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Ghrelin, Leptin and Insulin in Type 2 Diabetic Patients in Gaza Strip

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Declaration

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Ghrelin, leptin and insulin in type 2 diabetic patients in Gaza Strip

Abstract

Background: Recent studies on ghrelin in type 2 diabetes mellitus and its interactions with insulin or leptin is limited and controversial. However, it was shown that ghrelin and leptin recorded low and high levels, respectively with increasing insulin resistance. Clarifying and understanding of such tri-hormonal interplay in type 2 diabetes could be helpful in the intervention and control strategies of the disease.

Objective: to assess ghrelin, leptin and insulin in type 2 diabetic patients in Gaza Strip.

Materials and Methods: This case-control study comprised 80 diabetic patients selected from different Gaza Strip hospitals and diabetic clinics and 80 healthy controls. Controls and cases were matched for age and sex. Questionnaire interviews were applied. Serum ghrelin, leptin, insulin, glucose, cholesterol, triglycerides, high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), urea and creatinine were determined. Data were analyzed using SPSS version 18.0.

Results:

The mean ages of controls and cases were 49.8 ± 6.5 and 51.7 ± 7.0 years, respectively. Family history was found to be associated with diabetes. The number of diabetic patients who ate 3 and ≥ 4 meals/day were about twice higher than controls. Although half of patients had diabetes since less than 5 years, the majority of them had blood glucose test once/month, reflecting inadequate blood glucose monitoring. The most common self-reported complications among diabetic patients were retinopathy, neuropathy and cardiovascular disease. The prevalence of such complications were positively associated with the duration of diabetes. Body mass index of cases was significantly higher than that of controls. Serum glucose and insulin levels were significantly higher in diabetic patients than controls (187.4 ± 74.1 and

28.8±23.9 vs. 98.3±17.0 and 18.8±13.5, % difference=62.4 and 42.0, P=0.000 and P=0.025). Serum leptin showed no significant increase in diabetic patients compared to controls (7.1±2.7 vs. 5.9±2.2 ng/ml, % difference=18.5 and P= 0.081) whereas ghrelin was significantly lower in patients (1189±580 vs.1531±822 pg/ml, % difference=25.1 and P=0.038). The mean level of triglyceride was significantly higher in patients compared to controls (212.5±78.9 vs. 143.2±50.4 % difference=39.0 and P=0.000). In addition, urea showed no significant increase in patients (33.5±11.0 vs. 28.3±13.3, % difference=16.8 and P=0.059). The Pearson correlation test showed positive significant correlation of BMI with triglycerides (r=0.228, P=0.044). Insulin showed positive correlations with glucose, triglycerides and leptin (r=0.286, r=0.224 and r=0.187, respectively). However, this positive correlation was significant for glucose and triglycerides (P=0.011, P=0.049, respectively). On the other hand, ghrelin showed negative not significant correlation with insulin (r=-0.213 and P=0.057).

Conclusion: serum insulin and leptin levels were increased in diabetic patients compared to controls whereas ghrelin was decreased in patients. Insulin was positively correlated with leptin and negatively correlated with ghrelin confirming the tri-hormonal interplay in diabetes.

Key words: Ghrelin, insulin, leptin, diabetes, Gaza Strip.

حالة هرمون الجريلين وهرمون اللبتين وهرمون الانسولين في مرضى السكر

النوع الثاني في قطاع غزة

ملخص الدراسة:

مقدمة: الدراسات حديثا على هرمون الجريلين في مرض السكري من النوع 2، وتفاعل هذا الهرمون مع هرموني الأنسولين و اللبتين هي محدودة ومثيرة للجدل. ومع ذلك، فقد ظهر أن هرموني الغريلين واللبتين قد سجلت مستويات منخفضة وعالية، على التوالي مع زيادة مقاومة الانسولين. ويمكن توضيح وعليه فان توضيح وفهم مثل هذا التفاعل الثلاثي الهرموني في مرض السكر من النوع 2 قد يكون مفيدا في استراتيجيات التدخل والسيطرة على المرض.

الهدف: تقييم هرمونات الجريلين، اللبتين و الانسولين لدى مرضى السكري من النوع 2 في قطاع غزة.

المواد والطريقة: هذه دراسة الحالات والضوابط شملت 80 من مرضى السكري يتم اختيارهم من مختلف مستشفيات قطاع غزة، وعيادات مرضى السكري، و 80 من الاصحاء. وقد تم مطابقة الضوابط والحالات بالنسبة للعمر والجنس. تم تطبيق الاستبيان. وتم قياس مستويات هرمونات الانسولين في الدم، اللبتين، و الجريلين، وكذلك قياس الجلوكوز والكوليسترول والدهون الثلاثية وكذلك نسبة الكوليسترول على الكثافة (HDL-C)، وايضا قياس نسبة انخفاض نسبة الكوليسترول منخفض الكثافة (LDL-C) وكذلك نسبة اليوريا والكرياتينين. وقد تم تحليل البيانات باستخدام SPSS إصدار 18.0.

النتائج:

أظهرت النتائج أن: متوسط الأعمار للضوابط والحالات 6.5 ± 49.8 و 7.0 ± 51.7 سنوات. وكان تاريخ العائلة هو أحد عوامل المرتبطة بمرض السكري ووجد أن عدد مرضى السكري الذين يتناولون 3 وجبات و 4 وجبات فأكثر في اليوم كانت تقريبا أعلى مرتين من الضوابط، وعلى الرغم من أن نصف المرضى لديهم مرض السكري منذ أقل من 5 سنوات، كان معظمهم يجري فحص الجلوكوز في الدم مرة واحدة في الشهر، مما يعكس عدم كفاية مراقبة مستوى السكر في الدم. كانت المضاعفات الأكثر شيوعا لدى مرضى السكر مضاعفات اعتلال الشبكية، الاعتلال العصبي، أمراض القلب والشرابيين، وقد ارتبطت هذه المضاعفات بشكل ايجابي مع مدة مرض السكر. كان مؤشر كتلة الجسم في الحالات أعلى بكثير من تلك في الضوابط، وكانت نسبة الجلوكوز في مصل الدم ومستويات الأنسولين أعلى بشكل ملحوظ في مرضى السكري عنها في الضوابط (187.4 ± 74.1 و 28.8 ± 23.9 مقابل 98.3 ± 17.0 و 18.8 ± 13.5 ، الفرق = 62.4 و 42.0) ($P = 0.000$ و $P = 0.025$). أظهرت النتائج ان اللبتين سجل زيادة كبيرة في مرضى السكري مقارنة مع الضوابط (7.1 ± 2.7 مقابل 5.9 ± 2.2 ng/ml، فروق = 18.5 و $P = 0.081$) في حين ان هرمون الجريلين سجل انخفاضا ملحوظا في المرضى (1189 ± 580 مقابل 1531 ± 822 pg/ml، الفرق = 25.1 و $P = 0.038$). بينت النتائج بشكل ملحوظ ان متوسط مستوى الدهون الثلاثية كان اعلى بكثير لدى

المرضى مقارنة بالمجموعة الضابطة (78.9 ± 212.5 مقابل 50.4 ± 143.2 % الفرق = 39.0 و $P = 0.000$). وبالإضافة إلى ذلك، أظهرت اليوريا زيادة المرضى (11.0 ± 33.5 مقابل 13.3 ± 28.3 ، % الفرق = 16.8 و $P = 0.059$). وأظهر ارتباط بيرسون ان هناك ارتباط إيجابي كبير لمؤشر كتلة الجسم مع الدهون الثلاثية ($P = 0.044$ ، $r = 0.228$). والانسولين أظهر علاقة إيجابية مع الدهون الثلاثية، والجلوكوز وهرمون الليبتين ($r = 0.224$ ، $r = 0.286$ ، و $r = 0.187$ على التوالي). كانت هذه العلاقة ملحوظة بالنسبة للجلوكوز والدهون الثلاثية ($P = 0.011$ ، $P = 0.049$ على التوالي)، من ناحية أخرى، سجل هرمون الجريلين ارتباط سلبي مع هرمون الانسولين ($r = -0.213$ و $P = 0.057$).

الاستنتاج: كانت هناك زيادة في مستويات هرموني الانسولين والليبتين في مرضى السكري مقارنة مع الضوابط بينما انخفض مستوى هرمون الجريلين في المرضى. اظهر هرمون الانسولين ارتباطا إيجابيا مع الليبتين و ارتباطا سلبيا مع هرمون الجريلين مما يؤكد على وجود تفاعل ثلاثي هرموني في مرض السكري.

مفتاح الكلمات: هرمون الغريلين وهرمون الانسولين، وهرمون الليبتين، والسكري، في قطاع غزة.

Dedication

I dedicate this work to:

My beloved mother who has always been supporting to me

*My beloved nephew Mohammed who gives our life a new
beautifully shining thought and meaning*

*My brother Ibrahim, brother Ahmed and his wife Heba, to my
sister Manal and her sons Malek and Basem*

*To my greatest friend forever, Fatma El Ejla who has never
left my side, she always supports me whatever the difficulties
were, all the time she was there*

*And I thank Allah so much for their existence in my life, and I
hope Allah will bless them*

*To all those who have hope, patience, principles and the
tenacity to make a difference in our world*

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And to everyone help me to get this work done.

List of Abbreviation

α -MSH	Alpha-melanocyte stimulating hormone
BMI	Body mass index
CDC	Center for disease control and prevention
DM	Diabetes mellitus
ELISA	Enzyme linked immunosorbent assay
GH	Growth hormone
GIP	Glucose-dependent insulintropic peptide
GIR	Glucose infusion rate
GLP-1	Glucagon-like peptide-1
HbA1c	Glycosylated haemoglobin A1c
HDL-C	High density lipoprotein cholesterol
IR	Insulin resistant
LDL-C	Low density lipoprotein cholesterol
MC4R	Type 4 melanocortin receptor
NIR	Non insulin resistant
NPY	Neuropeptide Y
OHAs	Oral hypoglycaemic agents
PC-1	Proenzyme convertase
POMC	Prohormone proopiomelanocortin
T2DM	Type 2 Diabetes Mellitus
TC	Total cholesterol
TG	Triglyceride
The Ob (Lep) gene	Obese, Leptin gene

Table of Contents

Declaration	i
English Abstract	ii
Arabic Abstract	iv
Dedication	vi
Acknowledgement	vii
List of Abbreviations	viii
Table of Contents	ix
List of Tables	xii
List of Figures	xiii
List of Annex	xiv

Chapter One: Introduction

1.1 Overview	1
1.2 General objective	3
1.3 Specific objectives	3
1.4 Significance.....	3

Chapter Two: Literature Review

2.1 Definition of diabetes mellitus	5
2.2 Types of diabetes	5
2.2.1 Type 1 diabetes	5
2.2.2 Type 2 diabetes	5
2.2.3 Gestational diabetes	6
2.3 Prevalence and mortality rate of diabetes mellitus in Palestine	6
2.4 Type 2 diabetes	6
2.4.1 Metabolism in type 2 diabetes	6
2.4.2 Risk factors of type 2 diabetes	8
2.4.3 Complications of type 2 Diabetes	8
2.4.3.1 Retinopathy	8
2.4.3.2 Diabetic nephropathy	9
2.4.3.3 Diabetic neuropathy	9
2.4.3.4 Cardiovascular disease	10
2.5 Obesity	10
2.6 Ghrelin	11
2.6.1 Definition and site of secretion.....	11
2.6.2 Biological effects of ghrelin	11
2.6.2.1 Effect of ghrelin on the anterior pituitary gland	11
2.6.2.2 Effect of ghrelin on appetite and food intake	12
2.7 Leptin	12
2.7.1 Definition and site of secretion	12
2.7.2 Mechanism of leptin action	13

2.8 Insulin	14
2.8.1 Definition and site of secretion.....	14
2.8.2 Mechanism of insulin action	14
2.9 Related studies	16

Chapter Three: Materials and Methods

3.1 Study design	19
3.2 Target population	19
3.3 Sampling and sample size	19
3.4 Exclusion criteria	20
3.5 Ethical consideration	20
3.6 Data collection	20
3.6.1 Questionnaire interview	20
3.6.2 Body mass index	20
3.6.3 Specimen collection and biochemical analysis	21
3.7 Biochemical analysis	21
3.7.1 Determination of serum insulin	21
3.7.2 Determination of serum leptin	24
3.7.3 Determination of serum ghrelin.....	26
3.7.4 Determination of serum glucose	31
3.7.5 Determination of serum cholesterol	32
3.7.6 Determination of serum triglycerides	34
3.7.7 Determination of serum high density lipoprotein (HDL-C)	36
3.7.8 Determination of serum low density lipoproteins (LDL-C)	38
3.7.9 Determination of serum urea	39
3.7.10 Determination of serum creatinine	40
3.8 Statistical analysis.....	43

Chapter Four: Results

4.1 Sociodemographic characters of the study population	44
4.2 Meal frequency/day of the study population.....	46
4.3 Distribution of diabetic patients by the duration of the disease	47
4.4 Regular blood glucose testing among diabetic patients.....	48
4.5 Self-reported complications among the study population	49
4.6 Self-reported complications in relation to duration of diabetes	50
4.7 Anthropometric measurements of the study population	50
4.8 Serum glucose level of the study population	51
4.9 Serum insulin, leptin and ghrelin levels of the study population	52
4.10 lipid profile of the Study population	53
4.11 Serum urea and creatinine concentrations of the study population.....	54
4.12 Body mass index in relation to insulin, leptin, ghrelin and triglycerides of the study population.....	54
4.13 Insulin levels in relation to glucose, triglycerides, leptin and ghrelin levels of the study population	58

Chapter Five: Discussion

5.1 Sociodemographic characters of the study population.....	61
5.2 Meal frequency/day of the study population	62
5.3. Distribution of diabetic patients by disease duration and regular blood glucose testing	62
5.4 Self-reported complications among the study population.....	63
5.5 Anthropometric measurements of the study population	63
5.6 Glucose and insulin levels of the study population	64
5.7 Serum insulin, leptin and ghrelin levels of the study population	65
5.8 lipid profile of the Study population	66
5.9 Urea and creatinine concentrations of the study population	66

