagnostic criteria for diabetes (World Health Organization, 2006), were selected as cases from Medical Relief Center in Gaza Governorate. Healthy non diabetic women were served as controls. Cases and controls were matched with age. The sample size was 128 women (64 type 2 diabetic women and 64 healthy non diabetic women).

## 3.4 Exclusion criteria

Women aged <40 and >65 years

Women with blood pressure >140/90

Pregnant women

Type 1 diabetic women

## 3.5 Ethical Considerations

The necessary approval to conduct the study was obtained from the local ethical committee in the Gaza Strip.

## 3.6 Data collection

### 3.6.1 Questionnaire interview

A meeting interview was used for filling in a questionnaire which designed for matching the study need for both cases and controls (Annex 1). All interviews were conducted face to face by the researcher himself. During the survey the interviewer explained any of the questions that were not clear. The questionnaire was based on the questions of a previous study with some modifications (Altawil, 2009). Most questions were the yes/no questions which offer a dichotomous choice (Backestrom and Hursh-Cesar, 1981). The validity of the questionnaire was tested by six specialists in the fields of epidemiology, public health, endocrinology and nutrition. The questionnaire was piloted with 10 patients. The questionnaire included questions on the sociodemographic data of the study population (age, education and family history), diet and drug therapy, and clinical data including duration of DM and the most important complications of diabetes.

### 3.6.2 Body mass index

Body mass index was calculated as the ratio of body weight in Kg/height in square meter. Women were asked to remove heavy clothes and shoses before measurement of weight and height. Medical balance (Seca Model 762, Germany) was used for weight measurement. Women with BMI=18.5–24.9 were considered to have normal weight and women with BMI≥30.0 were considered obese (WHO, 2000).

### 3.6.3 Specimen collection and biochemical analysis

Twelve hours fasting overnight venous blood samples were collected from 64 type 2 diabetic women and 64 healthy non diabetic controls. Blood samples (6 ml each) were drawn by the researcher herself, who had a Bachelor Degree of Medical Technology, into vacutainer and plastic tubes from each control and diabetic women. About 2 ml blood was placed into EDTA vacutainer tube to perform HbA1C for cases and controls. One ml blood was placed into a plastic tube contained 10 µl protease inhibitor cocktail supplied by sigma-Aldrich Germany with catalog number (P8340) for serum obestatin assay. The remainder quantity of blood (3 ml) was placed in plastic tube for glucose, cholesterol, triglycerides, high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), urea and creatinine determination. The blood (one and 3 ml samples) was left for a while without anticoagulant to allow blood to clot. Serum samples were obtained by centrifugation at 3000 rpm for 10 minutes. Serum with protease inhibitor cocktail tubes were stored at –30±5° Cfor serum obestatin assay. However, serum analysis for glucose, lipid profile, urea and creatinine was done immediately.

## 3.7 Biochemical analysis

### 3.7.1 Determination of serum obestatin

Determination of human serum obestatin level was carried out by competitive enzyme immunoassay (ELISA) for the quantitative determination of obestatin in human serum and plasma Catalog number 48-OBEHU-E01 Size: 96 wells, Alpco diagnostics, USA.

**Assay principle**

This EIA kit for determination of obestatin in human plasma or serum samples is based on a competitive enzyme immunoassay using the combination of highly specific antibody to human obestatin and biotin–avidin affinity system. The 96 wells plate is coated with goat anti rabbit IgG, to which biotinylated human obestatin, human obestatin standard or samples and rabbit antihuman obestatin antibody are added for competitive immunoreaction. After incubation and plate washing, horse radish peroxidase (HRP) labeled streptavidin (SA) is

added, so that HRP labeled SA-biotinylated human obestatin-antibody complex is formed on the surface of the wells. Finally, HRP enzyme activity is determined by 3, 3,5,5'-Tetramethylbenzidine (TMB) and the concentration of human obestatin is calculated.

## Composition

| | Component | Form | Quantity | Main ingredient |
|---|---|---|---|---|
| 1 | Antibody Coated Plate | Microtiter plate | 1 Plate (96 wells) | Goat anti rabbit IgG |
| 2 | Standard | Lyophilized powder | 1 Vial (50ng) | Synthetic human obestatin |
| 3 | Labeled Antigen | Lyophilized powder | 1 Vial | Biotinylated human obestatin |
| 4 | Specific Antibody | Liquid | 1 Bottle (6mL) | Rabbit anti human obestatin antibody |
| 5 | SA-HRP Solution | Liquid | 1 Bottle (12mL) | HRP labeled streptavidin |
| 6 | TMB Substrate | Liquid | 1 Bottle (12mL) | 3,3',5,5'-Tetramethylbenzidine (TMB) |
| 7 | Reaction Stopping Solution | Liquid | 1 Bottle (12mL) | 1M H2SO4 |
| 8 | Buffer Solution | Liquid | 1 Bottle (25mL) | BSA containing saline buffer |
| 9 | Concentrated Wash Solution | Liquid | 1 Bottle (25mL) | Concentrated saline |
| 10 | Adhesive Foil | | 3 Sheets | |

## Method

Note: Before starting assay, all the reagents were brought to room temperature (20-25°C) except samples. Protease inhibitor added serum and plasma samples were kept in an ice-bath after separation or during thawing from freezing and were used in as soon as possible.

Equipment required

1. Photometer for microtiter plate (plate reader), which can read extinction 2.5 at 450nm.
2. Washing device for microtiter plate and dispenser with aspiration system.
3. Micropipettes, multi-channel pipettes for 8 wells or 12 wells and their tips (20µL-1mL).
4. Test tubes (glass or polypropylene) for preparation of standard solution

5. Graduated cylinder (500mL or 1,000mL).
6. Distilled or deionized water.
7. Lint free paper towel.
8. A microplate shaker if necessary.

**Preparatory work**

1. Preparation of standard solution:

The Standard (lyophilized human obestatin 50ng/vial) was reconstituted with 0.5mL of Buffer Solution, which affords 100ng/mL standard solution. The reconstituted standard solution (0.1mL) was diluted with 0.2mL of Buffer Solution that yields 33.333ng/mL standard solution. The same dilution to make each standard solution of 11.111, 3.704, 1.235, and 0.412 ng/mL. Buffer Solution was used as 0 ng/mL.

2. Preparation of labeled antigen solution: Reconstitute Labeled Antigen with 6mL of Buffer Solution was reconstituted.

3. Preparation of washing solution: A volume of 25mL of Concentrated Wash Solution was diluted to 500mL with distilled or deionized water.

4. Other reagents were ready for use.

**Procedure**

1. Three hundred microliter of washing solution was added to each well and kept for at least 30 seconds, then the washing solution was aspirated in the wells. The plate was inverted and tapped firmly on a lint free paper towel to ensure blotting free of most residual washing solution.

2. Fifty microliter of labeled antigen solution was filled into each well first, then 20μL of each of standard solutions (0, 0.412, 1.235, 3.704, 11.111, 33.333, 100ng/mL) or samples was introduced and finally 50μL of human obestatin antibody solution was added into each well.

3. The plate was covered with Adhesive Foil and was incubated at 4°C for 18 - 20 hours with additional 30 minutes at room temperature (still or shaking).

4. After incubation, the Adhesive Foil was taken off, the contents were aspirated, then 300μL of washing solution was added to each well and aspirated. The wash step was repeated for total of five times with approximately 300μL/well of washing solution each time and finally invert the

plate and tap it firmly on a lint free paper towel to ensure blotting free of most residual washing solution.

