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Ghrelin Status and Lipid Profile in Obese Women From Southern Gaza Strip

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(قل هل يستوي الذين يعلمون والذين لا يعلمون إنما يتذكّر أولو الألباب)

[الزمر9]

Declaration

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

Signature	**Name**	**Date**
Aliaa	**Aliaa H. Al-smairi**	**January,2012**

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Dedication

I dedicate this work to:

My beloved parents who have always supporting me

My husband who helped me to accomplish this thesis

The dearest to me; my son Mohammed

All researchers who are working to improve the quality of life

Ahlaa H Al-smairi

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Ghrelin Status and Lipid Profile in Obese Women from Southern Gaza Strip

Abstract

Background: Ghrelin is a novel hormone consisting of 28 amino acids, which causes weight gain by increasing appetite, food intake (hunger hormone), decreases fat utilization and increases fat accumulation. Therefore, assessment the status of ghrelin hormone in obesity could constitute a promising therapy of obesity.

Objective: To asses ghrelin status and some biochemical parameters in obese women from Southern Gaza Strip.

Materials and Methods: This case-control study comprised 94 obese women (mean body mass index, BMI=35.1±4.6 Kg/m^2) and 94 healthy normal weight women (mean BMI=22.6±1.8 Kg/m^2). Questionnaire interview was applied. Serum ghrelin, cholesterol, triglycerides, high density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LDL-C) were determined. Data were analyzed using SPSS version 18.0.

Results: The mean ages of controls and cases were 28.7±6.2 and 29.6±6.3 years, respectively. Obesity was more frequent among married, less educated, unemployed women as well as among women with family history of obesity compared to single, highly educated, employed women and women without family history of obesity (P=0.000). Drinking soft drink, not doing exercise and sedentary life style were risk factors of obesity (P=0.039, P=0.037 and P=0.002). The mean level of serum ghrelin was significantly decreased in cases compared to controls (1060±646.8 pg/ml vs. 1473.4±690.7 pg/ml, % difference=32.6 and P=0.005). The average levels of triglycerides and LDL-C were found to be significantly higher in cases (141.8±36.5 and 89.5±32.1 mg/dl) compared to controls (114.8±48.2 and 75.9±31.4mg/dl) with % differences of 21.0 and 16.4% and P=0.004, P=0.049, respectively. When related to sociodemographic characters of the study population, ghrelin showed lower levels in married, less educated, unemployed women as well as in women with family history of obesity

(P>0.05). Similar trend in ghrelin levels were found in women who did not do exercise and drunk soft drink. Life style revealed that the less active life style, the lower the level of ghrelin. This positive relationship was significant (P=0.015). The Pearson correlation test showed negative correlations between ghrelin levels and cholesterol, triglyceride and LDL-C levels and a positive correlation with HDL-C (P>0.05). Concerning BMI, there was a negative signifigant correlation between BMI and ghrelin levels (r=-0.279, P=0.009).

Conclusions: Serum ghrelin was significantly lower in obese women compared to normal weight women. The less active life style, the lower the level of ghrelin. There was a negative signifigant correlation between BMI and ghrelin levels.

Keywords: Ghrelin, lipid profile, obesity, Gaza strip.

هرمون الجريلين وصورة الدهون في النساء البدينات في جنوب قطاع غزة

ملخص الدراسة

مقدمة: الجريلين هرمون مكتشف حديثا يتكون من 28 حمضاً أمينياً يسبب زيادة الوزن عن طريق زيادة الشهية، زيادة الأكل (يسمى هرمون الجوع)، التقليل من أستهلاك دهون الجسم كمصدر للطاقة وزيادة تراكمها. بالتالي فأن تحديد مستوى هرمون الجريلين في حالات السمنة قد يشكل خطوة نحوعلاج واعد للسمنة.

الهدف: تهدف الدراسة الي قياس مستوى هرمون الجريلين و بعض المعايير البيوكميائية في النساء البدينات في جنوب قطاع غزة.

الطرق والادوات: منهج الدراسة (مجموعة مرضية- مجموعة ضابطة)، المجموعة المرضية تحتوي على 94 إمرأة بدينة (متوسط مؤشر كتلة الجسم =35.1±4.6 كجم/م^2) و المجموعة الضابطة تحتوي على 94 إمرأة ذات وزن طبيعي (متوسط مؤشر كتلة الجسم =22.6±1.8 كجم/م^2). البيانات المستخدمة في الدراسة تم الحصول عليها من خلال المقابلة المباشرة مع المرضى. تم قياس مستوى هرمون الجريلين، الكوليستيرول، الدهون الثلاثية، البروتين الدهني عالي الكثافة والبروتين الدهني منخفض الكثافة. تم تحليل البيانات والنتائج التي تم الحصول عليها باستخدام البرنامج الإحصائي SPSS-18.0.

النتائج: متوسط العمر للمجموعة الضابطة هو28.7±6.2 سنة ومتوسط العمر للمجموعة المرضية هو 29.6± 6.3 سنة. أظهرت النتائج أن السمنة كانت أكثر أنتشارا في النساء المتزوجات، الأقل تعليما، غير العاملات و ذوات تاريخ عائلي للسمنة بالمقارنة مع النساء غير المتزوجات، ذوات تعليم عالي،عاملات و بلا تاريخ عائلي للسمنة و كانت العلاقات ذات دلالة أحصائية. يعد شرب المشروبات الغازية و عدم ممارسة التمارين الرياضية و النمط الخامل للحياة كلها عوامل خطورة للأصابة بالسمنة بدلالة أحصائية. وقد أظهرت النتائج أن متوسط مستوي هرمون الجريلين كان أقل في المجموعة المرضية مقارنة بالمجموعة الضابطة وقد كانت النتائج ذات دلالة إحصائية . كانت نتيجة متوسط مستوي كل من الدهون الثلاثية والبروتين الدهني منخفض الكثافة أعلى بدلالة احصائية في المجموعة المرضية منها في المجموعة الضابطة. مستوى هرمون الجريلين كان أقل في النساء المتزوجات، الأقل تعليما، غير العاملات و ذات تاريخ عائلي للسمنة و العلاقة لم تكن ذات دلالة أحصائية. و بالمثل كان مستوى هرمون الجريلن لدى من لا يمارسن التمارين الرياضية و يتناولن المشروبات الغازية. و اظهرت النتائج وجود علاقة طردية ذات دلالة احصائية بين نمط الحياة و مستوى هرمون الجريلين حيث كلما قل نشاط نمط الحياة كلما قل مستوى هرمون الجريلين. و قد أظهرت النتائج وجود علاقة عكسية بين مستوى هرمون الجريلين و الكولسترول، الدهون الثلاثية والبروتين الدهني منخفض الكثافة و علاقة طردية مع البروتين الدهني عالي الكثافة والعلاقة لم تكن ذات دلالة أحصائية. فيما يخص مؤشر كتلة الجسم وجدت علاقة عكسية ذات دلالة احصائية بين هرمون الجريلين و مؤشر كتلة الجسم.

الاستنتاجات: مستوي هرمون الجريلين أقل بدلالة أحصائية في النساء البدينات مقارنة مع النساء ذوات الوزن الطبيعي.كلما قل نشاط نمط الحياة كلما قل مستوى هرمون الجريلين. توجد علاقة عكسية ذات دلالة احصائية بين هرمون الجريلين و مؤشر كتلة الجسم.

الكلمات المفتاحية: جريلين،الدهون، السمنة، قطاع غزة.

Chapter 1

Introduction

1.1 Overview

Obesity is a complex, multifactorial disease that develops from the interaction between genotype and the environment. It is characterized by an excess of adipose tissue (Kopelman, 2000 and Kantachuvessiri, 2005). The most commonly used measurement for determining obesity is the body mass index (BMI), which is calculated as the weight (kg)/height (m^2) (World Health Organization, WHO, 2000). Obesity can lead to serious medical, social, physical and psychological problems (da Mota and Zanesco, 2007).

The prevalence of obesity has been increased worldwide. The WHO estimates that at least 400 million adults (9.8% of world population) are obese, with higher rates among women than men (WHO, 2009). The rate of obesity also increases with age at least up to 50 or 60 years old and severe obesity in the United States, Australia, and Canada is increasing faster than the overall rate of obesity (Tjepkema, 2005; Sturm, 2007 and Howard et al., 2008). In Arab countries including Palestine the prevalence of obesity also recorded high rates (Musaiger et al., 2000 and Abdul-Rahim et al., 2001).

A combination of excessive food energy intake and a lack of physical activity are thought to explain most cases of obesity (Lau et al., 2007). A limited number of cases are due primarily to genetics, medical reasons, or psychiatric illness (Bleich et al., 2008). In contrast, increasing rates of obesity at a social level are felt to be due to an easily accessible and palatable diet, (Drewnowski and Specter, 2004), increased reliance on cars, and mechanized manufacturing (Nestle and Jacobson, 2000 and James, 2008). The risk of cardiovascular disease and diabetes is high in obese individuals (Flegal et al., 2007 and National Institute of Diabetes and Digestive and Kidney Diseases,

NIDDK, 2010). However, pathogenesis of obesity is complicated and not well known. The mortality rises exponentially with increasing body weight (Bovbjerg, 2008).

Some hormonal changes associated with obesity were addressed. Ghrelin is peptide hormone secreted mainly from stomach (Ariyasu et al., 2001 and Rindi et al., 2002). It increases appetite and food intake and stimulate the secretion of growth hormone in humans (Peino et al., 2000 and Wren et al., 2001). Ghrelin levels increase before and decrease after meals, potentially playing a role in meal initiation and satiety in an inverse pattern to that of insulin (Cummings et al., 2001, Bacha and Arslanian, 2005). Therefore, ghrelin has been implicated in the research on prevention and treatment of obesity. Several authors showed that low plasma ghrelin levels are associated with obesity (Tschöp et al., 2001 and Shiiya et al., 2002).