Chapter Six: Conclusions and Recommendations

6.1 Conclusions	68
6.2 Recommendation	69

Chapter seven: References

References	70
------------------	----

List of Tables

Table 4.1 General characteristics of the study population	45
Table 4.2 Meal frequency/day of the study population	46
Table 4.3 Distribution of diabetic patients by the duration of the disease.	47
Table 4.4 Blood glucose testing among diabetic patients	48
Table 4.5 The main self-reported complications among the study population .	49
Table 4.6 Self-reported complications among diabetic patients in relation to duration of the disease	50
Table 4.7 Anthropometric measurements of the study population	51
Table 4.8 Serum glucose of the study population.....	52
Table 4.9 Serum insulin, leptin and ghrelin levels of the study population	52
Table 4.10 lipid profile of the Study population	53
Table 4.11 Serum urea and creatinine concentrations of diabetic patients and controls	54
Table 4.12 Body mass index (BMI) in relation to insulin, leptin, triglycerides and ghrelin of the study population	55
Table 4.13 Insulin levels in relation to glucose, triglycerides, leptin and ghrelin levels of the study population	58

List of Figures

Figure 2.1 Effect of ghrelin on the anterior pituitary gland to release GH	12
Figure 2.2 Mechanism of action of leptin	13
Figure 2.3 The hypothalamic leptin-melanocortin system	14
Figure 2.4 Mechanism of insulin action	15
Figure 4.1 Meal frequency/day of the study population.....	46
Figure 4.2 Distribution of diabetic patients by the duration of the disease	47
Figure 4.3 Blood glucose testing among diabetic patients	48
Figure 4.4 Correlation between body mass index (BMI) with insulin hormone level of the study population	56
Figure 4.5 Correlation between body mass index (BMI) with leptin hormone level of the study population	56
Figure 4.6 Correlation between body mass index (BMI) and triglyceride level of the study population.....	57
Figure 4.7 Correlation between body mass index (BMI) with ghrelin hormone level of the study population.	57
Figure 4.8 Correlation between insulin hormone level with glucose level of the study population	59
Figure 4.9 Correlation between insulin hormone level with triglyceride level of the study population.....	59
Figure 4.10 Correlation between insulin hormone level with leptin level of the study population	60
Figure 4.11 Correlation between insulin hormone level with ghrelin level of the study population	60

List of Annexes

Annex 1	Helsinki committee approval letter	84
Annex 2	Ministry of Health permission letter	85
Annex 3	Interview questionnaire	86

Chapter 1

Introduction

1.1 Overview

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. Two major forms of diabetes were identified; type 1 and type 2. Lack of or severe reduction in insulin secretion due to autoimmune or viral destructions of β cells is responsible for type 1 diabetes, which accounts for 5-10% of diabetic patients. Type 2 diabetes is more prevalent and 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).

Obesity is one of the most significant risk factor of type 2 diabetes. It is estimated that the chance to develop diabetes doubles for every 20% increase over desirable body weight (Keslore, 2008). Chronic obesity leads to increased insulin resistance that can develop into diabetes (Camastra, 1999). Other risk factors include poor diet, sedentary lifestyle and family history; diabetes tends to run in families (Fujita, 2009 and Pijl et al., 2009). Over time, diabetes can lead to retinopathy, nephropathy, and neuropathy. These types of damage are the result of damage to small vessels, referred to as microvascular disease. Diabetes is also an important factor in accelerating the hardening and narrowing of the arteries (atherosclerosis), leading to strokes, coronary heart disease, and other large blood vessel diseases. This is referred to as macrovascular disease (Fowler, 2008).

Lack of insulin action and/or secretion in type 2 diabetes induces hepatic glucose output by inhibiting glycogen synthesis and stimulating glycogenolysis

and gluconeogenesis then increased rates of hepatic glucose production result in the development of overt hyperglycemia, especially fasting hyperglycemia (DeFronzo et al., 1992 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 (Botton 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).

Leptin, a 16 Kd a circulating protein hormone that consists of 167-amino acids, produced and released primarily by adipose tissue, exerts a regulatory control mechanism on food intake via inhibition of neuropeptide Y and increases the basal metabolism rate with selectively promoting fat metabolism (Prieur et al., 2008). The link of leptin with obesity and diabetes is unclear and controversial. Leptin could be regulated by insulin (Susan, 2000). However, secretion of leptin is impaired in diabetes. Many researchers found a decrease in leptin levels in Type 2 diabetic patients (Tatti et al., 2001; Abdelgadir et al., 2002 and Altawil, 2009) whereas others demonstrated increase in the hormone level (ref El Meligi et al., 2003; Chan et al., 2004; and Abu Mustafa, 2011).

Ghrelin is a multi-functional gut-derived peptide hormone that consists of 28 amino acids with a molecular weight of about 3.3kDa. At the protein level, the main source of ghrelin synthesis in all vertebrate species has been shown to be the stomach (Ariyasu et al. 2001). Ghrelin has been shown to increase appetite and food intake and stimulate the secretion of growth hormone in humans (Peino et al. 2000 and Wren et al. 2001). Several authors showed that low plasma ghrelin is associated with insulin resistance, hypertension, and the prevalence of type 2 diabetes (Pöykkö et al., 2003; McLaughlin et al., 2004; Erdmann et al. 2005 and Pulkkinen et al., 2010).

Research on ghrelin in type 2 diabetes mellitus and its interactions with insulin or leptin is limited and controversial. However, it was shown that ghrelin and leptin recorded low and high levels, respectively with increasing insulin resistance. Clarifying and understanding of such tri-hormonal interplay in type 2 diabetes could be helpful in the intervention and control strategies of the disease. Therefore, the present study is the first to assess ghrelin, leptin and insulin levels and their interactions with themselves as well as with other biochemical parameters in type 2 diabetic patients in Gaza Strip.

1.2 General objective

The general objective is to assess ghrelin, leptin and insulin in type 2 diabetic patients in Gaza Strip.

1.3 Specific objectives

1. To determine ghrelin, leptin and insulin levels in type 2 diabetic patients and compared them with non diabetic controls.
2. To calculate body mass index (BMI) of the study population.
3. To Measure lipid profile urea and creatinine in patients and controls.
4. To verify the interrelations between the three studied hormones as well as with other studied parameters.

1.4 Significance

1. Ghrelin is a recent discovered hormone and was shown to be lower among type 2 diabetics with insulin resistance compared to those without the disease. In contrast, leptin recorded high level with the progress of diabetes. Therefore, it is important to determine the levels of these hormones in diabetics and controls and to assess their interrelations. This may: a) Provide us with a clear picture on the roles and mechanisms of such hormones in diabetes particularly the novel hormone ghrelin and b) enable us to put new strategy for control of diabetes.

2. Type 2 diabetes is prevalent in Gaza Strip, as well as globally.
3. Research on ghrelin and leptin hormones in type 2 diabetes is a new and not much data are available globally. Of course, this will be the first study in Palestine to assess ghrelin status in type 2 diabetes.

Chapter 2

Literature Review

2.1 Definition of diabetes mellitus

Diabetes mellitus is defined solely in terms of elevated blood glucose concentrations and because of the vital role of insulin in regulating glucose metabolism a reduction in insulin secretion and/or insulin resistance is a common cause of diabetes (Nussey and Whitehead, 2001).

2.2 Types of diabetes

The most common types of diabetes mellitus are:

2.2.1 Type 1 diabetes

Type 1 or insulin-dependent diabetes develops when the body's immune system destroys pancreatic β cells resulting in failure of insulin production. This form of diabetes usually strikes children and young adults, although disease onset can occur at any age. Type 1 diabetes accounts for 5-10% of all diagnosed cases of diabetes (Olefsky, 2001 and Kumar et al., 2005).

2.2.2 Type 2 diabetes

Type 2 or non insulin-dependent diabetes results from insulin resistance, a condition in which the body fails to properly use insulin, combined with relative insulin deficiency (Robbins and Cotran, 2004). This form of diabetes accounts for about 90-95% of all diagnosed cases of diabetes. Type 2 diabetes is associated with older age, obesity, history of gestational diabetes, impaired

glucose metabolism, physical inactivity, and race/ethnicity (Centers for Disease Control and Prevention, CDC, 2007).

2.2.3 Gestational diabetes

Gestational diabetes develops in the course of pregnancy in some women. It is also more common among obese women and women with a family history of diabetes. During pregnancy, gestational diabetes requires treatment to optimize maternal blood glucose levels to lessen the risk of complications in the infant (CDC, 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. 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

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 et al., 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) whereas HDL-C was decreased (Altawil, 2009; Abu Mustafa, 2011 and Yassin et al., 2011).

2.4.2 Risk factors of type 2 diabetes

Cavallerano (2009) reported that the high-risk individual of type 2 diabetes is one who:

- is obese ($>120\%$ desirable body weight or body mass index $>25 \text{ kg/m}^2$)
- has a first-degree relative with diabetes.
- is a member of a high-risk ethnic population (i.e., African American, Hispanic, Native American).
- has delivered a baby weighing more than 4.5 kg or has been diagnosed with gestational diabetes.
- is hypertensive (blood pressure $>140/90$).
- has a high density lipoprotein cholesterol (HDL-C) level $<35 \text{ mg/dl}$ and/or a triglyceride level $>250 \text{ mg/dl}$.
- has had impaired glucose tolerance or impaired fasting glucose on previous testing.

2.4.3 Complications of type 2 diabetes

Type 2 diabetes is associated with long-term damage, dysfunction and failure of various organ systems in the body, especially the eyes, kidneys, nerves, heart and blood vessels (Rayner et al., 2001 and Adeghate et al., 2006).

2.4.3.1 Retinopathy

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 and has had diabetes for several years. Retinopathy is more likely to become a problem in people with diabetes if they have high blood sugar levels and high blood pressure over a long period of time. 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).

2.4.3.2 Diabetic nephropathy

One of the most severe complications of diabetes is the development of diabetic nephropathy. The earliest detectable change in the course of diabetic nephropathy is a thickening in the glomerulus. At this stage, the kidney may start allowing more serum albumin than normal in the urine, and this can be detected by sensitive medical tests for albumin. This stage is called microalbuminuria (urinary albumin 30-300 mg/24hr). It can appear 5 to 10 years before other symptoms develop. As diabetic nephropathy progresses, increasing numbers of glomeruli are destroyed by nodular glomerulosclerosis. Now the amounts of albumin being excreted in the urine increases, and may be detected by ordinary urinalysis techniques. The condition is called macroalbuminuria (urinary albumin >300 mg/24hr). At this stage, a kidney biopsy clearly shows diabetic nephropathy (Maeda and Shiigai, 2007). Further progress of the disease may lead to end stage kidney disease. Most diabetic patients with end stage kidney disease have type 2 diabetes (American Diabetic Association, 2004).

2.4.3.3 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).

2.4.3.4 Cardiovascular disease

People with type 2 diabetes developed 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: severe coronary atherosclerosis, prolonged hypertension, chronic hyperglycemia, microvascular disease, glycosylation of myocardial proteins, and autonomic neuropathy (Savage, 2005).

2.5 Obesity

Obesity is a condition in which excess body fat has accumulated to an extent that health may be negatively affected. Body mass index (BMI) is a measure of body fat based on a formula that calculates the ratio of body weight in Kg/height in meter square (National Heart, Lung and Blood Institute, 1998). Therefore, obesity is commonly defined as a BMI of 30 kg/m² or higher (World Health Organization, WHO, 2000).

High body mass lead to serious health consequences. Risk increases progressively as BMI increases. Raised body mass index is a major risk factor for chronic diseases including diabetes. About 55 percent of type 2 diabetics

are obese (Eberhart et al., 2004). Chronic obesity leads to increased insulin resistance that can develop into diabetes, most likely because adipose tissue is a recently identified as a source of several chemical signals (hormones and cytokines) to other tissues (Thévenod, 2008).

2.6 Ghrelin

2.6.1 Definition and site of secretion

Ghrelin is peptide hormone that consists of 28 amino acids with a molecular weight of about 3.3 kDa. It was identified in 1999 in a study which was designed to search for an endogenous ligand for an orphan receptor, the type 1a growth hormone secretagogue receptor, GHS-R1a receptor (Kojima et al., 1999). Ghrelin is secreted mainly from stomach (Ariyasu et al., 2001 and Rindi et al., 2002).

2.6.2 Biological effects of ghrelin

2.6.2.1 Effect of ghrelin on the anterior pituitary gland

Ghrelin acts directly on the anterior pituitary gland to release growth hormone in humans (Peino et al., 2000). It binds to its receptor GHS-R1a which activates a G-protein $G_{\alpha 11}$, and this activated G-protein then stimulates phospholipase C. The action of this lipase increases the intracellular concentration of inositol triphosphate, which causes the release of Ca^{2+} from intracellular stores. Increase in the intracellular Ca^{2+} leads to the release of growth hormone (Figure 2.1).

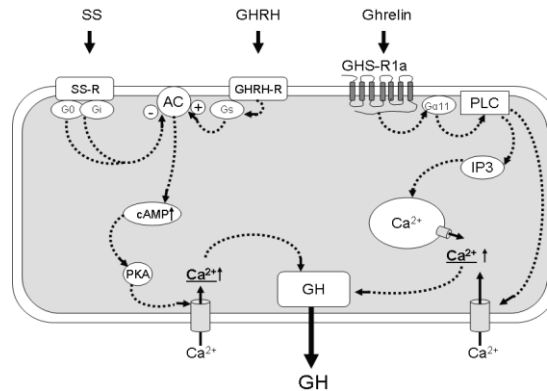


Figure 2.1. Effect of ghrelin on the anterior pituitary gland to release GH (Vartiainen, 2009). Growth hormone (GH), Somatostatin (SS), Growth hormone releasing hormone (GHRH), Binding of SS and GHRH to their receptors (SS-R and GHRH-R), Adenylate cyclase (AC), Cyclic AMP (cAMP), protein kinase A (PKA), GH binding to its receptor GHS-R1a, Phospholipase C (PLC), Inositol triphosphate (IP3).

2.6.2.2 Effect of ghrelin on appetite and food intake

Ghrelin increases appetite and food intake in humans (Wren et al., 2001). This effect is mainly mediated via the vagal afferents (le Roux et al., 2005). Ghrelin stimulates arcuate nucleus of the hypothalamus to release neuropeptide Y and agouti related protein into the synapse leading to decrease the production and release of alpha melanocyte stimulating hormone from the post-synaptic pro-opiomelanocortin neurons (Lawrence et al., 2002 and Wilding, 2002).