5. One hundred microliter of SA-HRP Solution into each well was pipetted.

6. The plate with Adhesive Foil was covered and incubated at room temperature for 1 hour (still or shaking).

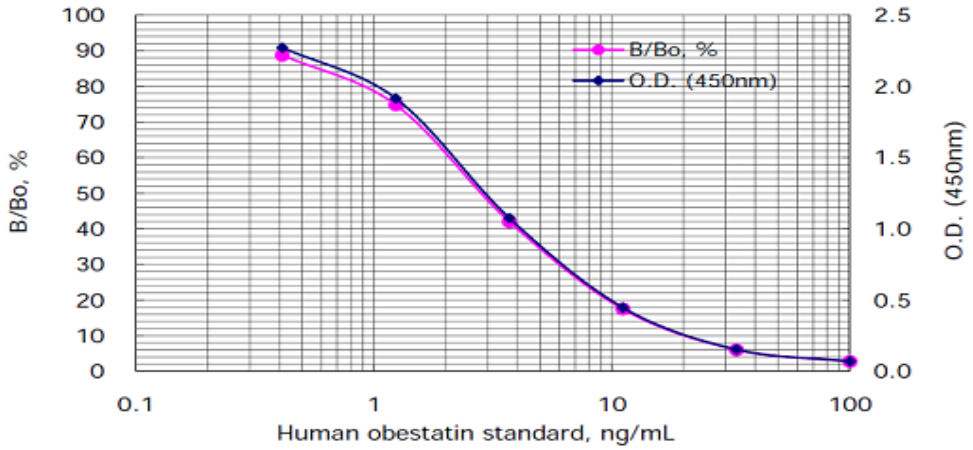
7. The Adhesive Foil was taken off, the wells were aspirated and washed five times as procedure 4.

8. One hundred microliter of TMB Substrate was added into each well; the plate was covered with Adhesive Foil and kept for 30minutes (still or shaking) at room temperature under a light proof condition.

9. One hundred microliter of reaction Stopping Solution was added into each well to stop coloring reaction.

10. The optical absorbance of the wells was read at 450nm.

11. Mean optical density values of wells containing standard solutions or their bound percentage (B/Bo%) to Bo wells (0 ng/mL standard as Bo) was calculated and a standard curve on a semi-logarithmic graph paper (abscissa: concentrations of standard; ordinate: optical density or B/Bo%) was plotted. The average optical density or B/Bo% of each sample was used to determine the corresponding value by simple interpolation from the standard curve.



**Assay range**

0.412 – 100ng/mL

## 3.7.2 Determination of glycohemoglobin (HbA1c) in whole blood

HbA1c was determined by the colorimetric determination of glycohemoglobin in whole blood using Stanbio Kit, Texas-USA.

**Principle**

A preparation of hemolyzed whole blood is mixed with a weakly binding cation exchange resin. The non-glycosylated hemoglobin HbA0 binds to the resin, leaving HbA1 free to be removed by means of a resin separator in the supernate. The percent of HbA1 is determined by measuring the absorbance values at 415 nm of the HbA1 fraction and of the total Hemoglobin fraction, calculating the ratio of absorbances (R), and comparing this ratio to that of a glycohemoglobin standard carried through the same procedure.

Results are express as HbA, but can be converted or derived as HbA1c by using a conversion factor or when using HbA1c value for the standard.

**Reagents**

| |
|---|
| Glycohemoglobin lon Exchange Resin. Each tube contains 3.0 mL cation exchange resin 8 mg/dL. pH 6.9 |
| Glycohemoglobin Lysing Reagent<br>contains potassium cyanide 10 mmol/L and surfactants. |
| Glycohemoglobin Standard (Lyophilized) (1 vial)<br>prepared from packed human erythrocytes. |

**Procedure**

**Hemolysate Preparation**

1. Five hundred microliter lysing reagent was pipetted into tubes labeled Standard (S), Unknown (U) and Control (C).
2. One hundred microliter of each was pipetted into appropriately labeled tube and mixed.

3. Tubes was allowed to stand for 5 minutes at room temperature (15-30°C) to complete hemolysis.

**Glycohemoglobin separation and assay**

1. Resin tubes was labeled Standard (S), Unknown (U) and Control (C).
2. One hundred microliter was pipetted of the prepared hemolysate into appropriately labeled resin tube.
3. A resin separator was positioned in the tube so rubber sleeve is approximately 1-2 cm above liquid level.
4. Tubes were mixed on a hematology rocker for 5 minutes. Alternatively tubes may be mixed by hand if held above the resin.
5. At the end of the 5 minute mixing, resin separator was pushed into tube until resin is firmly packed in bottom of the 13mm tube.
6. Each supernate was poured directly into separate cuvettes for absorbance measurements.
7. Absorbance was read (Agly) of Standard, Unknown and Control vs. water at 415 nm within 60 minutes.

**Total hemoglobin assay**

1. Five milliliter deionized water was pipetted into tubes labeled Standard (S), Unknown (U) and Control (C).
2. Twenty microliter of hemolysate was pipetted into appropriately labeled tube and mixed well and transferred to cuvette for absorbance reading.
3. Absorbance (Atot) of Standard was read, Unknown and Control vs. water at 415 nm within 60 minutes.

**Calculation**

For each Standard and Unknown calculate the ratio (R) of the glycohemoglobin absorbance to the hemoglobin absorbance as follows:

$$(R) = Agly / Atot$$

$$\text{Hemoglobin (\%)} = \frac{(R)\ \text{Unknown} \times \text{Hemoglobin Standard (\%)}}{(R)\ \text{Standard}}$$

Results may also be reported as HbA1c when compared to the reference A1c method, the Stanbio method showed a 98% correlation with an equation of:

Y (A1c value) = 0.838 x (Stanbio value) - 0.732

The value obtained by the Stanbio method may be converted to Calculated A1c value by use of this formula. For a direct calculated A1c value, the value of the standard may be changed to 7.6% in lieu of the 10.0% and the results will be A1c values.

## 3.7.3 Determination of serum glucose

**Principle**

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

$$\text{Glucose} + O_2 \xrightarrow{\text{GO}} \text{Gluconic acid} + H_2O_2$$

$$2\ H_2O_2 + \text{4-Aminoantipyrine} + \text{Phenol} \xrightarrow{\text{POD}} \text{Quinoneimine} + 4\ H_2O$$

**Reagents**

| **Reagent** | **Concentration** |
|---|---|
| Phosphate buffer (pH 7.5)<br>Phenol<br>4-Aminoantipyrine<br>Glucose oxidase (GOD)<br>Peroxidase (POD) | 250 mmol/l<br>5 mmol/l<br>0.5 mmol/l<br>≥ 15 ku/l<br>≥ 1 ku/l |
| **Standard** | 100 mg/dl |

**Assay procedure**

Wavelength: 500 nm

Optical path: 1 cm

Temperature: 37 °C

Measurement: Against reagent blank.

- 10 µl of standard (sample or control) was added to 1ml of the reagent and mixed well.
- The mixture was incubated for 10 min at 37 ºC.
- The absorbance was measured within 60 min.

**Calculation**

$$\text{Glucose [mg / dl]} = \frac{\Delta A \text{ sample} \times \text{concentration of standard}}{\Delta A \text{ standard}}$$

**Reference value** (fasting glucose)

(Palestinian clinical laboratory tests guide, PCLTG, 2005)

| | |
|---|---|
| Child | 60 – 100 mg/dl |
| Adult | 70 – 110 mg/dl |

## 3.7.4 Determination of serum cholesterol

Enzymatic colorimetric method for the quantitative determination of total cholesterol in serum or plasma, using Diasys Diagnostic Systems, Germany.