Ghrelin can decrease energy expenditure (Asakawa et al., 2001 and Kamegai et al. 2001), fat catabolism and lipolysis (Tschop et al., 2000; Muccioli et al., 2004 and Barazzoni et al., 2005) and adipocyte apoptosis (Kim et al. 2004). Hypercholesterolemia, hypertriglyceridaemia, high level of low density lipoprotein cholesterol, and low level of high density lipoprotein cholesterol were found in obese individuals (Kanazawa et al., 2002; Thorpe et al., 2004; Hwang et al., 2006 and Fox, 2011).

Although obesity is prevalent in Gaza Strip, the available studies are limited and focused on determination of leptin, Insulin and thyroid stimulating hormones in obesity (Zabut et al., 2007 and AL-jedi, 2011). No previous study investigated the role of the novel hormone ghrelin in obesity. Therefore, the present work is the first to assess ghrelin status in obese women from Southern Gaza Strip.

1.2 General objective

The general objective of the present study is to assess ghrelin status and lipid profile in obese women from Southern Gaza Strip.

1.3 Specific objectives

1. To determine ghrelin level in obese women and compare them with healthy normal weight controls.
2. To assess the risk factors of obesity in women from Southern Gaza Strip.
3. To measure lipid profile in obese and non obese women.
4. To verify the interrelations between ghrelin and the studied parameters of the study population.

1.4 Significance

Obesity is prevalent in developed and developing countries including Gaza Strip. Obesity is a risk factor for many diseases. In addition, there is lack of knowledge about obesity. Understanding the status of ghrelin hormone in obesity may: A) Provide us with a clear picture on the role and mechanism of such novel hormone in obesity and B) enable us to put new strategy for control of obesity. Research on ghrelin in obesity is a new and not much data are available globally. Of course, this will be the first study in Gaza Strip to assess ghrelin status in obese women from Southern Gaza Strip.

Chapter 2

Literature Review

2.1 Definition of obesity

Obesity is a condition in which excess body fat has accumulated to an extent that health may be negatively affected. Obesity is the consequence of an overall positive energy balance maintained over time, that is, the metabolizable energy intake exceeds the energy expenditure for basal metabolic requirements, thermoregulation, physical activity, and growth (Rosenbaum et al., 1997 and National Institutes of Health, 2004).

2.2 Assessment and classification of obesity

The initial step in evaluation of obesity is calculation of body mass index (BMI). To measure BMI, one begins by weighing the patient in light clothes and no shoes. Height is measured without shoes. BMI is calculated by dividing weight (in kilograms), by square height (in square meters). BMI has replaced percentage ideal body weight as a criterion for assessing obesity for several reasons. BMI correlates significantly with body fat, morbidity, and mortality and it can be calculated quickly and easily. Furthermore, recommendations for treatment of obesity are based on BMI. A BMI of 25 kg/m^2 is the generally accepted threshold for identifying a patient at higher risk for obesity-related diseases, most notably type 2 diabetes, hypertension, and cardiovascular disease (Lyznicki et al., 2001). Risk of death begins to increase at a BMI of 23 kg/m^2 when compared with the lowest risk group (BMI, 19.0 to 21.9 kg/m^2) (Aronne., 2001). Medical risk rises progressively with increasing degrees of obesity beginning with overweight, defined by BMI between 25.0 and 29.9 kg/m^2, through class I obesity (BMI, 30.0 to 34.9 kg/m^2), class II obesity (BMI, 35.0 to 39.9 kg/m^2), and class III or morbid obesity (BMI > 40 kg/m^2) (Hirsch et al., 2001). This classification system of obesity by BMI was developed by the World Health Organization Obesity

Task Force (Table 2.1) and has been adopted by researchers on the identification, evaluation, and treatment of overweight and obesity in adults (WHO, 2000).

Table 2.1 Different classes of body mass index (BMI), WHO (2000)

BMI groups	**BMI (kg/m^2)**
Underweight	<18.5
Normal	18.5-24.9
Overweight	25.0-29.9
Obese I	30.0-34.9
Obese II	35.0-39.9
Extreme obesity	≥ 40

2.3 Prevalence of obesity

The prevalence of obesity has reached alarming levels, affecting virtually both developed and developing countries. From 1976-1980 to 2005-2006, the prevalence of obesity increased from 13.4 to 35.1% in US adults aged 20 to 74 (National Center for Health Statistics, 2008). In European countries the prevalence of obesity has increased by about 10-40% in the majority of countries in the last decade. In Saudi Arabia, the prevalence of obesity rates at 23.6% in women and 14.2% in men, compared to 28.4% overweight females and 30.7% overweight males (Al-Othaimeen et al., 2007). In Northern Jordan, the prevalence of obesity was 28.1% for men and 53.1% for women (Khader et al., 2008). The prevalence of obesity in Syria was higher in women (46.3%) than men (28.4%) (Fouad et al., 2006). In urban Palestinian population the prevalence of obesity was high, 30% for men and 49% for women (Abdul-Rahim et al., 2001). Finally, WHO predicts there will be 2.3 billion overweight adults in the world by 2015 and that more than 700 million of them will be obese (WHO, 2008).

2.4 Causes of obesity

2.4.1 Diet

Consumption of an energy intake that is inappropriately large for a given energy expenditure leads to obesity. Overeating of certain specific dietary components may also lead to health risks. Obvious examples are saturated and trans-fatty acids. More recently attention has switched to high glycemic foods and to n-6 fatty acids (Prentice, 2001). In the last years, an increase occurred in the average amount of food energy consumed in US. For women, the average increase was 335 calories per day (1,542 calories in 1971 and 1,877 calories in 2004); while for men the average increase was 168 calories per day (2,450 calories in 1971 and 2,618 calories in 2004). Most of this extra food energy came from an increase in carbohydrate consumption rather than fat consumption (Wright et al., 2004). The primary sources of these extra carbohydrates are sweetened beverages, which now account for almost 25% of daily food energy in young adults in America (Caballero, 2007), and potato chips (Mozaffarian et al., 2011). Consumption of fast-food and sweetened drinks is believed to be contributing to the rising rates of obesity (Malik et al.; 2006, Rosenheck, 2008 and Olsen and Heitmann, 2009). In Europe and UK, agricultural policy and techniques led to lower price of processed food compared to fruits and vegetables (Pollan and Michael, 2007). In the Arab World, the spread of the Western lifestyle, defined as "the intake of attractive energy dense food with undesirable composition, increased consumption of animal fats and sugars and reduced consumption of dietary fiber, along with a lack of sufficient physical activity", is one of the leading causes of obesity (Kelishadi et al., 2003).

2.4.2 Physical activity

One of the major contributing factors of obesity is lack of exercise or sedentary lifestyle. According to the center for disease control and prevention (CDC) being overweight and obese result from an energy imbalance caused by eating too many calories and not getting enough exercise (CDC, 2009). Worldwide there has been a large shift towards less physically demanding

work (Ness-Abramof and Apovian, 2006 and WHO, 2009) and currently at least 60% of the world's population gets insufficient exercise (WHO, 2009). This is primarily due to increasing use of mechanized transportation and a greater prevalence of labor-saving technology in the home (Ness-Abramof and Apovian, 2006 and WHO, 2009). In children, there appear to be declines in levels of physical activity due to less walking and physical education (Salmon and Timperio, 2007). World trends in active leisure time physical activity are less clear. The World Health Organization indicates people worldwide are taking up less active recreational pursuits, while a study from Finland (Borodulin et al., 2008) found an increase and a study from the United States found leisure-time physical activity has not changed significantly (Brownson et al., 2005). In both children and adults, there is an association between television viewing time and the risk of obesity (Vioque et al., 2000 and Spence-Jones, 2003). A 2008 meta-analysis found 63 of 73 studies (86%) showed an increased rate of childhood obesity with increased media exposure, with rates increasing proportionally to time spent watching television (Emanuel, 2009).

2.4.3 Genetics

Like many other multifactorial medical conditions, obesity is the result of interplay between genetic and environmental factors. Polymorphisms in various genes controlling appetite and metabolism predispose to obesity when sufficient calories are present. The percentage of obesity that can be attributed to genetics varies widely, depending on the population examined, from 6% to 85% (Yang et al., 2007). As of 2006, more than 41 sites on the human genome have been linked to the development of obesity when a favorable environment is present (Poirier et al., 2006). Some studies have focused upon inheritance patterns without focusing upon specific genes. One study found that 80% of the offspring of two obese parents were obese, in contrast to less than 10% of the offspring of two parents who were of normal weight (Kolata, 2007). A commonly quoted genetic explanation for the rapid rise in obesity is the thrifty gene hypothesis which postulates that certain ethnic groups may be more prone to obesity in a given homogeneous

environment. In the past their ability to take advantage of rare periods of abundance by storing energy as fat would've been advantageous in an environment of varying food availability. Individuals with greater adipose reserves would be more likely to survive a famine. This tendency to store fat, however, seems to be a disadvantage in modern societies with a stable food supply (Chakravarthy and Booth, 2004). Numerous studies provided strong evidence that genetics plays an important role in obesity (Garland et al., 2011).

2.4.4 Other causes

Medical illnesses that increase obesity risk include some congenital or acquired conditions: hypothyroidism, Cushing's syndrome, growth hormone deficiency (Rosén et al., 1993) and the eating disorders: binge eating disorder and night eating syndrome. Certain medications may cause weight gain or changes in body composition; these include insulin, sulfonylureas, thiazolidinediones, atypical antipsychotics, antidepressants, steroids, certain anticonvulsants (phenytoin and valproate), pizotifen, and some forms of hormonal contraception (Haslam and James, 2005). Psychological factors may also contribute to the development of obesity (Aronne, 2001). An association between viruses and obesity has been found in humans and several different animal species. The amount that these associations may have contributed to the rising rate of obesity is yet to be determined (Falagas and Kompoti, 2006). There is an indication that gut flora in obese and lean individuals can affect the metabolic potential. This apparent alteration of the metabolic potential is believed to confer a greater capacity to harvest energy contributing to obesity. Whether these differences are the direct cause or the result of obesity has yet to be determined unequivocally (DiBaise et al., 2008).