2.7 Leptin

2.7.1 Definition and site of secretion

Leptin is a 16 kDa protein hormone that plays a key role in regulating energy intake and energy expenditure, including appetite and metabolism (Zhang et al., 1994). Leptin is one of the most important adipose derived hormones (Kiess, 1998). The Ob (Lep) gene (Ob for obese, Lep for leptin) is located on chromosome 7 in humans, the gene encodes adipose tissue mRNA with a

highly conserved 167-amino acids. The amino acid sequence of leptin is approximately 84% identical between human and mouse.

2.7.2 Mechanism of leptin action

Leptin binding induces dimerization of the leptin receptor, activating a kinase molecule (JAK-2) which phosphorylates tyrosine (Y) residues on the receptor. This creates phosphotyrosine docking sites on for Signal Transducers and Activators of Transcription (STAT) proteins (Figure 2.2). These STAT proteins then dimerize and return to the nucleus to control expression of response genes (Friedmann, 2000).

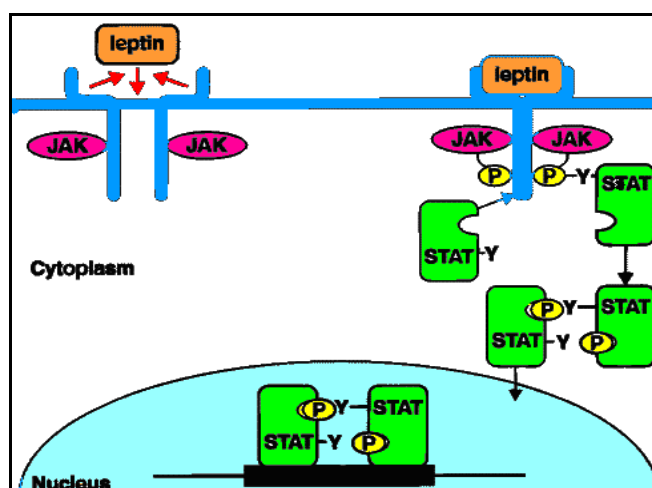


Figure 2.2. Mechanism of action of leptin (Friedmann, J.M. 2000). Just Activated Kinase (JAK), Signal Transducers and Activators of Transcription (STAT), Tyrosine (Y).

Genes expression in response to leptin action causes an increase in the prohormone proopiomelanocortin (POMC). One of the products of POMC is alpha-melanocyte stimulating hormone (α -MSH) which decreases hunger (Figure 2.3). The enzyme that processes POMC into α -MSH is called proenzyme convertase (PC-1). α -MSH activates a receptor on cell surfaces in the hypothalamus called type 4 melanocortin receptor (MC4R). Activation of this receptor is like flipping a switch, telling the cell to inhibit appetite (Janeckova, 2001 and Flier, 2004).

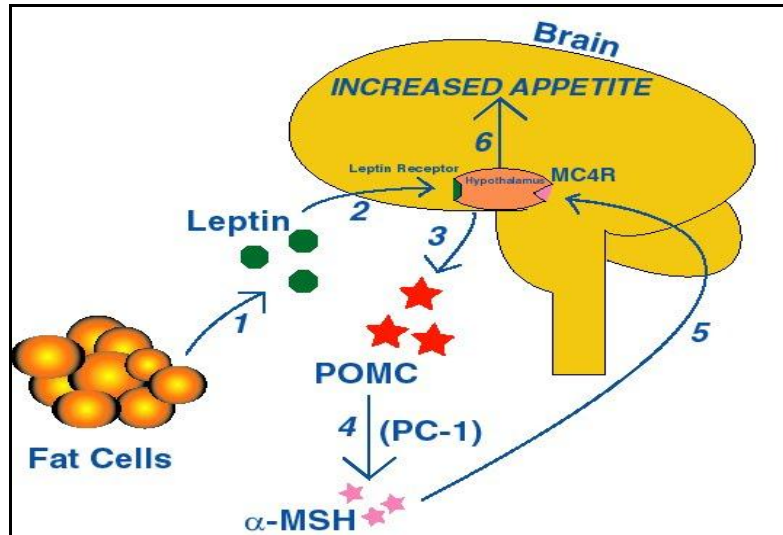


Figure 2.3. The hypothalamic leptin-melanocortin system. (Nussey and Whiteheds, 2001) Prohormone Proopiomelanocortin (POMC), Alpha-Melanocyte Stimulating Hormone (α -MSH), Proenzyme Convertase (PC-1), Type 4 Melanocortin Receptor (MC4R).

2.8 Insulin

2.8.1 Definition and site of secretion

Human insulin contains 51 amino acids (molecular weight 5700) and is structurally homologous to insulin-like growth factors 1 and 2 and also to the ovarian hormone, relaxin. It is composed of two amino acid chains A and B connected to each other by disulfide linkages. Insulin is synthesized in the beta cells of the pancreas by the usual cell machinery for protein synthesis.

2.8.2 Mechanism of insulin action

Insulin binds to the α -subunit (outside the cell membrane) of its receptor, which causes autophosphorylation of the β -subunit receptor (protruding into the cell cytoplasm), which in turn induces tyrosine kinase activity (Figure 2. 4). The receptor tyrosine kinase activity begins a cascade of cell phosphorylation that increases or decreases the activity of enzymes, including insulin receptor substrates, that mediate insulin effects. These effects include promotion of

glucose uptake and storage particularly into muscle and liver cells, stimulation of fat synthesis and storage, and activation of protein synthesis and storage.

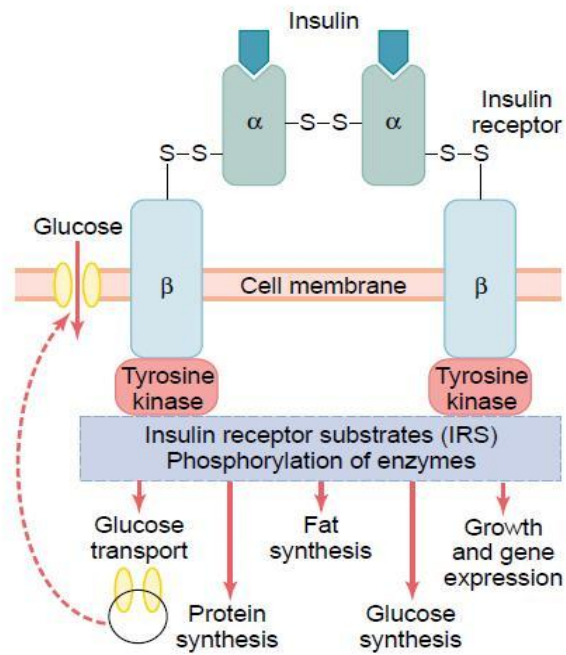


Figure 2.4. Mechanism of insulin action (Guyton and Hall, 2006)

2.9 Related studies

Anderwald et al. (2003) examined dose-dependent effects of insulin on plasma ghrelin and leptin concentrations during hyperinsulinemic (1, 2, and 4 mU kg⁻¹ min⁻¹)-euglycemic clamp tests in six type 2 diabetic patients. Patients were studied before and after prolonged (12-h and 67-h) variable intravenous insulin treatment aiming at near-normoglycemia (115±4 mg/dl). In patients without insulin treatment, ghrelin decreased by 18±7% (P < 0.05) only after 4 mU kg⁻¹ min⁻¹ insulin infusion and leptin increased by 19±6% (P < 0.05). After prolonged insulin treatment and near-normoglycemia, ghrelin and leptin remained unchanged during the clamps. In addition, Pöykkö et al. (2003) demonstrated that ghrelin concentrations were negatively associated with fasting insulin (P < 0.001), the prevalence of type 2 diabetes (P = 0.015) and with insulin resistance (P < 0.001).

The relationship between the circulating level of active ghrelin and abdominal adiposity, serum levels of insulin or insulin resistance in patients with type 2 diabetes mellitus was investigated (Katsuki et al., 2003). The plasma levels of the active form of ghrelin were measured in 18 obese and 18 nonobese patients with type 2 diabetes mellitus using a radioimmunoassay. Plasma levels of ghrelin in obese patients with type 2 diabetes mellitus were significantly decreased compared with nonobese patients. There were significant correlations between the plasma levels of ghrelin and BMI (r= 20.505, P, 0.01), visceral (r= 20.444, P, 0.01), subcutaneous (r= 20.506, P, 0.01) and total (r= 20.534, P, 0.01) fat area, serum levels of insulin (r= 20.513, P, 0.01) or GIR (r= 0.478, P, 0.01) in type 2 diabetic patients. The plasma level of ghrelin was significantly associated with serum levels of insulin (r= 8.468, P, 0.05) or glucose infusion rate (GIR) (r= 8.522, P, 0.05) after adjustment for BMI in patients with type 2 diabetes mellitus.

To gain further insight into the regulatory role of insulin and leptin on plasma ghrelin, 56 normal weight, 128 normoinsulinemic obese, 121 hyperinsulinemic

obese, and 30 type 2 diabetic normoinsulinemic and 75 type 2 diabetic hyperinsulinemic obese patients were examined (Erdmann et al., 2005). In the obese subjects, basal hyperinsulinemia was associated with significantly lower ghrelin independent of BMI, age, and leptin. In normoinsulinemic (normal weight and normoinsulinemic obese) subjects, ghrelin was inversely related to stepwise increasing leptin. Multiple regression analysis and matching for insulin revealed a significant negative interaction of ghrelin with leptin but not insulin. In type 2 diabetic normoinsulinemic subjects, ghrelin was significantly lower compared with that in normoinsulinemic obese subjects. In type 2 diabetic hyperinsulinemic subjects, ghrelin was significantly lower than in normoinsulinemic subjects, whereas no further reduction was observed compared with hyperinsulinemic obese subjects.

Stylianou et al. (2007) compared fasting ghrelin and leptin levels between obese and non-obese, adolescents and investigated possible correlations of these hormones with body fat percentage (BF%), as well as insulin resistance (IR). Twenty obese insulin resistant IR adolescents, twenty obese non IR (NIR) and fifteen healthy non-obese, age-matched adolescents were studied. In all participants, height, weight, body mass index (BMI) and BF % were measured. Fasting glucose, insulin, ghrelin and leptin levels were determined. IR was assessed using HOMA-IR index. BMI, BF %, insulin and HOMA-IR values were positively correlated with leptin and negatively with ghrelin levels. A negative correlation between circulating leptin and ghrelin levels was found. A suggestive positive correlation between leptin levels and BF %, independent of BMI, was also observed ($P=0.075$). Ghrelin levels were significantly correlated with insulin levels and HOMA-IR, independent of BMI ($P=0.077$).

Katsiki et al. (2011) evaluated the effects of insulin-induced improved glycaemic control on leptin, adiponectin, ghrelin, neuropeptide Y (NPY) levels and patient characteristics. Consecutive type 2 diabetic patients ($n = 90$) were divided into 2 groups: Group A: 45 insulin-naïve uncontrolled (glycosylated haemoglobin A1c; $HbA1c >7\%$) patients on oral hypoglycaemic agents (OHAs) who converted to insulin monotherapy. Group B: 45 well-controlled ($HbA1c <7\%$) patients on OHAs. Both groups were monitored at baseline, 3 and 6

months. In both genders, insulin therapy (Group A) was associated with significant ($P= 0.003$ to <0.001) increases in weight, body mass index and leptin levels and significant decreases in glucose, HbA1c and NPY levels. In male insulin treated patients a significant increase in adiponectin levels ($P=0.008$) was observed. There were significant correlations ($P=0.016$ to <0.001) between leptin levels, waist circumference and body fat in all patient groups, except group B males.

Chapter 3

Materials and Methods

3.1 Study design

Case control study

3.2 Target population

The target population was type 2 diabetic patients aged 40-60 years from different Governmental hospitals and diabetic clinics distributed in Gaza Strip.

3.3 Sampling and sample size

Type 2 diabetic patients, previously diagnosed according to the current WHO diagnostic criteria for diabetes (World Health Organization, 2006), were selected from different Gaza Strip hospitals including Al Shifa hospital (Gaza), Al Najjar and Nasser hospitals (Southern Gaza) and Kamal Edwan hospital (Northern Gaza) as well as from different diabetic clinics distributed in Gaza Strip. These major hospitals are representative to Gaza Strip. Controls were healthy persons selected from general population lived in different Governorates of the Gaza Strip. Controls and cases were matched for age and sex. The Sample size was 160 individuals: 80 type 2 diabetic patients (40 males and 40 females) and 80 healthy persons (40 males and 40 females).

3.4 Exclusion criteria

- Patients with hormonal complications.
- Pregnant women.

3.5 Ethical consideration

The necessary approval to conduct the study was obtained from Helsinki committee in the Gaza Strip (Annex 1). Coordination with the Ministry of Health was fulfilled (Annex 2).

3.6 Data collection

3.6.1 Questionnaire interview

A meeting interview was used for filling in a questionnaire which designated for matching the study need (Annex 3). All interviews were conducted face to face by the researcher herself. The questionnaire was based on diabetic clinics questions, and on that used in similar studies with some modifications (Shubair, 2008 and Abu Mostafa, 2011). Most questions were the yes/no questions, which offer a dichotomous Choice (Backstrom and Hursh-Cesar, 1981). The questionnaire was validated by seven experts in the fields of public health, epidemiology, endocrinology, physiology and nutrition. A questionnaire was piloted with 10 patients. During the study the interviewer explained to the participants any of the confused questions that is not clear to them. The questionnaire included questions on general characters of the study populations (age, education, employment, family history of diabetes and smoking), meal frequency/day, and clinical data including duration of DM, regular testing of blood glucose level and the most common complications of diabetes.