**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.

$$\text{Cholesterol ester} + H_2O \xrightarrow{\text{CHE}} \text{cholesterol} + \text{fatty acid}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{CHO}} \text{cholesterol-3-one} + H_2O_2$$

$$H_2O_2 + \text{4- aminoantipyrine} + \text{Phenol} \xrightarrow{\text{POD}} \text{Quinoneimine} + 4\ H_2O$$

**Reagents**

Concentrations are those in the final test mixture.

| **Reagent** | **Concentration** |
|---|---|
| Good's buffer (pH 6.7) | 50 mmol/l |
| Phenol | 5 mmol/l |
| 4- Aminoantipyrine | 0.3 mmol/l |
| Cholesterol esterase (CHE) | ≥ 200 u/l |
| Cholesterol oxidase (CHO) | ≥ 100 u/l |
| Peroxidase (POD) | ≥ 3 ku/l |
| **Standard** | 200 mg/dl |

**Assay procedure**

Wavelength: 500 nm

Optical path: 1cm

Temperature: 37 ºC

Measurement: against reagent blank.

- Ten µl of standard (sample or control) was added to 1ml of working reagent and mixed well.
- The mixture was incubated for 5 min at 37 ºC.
- The absorbance was measured within 60 min.

**Calculation**

$$\text{Cholesterol (mg/dl)} = \frac{\Delta A \text{ sample X concentration of standard}}{\Delta A \text{ standard}}$$

**Reference value**

| | |
|---|---|
| Child (desirable) | < 170 mg/dl |
| Adult (desirable) | <200 mg/dl |

## 3.7.5 Determination of serum triglycerides

Enzymatic colorimetric method for the quantitative determination of total Cholesterol in serum or plasma, using Diasys Diagnostic Systems, Germany.

**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.

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

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

$$\text{Glycerol-3-phosphate} + O_2 \xrightarrow{\text{GPO}} \text{Dihydroxyaceton phosphate} + H_2O_2$$

$$2H_2O_2 + \text{Aminoantipyrine + 4-Chlorophenol} \xrightarrow{\text{POD}} \text{Quinoneimine + HCl} + 4H_2O$$

**Reagents**

Concentrations are those in the final test mixture.

| **Reagent** | **Concentration** |
|---|---|
| Cood's buffer (pH 7.2) | 50 mmol/l |
| 4-Chlorophenol | 4 mmol/l |
| ATP | 2 mmol/l |
| $Mg^{2+}$ | 15 mmol/l |

| | | |
|---|---|---|
| Glycerokinase | (GK) | ≥ 0.4 KU/l |
| Peroxidase | (POD) | ≥ 2 KU/l |
| Lipoprotein lipase | (LPL) | ≥ 2 KU/l |
| 4-Aminoantipyrine | | 0.5 mmol/l |
| Glycerol-3-phosphate-oxidase (GPO) | | ≥ 0.5 KU/l |
| **Standard** | | 200 mg/dl |

**Assay Procedure**

Wavelength: 500 nm

Optical path: 1 cm

Temperature: 37 °C

Measurement: Against reagent blank.

- Ten µl of standard (sample or control) was added to 1ml of working reagent and mixed well.
- The mixture was incubated for 5 min at 37 ºC.
- The absorbance was measured within 60 min.

**Calculation**

$$\text{Triglycerides [mg / dl]} = \frac{\Delta A \text{ sample X concentration of standard}}{\Delta A \text{ standard}}$$

**Reference value**

| | |
|---|---|
| Child (desirable) | 30 - 150 mg/dl |
| Adult (desirable) M | 40 - 160 mg/dl |
| F | 35 - 135 mg/dl |

## 3.7.6 Determination of serum high density lipoprotein cholesterol (HDL-C)

Liquid HDL-C precipitant for the determination of HDL-C Cholesterol using Diasys Diagnostic Systems, Germany.

**Principle**

Chylomicrons, VLDL-C and LDL-Cwere precipitated by adding phosphotungstic acid and magnesium ions to the sample. Centrifugation leaves only the HDL-C in the supernatant, their cholesterol content is determined enzymatically using cholesterol reagent.

**Reagents**

| **Reagent** | **Concentration** |
|---|---|
| Monoreagent contain: Magnesium chloride<br>Phosphotungstic acid | 1.4 mmol/l<br>8.6 mmol/l |
| Choesterol standard | 200 mg/dl |

**Assay procedure**

**1- Precipitation**

- Two hundred µl of standard (sample or control) were added to 500 µl of the precipitation reagent and mixed well.
- The mixture was allowed to stand for 15 min at room temperature, and then centrifuged for 20 min at 4000 rpm.

**2- Cholesterol determination**

Wavelength: 500 nm

Optical path: 1cm

Temperature: 37 ºC

Measurement: against reagent blank.

- One hundred µl of the supernatant of standard (sample or control) was added to 1ml of the cholesterol reagent and mixed well.
- The mixture was incubated for 5min at 37 ºC.
- The absorbance was measured within 45 min.

**Calculation**

$$\text{HDL-C (mg/dl)} = \frac{\Delta A \text{ sample X concentration of standard}}{\Delta A \text{ standard}}$$

**Reference value**

| | |
|---|---|
| Child | 37 – 75 mg/dl |
| Adult: M | 35 – 65 mg/dl |
| F | 35 – 80 mg/dl |

## 3.7.7 Determination of serum low density lipoproteins cholesterol (LDL-C)

LDL-C can be calculated using the empirical relationship of Friedewald.

**Principle**

The ultracentrifugal measurement of LDL-C is time consuming and expensive and requires special equipment. For this reason, LDL-C is most commonly estimated from quantitative measurements of total and HDL-C and plasma triglycerides (TG) using the empirical relationship of Friedewald.

**The Equation**

LDL-C = Total Cholesterol - HDL-C - TG/5

## 3.7.8 Determination of serum urea

Serum Urea was determined by urease - glutamate dehydrogenase (GDH)/UV method using BioSystems kit, Spain.

**Principle**

Urea in the sample is consumed, by means of the coupled reactions described below. The decrease of NADH can be measured photometrically at 340 nm.

$$\text{Urea} + 2H_2O \xrightarrow{\text{Urease}} 2NH_4 + 2HCO$$

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

**Reagents**

Concentrations are those in the final test mixture.

| Reagent | Concentration |
|---|---|
| **R1:** TRIS | 120 mmol/l |
| 2- Oxoglutarate | 7 mmol/l |
| ADP | 0.6 mmol/l |
| Urease | ≥ 0.6 ku/l |
| GLDH | ≥ 1 ku/l |
| **R2:** NADH | 0.25 mmol/l |
| **Standard** | 50 mg/dl |

**Assay procedure**

The working solution was prepared by mixing 4 parts of R1 with 1 part of R2.

Wavelength: 340 nm

Optical path: 1cm

Temperature: 37 ºC

Measurement: against distilled water.

- 10 µl of standard (sample or control) was added to 1ml of working reagent and mixed well.
- The mixture was incubated for 30 sec then absorbance (A1) was recorded.
- After exactly further 60 sec the absorbance (A2) was measured.