2.5 Ghrelin

2.5.1 Definition and site of secretion

Ghrelin is a 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). The name ghrelin comes from this physiological effect: Ghre is the Proto-Indo-European root of the word “grows” (Kojima et al., 1999). Subsequently ghrelin was demonstrated to have other biological activities, such as increasing appetite and food intake, and it has been given the nickname, the “hunger hormone” (Vartiainen, 2009). Ghrelin is predominantly produced by the stomach, whereas substantially lower amounts are derived from the bowel, pancreas, pituitary, kidney, and placenta (Date et al., 2000, Hosoda et al., 2000; Ariyasu et al., 2001 and Korbonits et al., 2001).

2.5.2 Ghrelin gene and types

The ghrelin gene (GHRL, GeneID 51738) is located in the chromosome 3p26–25. It contains four exons and three introns (Wajnrajch et al., 2000). Ghrelin is synthesized as a precursor protein containing 117 amino acids, and is called prepro-ghrelin. Prepro-ghrelin is modified by the action of protein convertase enzymes into mature ghrelin, consisting of 28 amino acids (Zhang et al., 2005). After post-translational modifications, a fatty acyl group is attached to the third amino acid, serine (Ser3) of the mature ghrelin via an esteric-bond. The most common acyl group found attached on the circulating ghrelin molecules is an octanoyl group (Yang et al., 2008). Ghrelin could be classified into four groups by the type of acylation observed at Ser3: nonacylated (des-acyl ghrelin), octanoylated (C8:0), decanoylated (C10:0), and possibly decenoylated (C10:1) (Hosoda et al., 2000).

2.5.3 Ghrelin transport in blood

After its synthesis, ghrelin is stored in secretory vesicles inside the cell and released subsequently into the bloodstream where it circulates presumably as a free molecule. One study has suggested that ghrelin might be able to interact with high density lipoprotein cholesterol (HDL-C) in the circulation associated with the plasma esterase, paraoxonase. Both free ghrelin and paraoxon, a substrate for paraoxonase, can inhibit this interaction. This interaction links the orexigenic peptide hormone ghrelin to lipid transport and a plasma enzyme that breaks down oxidised lipids in low density lipoprotein

cholesterol (LDL-C). Furthermore, the interaction of the esterified ghrelin with a species containing an esterase suggests a possible mechanism for the conversion of ghrelin to des-acyl ghrelin (Beaumont et al., 2003).

2.5.4 Mechanism of action

The gherlin receptor or GHS-R is a typical G-protein coupled receptor with seven transmembrane domains, Figure 2.1 (Howard et al., 1996). Although it is located primarily in the hypothalamus and pituitary, it is also found in various peripheral organs, such as the stomach, heart, lung and liver, suggesting activity in these areas (Nakazato et al., 2001 and Gnanapavan et al., 2002). GHS-R specifically different from GHRH which known to act on the GHRH receptor to increase intracellular cyclic AMP through the protein kinase A pathway (Cheng et al., 1989). This indicates that the GHRH receptor is coupled to a Gs subclass of G protein. On the other hand, GHS stimulates the phospholipase C pathway, resulting in an increase in the intracellular Ca^{2+} through inositol 1,4,5-triphosphate-mediated signal transduction, indicating that the ghrelin receptor is coupled to a Gq subclass of G protein. The increase in Ca^{2+} concentration stimulates the release of GH (Cheng et al., 1991 and Akman et al., 1993).

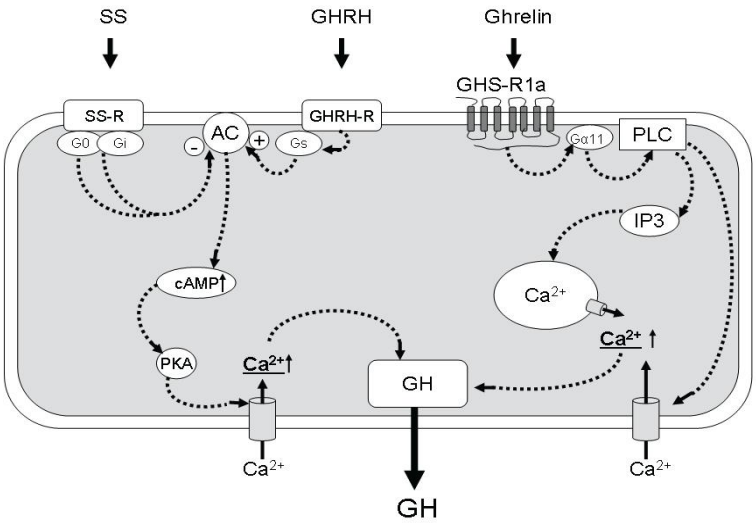


Figure 2.1 Effect of ghrelin on the anterior pituitary gland to release GH (Vartiainen, 2009).

The effects of ghrelin on energy balance are in a large part mediated by the hypothalamus (Figure 2.2.) Korbonits and his colleagues proposed three different pathways for the appetite-inducing effects of ghrelin (Korbonits et al., 2004). First, after release into the bloodstream by the stomach, ghrelin may cross the blood brain barrier (BBB) and bind to its receptors in the hypothalamus (Banks et al., 2002; Tortorella et al., 2003 and Korbonits et al., 2004). Second, ghrelin may reach the brain through the vagal nerve and nucleus tractus solitarus (Korbonits et al., 2004 and Ueno et al., 2005). Third, ghrelin is produced locally in the hypothalamus (arcuate and paraventricular nuclei), where it may directly affect the various hypothalamic nuclei (Cowley et al., 2003 and Korbonits et al., 2004). In the arcuate nucleus, ghrelin-containing neurons send efferent fibers onto neuropeptide Y (NPY)- and agouti-related protein (AgRP)-expressing neurons to stimulate the release of these orexigenic peptides and onto pro-opiomelanocortin (POMC) neurons to suppress the release of this anorexigenic peptide. In the PVN, ghrelin neurons also send efferent fibers onto NPY neurons, which in turn suppress γ-aminobutyric acid (GABA) release, resulting in the stimulation/suppresion of corticotrophin releasing hormone (CRH)-expressing neurons, leading to adrenocorticotropin (ACTH) and cortisol release (Kamegai et al., 2001; Nakazato et al., 2001; Sun et al., 2003 and Toshinai et al., 2003).

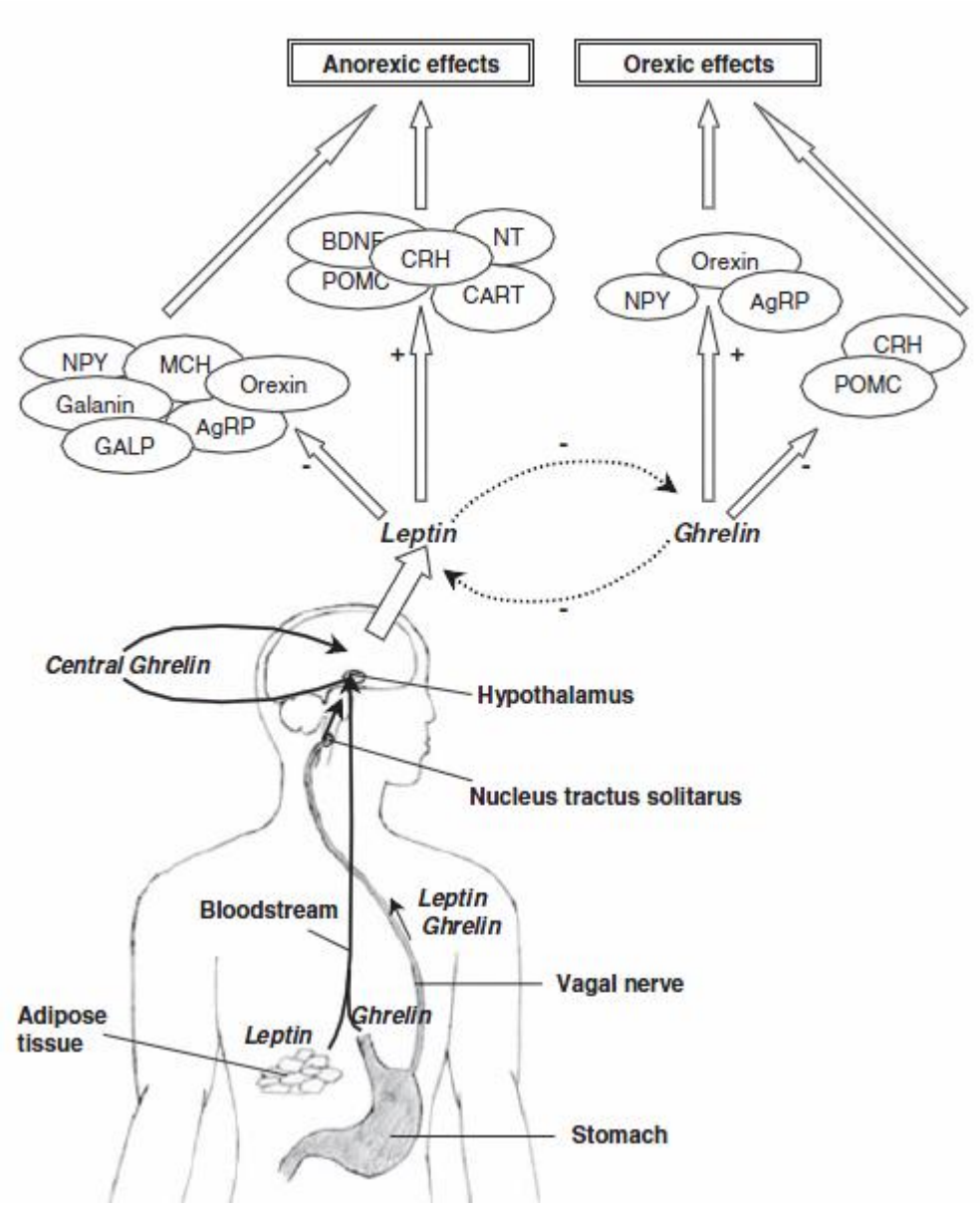


Figure 2.2 Pathways by which ghrelin may have effect on energy balance in humans (Klok et al., 2006). AgRP: agouti-related protein, BDNF: brain-derived neurotrophic factor, CART: cocaine- and amphetamine- regulated transcript, CRH: corticotropinreleasing hormone, GALP: galanin-like peptide, MCH: melanin-concentrating hormone, NPY: neuropeptide Y, NT: neurotensin, POMC: proopiomelanocortin.