3.6.2 Body mass index

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

3.6.3 Specimen collection and biochemical analysis

Blood samples were collected from 80 type 2 diabetic patient and 80 controls. Twelve hours fasting overnight venous blood sample (about 9 ml) was drawn by the researcher herself who had Bachelor Degree in Medical Technology into vacutainer tubes from each control and diabetic individual. Three ml blood were placed into plastic tube contained 15 µl protease inhibitor (pefabloc) supplied by sigma-Aldrich Germany with catalog number (76307) for serum ghrelin assay (Al Arabi laboratory, Gaza City). The remainder quantity of blood (6 ml) was placed in a separate plastic tube. The blood in the two plastic tubes was left for a while without anticoagulant to allow blood to clot. Then serum samples were obtained by centrifugation at room temperature at 4000 rpm for 10 minutes. One ml of serum with protease inhibitor was placed in plastic tube and 10 µl of 5 N HCl were added and samples were stored at -20±5°C for serum ghrelin assay. A quantity of serum obtained from the plastic tube without protease inhibitor was stored at -20±5°C for insulin and leptin assay. The remainder was used for glucose, cholesterol, triglycerides, high density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LDL-C), urea and creatinine analysis.

3.7 Biochemical analysis

3.7.1 Determination of serum insulin

Monobinds insulin MAPS ELISA test kit (Insulin-C peptide/ Product code 7325-300) was used for the quantitative determination of serum insulin.

Principle

The essential reagents required for an immunoenzymometric assay include high affinity and specificity antibodies (Ab) (enzyme conjugated and immobilized), with different and distinct epitope recognition, in excess, and native antigen (Ag). In this procedure the immobilization takes place during the assay at the surface of the microplate well through the interaction of streptavidin coated on the well and exogenously added biotinylated monoclonal insulin antibody. Upon mixing monoclonal biotinylated antibody, the enzyme labeled antibody and a serum containing the native antigen, reaction results between the native antigen and the antibodies without competition or steric hindrance, to form soluble sandwich complex. Simultaneously the complex is deposited to the well through the high affinity reaction of streptavidin and biotinylated antibody, after equilibrium is attained the antibody-bound fraction is separated from unbound antigen by decantation or aspiration. The enzyme activity in the antibody-bound fraction is directly proportional to the native antigen concentration .

Reagents and materials supplied

1. Insulin calibrators-2ml/vial(dried), six (6)vials of references for insulin antigens at levels of 0(A), 5(B), 25(C), 50(D), 100(E), and 300(F) (μ IU/ml). Reconstitute each vial with 2 ml of distilled or deionized water.
2. Insulin enzyme reagent (13ml/vial), one (1)vial containing enzyme labeled affinity purified monoclonal mouse x-insulin IgG, Biotinylated monoclonal mouse x-insulin IgG in buffer, dye, and preservative.

3. Streptavidin coated plate (96wells), one 96 well microplate coated with streptavidin and packaged in an aluminum bag with a drying agent. Store at 2-8°C.
4. Wash solution concentrate (20 ml), one (1)vial containing a surfactant in phosphate buffered saline, a preservative has been added. Store at 2-30°C.
5. Substrate A (7ml/vial), one bottle containing tetramethylbenzidine (TMB) in buffer store at 2-8°C.
6. Substrate B (7ml/vial), one bottle containing hydrogen peroxide (H₂O₂) in buffer, store at 2-8°C.
7. Stop solution (8ml /vial), one bottle containing a strong acid (1NHCL).Store at 2-30°C.

Assay procedure

Before proceeding with the assay bring all reagents , serum, references and controls to room temperature (20-27°C).

1. Formate the microplates wells for calibrator, control and patient specimen to be assayed in duplicate .
2. Pippete 50 µl of the appropriate calibrators, controls and samples into the assigned wells.
3. Add 100 µl of the insulin enzyme reagent to each well.
4. Swirl the microplate gently for 20-30 seconds to mix. Cover with plastic wrap.
5. Incubate for 120 minutes at room temperature (20-27°C).
6. Discard the contents of the microplate by decantation or aspiration. If decantation tab and blot the plate dry with absorbent paper .

7. Add 300 μ l of wash buffer, decant or aspirate. Repeat two additional times for a total of three washes. An automatic washer can be used.
8. Add 100 μ l of working substrate solution to all wells.
9. Incubate at room temperature for 15 minutes.
10. Add 50 μ l of stop solution to each well and mix gently for 15-20 seconds.
11. Read the absorbance in each well at 450 nm in a microplate reader. The result should be read within 30 minutes of adding stop solution.

Insulin normal range

Children < 12	< 10 μ IU/ml
Adult normal	0.7-9 μ IU/ml
Diabetic (type2)	0.7-25 μ IU/ml

3.7.2 Determination of serum leptin

Determination of serum leptin level was carried out by competitive enzyme immunoassay Diagnostic System Laboratories (DSL), USA technique.

Principle

The DSL-10-23100 ACTIVE Human Leptin ELISA is an enzymatically amplified "twostep" sandwich-type immunoassay. In the assay, standards, controls and unknown serum or plasma samples were incubated in microtitration wells, which have been coated with anti-human leptin antibody. After incubation and washing, the wells were treated with another anti-human leptin detection antibody labeled with the enzyme horseradish peroxidase (HRP). After a second incubation and washing step, the wells were incubated with the substrate tetramethylbenzidine (TMB). An acidic stopping solution was then added and the degree of enzymatic turnover of the substrate was determined by dual wavelength absorbance measurement at 450 and 620 nm. The absorbance measured was directly proportional to the concentration of human

leptin present. A set of human leptin standards was used to plot a standard curve of absorbance versus human leptin concentration from which the human leptin concentrations in the sample can be calculated.

Assay procedure

Annabel all specimens and reagents to reach room temperature (~25°C) and mix thoroughly by gentle inversion before use. Standards, controls and samples should be assayed in duplicate.

1. The microtitration strips were marked to be used.
2. Twenty five microliters of the standards, controls and samples were pipeted into the appropriate wells.
3. One hundred microliters of the assay buffer E were added to each well using a semi-automatic dispenser.
4. Incubate the wells, shaking at a fast speed (500-700 rpm) on an orbital microplate shaker, at room temperature (~25 °C) for 2 hours.
5. Aspirate and wash each well 5 times with the wash solution using an automatic microplate washer. Blot dry by inverting plate on absorbent material.
6. The antibody-enzyme conjugate solution was prepared by diluting the antibody-enzyme conjugate concentrate in the assay buffer.
7. One hundred microliters of the antibody-enzyme conjugate solution was added to each well using a semi-automatic dispenser.
8. The wells were incubated, shaken at a fast speed (500-700 rpm) on an orbital microplate shaker, at room temperature (~25 °C) for 1 hour.
9. Aspirate and wash each well 5 times with the wash solution using an automatic microplate washer. Blot dry by inverting plate on absorbent material.
10. One hundred microliters of the TMB Chromogen solution was added to each well using a semi-automatic dispenser.

11. Incubate the wells, shaking at a fast speed (500-700 rpm) on an orbital microplate shaker, at room temperature (~25°C) for 10 minutes. Avoid exposure to direct sunlight.

12. One hundred microliters of the stopping solution (0.2M sulfuric acid) was added to each well using a semi-automatic dispenser.

13. The absorbance of the solution in the wells was read within 30 minutes, using a microplate reader set to 450 nm.

Calculation

1. The mean absorbance for each standard, control and samples were calculated.

2. Plot the log of the human leptin concentrations in ng/mL along the x-axis versus the mean absorbance readings for each of the standards along the y-axis versus, using a linear curve-fit. Alternatively, the data can be plotted linear vs. linear and a smoothed spine curve-fit can be used.

3. Determine the human leptin concentrations of the controls and samples from the standard curve by matching their mean absorbance readings with the corresponding human leptin concentrations.

3.7.3 Determination of serum ghrelin

Determination of human serum ghrelin level was carried out by Enzyme Immunoassay for the quantitative determination of total human ghrelin (both intact and des-octanoyl forms) in serum and plasma catalog number EIA-4709, DRG Instruments GmbH, Germany.

Principle

This assay is a Sandwich ELISA based on:

1. Capture of human ghrelin molecules (both active and des-octanoyl forms) in the sample by anti-human ghrelin IgG and immobilization of the resulting

complex to the wells of a microtiter plate coated by a pre-titered amount of anchor antibodies,

2. and the simultaneous binding of a second biotinylated antibody to ghrelin,
3. Wash away of unbound materials, followed by conjugation of horseradish peroxidase to the immobilized biotinylated antibodies,
4. Wash away of free enzyme, and
5. Quantification of immobilized antibody-enzyme conjugates by monitoring horseradish peroxidase activities in the presence of the substrate 3,3',5,5'-tetra-methylbenzidine. The enzyme activity is measured spectrophotometrically by the increased absorbency at 450 nm, corrected from the absorbency at 590 nm, after acidification of formed products. Since the increase in absorbency is directly proportional to the amount of captured total human ghrelin in the unknown sample, the concentration of total ghrelin can be derived by interpolation from a reference curve generated in the same assay with reference standards of known concentrations of human ghrelin.

Reagents and materials supplied

1. Microtiter Plate: Coated with pre-titered anchor antibodies.
2. Adhesive Plate Sealer.
3. 10X HRP Wash Buffer Concentrate: 10X concentrate of 50 mM Tris Buffered Saline containing Tween-20.
4. Human Ghrelin (Total) Standard: Human Ghrelin (total) reference standard, 5 ng/ml, lyophilized.
5. Human Ghrelin (Total) Quality Controls 1 and 2: One vial each, lyophilized, containing human ghrelin (total) at two different levels.
6. Human Ghrelin (Total) Matrix: Processed serum matrix containing 0.08% Sodium Azide

7. Assay Buffer: 0.05 M phosphosaline, pH 7.4, containing 0.025 M EDTA, 0.05 % Triton X-100, 0.08% sodium azide, and 0.1% BSA.
8. Human Ghrelin (Total) Capture Antibody: Pre-titered capture antibody solution in buffer
9. Human Ghrelin (Total) Detection Antibody: Pre-titered detection antibody solution in buffer
10. Enzyme Solution: Pre-titered streptavidin-horseradish peroxidase conjugate in buffer.
11. Substrate: 3, 3',5,5'-tetramethylbenzidine in buffer.
12. Stop Solution: 0.3 M HCl; Caution: Corrosive Solution.

Assay procedure

Pre-warm all reagents to room temperature immediately before setting up the assay.

1. Dilute the 10X concentrated HRP wash buffer 10 fold by mixing the entire contents of both buffer bottles with 900 mL de-ionized or glass distilled water.
2. Remove the required number of strips from the microtiter assay plate. Unused strips should be resealed in the foil pouch and stored at 2-8°C. Assemble the strips in an empty plate holder and fill each well with 300 μ L diluted wash buffer. Decant wash buffer and remove the residual amount by inverting the plate and tapping it smartly onto absorbent towels several times. Wash assay plate using this procedure 2 additional times. Do not let wells dry before proceeding to the next step. If an automated machine is used for the assay, follow the manufacturer's instructions for all washing steps described in this protocol.
3. Add 20 μ L matrix solution to Blank, standards and quality control wells (refer to 10 for suggested well orientations).
4. Add 30 μ L assay buffer to each of the blank and sample wells.

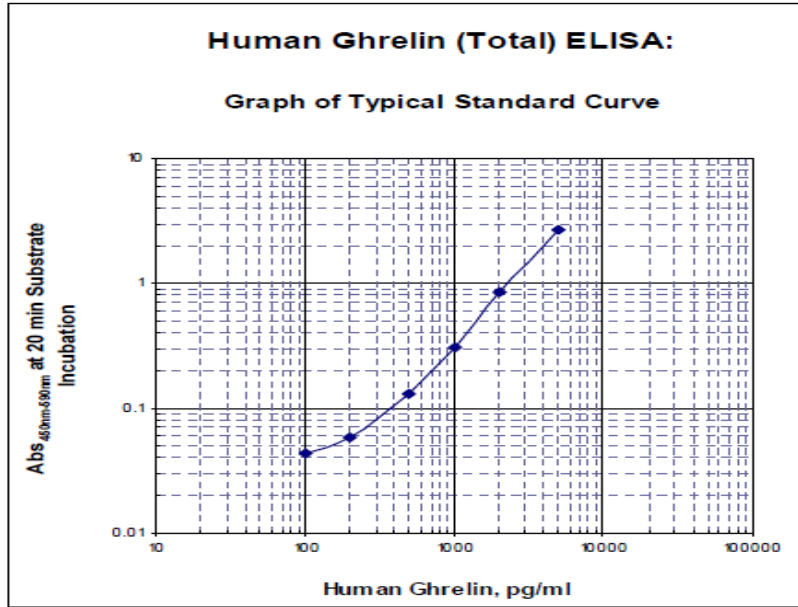
5. Add 10 μL assay buffer to each of the standard and quality control wells.
6. Add in duplicate 20 μL ghrelin standards in the order of ascending concentrations to the appropriate wells.
7. Add in duplicate 20 μL QC1 and 20 μL QC2 to the appropriate wells.
8. Add sequentially 20 μL of the unknown samples in duplicate to the remaining wells.
9. Transfer the antibody solution mixture (1:1 mixture of capture and detection antibody) to a buffer/reagent reservoir and add 50 μL to each well with a multi-channel pipette.
10. Cover the plate with plate sealer and incubate at room temperature for 2 hours on an orbital microtiter plate shaker set to rotate at moderate speed, about 400 to 500 rpm.
11. Remove plate sealer and decant solutions from the plate. Tap as before to remove residual solutions in well.
12. Wash wells 3 times with diluted wash buffer, 300 μL per well per wash. Decant and tap after each wash to remove residual buffer.
13. Add 100 μL enzyme solution to each well. Cover plate with sealer and incubate with moderate shaking at room temperature for 30 min on the micro-titer plate shaker.
14. Remove sealer, decant solutions from the plate and tap plate to remove the residual fluid.
15. Wash wells 6 times with diluted wash buffer, 300 μL per well per wash. Decant and tap after each wash to remove residual buffer.
16. Add 100 μL of Substrate solution to each well, cover plate with sealer and shake in the plate shaker for approximately 5~20 minutes. Blue color should be formed in wells of ghrelin standards with intensity proportional to increasing concentrations of ghrelin. (Note: Please be aware that the color may develop

more quickly or more slowly than the recommended incubation time depending on the localized room temperature. Please visually monitor the color development to optimize the incubation time.)

17. Remove sealer and add 100 μ L stop solution [CAUTION: CORROSIVE SOLUTION] and shake plate by hand to ensure complete mixing of solution in all wells. The blue color should turn into yellow after acidification. Read absorbance at 450 nm and 590 nm in a plate reader within 5 minutes and ensure that there is no air bubbles in any well.