**Calculation**

$\Delta A$ = (A1 –A2) sample or standard

$$\text{Urea (mg/dl)} = \frac{\Delta A \text{ sample X concentration of standard}}{\Delta A \text{ standard}}$$

**Reference value**

(PCLTG, 2005)

| | |
|---|---|
| Child | 5 - 30 mg/dl |
| Adult | 13 - 43 mg/dl |

## 3.7.9 Determination of serum creatinine

Serum Creatinine was determined by Alkaline Picrate method using BioSystems kit, Spain.

**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 $\longrightarrow$ creatinine picrate complex

**Reagents**

Concentrations are those in the final test mixture.

| **Reagent** | **Concentration** |
|---|---|
| **R1:** Sodume hydroxide (pH approx. 13) | 0.16 mol/l |
| **R2:** Picric acid (pH approx. 1.2) | 4.0 mmol/l |
| **Standard** | 2.0 mg/dl |

**Assay procedure**

The working solution was prepared by mixing 4 parts of R1 with 1 part of R2.

Wavelength: 490 nm

Optical path: 1cm

Temperature: 37 ºC

Measurement: against distilled water.

- 50 µl of standard (sample or control) was added to 1ml of working reagent add and mixed well.

- The Mixture was incubated for 60 sec then absorbance(A1) was recorded.
- After exactly further 120 sec the absorbance (A2) was measured.

**Calculation**

$\Delta A$ = (A1 –A2) sample or standard

$$\text{Creatinine (mg/dl)} = \frac{\Delta A \text{ sample X concentration of standard}}{\Delta A \text{ standard}}$$

**Reference value** (PCLTG, 2005)

| | |
|---|---|
| Infant | 0.2 – 0.4 mg/dl |
| Child | 0.3 - 0.7 mg/dl |
| Adolescent | 0.5 - 1.0 mg/dl |
| Adult: M<br>F | 0.6 - 1.2 mg/dl<br>0.5 -1.1 mg/dl |

## 3.8 Statistical analysis

Data were computer analyzed using SPSS/ PC (Statistical Package for the Social Science Inc. Chicago, Illinois USA, version 18.0) statistical package.

- Simple distribution of the study variables and the cross tabulation were applied.
- Chi-square ($\chi^2$) was used to identify the significance of the relations, associations, and interactions among various variables. Yates's continuity correction test, $\chi^2_{(corrected)}$, was used when not more than 20% of the cells had an expected frequency of less than five and when the expected numbers were small.
- The independent sample t-test procedure was used to compare means of quantitative variables by the separated cases into two qualitative groups such as the relationship between cases and controls obestatin hormone.
- Pearson's correlation test was applied.
- The results in all the above mentioned procedures were accepted as statistical significant when the p-value was less than 5% ($p<0.05$).
- Range as minimum and maximum values was used.
- The percentage difference was calculated according to the formula: Percentage difference equals the absolute value of the change in value, divided by the average of the 2 numbers, all multiplied by 100.

  Percent difference = (| (V1 - V2) | / ((V1 + V2)/2)) * 100.
- Microsoft Excel program version 11.0 was used for correlation graph plotting, between obestatin level and HbA1C, glucose and triglyceride levels.

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# Chapter 4

# Results

## 4.1 Sociodemographic data of the study population

The present study is a case control design. The study population comprised 64 diabetic women (case group) and 64 healthy control women. Table 4.1 summarizes the sociodemographic data of the study population. Age classification showed that 28 (43.7%) controls and 27 (42.2%) cases were 40-50 years old. Age group 51-60 years comprised 24 (37.5%) controls and 26 (40.6%) cases. Controls and cases aged >60 years old were 12 (18.8%) and 11 (17.1%), respectively. The difference between controls and cases in term of age distribution was not significant ($\chi^2$=0.142, P=0.931). The mean ages of controls and cases were 52.7±8.9 and 52.9±8.3 years old with ranges of 40-65 and 40-64 years, respectively. The independent sample t-test also showed no significant difference between mean ages of controls and cases (t=0.113, P=0.911). Analysis of the educational status of the study population showed that 13 (20.3%) controls and 20 (31.3%) cases had diploma or university degree, 14 (21.9%) and 14 (21.9%) had finished secondary school, 25 (39.1%) and 16 (25.0%) had finished preparatory school, 3 (4.7%) and 8 (12.5%) had passed primary school, and 9 (14.1%) and 6 (9.4%) were illiterate, respectively. The difference between various educational levels of controls and cases was not significance ($\chi 2_{(corrected)}$=4.409, P=0.353).Regarding family history, 41 (64.1%) controls and 52 (81.2%) cases had family history of diabetes whereas 23 (35.9%) and 12 (18.8%) had not. The difference between the two groups was significance ($\chi^2$=4.758, P=0.029), indicating that family history is associated with diabetes.

Table 4.1. Sociodemographic data of the study population

| Character | Controls (n=64) | | Cases (n=64) | | test | | P-value |
|---|---|---|---|---|---|---|---|
| | No. | % | No. | % | | | |
| **Age (Year)** | | | | | | | |
| 40-50 | 28 | 43.7 | 27 | 42.2 | $\chi^2$ | 0.142 | 0.931 |
| 51-60 | 24 | 37.5 | 26 | 40.6 | | | |
| >60 | 12 | 18.8 | 11 | 17.1 | | | |
| Mean±SD | 52.7±8.9 | | 52.9±8.3 | | **t** | 0.113 | 0.911 |
| Range (min-max) | 40-65 | | 40-64 | | | | |
| **Education** | | | | | | | |
| University or diploma | 13 | 20.3 | 20 | 31.3 | $\chi^2$ | 4.409 | 0.353* |
| Secondary | 14 | 21.9 | 14 | 21.9 | | | |
| Preparatory | 25 | 39.1 | 16 | 25.0 | | | |
| Primary | 3 | 4.7 | 8 | 12.5 | | | |
| Illiterate | 9 | 14.1 | 6 | 9.4 | | | |
| **Family history** | | | | | | | |
| Yes | 41 | 64.1 | 52 | 81.2 | $\chi^2$ | 4.758 | 0.029 |
| No | 23 | 35.9 | 12 | 18.8 | | | |

*P-value of $\chi^2_{(corrected)}$ test
P>0.05:not significant, P<0.05:significant.

## 4.2 Diet and drug therapy among the study population

Table 4.2 illustrates diet and drug therapy among the study population. The number of controls and cases on diet were 16 (25.0%) and 30 (46.9%), respectively whereas those who were not were 48 (75.0%) and 34 (53.1). The difference between the two groups was significant ($\chi^2$=6.651, P=0.010). Almost all cases 63 (98.4%) were administered the antidiabetic drug Glucophage.

Table 4.2.Diet and drug therapy among the study population

| Character | Controls (n=64) | | Cases (n=64) | | $\chi^2$ | P-value |
|---|---|---|---|---|---|---|
| | No. | % | No. | % | | |
| **Diet** | | | | | | |
| Yes | 16 | 25.0 | 30 | 46.9 | 6.651 | 0.010 |
| No | 48 | 75.0 | 34 | 53.1 | | |
| **Therapy drug** | | | | | | |
| Glucophage | - | - | 63 | 98.4 | NA | NA |
| Glucophage and Daonail | - | - | 1 | 1.6 | | |

P<0.05:significant. NA: Non Applicable.

## 4.3 Distribution of diabetic patients by the duration of the disease

Table 4.3 demonstrates the distribution of diabetic patients by the duration of the disease. Patients with diabetes since less than 5 years were 38 (59.4%), whereas those with diabetic duration of 5-10 years were 14 (21.9%). The rest of patients 12 (18.7%) had diabetes for more than 10 years.