2.5.5 Ghrelin and regulation of energy homeostasis

There are two systems that operate in the regulation of the quantity of food intake: short-term regulation, that is concerned primarily with preventing overeating at each meal and long-term regulation, which is primarily related with the maintenance of normal quantities of energy stores in the form of fat in the body (Konturek et al., 2005).

2.5.5.1 Roles of ghrelin in appetite and short-term food intake

Plasma ghrelin levels are dependent on recent food intake; they are increased by fasting and decline after eating (Tschop et al., 2001). For that reason, it has been suggested that they may play a major role in meal initiation (Cummings et al., 2001). The main factors promoting ghrelin production are fasting, hypoglycaemia and leptin, whilst the main inhibiting factors are food intake, hyperglycaemia and obesity (Cummings et al., 2001; Toshinai et al.; 2001, Tschop et al., 2001 and Shiiya et al., 2002). High glucose levels and consequently hyperinsulinaemia reduce ghrelin secretion; however, stomach expansion does not display this effect (Shiiya et al., 2002). The depth and duration of prandial ghrelin suppression are dose dependently related to the energy intake (Callahan et al., 2004). That means that large meals suppress both ghrelin and hunger more thoroughly than do small meals. Ingested energy suppresses ghrelin with an efficacy order of: carbohydrates > proteins > lipids (Cummings et al., 2005). Glucose and amino acids suppress ghrelin more rapidly and strongly (by approximately 70 %) than do lipids (by approximately 50 %); the relatively weak suppression of ghrelin by lipids compared with glucose or amino acids could represent one mechanism promoting high-fat dietary weight gain (Overduin et al., 2005). Regardless of the effect of ghrelin on appetite in healthy human subjects, this hormone appears to enhance the perceived palatability of the food offered (Druce et al., 2006). Daytime secretion of stomach ghrelin appears to be suppressed by food intake, whilst it is augmented by night-time fasting. By contrast, the daytime secretion of hypothalamic ghrelin is increased, and nocturnal secretion decreased, thus regulating food intake (Bowers, 2001). Table 2.2

summarises the observations which support the role of ghrelin in appetite and short-term food intake.

Table 2.2 Features which support the role of ghrelin in appetite and short-term food intake (Gil-Campos et al., 2006).

1. Most ghrelin is synthesized in the stomach, a well-positioned organ to detect recent intake of food.
2. The main effects of intraventricular and blood system ghrelin injection at times of minimal spontaneous intake is to trigger eating and to decrease the latency of feeding.
3. Human ghrelin secretion is suppressed immediately after a meal, the depth and duration of the suppression being proportional to the energy intake.
4. Ghrelin stimulates gastrointestinal motility, and gastric and exocrine pancreatic secretions.
5. Ghrelin stimulates the secretion of neuropeptide Y and agouti-related protein, two well-known orexigens, in the arcuate nucleus of the hypothalamus.
6. Some ghrelin gene polymorphisms are associated with alterations in eating patterns.

2.5.5.2 Roles of ghrelin in the regulation of body weight and long-term energy balance

Ghrelin concentrations are inversely correlated with certain obesity related parameters such as BMI, waist circumference and percentage of body fat. Obesity is characterized by decreased ghrelin levels whereas lean and anorectic patients have increased ghrelin concentrations as compared with normal weight controls (Tschöp et al., 2001 and Shiiya et al., 2002). The decline in plasma ghrelin levels recorded in lean subjects after eating a test meal is not observed in adult obese subjects (English et al., 2002). Also,

bariatric surgery is associated with reduced ghrelin plasma levels (Cummings and Shannon, 2003 and Holdstock et al., 2003). Peripheral or central chronic administration of ghrelin increases body weight (Tschop et al., 2000). Gherlin can also decrease energy expenditure (Asakawa et al., 2001 and Kamegai et al., 2001), fat catabolism and lipolysis (Tschop et al., 2000; Muccioli et al., 2004 and Barazzoni et al., 2005) and adipocyte apoptosis (Kim et al., 2004). Table 2.3 summarizes the observations which support the role of ghrelin in the control of body weight and energy expenditure.

Table 2.3 Features which support the role of ghrelin in the regulation of energy balance (Gil-Campos et al., 2006).

1. Serum ghrelin levels are inversely correlated with BMI, age and insulin concentrations.
2. Ghrelin levels are influenced by nutritional status, increasing in response to weight loss resulting from low-energy diets, life-style modifications and diseases leading to malnutrition.
3. Obese children and adults have lower ghrelin plasma levels than lean subjects, and they exhibit a lower postprandial decline and a more rapid returning towards baseline levels.
4. Bariatric surgery is associated with reduced ghrelin plasma levels.
5. Peripheral or central chronic administration of ghrelin increases body weight and reverses the effects of leptin.
6. Ghrelin decreases energy expenditure limiting fat catabolism, lipolysis and adipocyte apoptosis.

2.5.6 Ghrelin in blood and its degradation

Adult human plasma has been reported to contain 100-120 fmol total inmunoreactive ghrelin/ml (Kojima et al., 2001) and, more recently, 61-293 fmol/ml (Murashita et al., 2005). However, the concentration of active acyl ghrelin is about five to twenty times less than total ghreline in human plasma; 3·6 to 23·9 fmol/ml with an average of 9·1 fmol/ml (Date et al., 2000, Gauna et

al., 2004 and Murashita et al., 2005). Acylated ghrelin is very rapidly cleared from the circulation because hardly any of it can be found in serum in the following hour after injection (Gauna et al., 2004). The half-life of human ghrelin has been reported to be about 10 minutes (Hosoda et al., 2003**)**. Recently, the half life for hydrolysis was reported to be 45 minutes and that for degradation was 204 minutes (Rauh et al., 2007). Carboxylesterase appears to be the enzyme responsible for des-octanoylation. In addition, several esterases, including butyrylcholinesterase, contribute to ghrelin des-octanoylation in human serum (De Vriese et al., 2004). Since ghrelin is an unstable molecule in a blood or plasma sample, if the sample is not acidified and treated with a proteinase inhibitor, about one third is degraded in one hour at 37^{o}C. Freezing and thawing cycles also affect ghrelin molecules, e.g. after 3 cycles, up to 40 % of the ghrelin molecules might be lost, according to Hosoda et al. (2004).

2.6 Related studies

Tscho¨p et al. (2001) investigated the possible involvement of ghrelin in the pathogenesis of human obesity. Body composition (by dual X-ray absorption) as well as fasting plasma ghrelin concentrations (radioimmunoassay) in 15 Caucasians (8 men and 7 women, 31±9 years of age, 92±24 kg body weight, and 29±10% body fat) and 15 Pima Indians (8 men and 7 women, 33±5 years of age, 97±29 kg body weight, and 30±8% body fat) were measured. Fasting plasma ghrelin was negatively correlated with percent body fat (r=-0.45, P=0.01), fasting insulin (r=-0.45, P=0.01) and leptin (r=-0.38, P=0.03) concentrations. Plasma ghrelin concentration was decreased in obese Caucasians as compared with lean Caucasians (P<0.01). Also, fasting plasma ghrelin was lower in Pima Indians, a population with a very high prevalence of obesity, compared with Caucasians (87±28 vs. 129±34 fmol/ml, P<0.01). This result did not change after adjustment for fasting plasma insulin concentration. There was no correlation between fasting plasma ghrelin and height.

The effects of a test meal on plasma ghrelin and leptin concentrations in 13 lean and 10 obese subjects were investigated (English et al., 2002). Fasting ghrelin was significantly higher in lean than in obese subjects (857 pmol/l vs. 325 pmol/l, (P=0.002) and fell by 39.5% thirty minutes after eating in the lean group before returning rapidly towards baseline values: (P=0.003). There was no change in circulating ghrelin in the obese group. Circulating leptin concentration also fell acutely 15 minutes following food intake in lean but not obese subjects (P<0.0001).

Shiiya et al. (2002) determined plasma ghrelin concentrations in patients with simple obesity, anorexia nervosa, and type 2 diabetes mellitus by radio immune assay. The authors also studied plasma ghrelin responses to glucose load and meal intake and obtained a 24-hours profile of circulating ghrelin in humans. Plasma ghrelin concentrations in patients with simple obesity and anorexia nervosa were lower and higher, respectively, than those of healthy subjects with normal body weight. Among those with type 2 diabetes mellitus, obese patients had lower and lean patient's higher fasting plasma ghrelin concentrations than normal-weight patients. Fasting plasma ghrelin concentration was negatively correlated with body mass index in both non diabetic and diabetic patients. Plasma ghrelin concentrations of normal subjects decreased significantly after oral and intravenous glucose administration; a similar response was also observed in diabetic patients after a meal tolerance test, reaching a nadir of 69% of the basal level after the meal. Circulating plasma ghrelin showed a diurnal pattern with preprandial increases, postprandial decreases, and a maximum peak at 0200 hour.

Plasma active and serum total ghrelin levels in 20 obese (ages, 22-42 years; BMI, 41.3±1.1 kg/m^2) and 20 lean subjects (ages, 22-43 years; BMI, 22.4±0.6 kg/m^2) as well as their relationship to measures of glucose homeostasis, body fat, and resting energy expenditure (REE) were analyzed (Marzullo et al., 2004). The measured/predicted REE percentage ratio was calculated to subdivide groups into those with positive (≥100%) and negative (<100%) ratio values. In obese patients, plasma active (180±18 vs. 411±57 pg/ml, P<0.001) and serum total ghrelin levels (3650±408 vs. 5263±643 pg/ml, P<0.05) were

significantly lower when compared with lean subjects. Hence, ghrelin activity, defined as the proportion of active over total ghrelin levels, was similarly reduced in the obese state (6.1±0.9% vs. 8.4±1%, P<0.05). There was a significant correlation between active and total ghrelin (r=0.62, P<0.001), and between total ghrelin and insulin (r=-0.53, P<0.001) or insulin resistance (r=-0.49, P<0.001) approach. Significantly higher active ghrelin levels (214±22 vs. 159±30 pg/ml, P<0.05) and ghrelin activity (8±1.7% vs. 4.9±0.9%, P<0.05) were observed in patients with positive compared with negative measured/predicted REE ratio values.