Calculations

Graph a reference curve by plotting the absorbance unit of 450 nm, less unit at 590 nm, on the Y-axis against the concentrations of ghrelin standard on the X-axis. The dose-response curve of this assay fits best to a sigmoidal 4- or 5-parameter logistic equation. The results of unknown samples can be calculated with any computer program having a 4- or 5-parameter logistic function. The appropriate range of this assay is 100 pg/mL to 5,000 pg/mL Total Ghrelin (20 μ L sample size).



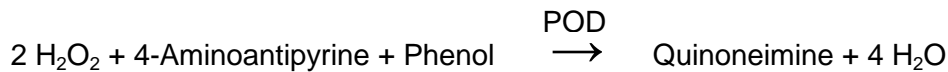
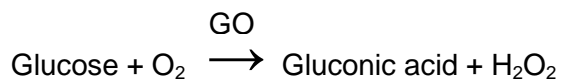
Graph of typical reference curve

3.7.4 Determination of serum glucose

Serum glucose was determined by glucose oxidase (GOD)/glucose peroxidase (POD) method using Labkit Kit, Spain.

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.



Reagents

Reagent	Concentration
Phosphate buffer (pH 7.5)	250 mmol/l
Phenol	5 mmol/l
4-Aminoantipyrine	0.5 mmol/l
Glucose oxidase (GOD)	≥ 15 ku/l
Peroxidase (POD)	≥ 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 1 ml of the reagent and mixed well.
- The mixture was incubated for 10 min at 37 °C.
- 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

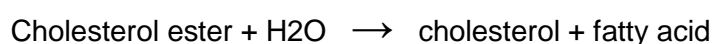
3.7.5 Determination of serum cholesterol

Serum cholesterol was determined by 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.

CHE



CHO



POD



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 °C

Measurement: against reagent blank.

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

Calculation

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

Reference value

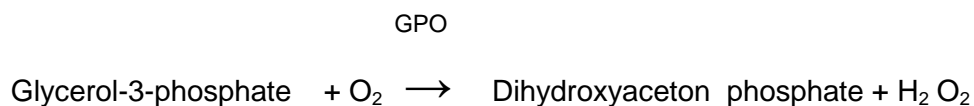
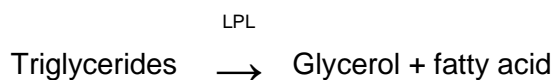
Child (desirable)	< 170 mg/dl
Adult (desirable)	<200 mg/dl

3.7.6 Determination of serum triglycerides

Serum triglycerides were determined by enzymatic colorimetric method for the quantitative determination of triglycerides 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.



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.

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

Calculation

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

Reference value

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

3.7.7 Determination of serum high density lipoprotein (HDL-C)

Serum HDL-C was determined by liquid HDL-C precipitant for the determination of HDL-C Cholesterol using Diasys Diagnostic Systems, Germany.

Principle

Chylomicrons, VLDL-C and LDL-C are 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	1.4 mmol/l
Phosphotungstic acid	8.6 mmol/l
Cholesterol standard	200 mg/dl

Assay procedure

1- Precipitation

- 200 µl of standard (sample or control) was 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 °C

Measurement: against reagent blank.

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

Calculation

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

Reference value

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

3.7.8 Determination of serum low density lipoproteins (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 using the empirical relationship of Friedewald.

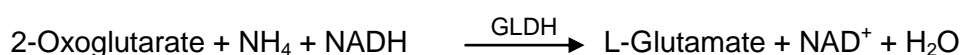
The Equation

$$\text{LDL-C} = \text{Total Cholesterol} - \text{HDL-C} - \text{TG}/5$$

3.7.9 Determination of serum urea

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

Principle



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 °C

Measurement: against distilled water.

- 10 µl of standard (sample or control) was added to 1 ml 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

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

$\Delta A \text{ standard}$

Reference value (PCLTG, 2005)

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

3. 7.10 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 → 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 °C

Measurement: against distilled water.

- 50 µl of standard (sample or control) was added to 1 ml 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

$$\Delta A = (A1 - A2) \text{ sample or standard}$$

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

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	0.6 - 1.2 mg/dl
F	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 (χ^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 ghrelin 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 were 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.

$$\text{Percent difference} = (| (V1 - V2) | / ((V1 + V2)/2)) \times 100.$$

- Microsoft Excel program version 11.0 was used for correlation graph plotting.

Chapter 4

Results

4.1 Sociodemographic characters of the study population

As indicated in Table 4.1, the study population included 80 controls (40 males and 40 females) and 80 cases (Diabetic patients: 40 males and 40 females). The mean ages of controls and cases were 49.8 ± 6.5 and 51.7 ± 7.0 years. The independent sample t- test showed no significant difference between mean ages of control and cases ($t=1.220$, $P=0.226$). Analysis of educational status of the study population showed that 22 (27.5%) control and 12 (15.0%) cases had university degree, 28 (35.0%) and 26 (32.5%) had finished secondary school, 18 (22.5%), 20 (25.0%) had finished preparatory school, and 12 (15.0%), and 22 (27.5%) had passed primary school only. The difference between various educational levels of controls and cases was not significant ($\chi^2=6.062$, $P=0.109$). The employed controls and cases were 48 (60%) and 40 (50%) whereas 32 (40%) controls and 40 (50%) cases were unemployed ($\chi^2=1.616$, $P=0.204$). Family history of the study population revealed that 27 (33.8%) controls and 44 (55.0%) cases have family history of diabetes whereas 53 (66.3%) controls and 36 (45.0%) cases have not. The difference between the two groups was significant ($\chi^2=7.318$, $P=0.007$), indicating that family history is a risk factor of diabetes. In addition, 13 (16.3%) controls were smokers compared to 9 (11.3%) cases ($\chi^2=0.843$, $P=0.359$).

Table 4.1. Sociodemographic characteristics of the study population

General characteristics	Controls (n=80)	Cases (n=80)	Statistical		P-value
	n (%)	n (%)	test		
Gender					
Male	40 (50.0)	40 (50.0)	χ^2	0.000	1.000
Female	40 (50.0)	40 (50.0)			
Age (Year)					
Mean±SD	49.8±6.5	51.7±7.0	T	1.220	0.226
Education					
University	22 (27.5)	12 (15.0)	χ^2	6.062	0.109
Secondary school	28 (35.0)	26 (32.5)			
Preparatory school	18 (22.5)	20 (25.0)			
Primary school	12 (15.0)	22 (27.5)			
Employment					
Employed	48 (60.0)	40 (50.0)	χ^2	1.616	0.204
Unemployed	32 (40.0)	40 (50.0)			
Family history					
Yes	27 (33.8)	44 (55.0)	χ^2	7.318	0.007
No	53 (66.3)	36 (45.0)			
Smoking					
Yes	13 (16.3)	9 (11.3)	χ^2	0.843	0.359
No	67 (83.7)	71 (88.7)			

P>0.05: not significant, P<0.05: significant.

4.2 Meal frequency per day of the study population

Table 4.2 and Figure 4.1 illustrate meal frequency/day of the study population. A total of 4 (5.0%), 40 (50.0%), 32 (40.0%) and 4 (5.0%) controls eat one, two, three, and four and more meals/day, respectively compared to 0 (0.0%), 16 (20.0 %), 50 (62.5%) and 14 (17.5%) cases. The difference between the two groups was significant with higher numbers of meals per day were eaten by cases ($\chi^2=19.721$ and $P=0.000$).

Table 4.2. Meal frequency/day of the study population

Meal frequency/day	Controls (n=80)	Cases (n=80)	χ^2	P-value
	n (%)	n (%)		
One	4 (5.0)	0 (0.0)	19.721	0.000*
Two	40 (50.0)	16 (20.0)		
Three	32 (40.0)	50 (62.5)		
Four and more	4 (5.0)	14 (17.5)		

*p-value of χ^2 (corrected) test. $P<0.05$: significant.

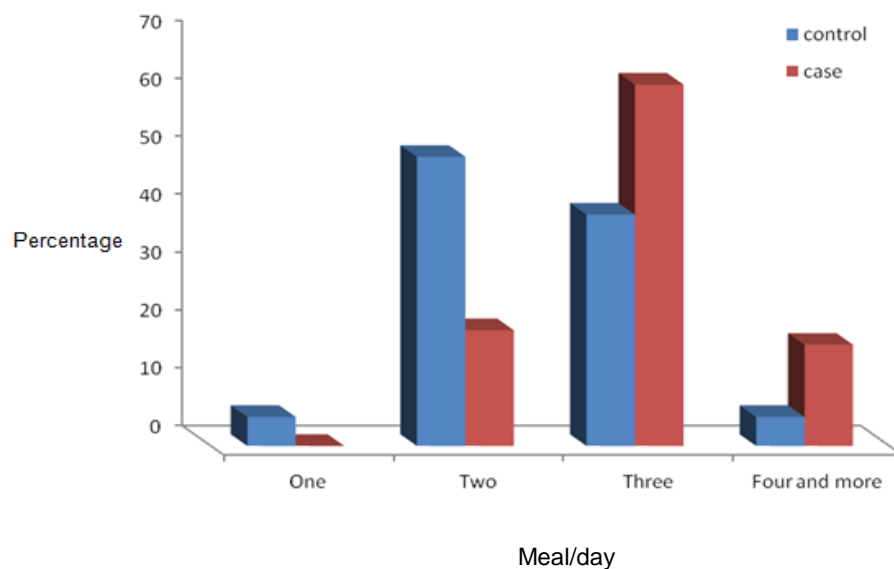


Figure 4.1. Meal frequency/day of the study population

4.3 Distribution of diabetic patients by the duration of the disease

Distribution of diabetic patients by the duration of the disease is shown in Table 4.3 and Figure 4. 2. Diabetic patients since less than 5 years were 40 (50.0%), whereas those with diabetic duration of 5-10 years were 14 (17.5%). The rest of the patients 26 (32.5%) had diabetes for more than 10 years.

Table 4.3. Distribution of diabetic patients (n=80) by the duration of the disease.

Duration of diabetes (Year)	No.	%
< 5	40	50.0
5-10	14	17.5
>10	26	32.5

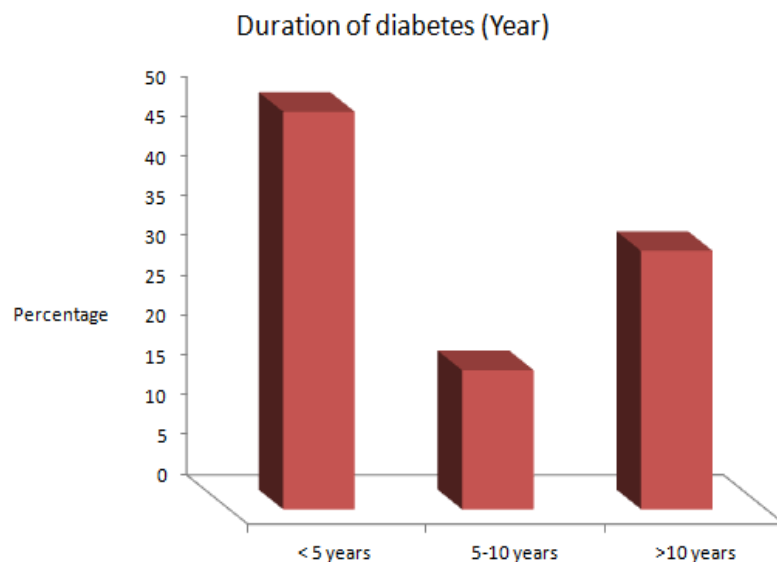


Figure 4.2. Distribution of diabetic patients by the duration of the disease.

4.4 Regular blood glucose testing among diabetic patients

Table 4.4 present the regular blood glucose testing among diabetic patients. Sixty six (82.5%) patients reported that they had regular blood glucose testing; 4 (6.1%), 22 (33.3%), and 40 (60.6%) had the test daily, weekly and monthly, respectively (Figure 4.3).

Table 4.4. Regular blood glucose testing among diabetic patients (n=80).

Variable	No.	%
Regular blood glucose testing		
Yes	66	82.5
No	14	17.5
If yes		
Daily	4	6.1
Weekly	22	33.3
Monthly	40	60.6

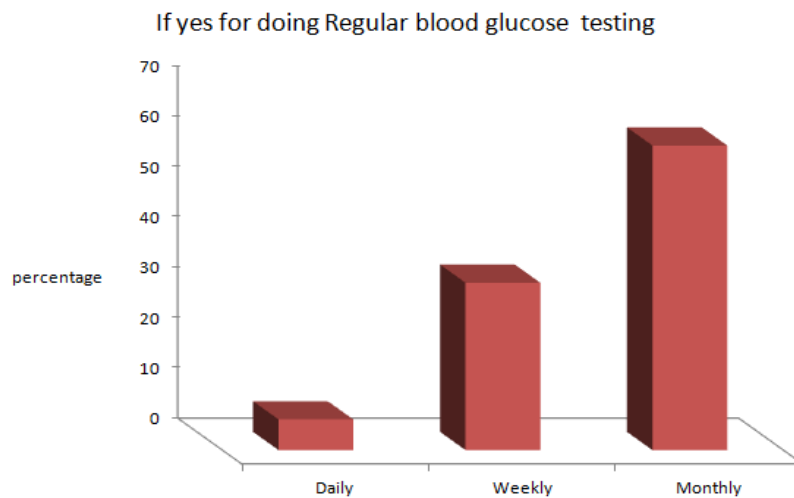


Figure 4.3. Regular blood glucose testing among diabetic patients

4.5 Self-reported complications among the study population

The main self-reported complications among the study population are pointed out in Table 4.5. The percentages of self-reported retinopathy, CVD and neuropathy were higher in diabetic patients compared to controls (45.0%, 15.0% and 42.5% vs. 22.5%, 2.5% and 20.0%, respectively), with statically significant differences of $\chi^2=9.057$ and $P=0.003$, $\chi^2_{(corrected)}=6.341$ and $P=0.012$, $\chi^2=9.425$ and $P=0.002$, respectively.

Table 4.5. The main self-reported complications among the study population.