Table 4.3. Distribution of diabetic patients (n=64) by the duration of the disease

| Duration of diabetes (Year) | No. | % |
|---|---|---|
| **< 5** | 38 | 59.4 |
| **5-10** | 14 | 21.9 |
| **>10** | 12 | 18.7 |

## 4.4 Self-reported complications of the study population

The main self-reported complications among diabetic patients and controls are summarized in Table 4.4. The percentages of retinopathy and neuropathy were higher in cases compared to controls (37.5 and 43.8% vs 12.5 and 14.1%, respectively) with statically significant differences ($\chi 2_{(corrected)}$=10.667, P=0.001 and $\chi 2_{(corrected)}$=13.724, P=0.000,respectively). However, no significant difference was found between cases (6.3%) and controls (1.6%) in term of CVD ($\chi 2_{(corrected)}$=0.833, P=0.361).

Table 4.4. The main self-reported complications among the study population (n=128)

| **Complication** | **Control** (n=64) **n (%)** | **Cases** (n=64) **n (%)** | $\chi^2$ | **P-Value** |
|---|---|---|---|---|
| **Retinopathy** | | | 10.667 | 0.001 |
| Yes | 8(12.5) | 24(37.5) | | |
| No | 56 (87.5) | 40 (62.5) | | |
| **CVD**** | | | 0.833 | 0.361***** |
| Yes | 1 (1.6) | 4(6.3) | | |
| No | 63 (98.4) | 60 (93.8) | | |
| **Neuropathy** | | | 13.724 | 0.000 |
| Yes | 9(14.1) | 28(43.8) | | |
| No | 55 (85.9) | 36 (56.2) | | |

*P-value of $\chi^2_{(corrected)}$ test. **CVD: Cardiovascular diseases. P>0.05: not significant, P<0.05: significant.

## 4.5 Self-reported complications among patients in relation to the duration of diabetes

Self-reported complications among patients in relation to the duration of diabetes is provided in Table 4.5. In general, the longer the duration of diabetes mellitus, the higher the percentage of self-reported complications

among patients. This positive relationship was statically significant for retinopathy ($\chi$2=6.210, P=0.045) and neuropathy ($\chi$2=6.934, P=0.031). On the other hand, such relationship was not statically significant for CVD ($\chi 2_{(corrected)}$=0.267,P=0.875).

Table 4.5. Self-reported complications among patients in relation to the duration of diabetes

| **Complication** | **Duration of diabetes (Year)** | | | $\chi^2$ | ***P-Value*** |
|---|---|---|---|---|---|
| | **< 5** (n=38) **n (%)** | **5-10** (n=14) **n (%)** | **> 10** (n=12) **n (%)** | | |
| **Retinopathy***(n=24)* | 9 (23.7) | 7 (50.0) | 8(66.7) | 6.210 | 0.045 |
| **CVD*****(n=4)* | 2 (5.3) | 1 (7.1) | 1 (8.3) | 0.267 | 0.875***** |
| **Neuropathy***(n=28)* | 11(28.9) | 8(57.1) | 9(75.0) | 6.934 | 0.031 |

*P-value of $\chi^2_{(corrected)}$ test. **CVD: Cardiovascular diseases. P>0.05: not significant, P<0.05: significant.

## 4.6 Anthropometric measurements of the study population

Table 4.6 shows the anthropometric measurements of the study population. The mean weight of controls was 88.3±21.6 kg compared to 88.5±17.4 kg of cases. The weight difference was not significant (t=0.173 and P=0.864) with % difference=0.2. There was also a none significant difference in the mean height of cases compared to controls (1.58±0.06 vs.1.59±0.06 m, % difference=0.6, t=0.894 and P=0.375). Therefore, no significant difference was found in BMI of cases versus controls (35.2±6.5 vs. 34.4±7.7, % difference=2.3, t=0.281 and P=0.780).

Table 4.6. Anthropometric measurements of the study population

| Anthropometric measurement | Controls (n=64) mean±SD | Cases (n=64) mean±SD | % difference | t | P-value |
|---|---|---|---|---|---|
| **Weight (kg)*** *(min-max)* | 88.3±21.6 (60-135) | 88.5±17.4 (59-140) | 0.2% | 0.173 | 0.864 |
| **Height (m)**** *(min-max)* | 1.59±0.06 (1.49-1.75) | 1.58±0.06 (1.46-1.71) | 0.6% | 0.894 | 0.375 |
| **BMI***** *(min-max)* | 34.4±7.7 (24-53) | 35.2±6.5 (25-59) | 2.3% | 0.281 | 0.780 |

*Kg: kilogram,** m: meter. ***BMI: Body mass index: Normal=18.5-24.9, Obese≥30 (WHO, 2000).All values are expressed as mean ±SD. P>0.05: not significant.

## 4.7 Serum obestatin levels of the study population

The average serum obestatin levels in controls and cases is presented in Table 4.7. There was a significant decrease in the mean level of obestatin in cases compared to controls (3.4±0.8 vs. 4.1±1.5 ng/ml, % difference=18.6, t=2.328 and P=0.023).

Table 4.7. Serum obestatin of the Study population

| Parameter | Controls (n=64) mean±SD | Cases (n=64) mean±SD | % difference | t | P-value |
|---|---|---|---|---|---|
| **obestatin(ng/ml)** *(min-max)* | 4.1±1.5 (2.2-7.3) | 3.4±0.8 (1.8-4.8) | 18.6 | 2.328 | 0.023 |

Assay range:0.412-100 ng/mL
P<0.05: significant.

## 4.8 HbA1c and glucose of the study population

As indicated in Table 4.8, the mean HbA1c and glucose levels in cases were significantly higher than that in controls (6.4±0.9% and 190.7±86.7 mg/dl vs. 4.7±0.6% and 101.6±21.0 mg/dl, % difference= 30.6 and 60.9, t=9.136 and 5.726, P=0.000).

Table 4.8. HbA1c and glucose of the Study population

| **Parameter** | **Controls (n=64) mean±SD** | **Cases (n=64) mean±SD** | **% difference** | **t** | **P-value** |
|---|---|---|---|---|---|
| **HbA1c (%)** *(min-max)* | 4.7±0.6 (3.8-6.0) | 6.4±0.9 (5.1-8.4) | 30.6 | 9.136 | 0.000 |
| **glucose (mg/dl)** *(min-max)* | 101.6±21.0 (71-154) | 190.7±86.7 (84-472) | 60.9 | 5.726 | 0.000 |

Reference range: 4-6% and 70-115mg/dL
P<0.05: significant.

## 4.9 Serum lipid profile of the study population

Table 4.9 illustrates serum lipid profile of the study population including cholesterol, triglycerides, HDL-C and LDL-C of cases and controls. The average levels of cholesterol and triglycerides were found to be higher in cases (194.8±44.7 and 183.3±80.0 mg/dl, respectively) compared to controls (188.1±38.3 and 122.4±42.9 mg/dl, respectively) with % differences of 3.5 and 39.8%, respectively). However, this increase was statistically significant for triglycerides (t=3.841, P=0.000). No statistically significant change was found for LDL-C between cases and controls. On the other hand, HDL-C was significantly lower in cases compared to controls (40.7±11.2 vs. 47.2±12.0 mg/dl, % difference=14.8, t=2.228 and P=0.030).