Rosická et al. (2003) characterized changes in serum ghrelin levels in obese subjects and their relationship to the serum levels of leptin and soluble leptin receptor. Eight obese patients (6 women and 2 men) with BMI 40.3±13.4 kg/m^2 and eight healthy controls (5 women and 3 men) with BMI 22.7±1.3 kg/m^2 were examined. The ghrelin serum levels (165.0±58.1 vs. 343.37±81.96, p<0.001) and soluble leptin receptor serum levels (7.25±3.44 vs. 21.80±4.99, p<0.0001) were significantly lower in obese patients. The leptin serum levels (23.45±12.90 vs. 6.41±2.96, p<0.005) were significantly higher compared to the lean subject group. In both measured groups the levels of serum leptin significantly positively correlated with BMI.

Plasma ghrelin levels and their relationship to measured parameters in obese adults were evaluated (Yang et al., 2005). A total of 116 adults (male: 28, female: 76) was divided into two groups by their BMI: obese (BMI >25, n=75) and control (BMI <25, n=41) groups. Height, weight, BMI, waist hip ratio (WHR), % body fat, and skinfold thickness were higher in obese group than that of control group. Serum total cholesterol, LDL-C, HDL-C, triglyceride, glucose and insulin levels in obese group were significantly higher, compared to the control group. However, plasma ghrelin concentration in obese group was lower than that of control group. The plasma ghrelin concentration was negatively associated with BMI, subscapular skinfold thickness, WHR، cholesterol, triglyceride and insulin in total subjects including control and

obese subjects. However, only height, weight and triceps skinfold thickness had significant relationships with plasma grhrelin levels in obese subjects.

Vicennati et al. (2007) measured blood levels of obestatin, total ghrelin, and the ghrelin/obestatin ratio and their relationship with anthropometric and metabolic parameters, adiponectin and insulin resistance, in 20 overweight/obese and 12 normal-weight Italian women. Obese women had higher obestatin and lower ghrelin blood levels, and a lower ghrelin/obestatin ratio compared with controls. In all subjects, obestatin was significantly and positively correlated with total cholesterol and triglycerides, but not with ghrelin, anthropometric, and metabolic parameters. In the obese women, however, obestatin and ghrelin concentrations were positively correlated. By contrast, the ghrelin/obestatin ratio was significantly and negatively correlated with BMI, waist, WHR, fasting insulin, and insulin resistance, and positively with insulin sensitivity index but not with adiponectin. None of these parameters were correlated with the ghrelin/obestatin ratio in the obese.

Serum ghrelin levels have been studied in two groups of people (Saed et al., 2009). First group included normal weight people (11 males and 9 females), they were none obese, none diabetic and they don't take any drugs, their mean BMI was 23.8 Kg/m^2, and their mean of age 38±12 years. The second group included obese people (18 males and 13 females), they were apparently normal and they don't take any oral hypoglycemic drug; their mean BMI was 36.34 Kg/m^2, and their mean age 40±10.9 years. Ghrelin levels in obese people were less than their levels in normal weight people. A statically significant reverse correlation between ghrelin levels and BMI was found in obese subjects.

Gueugnon et al. (2011) investigated (a) changes in ghrelin and peptide YY (PYY) levels during a weight reduction programme (months 0, 3, 6, 9) and (b) baseline ghrelin and PYY levels as predictors of weight loss in 32 severely obese adolescents. In addition, 15 normal-weight teenagers served as reference for the baseline assessments. At baseline, obese teenagers had

lower ghrelin and PYY levels than normal-weight adolescents ($P<0.05$). Moreover, they showed significantly higher leptin and insulin levels ($P<0.0001$). During the lifestyle modification, there was a significant decrease in body weight among obese teenagers, associated with an increase in ghrelin (from month 6, $P<0.05$), a decrease in leptin (from month 3, $P<0.05$) and a decrease in insulin (from month 3, $P<0.0001$), without any significant change in PYY. Anthropometrical changes were correlated neither with baseline ghrelin levels nor with changes in ghrelin and PYY after the lifestyle modification. However, higher baseline PYY tended to correlate with greater anthropometrical changes ($P<0.1$). In this context, Alvarez-Castro et al. (2011) reported that ghrelin is the only known circulating orexigenic factor, and has been found to be decreased in obese people.

Chapter 3

Materials and Methods

3.1 Study design

Non experimental case control study design.

3.2 Study population

The target population comprised obese women (body mass index, BMI≥30 Kg/m^2) aged 20-40 years from Southern Gaza Strip.

3.3. Sampling and sample size

Non probability accidental sample of obese women from southern Gaza Strip were served as cases during the period from September, 2011 to December, 2011. The controls were healthy normal weight women (BMI=18.5-24.9 Kg/m^2) selected from the same area. Cases and controls were matched with age. The sample size calculation was based on the formula for case-control studies. EPI-INFO statistical package version 3.5.1 (EPI-INFO, 2008) was used with 95% CI, 80% power and 50% proportion as conservative and OR > 2. The sample size in case of 1:1 ratio of case control was found to be 81:81. For a no-response expectation, the sample size was increased to 94 obese women. The controls were also consisted of 94 normal weight women.

3.4 Exclusion criteria

Pregnant women

Women with history of other diseases

Women under hormonal therapy

3.5 Data collection

3.5.1 Body mass index

The body weight of each women dressed in light clothing without shoes was weighed using a carefully calibrated electrical balance (seca model 762, Germany). The height of each woman was measured using vertical measuring rod. Body mass index was calculated as the ratio of body weight in Kg/height in meter square. Women with BMI=18.5–24.9 was considered to have normal weight and women with BMI≥30.0 was considered obese (WHO, 2000).

3.5.2 Questionnaire interview

A meeting interview was used for filling in the questionnaire for both cases and controls (Annex 1). All interviews were conducted face to face by the researcher himself. During the survey the interviewer explained any of the questions that were not clear. Most questions were one of two types: the yes/no question, which offers a dichotomous choice; and the multiple choice questions, which offer several fixed alternatives (Backestrom and Hursh-Cesar, 1981). The validity of the questionnaire was tested by six specialists in the fields of epidemiology, public health, biochemistry and nutrition. The questionnaire included personal information (age, marital status and education), socioeconomic characters (occupation, family members, family income/month and family history of obesity), feeding and feeding habits (drinking soft drink, eating sweets, eating potato chips, number of meals/day and eating while watching television), physical activity and life style. A pilot study was done prior to beginning real data collection to know the length and clarity of the questionnaire and to evaluate the outcome. Ten women were interviewed. At the end of the pilot study, a comprehensive revision to questionnaire was made and modified as necessary. The pilot subjects were not included in the study.

3.5.3 Specimen collection and processing

Blood samples were collected from 94 obese women and 94 healthy normal weight controls. Twelve hours fasting overnight venous blood sample (6 ml) was drawn by a well trained medical technologist into plastic tubes from each control and obese women. Three ml blood was placed into plastic tube contained 15 µl protease inhibitor (pefabloc) supplied by sigma-Aldrich Germany with catalog number (76307) for serum ghrelin assay (Care laboratory, Gaza City). The remainder quantity of blood was placed in plastic tube for cholesterol, triglycerides, high density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LDL-C) determination (Barhoom laboratory, Rafah). The blood was left for a while without anticoagulant to allow blood to clot. Serum samples were obtained by centrifugation at 3000 rpm/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. However, serum analysis for lipid profile was done immediately.

3.6 Biochemical analysis

3.6.1 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.

Principles of assay

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 supplied

Each kit is sufficient to run one 96-well plate and contains the following reagents:

1. Microtiter Plate

Coated with pre-titered anchor antibodies.

Quantity: 1 Strip Plate

Preparation: Ready to use.

Note: Unused strips should be resealed in the foil pouch with the dessicant provided and stored at 2-8 °C.

2. Adhesive Plate Sealer

Quantity: 2 sheets

Preparation: Ready to use.

3. 10X HRP Wash Buffer Concentrate

10X concentrate of 50 mM Tris Buffered Saline containing Tween-20.

Quantity: 2 bottles containing 50 mL each

Preparation: Dilute 1:10 with distilled or de-ionized water.

4. Human Ghrelin (Total) Standard

Human Ghrelin (total) reference standard, 5 ng/ml, lyophilized

Quantity: 1 bottle, 5 ng/ml after reconstitution with appropriate amount of water

Preparation: Hydrate thoroughly in distilled or de-ionized water immediately before use. Please refer to the analysis sheet for exact amount of water to be used since it will be lot dependent.

After hydration dilute with Assay Buffer according to 8.1.

5. Human Ghrelin (Total) Quality Controls 1 and 2

One vial each, lyophilized, containing human ghrelin (total) at two different levels.

Quantity: 0.5 mL/vial upon hydration

Preparation: Reconstitute each vial with 0.5 mL de-ionized water immediately before use.

Aliquot unused portion in smaller quantity and freeze at -20°C for later use. Avoid further freeze and thaw.

6. Human Ghrelin (Total) Matrix

Processed serum matrix containing 0.08% Sodium Azide

Quantity: 1 mL/vial

Preparation: Ready to use.

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.

Quantity: 15 mL/vial

Preparation: Ready to use.

8. Human Ghrelin (Total) Capture Antibody

Pre-titered capture antibody solution in buffer

Quantity: 3 mL/vial

Preparation: Mix thoroughly with Human Ghrelin (Total) Detection Antibody before use according to 8.3

9. Human Ghrelin (Total) Detection Antibody

Pre-titered detection antibody solution in buffer

Quantity: 3 mL/vial

Preparation: Mix thoroughly with Human Ghrelin (Total) Capture Antibody before use according to 8.3

10. Enzyme Solution

Pre-titered streptavidin-horseradish peroxidase conjugate in buffer.

Quantity: 12 mL/vial

Preparation: Ready to use

11. Substrate

3, 3',5,5'-tetramethylbenzidine in buffer.