Complication	Control (n=80) n (%)	Cases (n=80) n (%)	χ^2	P-Value
Retinopathy				
Yes	18 (22.5)	36 (45.0)	9.057	0.003
No	62 (78.5)	44 (55.0)		
CVD*				
Yes	2 (2.5)	12 (15.0)	6.341	0.012**
No	78 (97.5)	68 (85.0)		
Neuropathy				
Yes	16 (20.0)	34 (42.5)	9.425	0.002
No	64 (80.0)	46 (58.5)		

*CVD: Cardiovascular disease. **p-value of $\chi^2_{(corrected)}$ test. $P<0.05$: significant.

4.6 Self-reported complications in relation to duration of diabetes

Table 4.6 demonstrates self-reported complications among diabetic patients in relation to duration of the disease. In general the long the duration of diabetes, the higher percentage of self-reported complications among patients. This positive relationship was significant for retinopathy and neuropathy ($\chi^2=22.653$, $P=0.000$ and $\chi^2=6.621$, $P=0.036$, respectively).

Table 4.6. Self-reported complications among diabetic patients in relation to duration of the disease.

Complication	Duration of diabetes (Year)			χ^2	P-Value
	< 5 (n=40)	5-10 (n=14)	> 10 (n=26)		
	n (%)	n (%)	n (%)		
Retinopathy (n=36)	10 (25.0)	5 (35.7)	21 (80.7)	22.653	0.000
CVD* (n=12)	4 (10.0)	3 (21.4)	5 (19.2)	1.712	0.425**
Neuropathy (n=34)	11 (27.5)	6 (42.9)	17 (65.4)	6.621	0.036

*CVD: Cardiovascular diseases. **p-value of χ^2 (corrected) test. $P>0.05$: not significant, $P<0.05$: significant.

4.7 Anthropometric measurements of the study population

Anthropometric measurements of the study population are provided in Table 4.7. The mean weight of controls was 78.1 ± 7.6 kg compared to 84.1 ± 12.5 kg of cases. The weight difference was significant ($t=2.584$ and $P=0.012$) with % difference=7.4% higher in cases. There was no significant difference in the

mean height of cases compared to controls (1.70 ± 0.1 vs. 1.69 ± 0.09 m, % difference=0.53, $t=0.396$ and $P=0.693$). Therefore, BMI of cases was significantly higher than that of controls (29.5 ± 4.0 vs. 27.4 ± 3.3 , % difference=7.4, $t=2.514$ and $P=0.014$).

Table 4.7. Anthropometric measurements of the study population.

Anthropometric measurement	Control (n=80) mean± SD	Case (n=80) mean± SD	% difference	t	P-value
Weight (kg)* (min-max)	78.1±7.6 (59-90)	84.1±12.5 (60-120)	7.4	2.584	0.012
Height (m)** (min-max)	1.69.0±0.09 (1.50-1.85)	1.70±0.1 (1.53-1.96)	0.53	0.396	0.693
BMI*** (min-max)	27.4±3.3 (20.3-33.3)	29.5±4.0 (22.1-39.5)	7.4	2.514	0.014

*Kg: kilogram, ** m: meter. ***BMI: Body mass index: People with BMI=18.5–24.9 were considered to have normal weight, people with BMI=25.0–29.9 were classified overweight, people with BMI≥30.0 were considered obese (WHO, 2000). All values are expressed as mean±standard deviation (SD). $P>0.05$: not significant, $P<0.05$: significant.

4.8 Serum glucose level of the study population

Table 4.8 points out that the mean serum glucose level in cases was significantly higher than that in controls (187.4 ± 74.1 vs. 98.3 ± 17.0 , % difference=62.4, $t=6.467$ and $P=0.000$).

Table 4.8. Serum glucose level of the study population.

Parameter	Control (n=80) mean±SD	Case (n=80) mean±SD	% difference	t	P- value
Glucose (mg/dl) <i>(min-max)</i>	98.3±17.0 <i>(70-158)</i>	187.4±74.1 <i>(82-365)</i>	62.4	6.467	0.000

P<0.05: significant.

4.9 Serum insulin, leptin and ghrelin levels of the study population

Serum insulin, leptin and ghrelin levels of the study population are shown in Table 4.9. The average levels of insulin and leptin were found to be higher in cases (28.8±23.9 µU/ml and 7.1±2.7 ng/ml, respectively) compared to controls (18.8±13.5 µU/ml and 5.9±2.2 ng/ml, respectively) with % differences of 42.0 and 18.5%, respectively). This increase was statically significant for insulin (t=2.285, P=0.025). In contrast, serum ghrelin level was lower in cases than in controls (1189±580 vs.1531±822 pg/ml, % difference=25.1). This change was significant (t=2.111, P=0.038).

Table 4.9. Serum insulin, leptin and ghrelin levels of the study population.

Parameter	Control (n=80) mean±SD	Case (n=80) mean±SD	% difference	t	P-value
Insulin(µU/ml) <i>(min-max)</i>	18.8±13.5 <i>(2.9-61.1)</i>	28.8±23.9 <i>(4.9-103.4)</i>	42.0	2.285	0.025
Leptin (ng/ml) <i>(min-max)</i>	5.9±2.2 <i>(3.8-10.4)</i>	7.1±2.7 <i>(5.1-13.3)</i>	18.5	1.737	0.081
ghrelin (pg/ml) <i>(min-max)</i>	1531±822 <i>(833-6244)</i>	1189±580 <i>(692-3850)</i>	25.1	2.111	0.038

P>0.05: not significant, P<0.05: significant.

4.10 lipid profile of the Study population

Table 4.10 demonstrates lipid profile including cholesterol, triglycerides, low density lipoprotein cholesterol (LDL-C) and high density lipoprotein cholesterol (HDL-C) of the study population. The average levels of cholesterol, triglycerides and LDL-C were found to be higher in cases (195.2±44.6, 212.5±78.9 and 115.4±45.5 mg/dl, respectively) compared to controls (183.5±31.5, 143.2±50.4 and 111.3±26.7 mg/dl, respectively) with % differences of 6.2, 39.0 and 3.6%, respectively). This increase was statically significant only for triglycerides (t=4.651, and P=0.000). In contrast, HDL-C was lower in cases than in controls (41.0±7.9 vs. 42.2±6.4 mg/dl, % difference=2.9). However, this change was not significant (t=0.745, P=0.459).

Table 4.10. Lipid profile of the study population.

Lipid profile (mg/dl)	Control (n=80) mean±SD	Case (n=80) mean±SD	% difference	t	P- value
Cholesterol <i>(min-max)</i>	183.5±31.5 <i>(127-256)</i>	195.2±44.6 <i>(121-304)</i>	6.2	1.352	0.180
Triglycerides <i>(min-max)</i>	143.2±50.4 <i>(42-239)</i>	212.5±78.9 <i>(90-406)</i>	39.0	4.651	0.000
LDL-C * <i>(min-max)</i>	111.3±26.7 <i>(63-118)</i>	115.4±45.5 <i>(40-227)</i>	3.6	0.491	0.625
HDL-C ** <i>(min-max)</i>	42.2±6.4 <i>(32-63)</i>	41.0±7.9 <i>(29-68)</i>	2.9	0.745	0.459

*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.

4.11 Serum urea and creatinine concentrations of the study population

As illustrated in Table 4.11, serum urea concentration is higher in cases than in controls (33.5 ± 11.0 vs. 28.3 ± 13.3 , % difference=16.8%, $t=1.916$ and $P=0.059$). Also, no significant change was found for creatinine concentrations between cases and controls (0.81 ± 0.20 vs. 0.89 ± 0.24 mg/dl, % difference=9.4, $t=1.574$ and $P=0.119$).

Table 4.11. Serum urea and creatinine concentrations of the study population.

Parameter (mg/dl)	Controls (n=80) mean \pm SD	Cases (n=80) mean \pm SD	% difference	t	P- value
Urea <i>Range (min –max)</i>	28.3 ± 13.3 (10-60)	33.5 ± 11.0 (15-65)	16.8	1.916	0.059
Creatinine <i>Range (min –max)</i>	0.89 ± 0.24 (0.5-1.6)	0.81 ± 0.20 (0.5-1.3)	9.4	1.574	0.119

$P > 0.05$: not significant

4.12 Body mass index in relation to insulin, leptin, triglycerides and ghrelin of the target population

Table 4.12 shows the correlations of body mass index with insulin, leptin, ghrelin and triglycerides of the target population. The Pearson correlation test showed positive correlation of BMI with insulin, leptin and triglycerides of the target population (Figures 4.4, 4.5 and 4.6, $r=0.183$, $r=0.207$ and $r=0.228$, respectively). However, this positive correlation was significant only for triglycerides ($P=0.044$). On the other hand, ghrelin showed negative not significant correlation with BMI (Figure 4.7, $r=-0.149$ and $P=0.192$).

Table 4.12. Body mass index (BMI) in relation to insulin, leptin, triglycerides and ghrelin of the target population.

Parameter	BMI (kg/m ²)	
	Pearson correlation (r)	P-value
Insulin (μU/ml)	0.183	0.104
Leptin (ng/ml)	0.207	0.063
Triglycerides(mg/dl)	0.228	0.044
Ghrelin (pg/ml)	-0.149	0.192

P>0.05: not significant, P<0.05: significant.

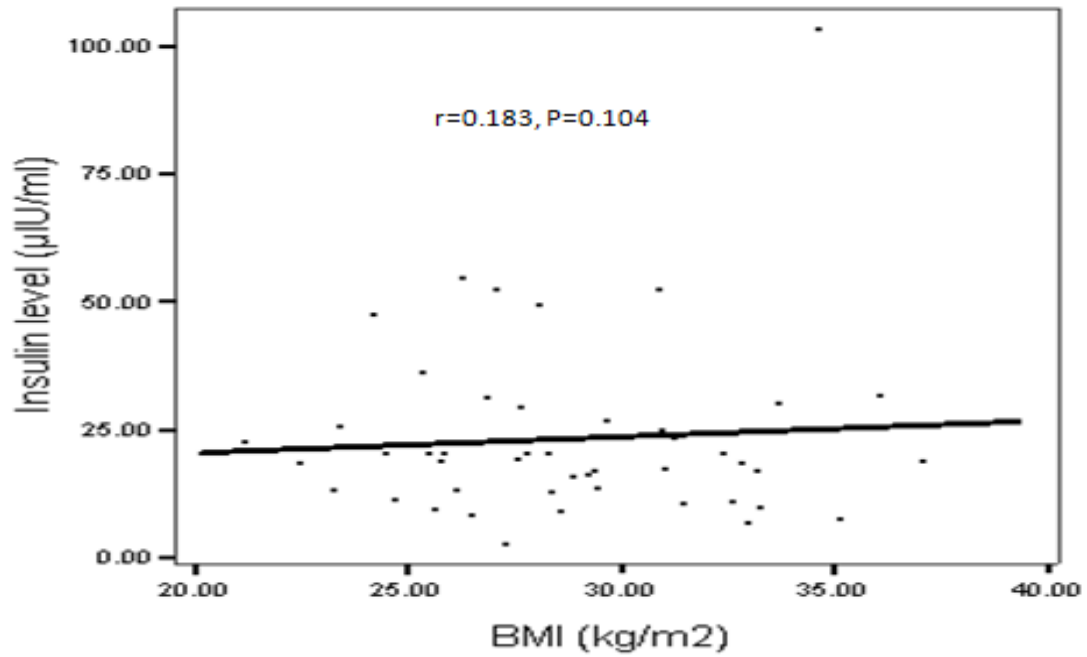


Figure 4.4. Correlation between body mass index (BMI) with insulin hormone level of the target population.

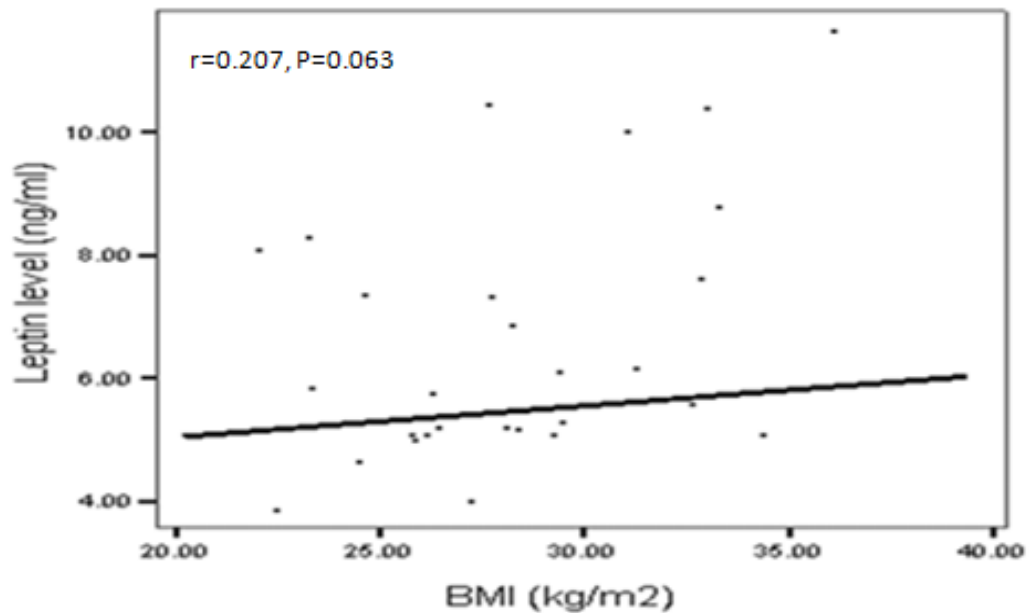


Figure 4. 5. Correlation between body mass index (BMI) with leptin hormone level of the target population.