Table 4.9. ipid profile of the Study population

| Parameter | Controls (n=64) mean±SD | Cases (n=64) mean±SD | % difference | t | P-value |
|---|---|---|---|---|---|
| **Cholesterol (mg/dl)** *(min-max)* | 188.1±38.3 (136-273) | 194.8±44.7 (116-295) | 3.5 | 0.640 | 0.524 |
| **Triglycerides (mg/dl)** *(min-max)* | 122.4±42.9 (67-265) | 183.3±80.0 (75-391) | 39.8 | 3.841 | 0.000 |
| **LDL-C (mg/dl) *** *(min-max)* | 116.5±37.1 (61-203) | 115.3±36.0 (39-191) | 1.0 | 0.101 | 0.920 |
| **HDL-C (mg/dl) **** (min-max) | 47.2±12.0 (24-68) | 40.7±11.2 (21-68) | 14.8 | 2.228 | 0.030 |

*LDL-C: Low density lipoprotein cholesterol, **HDL-C: High density lipoprotein cholesterol. All values are expressed as mean ±SD. P>0.05: not significant, P<0.05: significant.

## 4.10 Serum urea and creatinine of the study population

Serum urea and creatinine concentrations of the study population are clarified in Table 4.10. There was a border line significant increase in urea concentration in cases compared to controls (26.0±8.1 vs 23.1±3.7mg/dl, % difference=11.8, t=1.871, P=0.066). However, such increase was not statistically significant for creatinine (0.74±0.2 vs 0.73±0.1 mg/dl, % difference= 1.4, t= 0.108, P= 0.914).

Table 4.10. Serum urea and creatinine concentration of the study population

| **Parameter (mg/dl)** | **Controls** (n=64) mean±SD | **Cases** (n=64) mean±SD | **% difference** | **t** | **P-value** |
|---|---|---|---|---|---|
| **Urea** Range (min –max) | 23.1±3.7 (17.0-32.0) | 26.0± 8.1 (11.0-58.0) | 11.8 | 1.871 | 0.066 |
| **Creatinine** Range (min –max) | 0.73±0.1 (0.50-0.90) | 0.74±0.2 (0.50-1.70) | 1.4 | 0.108 | 0.914 |

Reference range: urea=15-43 mg/dl (Thomus, 1998) and creatinine=0.6-1.1 mg/dl(Newman and Price, 1999).

## 4.11 Obestatin levels in relation to BMI of the study population

Obestatin level in relation to BMI of the study population is presented in Table 4.11. The Pearson correlation test showed that the higher the BMI, the lower the level of obestatin. However, this negative correlation was not statistically significant (r=-0.068, P= 0.590).

Table 4.11. Obestatin levels in relation to BMI of the study population

| | Obestatin | |
|---|---|---|
| | Pearson correlation (r) | P-value |
| BMI* | -0.068 | 0.590 |

* Women with BMI=18.5–24.9 were considered to have normal weight, Women with BMI≥30.0 were considered obese (WHO, 2000).P>0.05: not significant.

## 4.12 Obestatin levels in relation to HbA1c and glucose of the study population

Table 4.12 and Figures 4.1 and 4.2 present the relationship between obestatin level with HbA1c and glucose of the study population. Obestatin

showed significant negative correlation with HbA1c and glucose (r=-0.320, P= 0.009 and r=-0.469, P= 0.000, respectively).

Table 4.12. Obestatin levels in relation to HbA1c and glucose of the study population

| (mg/dl) | Obestatin | |
|---|---|---|
| | Pearson correlation (r) | P-value |
| HbA1c | -0.320 | 0.009 |
| Glucose | -0.469 | 0.000 |

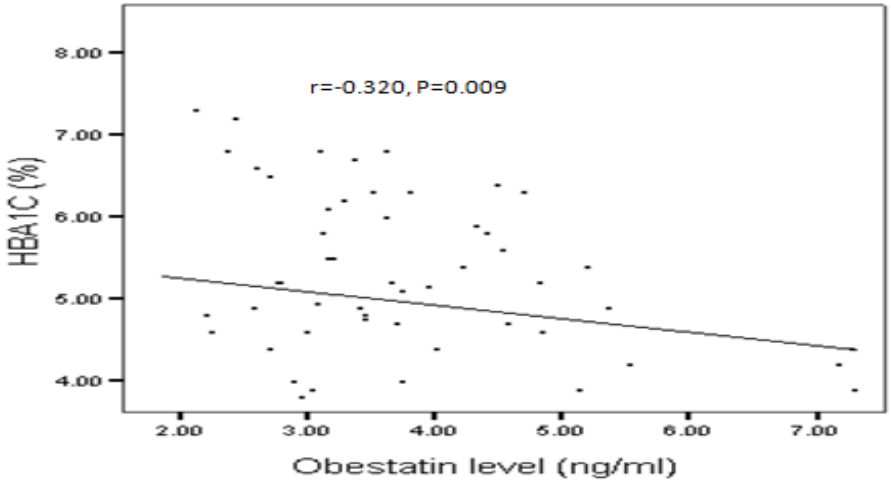


Figure 4.1. Correlation between obestatin hormone level with HbA1c of the study population.

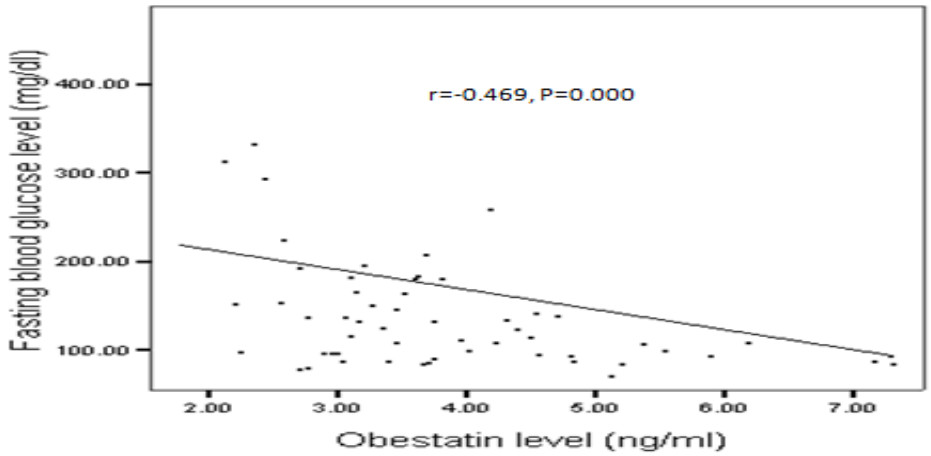


Figure 4.2. Correlation between obestatin hormone level with fasting blood glucose level of the study population.

## 4.13 Obestatin levels in relation to lipid profile of the study population

The relationship between obestatin and lipid profile of the study population is demonstrated in Table 4.13. The Pearson correlation test showed that with decreasing obestatin levels there are increases in cholesterol, triglycerides and LDL-C levels. Such negative correlation was significant only for triglycerides (Figure 4.3, r=-0.275, P=0.026). On the other hand, there is a positive correlation between obestatin level and HDL-C and this correlation was also not significant (r=0.024, P=0.848).

Table 4.13. Obestatin levels in relation to lipid profile of the study population

| Lipid Profile (mg/dl) | Obestatin | |
|---|---|---|
| | Pearson correlation (r) | P-value |
| Cholesterol | -0.175 | 0.164 |
| Triglycerides | -0.275 | 0.026 |
| *LDL-C | -0.090 | 0.474 |
| **HDL-C | 0.024 | 0.848 |

P>0.05: not significant. *LDL-C: Low density lipoprotein cholesterol, **HDL-C: High density lipoprotein cholesterol.