Quantity: 12 mL/vial

Preparation: Ready to use. Minimize the exposure to light.

12. Stop Solution

0.3 M HCl; Caution: Corrosive Solution

Quantity: 12 mL/vial

Preparation: Ready to use.

Reagent preparation

Standard preparation

1. Use care in opening the lyophilized Standard vial. Using a pipette, reconstitute the Human Ghrelin (Total) Standard with the amount of distilled or deionized water specified in the data sheet supplied with this kit to give a final concentration of 5 ng/ml (or 5000 pg/mL) of Total Ghrelin Standard. Invert and mix gently until completely in solution.

2. Label five tubes with the additional concentrations of standards to be prepared:

100 pg/mL, 200 pg/mL, 500 pg/mL, 1000 pg/mL, and 2000 pg/mL.

Add Assay Buffer to each of the five tubes according to the volumes outlined in the chart below. Dilute the reconstituted 5 ng/ml standard stock according to the chart below. Vortex each tube briefly to ensure complete mixing.

Note: Change tip for every dilution. Wet tip with standard before dispensing.

Unused portions of standard should be stored in small aliquots at ≤ -20°C. Avoid multiple freeze/thaw cycles.

Concentration of Standards	**Volume of 5 ng/ml Stock to Add**	**Volume of Assay Buffer to Add**
100 pg/mL	0.020 mL	0.980 mL
200 pg/mL	0.040 mL	0.960 mL
500 pg/mL	0.100 mL	0.900 mL
1,000 pg/mL	0.200 mL	0.800 mL
2,000 pg/mL	0.400 mL	0.600 mL
5,000 pg/mL	----	----

Quality control 1 and 2 preparation

Use care in opening the lyophilized Quality Control vials. Reconstitute each Human Ghrelin (Total) Quality Control 1 and Quality Control 2 with 0.5 mL distilled or deionized water and gently invert to ensure complete hydration. Unused portions of the reconstituted Quality Controls should be stored in small aliquots at ≤ -20°C.

Avoid further freeze/thaw cycles.

Preparation of capture and detection antibody mixture

Prior to use, combine the entire contents of Human Ghrelin (Total) Capture Antibody (3 mL) and Human Ghrelin (Total) Detection Antibody (3 mL) at a 1:1 ratio and invert to mix thoroughly.

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 µL 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 µL Matrix Solution to Blank, Standards and Quality Control wells (refer to 10 for suggested well orientations).
4. Add 30 µL 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.)

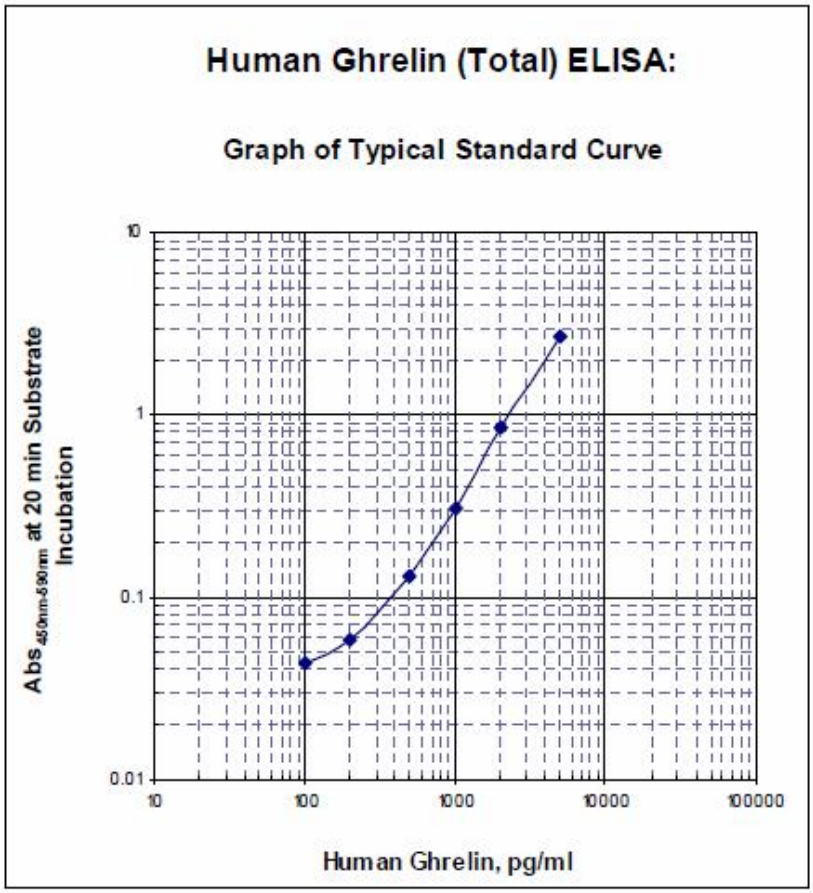
17. Remove sealer and add 100 µL stop solution [CAUTION: CORROSIVE SOLUTION] and shake plate by hand to ensure complete mixing of solution in all wells. The blue color should turn into yellow after acidification.

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 µL sample size).

Graph of typical reference curve



3.6.2 Determination of serum cholesterol

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

Principle

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

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

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

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

Reagents

Concentrations are those in the final test mixture.

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

Assay procedure

Wavelength: 500 nm

Optical path: 1cm

Temperature: 37 ºC

Measurement: against reagent blank.

- 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 ºC.
- The absorbance was measured within 60 min.

Calculation

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

Reference value

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

3.6.3 Determination of serum triglycerides

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

Principle

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

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

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

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

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

Reagents

Concentrations are those in the final test mixture.

Reagent	Concentration
Cood's buffer (pH 7.2)	50 mmol/l
4-Chlorophenol	4 mmol/l
ATP	2 mmol/l
Mg^{2+}	15 mmol/l
Glycerokinase (GK)	≥ 0.4 KU/l
Peroxidase (POD)	≥ 2 KU/l
Lipoprotein lipase (LPL)	≥ 2 KU/l
4-Aminoantipyrine	0.5 mmol/l
Glycerol-3-phosphate-oxidase (GPO)	≥ 0.5 KU/l
Standard	200 mg/dl

Assay Procedure

Wavelength: 500 nm

Optical path: 1 cm

Temperature: 37 °C

Measurement: Against reagent blank.

- 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 ºC.
- The absorbance was measured within 60 min.

Calculation

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

Reference value

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

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

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 Phosphotungstic acid	1.4 mmol/l 8.6 mmol/l
Choesterol 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 ºC

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 ºC.
- The absorbance was measured within 45 min.

- **Calculation**

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

Reference value

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

3.6.5 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-cholesterol and plasma triglycerides (TG) using the empirical relationship of Friedewald.

The Equation

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

3.7 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.
- The one-way ANOVA test was used for analysis of variance.
- Pearson's correlation test was applied.
- The results in all the above mentioned procedures were accepted as statistical significant when the p-value was less than 5% ($p<0.05$).
- Range as minimum and maximum values was used.
- The percentage difference was calculated according to the formula: Percentage difference equals the absolute value of the change in value, divided by the average of the 2 numbers, all multiplied by 100.

  Percent difference = (| (V1 - V2) | / ((V1 + V2)/2)) X 100.

- Microsoft Excel program version 11.0 was used for correlation graph plotting between ghrelin and BMI.

Chapter 4

Results

4.1 Anthropometric measurements of the study population

The study population comprised 94 obese women (case group). A total of 94 healthy normal weight women were served as controls. Table 4.1 shows the anthropometric measurements of the study population. The mean weight of controls was 57.9±6.8 kg compared to 91.7±13.6 kg of cases. The weight difference was significant (t=14.267 and P=0.000) with % difference=45.2% higher in cases. There was no significant difference in the mean height of cases compared to controls (1.61±0.07 vs. 1.60±0.06 m, % difference=0.62, t=1.199 and P=0.234). Therefore, body mass index (BMI) of cases was significantly higher than that of controls (35.1±4.6 vs. 22.6±1.8, % difference=43.3, t=15.982 and P=0.000).

Table 4.1 Anthropometric measurements of the study population.

Anthropometric measurement	Control (n=94) mean± SD	Case (n=94) mean± SD	% difference	t	P-value
Weight (kg)* *(min-max)*	57.9±6.8 (45.0-74.0)	91.7±13.6 (69.0-132.0)	45.2%	14.267	0.000
Height (m)** *(min-max)*	1.60±0.06 (1.49-1.73)	1.61±0.07 (1.48-1.76)	0.62%	1.199	0.234
BMI*** *(min-max)*	22.6±1.8 (18.6-25.0)	35.1±4.6 (30.1-51.0)	43.3%	15.982	0.000

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

4.2 Personal profile of the study population

Personal profile of the study population is illustrated in Table 4.2. Age classification showed that 34 (36.2%) controls and 30 (31.9%) cases were <25 years old. Age group 25-32 years comprised 24 (25.5%) controls and 22 (23.4%) cases. Controls and cases aged >32 years old were 36 (38.3%) and

42 (44.7%), respectively. The difference between controls and cases in term of age distribution was not significant (χ^2=0.798, P=0.671). The mean ages of controls and cases were 28.7±6.3 and 29.6±6.3 years old with ranges of 20-40 and 21-40 years, respectively. The independent sample t-test also showed no significant difference between mean ages of controls and cases (t=0.762, P=0.448). Fifty nine (62.7%) and 35 (37.2%) of controls were single and married, respectively compared to 30 (31.9%) and 64 (68.1%) of cases. The difference between the two groups was significant (χ^2=17.944, P=0.000) with obesity was more found in married women. In addition, previous pregnancy was not significantly higher in obese women 61 (95.3%) than controls 33 (94.3%) with (χ^2=0.066, P=0.797). Analysis of the educational status of the study population showed that 6 (6.4%) controls and 30 (31.9%) cases passed preparatory school and less, 34 (36.2%) and 34 (36.2%) finished secondary school 54 (57.5%) and 30 (31.9) had a university degree. The difference between various educational levels of controls and cases was significance (χ^2 =22.857, P=0.000) indicating that obesity was less prevalent among university-educated women.