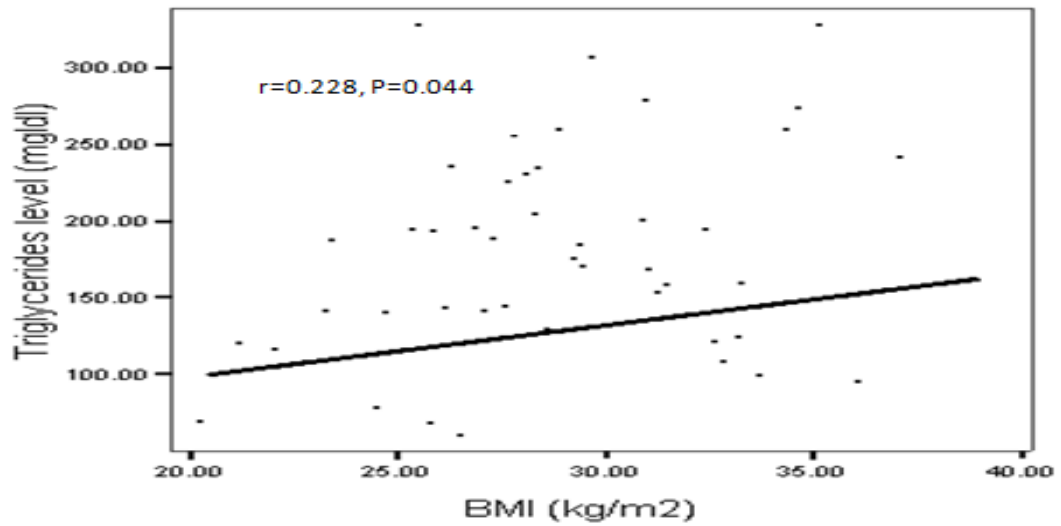


Figure 4. 6. Correlation between body mass index (BMI) and triglyceride level of the target population.

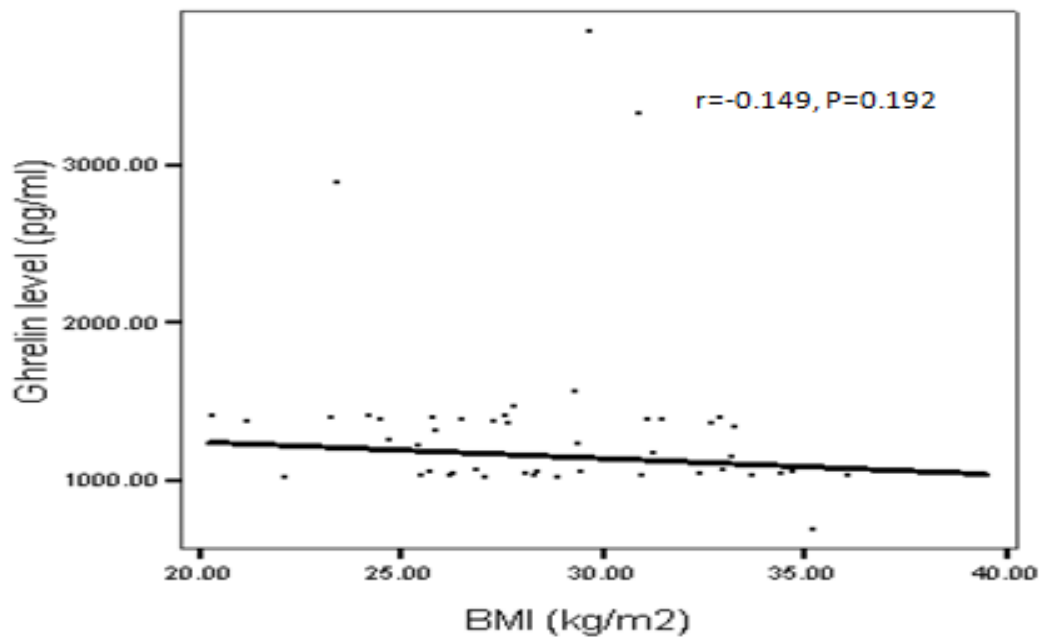


Figure 4. 7. Correlation between body mass index (BMI) with ghrelin hormone level of the target population.

4.13 Insulin levels in relation to glucose, triglycerides, leptin and ghrelin levels of the target population

Insulin levels in relation to glucose, triglycerides, leptin and ghrelin levels of the target population is presented in Table 4.13. The Pearson correlation test showed positive correlations of insulin with glucose, triglycerides and leptin (Figures 4.8, 4.9 and 4.10, $r=0.286$, $r=0.224$ and $r=0.187$, respectively). However, this positive correlation was significant for glucose and triglycerides ($P=0.011$, $P=0.049$, respectively). On the other hand, ghrelin showed negative not significant correlation with insulin (Figure 4.11, $r=-0.213$ and $P=0.057$).

Table 4.13. Insulin levels in relation to glucose, triglycerides, leptin and ghrelin levels of the target population

Parameter	Insulin ($\mu\text{U/ml}$)	
	Pearson correlation (r)	P-value
Glucose (mg/dl)	0.286	0.011
Triglycerides (mg/dl)	0.224	0.049
Leptin (ng/ml)	0.187	0.098
Ghrelin (pg/ml)	-0.213	0.057

$P>0.05$: not significant , $P<0.05$: significant.

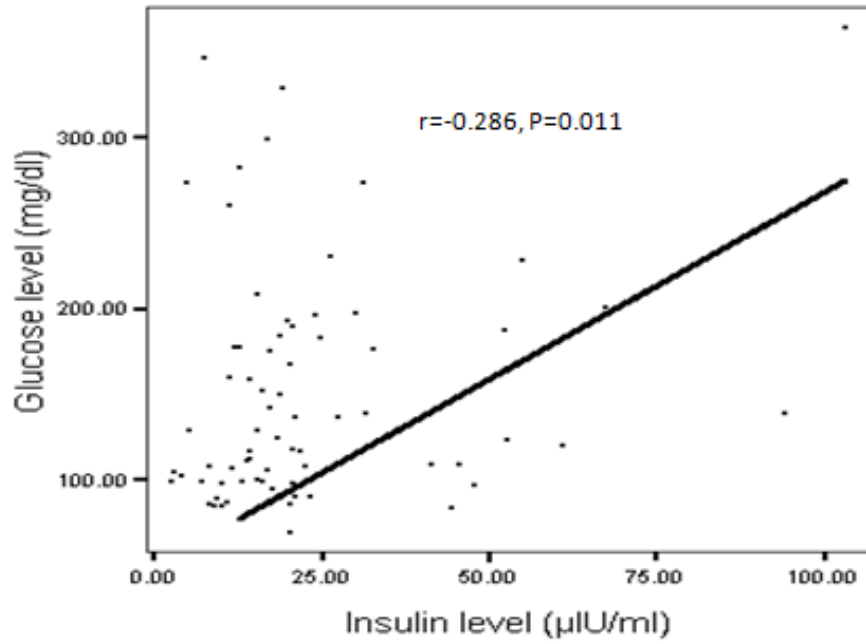


Figure 4.8. Correlation between insulin hormone level with glucose level of the target population.

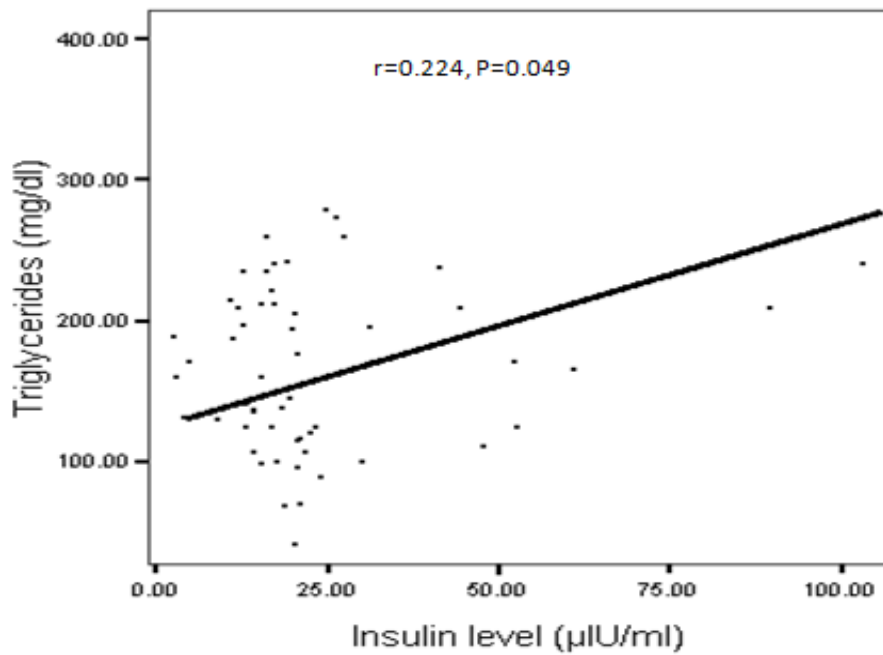


Figure 4.9. Correlation between insulin hormone level with triglyceride level of the target population.

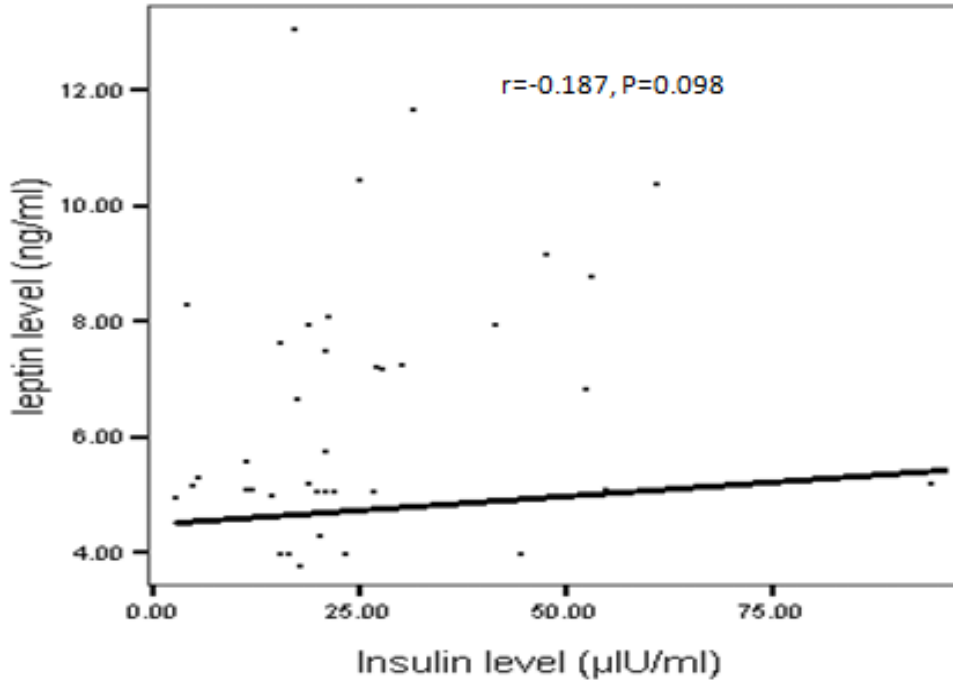


Figure 4.10. Correlation between insulin hormone level with leptin level of the target population.

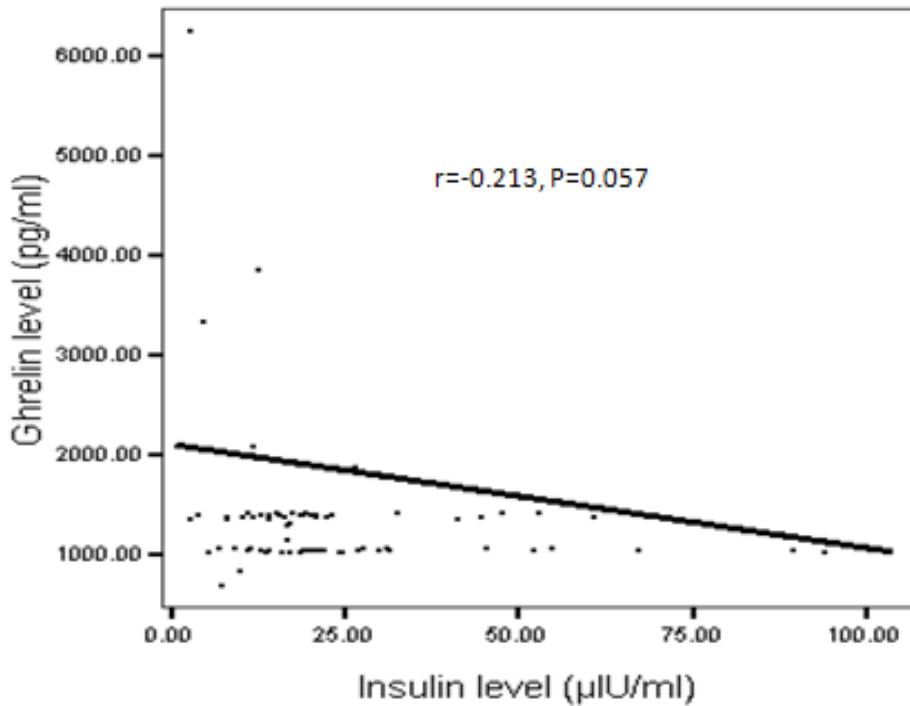


Figure 4.11. Correlation between insulin hormone level with ghrelin level of the target population.

Chapter 5

Discussion

Diabetes mellitus is prevalent globally as well as in the Gaza Strip. The total number of people with diabetes worldwide is projected to rise from 171 million in 2000 to 366 million in 2030 (Wild et al., 2004). Although extensive research has been conducted globally on diabetes mellitus, data on the disease is limited in the Gaza strip. Just recently, few studies have been carried out on type 2 diabetic patients in the Gaza Strip (Shubair 2008, Abu Hilal 2009 and Yassin et al., 2011). In addition, only two studies speculate the status of leptin hormone in type 2 diabetic patients in the Gaza Strip (Altawil, 2009 and Abu Mustafa, 2011). However, the role of ghrelin in such studies was not investigated. Therefore, the present study is the first to assess ghrelin as well as leptin and insulin levels in type 2 diabetic patients in the Gaza Strip. Assessment of such tri-hormonal interplay in diabetes could a) Provide us with a clearer picture on the role of such hormones in the disease and b) enable us to develop treatment strategy of the disease.

5.1 Sociodemographic characters of the study population

The target population was 80 type 2 diabetic patients selected from different hospitals and diabetic clinics which are representative for diabetic patients in the Gaza Strip. The mean ages of controls and cases (type 2 diabetic patients) were 49.8 ± 6.5 and 51.7 ± 7.0 years, respectively. Rodger, (1991) and Umpierrez et al. (2006) reported that type 2 diabetes mellitus usually develops after age 40 years. Diabetes was found to be associated with family history. This finding is in agreement with other studies who reported that family history is a risk factor of diabetes (Annis et al., 2002; Harrison et al., 2003; Pijl et al., 2009 and Yassin et al., 2011).