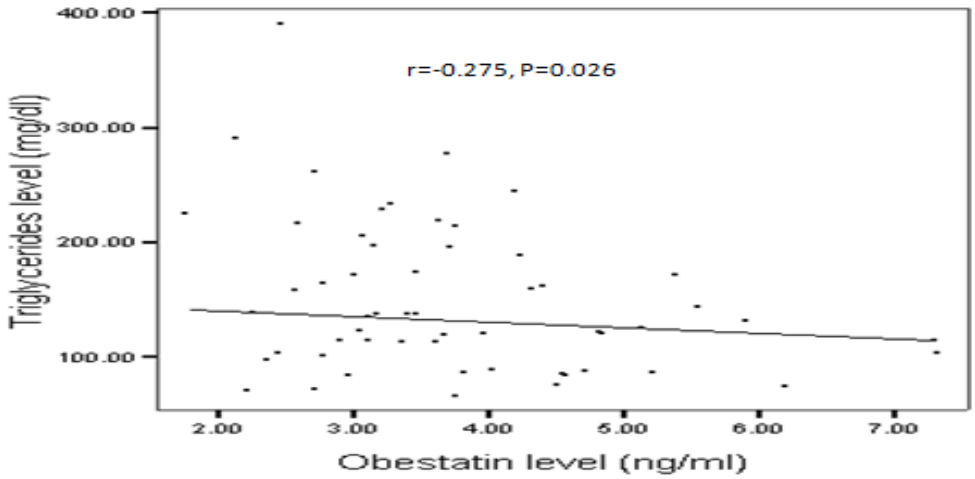


Figure 4.3. Correlation between obestatin hormone level with triglyceride level of the study population.

## 4.14 Obestatin levels in relation to urea and creatinine of the study population

As illustrated in Table 4.14, there were no significant positive correlations between obestatin levels and urea and creatinine concentrations (r=0.000, P= 0.997 and r=0.209, P= 0.096,respectively).

Table 4.14. Obestatin levels in relation to urea and creatinine of the study population

| (mg/dl) | Obestatin | |
|---|---|---|
| | Pearson correlation (r) | P-value |
| Urea | 0.000 | 0.997 |
| Creatinine | 0.209 | 0.096 |

# Chapter 5

# Discussion

Diabetes mellitus has in the last decade become a global problem. The total number of people with diabetes is projected to rise from 171 million in 2000 to 366 million in 2030. There are more women with diabetes than men (Wild et al., 2004). Despite its high prevalence and the subsequent health problems, there are under-diagnosis and under-reporting of diabetes mellitus in the Gaza Strip. Biochemical tests of the disease were restricted to monitoring blood glucose level when the patient visits the clinic. Recently, few studies have been focused on the determination of leptin, insulin and ghrelin in type 2 diabetic patients in Gaza Strip (Altawil, 2009 and Abu Snayma, 2012). However, no previous study investigated the role of the novel hormone obestatin in diabetes mellitus. Therefore, the present work is the first to assess obestatin status in type 2 diabetic women from Gaza.

## 5.1 Family history and diet among the study population

The Chi square test showed that the number of cases who had family history of diabetes was significantly higher than that of controls. This indicates that family history is associated with type 2 diabetes. Such finding is in agreement with that obtained by Annis et al., (2005); Pijl et al., (2009) and Abu Mustafa, (2011) who reported that family history is a risk factor for type 2 diabetes. Although there was a significant association between diabetes and diet, about half of the patients were not on diet. Previous work carried out in Gaza Strip showed such significant association(Altawil, 2009 and Abu Mustfa). This necessitates launching of educational programs to show the importance of diet in controlling the disease. Watkins (2003) reported that healthy eating is

the cornerstone of diabetic treatment, and control of the diet should always be the first treatment offered to Type 2 diabetic patients before drugs are considered. In this context, the most common used antidiabetic drug among type 2 diabetic patients in Gaza is glucophage.

## 5.2 Diabetes duration and Self-reported complications

The finding that more than half of patients had diabetes since less than 5 years do confirm the idea that type 2 diabetes has long asymptomatic pre-clinical phase which frequently goes undetected. At the time of diagnosis, the patient could have one or more diabetes complications i.e. there is a latent phase before diagnosis of Type 2 diabetes. During this period of undiagnosed disease, risk factors for diabetic micro- and macrovascular complications are markedly elevated and diabetic complications are developing (Canadian Diabetes Association Clinical Practice Guidelines Expert Committee, 2003 and Watkins, 2003). The relation between diabetes duration and self-reported complications confirmed this view. However, this point still needs further investigation. The most self-reported symptoms among diabetic patients were neuropathy and retinopathy. The prevalence of such symptoms was positively associated with the progress of the disease i.e. the longer the duration of diabetes mellitus, the higher the percentage of self-reported complications among patients. Several studies reported similar diabetic complications with increasing rates upon disease progress (Savage, 1996, Dyck et al., 2002, Marshall, 2006 and The National Eye Institute, 2006, Altawil, 2009 and Abu Sunayma, 2012).

## 5.3 Diabetes and body mass index

Body mass index provides a reliable indicator of body fatness for most people and it is used to screen for weight categories that may lead to health problems (CDC, 2007). Therefore, obesity is commonly defined as a BMI of 30 $kg/m^2$ or higher. This definition distinguishes obesity from being pre-obese or overweight, which is classified as a BMI of 25 $kg/m^2$ but less than

30 kg/m2 (WHO, 2000). In the present study, both controls and cases were obese. This masked the prospective significant association between BMI and diabetes which was mentioned by Marshall (2006) and Yassin et al. (2011). Nevertheless, matching this confounder factor may reveal the real status of obestatin in diabetic patients regardless of their body mass index.

## 5.4 Serum obestatin of the study population

Result presented in this study revealed that the mean level of obestatin was significantly decreased in type 2 diabetic women compared to healthy non diabetic controls. This finding is in agreement with that observed by Catalán et al. (2007); Qi et al. (2007); St-Pierre et al. (2010) and Jack, (2012). On the other hand, Lippl et al. (2008) reported that basal obestatin levels in patients with type 2 diabetes were not different compared to nondiabetic subjects. This contradiction may be explained on basis of different sample size, gender, age, BMI and pre- or postprandial periods. Therefore, well-controlled human studies collecting multiple measures of obestatin before and after a meal are needed. In addition, a better understanding of the biological mechanism of action of obestatin, i.e., through identification of its receptor, could help to explain these findings.

## 5.5 HbA1c and glucose of the study population

As indicated in the present results, the mean HbA1c and glucose levels in cases were significantly higher than that in controls. Similar results were obtained by Qi et al. (2007) and Yassin et al. (2011) who found that type 2 diabetic patients had higher fasting glucose and HbA1C levels than non-diabetics. In diabetes, prolonged hyperglycemia superdrives nonenzymatic protein glycation, which forms reversible Schiff bases and Amadori compounds. A series of further complex molecular rearrangements then yield irreversible advanced glycosylated end-products (AGEs). AGEs accumulate in the circulating blood and in various tissues (Furth, 1997). It is reported that the

levels of HbA1c in the blood reflect the glucose levels to which the erythrocyte has been exposed during its lifespan (Goldstein, 2004). Therefore, the HbA1c test is attractive as it measures chronic glycaemia, rather than instantaneous blood glucose levels. HbA1c has been used as an objective marker of average glycaemic control for many years, has an accepted place in the monitoring of patients with diabetes, and is relied on for significant management decisions, such as initiation of insulin therapy (d´Emden et al., 2012). In the present study, the Pearson correlation test showed negative significant correlations between obestatin levels and HbA1c and glucose. Similar result was documented by Qi et al. (2007) and Lippl et al.(2008). This negative relationship reflects the change of the glycemic parameter with obestatin and suggests an involvement of obestatin signalling in glucose homeostasis and type 2 diabetes development. This point needs further investigation.