Table 4.2 Personal profile of the study population

Personal character	**Controls (n=94)**		**Cases (n=94)**		**test**		**p-value**
	No.	**%**	**No.**	**%**			
Age (Year)							
<25	34	36.2	30	31.9	χ^2	0.798	0.671
25-32	24	25.5	22	23.4			
>32	36	38.3	42	44.7			
Mean±SD	28.7±6.2		29.6±6.3		**t**	0.762	0.448
Range (min-max)	20-40		21-40				
Marital status							
Single	59	62.7	30	31.9	χ^2	17.944	0.000
Married	35	37.2	64	68.1			
Previous pregnancy							
Yes	33	94.3	61	95.3	χ^2	0.066	0.797*
No	2	5.7	3	4.6			
Education							
Preparatory school and less	6	6.4	30	31.9	χ^2	22.857	0.000
Secondary school	34	36.2	34	36.2			
Diploma or University	54	57.5	30	31.9			

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

4.3 Socioeconomic characters of the study population

Table 4.3 provides socioeconomic characters of the study population. The employed controls and cases were 32 (34.0%) and 10 (10.6%) whereas 62 (65.9%) controls and 84 (89.4%) cases were unemployed. The difference between the two groups was significant (χ^2=14.839, P=0.000) with increasing obesity among unemployed women. Regarding family members and family income\month, no significant difference was recorded between controls and cases (P>0.05). However, family history revealed that 48 (51.1%) controls and 76 (80.8%) cases reported that they have family history of obesity whereas 46 (48.9%) controls and 18 (19.1%) cases did not (χ^2=18.573, P=0.000) indicating that family history is a risk factor of obesity.

Table 4.3 Socioeconomic characters of the study population

Family character	**Controls (n=94)**		**Cases (n=94)**		**χ^2**	**p-value**
	No.	**%**	**No.**	**%**		
Occupation						
Employed	32	34.0	10	10.6	14.839	0.000
Unemployed	62	65.9	84	89.4		
Family members						
<5	20	21.3	28	29.8	4.148	0.126*
5-10	70	74.5	56	59.6		
>10	4	4.2	10	10.6		
Family income/month (NIS)**						
<1000	34	36.2	28	29.8	4.425	0.109
1000-2000	34	36.2	48	51.1		
>2000	26	27.6	18	19.1		
Family history of obesity						
Yes	48	51.1	76	80.8	18.573	0.000
No	46	48.9	18	19.1		

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

4.4 Feeding and feeding habits of the study population

4.4.1 Drinking and eating among the study population

Drinking and eating among the study population are summarized in Table 4.4. A total of 66 (70.2%) controls drunk soft drink compared to 78 (82.9%) cases (χ^2=4.273, P=0.039) implying that soft drink contributes to obesity. Eighty four (89.4%) controls ate sweets versus 80 (85.1%) cases (χ^2=0.764, P=0.382). In addition, 64 (68.1%) controls ate chips compared to 58 (61.7%) case (χ^2=0.841, P=0.359).

Table 4.4 Drinking and eating among the study population

Feeding items	Controls (n=94)		Cases (n=94)		χ^2	P-value
	No.	%	No.	%		
Drinking soft drink						
Yes	66	70.2	78	82.9	4.273	0.039
No	28	29.7	16	17.0		
Eating sweets						
Yes	84	89.4	80	85.1	0.764	0.382
No	10	10.6	14	14.9		
Eating potato chips						
Yes	64	68.1	58	61.7	0.841	0.359
No	30	31.9	36	38.3		

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

4.4.2 Feeding habits of the study population

Table 4.5 provides feeding habits of the study population. A total of 15 (15.9%), 72 (76.6%) and 7 (7.4%) controls ate1, 2-3 and > 3 meals/day, respectively compared to 16 (17.0%), 74 (78.7%) and 4 (4.2%) cases (χ^2=0.370 and P=0.831). Also, no significant difference was found between controls 36 (38.3%) and cases 30 (31.9%) who admitted eating while watching television (χ^2=0.841 and P=0.359).

Table 4.5 Feeding habits of the study population

Feeding habits	Controls (n=94)		Cases (n=94)		χ^2	P-value
	No.	%	No.	%		
meals/day						
1	15	15.9	16	17.0	0.370	0.831*
2-3	72	76.6	74	78.7		
>3	7	7.4	4	4.2		
Eating while watching television						
Yes	36	38.3	30	31.9	0.841	0.359
No	58	61.7	64	68.1		

*P-value of χ^2 (corrected) test, P>0.05: not significant.

4.5 Physical activity among the study population

Physical activity among the study population is presented in Table 4.6. The number of cases who doing exercise 6 (6.3%) was found to be lower than that of controls 15 (15.9%). The difference between the two group was statistically significant (χ^2=4.342 and P=0.037) indicating that doing exercise is a protective factor against the development of obesity. Although the number of controls 35 (37.2%) was higher than cases 27 (28.7%) in terms of walking regularly, the difference between the two groups was not significant (χ^2=1.540 and P=0.215).

Table 4.6 Physical activity among the study population

Physical activity	Controls (n=94)		Cases (n=94)		χ^2	P-value
	No.	%	No.	%		
Doing exercise						
Yes	15	15.9	6	6.3	4.342	0.037
No	79	84.1	88	93.6		
Walking regularly						
Yes	35	37.2	27	28.7	1.540	0.215
No	59	62.8	67	71.2		

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

4.6 life style of the study population

Table 4.7 points out the life style of the study population. A total of 24 (25.5%), 30 (31.9%), 28 (29.8%) and 12 (12.8%) cases had sedentary, lightly, moderately and highly active life style, respectively compared to controls of 8 (8.5%), 28 (29.8%), 30 (31.9%) and 28 (29.8%). The difference between the different groups was statistically significant (χ^2=14.538 and P=0.002), indicating that the frequency of obesity was decreased with increased life style activity.

Table 4.7 Life style of the study population

Life style	Controls (n=96)		Cases (n=96)		χ^2	P-value
	No.	%	No.	%		
Sedentary	8	8.5	24	25.5	14.538	0.002
Lightly active	28	29.8	30	31.9		
Moderately active	30	31.9	28	29.8		
Highly active	28	29.8	12	12.8		

P<0.05:significant.

4.7 Serum ghrelin levels of the study population

Table 4.8 shows the average serum ghrelin levels of the study population. There was a significant decrease in the mean level of ghrelin in cases compared to controls (1060.0±646.8 vs. 1473.4±690.7 pg/ml, % difference=32.6, t=2.879 and P=0.005).

Table 4.8 Serum ghrelin levels of the study population

Parameter	Control (n=94) mean±SD	Case (n=94) mean±SD	% difference	t	P-value
ghrelin (pg/ml) *(min-max)*	1473.4±690.7 (379.0-3187.0)	1060.0±646.8 (201.4-3692.0)	32.6	2.879	0.005

Assay range: 100-5500 pg/ml.
P<0.05: significant.

4.8 Serum lipid profile of the study population

Serum lipid profile including cholesterol, triglycerides, high density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LDL-C) of the study population is illustrated in Table 4.9. The average levels of cholesterol, triglycerides and LDL-C were found to be higher in cases (142.9±50.9, 141.8±36.5 and 89.5±32.1 mg/dl, respectively) compared to controls (139.9±35.3, 114.8±48.2 and 75.9±31.4 mg/dl, respectively) with % differences of 2.1, 21.0 and 16.4%, respectively). This increase was statically significant for triglycerides and LDL-C (t=2.983, P=0.004 and t=1.994, P=0.049, respectively). In contrast, HDL-C was lower in cases than in controls (54.5±15.2 vs. 60.6±14.9 mg/dl, % difference=10.6). However, this change was not significant (t=1.724, P=0.088).

Table 4.9 lipid profile of the Study population

Parameter	**Control (n=94) mean±SD**	**Case (n=94) mean±SD**	**% difference**	**t**	**P-value**
Cholesterol (mg/dl) *(min-max)*	139.9±35.3 (43-197)	142.9±50.9 (49-227)	2.1	0.243	0.809
Triglycerides (mg/dl) *(min-max)*	114.8±48.2 (67-223)	141.8±36.5 (70-235)	21.0	2.983	0.004
LDL-C (mg/dl) * *(min-max)*	75.9±31.4 (17-148)	89.5±32.1 (16-153)	16.4	1.994	0.049
HDL-C (mg/dl) ** (min-max)	60.6±14.9 (28-94)	54.5±15.2 (29-91)	10.6	1.724	0.088

*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.

4.9 Ghrelin relations

4.9.1 Ghrelin levels in relation to sociodemographic characters of the study population

Ghrelin level in relation to sociodemographic characters of the study population is presented in Table 4.10. The t-test showed that married women had slightly lower ghrelin levels than single women, but this change was not significant (1243.9±776.0 vs. 1256.6±610.3 t=0.085, P=0.933). Regarding education, ANOVA test also revealed no significant differance in ghrelin levels among different educational levels (F=0.050, P=0.943). Similarly, ghrelin levels in unemployed women and those with family history of obesity were not significantly lower than that in employed women and those without family history of obesity (1188.9±688.3 and 1183.6±719.4 vs. 1469.5±692.3 and 1398.3±624.7; t=1.569, P=0.120 and t=1.340, P= 0.184).

Table 4.10 Ghrelin levels in relation to sociodemographic characters of the study population

Sociodemographic character	Ghrelin level (pg/ml) Mean±SD	Statistical test		p-value
Marital status				
Single	1256.6±610.3	t	0.085	0.933
Married	1243.9±776.0			
Education				
Preparatory school and less	1203.9±728.3	F	0.050	0.943
Secondary school	1275.1±829.8			
Diploma or University	1251.8±564.8			
Occupation				
Employed	1469.5±692.3	t	1.569	0.120
Unemployed	1188.9±688.3			
Family history of obesity				
Yes	1183.6±719.4	t	1.340	0.184
No	1398.3±624.7			

P>0.05:not significant.

4.9.2 Ghrelin levels in relation to physical activity of the study population

Table 4.11 points out ghrelin levels in relation to physical activity of the study population. The interaction of ghrelin levels with physical activity of the study population was not significant, with lower ghrelin levels in women who did not do exercise and those who did not walk regularly (1245.5±640.0 and 1196.8±627.4 vs. 1251.0±708.3 and 1362.7±820.7; t=0.026, P=0.979 and t=1.041, P=0.301, respectively).