5.2 Meal frequency/day of the study population

The present data showed that the number of cases who ate 3 and ≥ 4 meals/day were about twice higher than controls. Similar result was obtained by Dwivedi and Shukla (2008). This coincides with the fact that diabetic patients express feeling of dietary deprivation (Yannakoulia. 2006). Therefore, health professionals need to take into account these difficulty in their collaboration with the patients in order to improve the effectiveness of nutrition intervention.

5.3. Distribution of diabetic patients by disease duration and regular blood glucose testing

The result that 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 (Canadian Diabetes Association Clinical Practice Guidelines Expert Committee, 2003). This was supported by the present finding that the prevalence of diabetic complications among cases was significantly higher than that among controls. Although self blood glucose monitoring is important in the management of people living with diabetes (Benjamin, 2002 and porksen et al., 2007) inadequate practice of self blood glucose monitoring was reported by our patients. Small number of patients (6.1%) had blood glucose test once daily whereas the majority of them (60.6%) had blood glucose test once/month. Such finding is in agreement with that of Aghamollaei et al. (2003) and Unachukwu et al. (2011) who reported that only 6.3% and 6.7% of Iranian and Nigerian diabetic patients had glucose monitoring once daily. The present result necessitates launch of educational program on the importance of self blood glucose monitoring in the management of diabetes mellitus.

5.4 Self-reported complications among the target population.

As indicated by the present results, the most common self-reported complications among diabetic patients were retinopathy, neuropathy and cardiovascular disease. The prevalence of such symptoms was positively associated with the progress of the disease i.e. the longer the duration of diabetes, 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; The National Eye Institute, 2006; Altawil, 2009 and Roaeid and kadiki, 2011). In addition, it was concluded that the duration of diabetes plays a central role in the development of diabetic complications mainly diabetic retinopathy and diabetic neuropathy (Subhan et al., 2009; Niazi et al., 2010 and Jambart et al., 2011). Diabetic neuropathy also appears to be more common in people who have had problems controlling their blood glucose levels.

5.5 Anthropometric measurements of the study population

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/m² or higher. This definition distinguishes obesity from being pre-obese or overweight, which is classified as a BMI of 25 kg/m² but less than 30 kg/m² (WHO, 2000). In the present study, BMI of cases was significantly higher than that of controls. In other words, obese individuals are at higher risk for diabetes (Boffetta et al., 2011). The literature supported the present results in that obesity is a major risk factor for chronic diseases including diabetes (El-Hazmi et al., 1997, Marshall, 2006; Slynkova et al., 2006 and Yassin et al., 2011). It was reported that about 55% of type 2 diabetics are obese (Eberhart et al., 2004). Chronic obesity leads to increased insulin

resistance that can develop into diabetes, most likely because adipose tissue is a source of several chemical signals to other tissues. This was supported by the observed result that insulin level was significantly increased in diabetic patients compared to controls. Other research showed that type 2 diabetes causes obesity as an effect of the changes in metabolism and other deranged cell behavior attendant on insulin resistance (Camastra, 1999; Bavenholm et al., 2003 and Micic and Cvijocic 2008). In addition, metabolic changes in obesity is characterized by elevation of triglycerides (Hwang et al., 2006) which was observed in the present study, and reinforced by the positive significant correlation of BMI with triglycerides levels.

5.6 Glucose and insulin levels of the study population

Serum glucose and insulin levels were significantly higher in diabetic patients than controls, confirming the development of insulin resistance in type 2 diabetic patients. This concomitant elevation was manifested in the recorded positive significant correlation between insulin and glucose. Insulin resistance is a feature of type 2 diabetic patients. Thevenod (2008) showed that insulin resistance precedes the development of hyperglycemia in subjects that eventually develop type 2 diabetic patients. In addition, it was concluded that insulin resistance could significantly predict development of diabetic nephropathy in type 2 diabetic patients (Hsu et al., 2011). This may be explained in part by the finding that most of patients did not compliance with diet regime and displayed poor monitoring of self blood glucose level. High-fat diet was found to increase risk of type 2 diabetes (Lovejoy, 2002). In a previous study to assess non-compliance among 216 type 2 diabetic patients in Gaza Strip, Zakout (2006) reported that three quarters of the study population had poor glycemic control and half of them were not compliance with the medication regime.

5.7 Serum insulin, leptin and ghrelin levels of the study population

Data presented in this study revealed that the average levels of insulin and leptin were higher in cases compared to controls whereas ghrelin level was lower in cases than in controls. Such finding is coincided with that obtained by Erdmann et al. (2005) and Abu Mustafa (2011) who found higher levels of insulin and leptin in diabetic patients compared to non diabetics. On the other hand, lower ghrelin level was registered in diabetic patients (pöykko et al., 2003; Erdmann et al., 2005; pulkkinen et al., 2010 and Al-Hakeim and Ali 2012). The Pearson correlation test in the present study showed positive correlations of insulin with leptin and negative with ghrelin. Similar results were obtained by Anderwald et al. (2003), Mohiti and Qjug (2005) and Turki et al. (2012). Despite the evidence favoring a role for ghrelin in long-term energy balance, it is unclear how a hormone secreted by the gastrointestinal tract can sense and respond to changes in body energy stores. This raises the possibility that a circulating adiposity signal, such as leptin or insulin, may regulate the production of ghrelin. Several lines of evidence suggest that leptin does not sub-serve this function (Cummings and Foster, 2002). Insulin, a hormone that circulates in high concentrations in individuals who are insulin resistant, typically in the setting of obesity (Abbasi et al., 2002), is an attractive candidate for this signal. Short-term insulin infusions have been shown to decrease ghrelin concentrations in humans (Saad et al., 2002; Mohling et al., 2002, Lucidi et al., 2002; Flanagan et al., 2003 and Anderwald et al., 2003), consistent with a possible causal role of insulin in suppressing ghrelin secretion. The data implicating chronic hyperinsulinemia and/or insulin resistance in the negative regulation of ghrelin are suggestive, but inconsistent and need further investigation. Nevertheless, this tri-hormonal interplay scenario could play a role in metabolic alterations and appetite behavior in type 2 diabetic patients. Therefore, assessment of these three fat hormones interactions could constitute a promising therapy of diabetes mellitus type 2.

5.8 lipid profile of the Study population

The present results demonstrate significant increase in triglyceride levels of cases compared to controls. Such increase was not significant for cholesterol and LDL-C. Also, the observed decrease in HDL-C in cases was not significant. 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. These three substances with protein may be discharged into blood in the form of lipoproteins (Taskinen, 1992 and Jaworski et al., 2007). The positive correlation between insulin and triglycerides recorded in the present study do support the above results. Katsuki et al. (2003) and Hsu et al. (2011) reported positive correlation between triglycerides and insulin resistance.

5.9 Urea and creatinine concentrations of the study population

Data provided in the present study showed a border line significant increase in urea in diabetic patients compared to controls. However creatinine exhibited no significant change. Similar results are documented by Altawil (2009). 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 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

1. The study population included 80 controls (40 males and 40 females) and 80 cases (Diabetic patients: 40 males and 40 females). The mean ages of controls and cases were 49.8 ± 6.5 and 51.7 ± 7.0 years.
2. Family history was found to be associated with diabetes. The number of diabetic patients who ate 3 and ≥ 4 meals/day were about twice higher than controls.
3. Although half of patients had diabetes since less than 5 years, the majority of them had blood glucose test once/month.
4. The most common self-reported complications among diabetic patients were retinopathy, neuropathy and cardiovascular disease. The prevalence of such complications was positively associated with the duration of diabetes.
5. Body mass index of cases was significantly higher than that of controls.
6. Serum glucose and insulin levels were significantly higher in diabetic patients than controls.
7. Serum leptin showed no significant increase in diabetic patients compared to controls whereas ghrelin was significantly lower in patients.
8. The mean level of triglyceride was significantly higher in patients compared to controls. In addition, urea showed no significant increase in patients.
9. The Pearson correlation test showed positive significant correlation of BMI with triglycerides.

10. Insulin showed positive correlations with glucose, triglycerides and leptin. However, this positive correlation was significant for glucose and triglycerides. On the other hand, ghrelin showed negative not significant correlation with insulin confirming the presence of the tri-hormonal interplay in diabetes mellitus.

6.2 Recommendation

1. Enhancement of awareness of diabetic people in terms of the importance of diet control and regular monitoring of blood glucose level.
2. Weight control among obese individuals particularly those with family history of diabetes are highly recommended.
3. Regular visits to optical and neurological clinics to take early steps to avoid and manage diabetic complications concerning diabetic retinopathy, neuropathy and CV disease.
4. Further researches are highly recommended on the mechanism underlying the tri-hormonal interplay particularly the inhibitory action of ghrelin on insulin, which could be of diagnostic and therapeutic values in the course of diabetes mellitus.

Chapter 7

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Annex 1

التاريخ: 5/9/2011

Name: **Fatma Abu Snayma**

الاسم: فاطمة أبو سنيمة

I would like to inform you that the committee has discussed your application about:

نفيدكم علماً بأن اللجنة قد ناقشت مقترح دراستكم حول:-

“Ghrelin,leptin and insulin in type 2 diabetic patients in Gaza Strip”.

In its meeting on September 2011 and decided the Following:-

و ذلك في جلستها المنعقدة لشهر 9 2011

To approve the above mention research study.

و قد قررت ما يلي:-

الموافقة على البحث المذكور عاليه.



Signature

توقيع

Member

Member

Chairperson


عضو

عضو
محمد

Conditions:-

- ❖ Valid for 2 years from the date of approval to start.
- ❖ It is necessary to notify the committee in any change in the admitted study protocol.
- ❖ The committee appreciate receiving one copy of your final research when it is completed.

The Palestinian National Authority
Ministry of Health
Directorate General of Human Resources Development



السلطة الوطنية الفلسطينية
وزارة الصحة
الإدارة العامة لتنمية القوى البشرية

الرقم:

الأخ / د. مدحت محيسن
مدير عام المستشفيات
تحية طبية وبعد،،،

الموضوع/ تسهيل مهمة باحث

بخصوص الموضوع أعلاه، يرجى تسهيل مهمة الباحثة / فاطمة محمد
ابوسنينة والمتحققة ببرنامج ماجستير العلوم الحياتية - كلية العلوم - الجامعة
الإسلامية في إجراء بحث بعنوان :-

**"Ghrelin, Leptin and Insulin Type 2 Diabetes Patients
in Gaza Strip "**

حيث ستقوم الباحثة بتعبئة استبانته وأخذ جزء من عينات دم سحبت لأغراض
تشخيصية من مرضى السكر النوع الثاني من المراجعين لمستشفيات وزارة
الصحة .

كما نأمل توجيهاتكم لذوي الاختصاص بعدم السماح للباحث بأخذ عينات الدم إلا بعد الحصول على
الموافقة المستبصرة من المشاركين في البحث ، و بما لا يتعارض مع مصلحة العمل وضمن
أخلاقيات البحث العلمي، و دون تحمل الوزارة أي أعباء.

وتفضلوا بقبول التحية والتقدير،،،

مرفق طيه/

1. نموذج طلب تسهيل مهمة باحث
2. نموذج الموافقة المستبصرة

صورة/
- صاحبة العلاقة

الإدارة العامة للمستشفيات
صادر
رقم: 16573
التاريخ: 21/8

وزارة الصحة
تنمية القوى البشرية
مصادره: 11/1632
التاريخ: 17/8

د. ناصر رأفت أبو شعبان
مدير عام تنمية القوى البشرية

الإدارة العامة للمستشفيات
وارد
رقم: 18336
التاريخ: 17/8

Gaza Tel / 08-2827298 Fax / 08-2868109 Email / gdhrd@moh.gov.ps

Questionnaire

I am researcher/ **Fatma Abu Snayema** (Master student at the Islamic University of Gaza) needs your help to fill in this questionnaire in order to conduct research on the detection of certain hormones associated with disease morbidity in patients with diabetes mellitus type II in the Gaza Strip.

Personal data

1. Name: _____(optional) Phone Number: _____
2. Gender: Male Female
3. Age: _____.
4. Education: University Secondary school Preparatory school Primary school Illiterate
5. Employment : Yes No
6. Do you have one of your family members with type 2 diabetes?

 Yes No
7. Do you smoke? Yes No
8. Meal frequency/day One Two Three Four and more

Clinical data

1. Duration of diabetes (Years): _____

2. Regular blood glucose test Yes No

If yes Daily Weekly Monthly

3. Self-reported complications:

Retinopathy Yes No

Cardiovascular disease Yes No

Neuropathy Yes No

Agreement: I agree to complete this questionnaire concerning my health statement.

Signature:

Date:.....

Thank you for your help

Researcher/ Fatma Abu Snayma

استبانة

أنا الباحثة/ فاطمة أبو سنيمة (طالبة ماجستير بالجامعة الإسلامية) أرجو المساعدة في تعبئة هذا الاستبيان من أجل عمل بحث حول الكشف عن بعض الهرمونات المصاحبة لمرض الاعتلال السكري عند مرضى السكري من النوع الثاني في قطاع غزة .

معلومات شخصية

1. الاسم: _____ (اختياري) رقم الهاتف: _____
2. الجنس ذكر انثى
3. العمر: _____
4. التعليم: جامعي ثانوى اعدادى ابتدائى غير متعلم
5. موظف: نعم لا
6. هل يعانى احد أفراد عائلتك من مرض السكري من النوع الثاني؟
 نعم لا
7. هل تدخن؟ نعم لا
8. عدد الوجبات فى اليوم واحدة اثنتين ثلاثة اربعة فما فوق

بيانات طبية

1. مدة مرض السكرى(سنوات) : _____ .
2. هل تفحص السكر في الدم لديك بشكل دوري نعم لا
اذا كانت الاجابة نعم يومي اسبوعي شهري
3. هل تعاني اى من مضاعفات السكر:
مشاكل في العيون نعم لا
أمراض القلب والشرابين نعم لا
مشاكل في الأعصاب نعم لا

أنا موافق على تعبئة هذا الاستبيان الذي يتعلق بصحتي.

التوقيع:

التاريخ:

شكرا لكم على حسن تعاونكم

الباحثة/ فاطمة ابو سنينة