## 5.6 lipid profile of the study population

The present results demonstrate significant increase in triglyceride levels of cases compared to controls. On the other hand, HDL-C level was significantly decreased in cases. Elevation of triglycerides in diabetic patients was documented by several authors (Chen et al., 2009; Katsiki et al., 2011 and Al-Hakeim and Ali 2012). The general increase levels of serum lipids in diabetic patients may be mainly attributed to increase in the mobilization of free fatty acids from fat depots, since elevation of insulin inhibits the hormone sensitive lipase. Then, excess fatty acids in serum are converted into triglycerides, phospholipids and cholesterol in liver (Scheen, 2003). The Pearson correlation test showed negative significant correlation between obestatin and triglycerides level. Similar finding was observed by Nagaraj et al. (2008 and 2009) and Agnew et al. (2011). It was reported that obestatin is a metabolic hormone capable of binding to GPR39 receptor to regulate the functions of adipose tissues (Zahng, 2008). In this context, Miegueu et al. (2011) showed that fatty acid uptake was increased three- to fourfold in a

concentration-dependent manner with obestatin. Therefore, the decreased level of obestatin observed in the present study is expected to increase serum triglycerides in diabetic patients.

## 5.7 Urea and creatinine concentrations of the study population

Data provided in the present study showed no significant increase in urea and creatinine concentrations in diabetic patients compared to controls. Similar results were documented by Altawil (2009) and Abu Snayma. Urea is formed by the liver as an end product of protein breakdown and creatinine is a waste product that is normally filtered from the blood and excreted with the urine. Creatinine and urea are markers of the kidney function and changes in their levels are indicating renal diseases (Debra Manzella, 2008). Therefore, the results indicated that many of the studied diabetic patients are still in the early stages of the disease. This was obvious in the finding that more than half of the patients had diabetes since less than 5 years. However, it was difficult to determine the onset of such changes in urea and creatinine concentrations and this may lead to controversial results (Varghese et al., 2001 and El Meligi et al., 2003). Therefore, the creatinine levels must be watched carefully to determine how much function the kidneys have and this does vary slightly.

# Chapter 6

# Conclusions and Recommendations

## 6.1 Conclusions

- The mean ages of controls and cases were 52.7±8.9 and 52.9±8.3 years.
- Type 2 diabetes was found to be associated with family history and diet.
- More than half of patients had diabetes since less than 5 years.
- The main self-reported complications among patients were neuropathy, retinopathy and cardiovascular disease. The longer the duration of diabetes, the higher the prevalence of self-reported complications.
- The mean level of serum obestatin was significantly lower in diabetic women compared to healthy non diabetic controls (3.4±0.8 ng/ml vs. 4.1±1.5 ng/ml, P=0.023).
- The mean HbA1c and glucose levels in cases were significantly higher than that in controls (6.4±0.9 and 190.7±86.7 vs 4.7±0.6 and 101.6±21.0) with P=0.000. The Pearson correlation test showed negative significant correlations between obestatin levels and HbA1c and glucose (r=-0.320, P=0.009 and r=-0.469, P=0.000, respectively).
- The average level of triglycerides was significantly increased in diabetic women (183.3±80.0mg/dl) compared to controls (122.4±42.9 mg/dl) with P=0.000. On the other hand, HDL-C was significantly lower in cases (40.7±11.2 vs. 47.2±12.0 mg/dl, % difference=14.8 and t=2.228, P=0.030). The Pearson correlation test showed negative significant correlations between obestatin levels and triglycerides levels (r=-0.275, P=0.026)

## 6.2 Recommendations

- Assessment the status of obestatin hormone in diabetes could constitute a promising therapy of the disease. In this context, multiple measures of obestatin in pre- and post-prandial periods are needed.

- Launching of health education programs on diabetes particularly among women with family history of diabetes and those who are not on diet are highly recommended.

- Regular visits to neurological and optical clinics to take early steps to avoid and manage diabetic complications concerning diabetic neuropathy and retinopathy.

- Further research is highly recommended on obestatin and the development of diabetes in terms of HbA1c and glucose.

***Annex 1***

**Questionnaire**

I am a researcher / Mona M. Hamam (Master student/ The Islamic University of Gaza) will be very grateful if you help me in completing this study which focuses on the role of a novel hormone obestatin in type 2 diabetes in women from Gaza Governorate, Gaza strip.

| **Personal data** | | |
|---|---|---|
| Tel. No. | | |
| Name*(Optional)* | | |
| Age | | |
| Education | ☐University or diploma ☐Secondary school ☐Preparatory school ☐Primary school ☐illiterate | |
| Family history of diabetes | ☐ Yes☐No | |
| BMI | Weight: Kg | Height: cm |
| Diet | ☐ Yes☐No | |
| **Clinical data***(Only for diabetic patients)* | | |
| Age at diagnosis | | |
| Duration of diabetes | | |
| Type of drug used | | |
| **Complications** | | |
| Retinopathy | ☐ Yes☐No | |
| Cardiovascular diseases | ☐ Yes☐No | |
| Neuropathy | ☐ Yes☐No | |
| Recurrent infections | ☐ Yes☐No | |
| Skin lesions | ☐ Yes☐No | |
| Oral cavity lesions | ☐ Yes☐No | |

Thank you for your cooperationResearcher / Mona M. Hamam

***Annex 1***

## Questionnaire

أنا الباحثة /منى حمام (طالبة ماجستير بالجامعة الإسلامية). أرجو المساعدة في تعبئة هذا الاستبيان من أجل عمل بحث حول دور هرمون الاوبستاتين عند مرضى السكري من النوع الثاني من الاناث في غزة.

| **بيانات شخصية** | |
|---|---|
| رقم التليفون | |
| الاسم | |
| العمر | |
| المرحلة الدراسية | □ جامعة او دبلوم □ ثانوية عامة<br>□ المرحلة الاعدادية□المرحلة الابتدائية □أمي |
| هل يعاني أحد أفراد عائلتك من مرض السكري من النوع الثاني؟ | ☐ نعم ☐ لا |
| BMI | الوزن: الطول: |
| هل تتبع حمية غذائية ؟ | ☐ نعم ☐ لا |
| **بيانات طبية (خاص بمرضى السكري)** | |
| كم كان عمرك وقت تشخيص المرض؟ | |
| في أي عام تم تشخيص السكري لأول مرة لديك؟ | |
| ما هو نوع العلاج الذي تتناوله؟ | |
| **مضاعفات مرض السكري** | |
| مشاكل في العين | ☐ نعم ☐ لا |
| أمراض القلب | ☐ نعم ☐ لا |
| مشاكل في الأعصاب | ☐ نعم ☐ لا |
| عدوى متكررة | ☐ نعم ☐ لا |
| مشاكل في الجلد | ☐ نعم ☐لا |
| تقرحات في الفم | ☐ نعم ☐ لا |

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