Table 4.11 Ghrelin levels in relation to physical activity of the study population

Physical activity	Ghrelin level (pg/ml) Mean±SD	Statistical test		p-value
Doing exercise				
Yes	1251.0±708.3	t	0.026	0.979
No	1245.5±640.0			
Walking regularly				
Yes	1362.7±820.7	t	1.041	0.301
No	1196.8±627.4			

P>0.05:not significant.

4.9.3 Ghrelin levels in relation to soft drink and life style of the study population

Ghrelin levels in relation to soft drink and life style of the study population is illustrated in table 4.12. Ghrelin levels was not significantly lower among women who drunk soft drink than who did not (1132.7±348.3 vs. 1283.0±763.2, t=0.832, P=0.408). On the other hand, ANOVA test of the study population life style revealed that the less active life style, the lower the level of ghrelin. This positive relationship was statistically significant (F=3.682, P=0.015).

Table 4.12 Ghrelin levels in relation to soft drink and life style of the study population

Soft drink and life style	Ghrelin level (pg/ml) Mean±SD	Statistical test		p-value
Soft drink				
Yes	1132.7±348.3	t	0.832	0.408
No	1283.0±763.2			
Life style				
Sedentary	748.9±294.7	F	3.682	0.015
Lightly active	1278.6±714.4			
Moderately active	1382.7±592.2			
Highly active	1449.2±913.5			

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

4.9.4 Ghrelin levels in relation to lipid profile of the study population

Table 4.13 shows the correlation between ghrelin and lipid profile of the study population. The Pearson correlation test showed that with decreasing ghrelin levels there are increases in cholesterol, triglyceride and LDL-C levels. Such negative correlation was not significant (r=-0.131, P=0.373; r=-0.196, P=0.187 and r=-0.137 and P=0.360, respectively). On the other hand, there is a positive correlation between ghrelin level and HDL-C and this correlation was also not significant (r=0.050, P=0.742).

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

Lipid Profile (mg/dl)	Ghrelin	
	Pearson correlation (r)	P-value
Cholesterol	-0.131	0.373
Triglycerides	-0.196	0.187
LDL-C*	-0.137	0.360
HDL-C**	0.050	0.742

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

4.9.5 Ghrelin levels in relation to BMI of the study population

Ghrelin level in relation to BMI of the study population is presented in Table 4.14 and figure 4.1. The Pearson correlation test showed that the higher the BMI, the lower the level of ghrelin. This negative correlation was statistically significant (r=-0.279, P= 0.009).

Table 4.14 Ghrelin levels in relation to BMI of the study population

	Ghrelin	
	Pearson correlation (r)	P-value
BMI*	-0.279	0.009**

* Women with BMI=18.5–24.9 were considered to have normal weight, women with BMI≥30.0 were considered obese (WHO, 2000).
**P<0.05:significant.

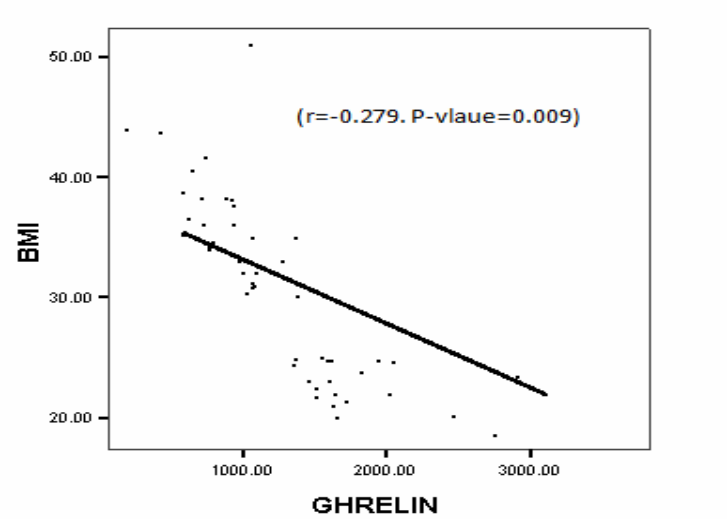


Figure 4.1 Correlation between ghrelin hormone level with BMI of the study population

Chapter 5

Discussion

Obesity and overweight have in the last decade become a global problem. By 2015, the foresight report estimates that 36% of males and 28% of females (aged between 21 and 60) will be obese. By 2025 it is estimated that 47% of men and 36% of women will be obese (National Health Service, 2011). Despite its high prevelance and the subsequent health problems, there are under-diagnosis and under-reporting of obesity in the Gaza Strip. Data on obesity were limited to annual reports emerged from the Palestinian Ministry of Health. Recently, few studies have been focused on determination of leptin, Insulin and thyroid stimulating hormones in obesity (Zabut et al., 2007, Zabut et al., 2009 and AL-jedi, 2011). However, no previous study investigated the role of the novel hormone ghrelin in obesity. Therefore, the present work is the first to assess ghrelin status in obese women from Southern Gaza Strip.

5.1 Personal profile of the study population

The present study is a case control investigation included 94 obese women (body mass index, BMI mean=35.1±4.6 kg/m^2) and 94 control (healthy normal weight women, BMI mean=22.6±1.8 kg/m^2) matched with age. The obesity was significantly higher among married women than among single ones. Such result is in agreement with that reported by Al-Malki et al. (2003) and Khader et al. (2008). The higher frequency of obesity among married women coincides with the fact that married women are more likely to put on weight gain as a result of their pregnancy and higher parity (Sotoudeh et al., 2005). This is obvious in the present result that the number of obese women who had pregnancy was higher than controls. In addition, it is possible that marriage increases cues and opportunities for eating because married people tend to eat together and thus reinforce each other's increased intake (Jeffery and Rick, 2002). Analysis of the educational status of the study population showed that obesity was less prevalent among university-educated women. Sotoudeh et al. (2005) reported that the mean BMI was significantly higher in

women with less than 8 years of formal education. This may be due to higher nutritional knowledge among highly educated women. Higher educational level has been associated with healthier dietary patterns and decreased prevalence of obesity (Rasheed, 1998 and Fouad et al., 2006).

5.2 Socioeconomic characters of the study population

Results in the present study showed a significant increase in obesity among unemployed women compared to employed ones. Similar result was obtained by Ahmad et al., (2006) and Fouad et al., (2006). The reason for this may be attributed to that employed people being more exposed to society, and therefore more interested in taking care of their weight. In general the association of BMI with employment (not working) may be explained in terms of the relationship between physical activity and obesity (Ahmad et al., 2006). Regarding family history, the present data indicated that family history is a risk factor of obesity. Such finding is in accordance with that found by Sibai et al., (2003) and Nasreddine et al., (2010) who reported that obesity was significantly greater among subjects reporting family history of obesity.

5.3 Feeding and feeding habits of the study population

As depicted in the present results, the number of obese individuals who drunk soft drink and ate 2-3 meals/day was higher than non obese individuals. The differences between both obese and controls groups was significant only for soft drink implying that soft drink in this study may contribute to obesity. This finding is concurrent with that of Ludwig et al., (2001) who pointed out that for each additional serving of sugar-sweetened drink consumed, both BMI and frequency of obesity increased. In addition, Babey et al., (2009) found that adults who drink soda occasionally (not every day) are 15% more likely to be overweight or obese, and adults who drink one or more sodas per day are 27% more likely to be overweight or obese than adults who do not drink soda.
As sugar- sweetened beverage consumption has increased, it has replaced other beverages, particularly milk and fruit juice, in the diet of children and adolescents, leading to an increase in caloric consumption (Harnack et al., 1999). According to one study, approximately 67% of sugar- sweetened

beverage consumed is soda (Powell et al., 2009). Increase in soda consumption has paralleled the rise of obesity (Fletcher et al., 2010).

5.4 Physical activity among the study population

Physical activity particularly doing exercise rather than walking regularly was found to be a protective factor against the development of obesity. The negative association between obesity and physical activity was previously documented (Al-Mahroos and Al-Roomi, 2001; Abdul-Rahim et al., 2003 and Fouad et al., 2006). This may be explained by the idea that obese women are more likely to be confined to their homes more than the non obese ones making them have little chance for recreational or sporting activities. The previous finding that obesity was higher among unemployed women does support this idea. On the other hand, occupations that require physical activity such as manual jobs were reported to be associated with lower rates of obesity (Ibrahim, 2009).

5.5 life style of the study population

The current data show that the frequency of obesity was decreased with increased life style activity ranging from sedentary, light, moderate and high life style activities. This negative association between obesity and life style activities was found by Al-Mahroos and Al-Roomi, (2001), Al-Othaimeen et al., (2007) and Khader et al., (2008). This may be explened on the basis that increased modernization and a westernized diet and lifestyle could be associated with an increased prevalence of obesity in Gaza strip as well as in many developing countries. In addition, sedentary life style is characterize by spending most of time indoors particularly for females and consumption of more food (Al-othaimeen et al., 2007).

5.6 Serum ghrelin of the study population

Results presented in this study revealed that mean level of ghrelin was significantly decreased in obese women compared to healthy normal weight controls. This finding was in agreement with that observed by Tscho¨p et al. (2001); Yang et al. (2005); Guo et al. (2007); Zamrazilová et al. (2008); Beasley et al. (2009) and Balagopal et al. (2010). However, Abdolali et al. (2011) reported no significant difference in the level of ghrelin between obese and none obese men. This discrepancy may be related to different experimental protocols of food intake. However this point needs further investigation. The lower level of ghrelin in obese women could be attributed to:

1. Physiological adaptation to energy balance associated with obesity, which means up-regulation of ghrelin expression under conditions of negative energy balance and down-regulation in the setting of positive energy balance that appear to represent a negative feed- back mechanism to maintain energy homeostasis (Tscho¨p et al., 2001 and Shiiya et al., 2002).
2. The fall in the high plasma ghrelin level (about 39.5% of the baseline) after a meal in lean subjects may represent suppression of a hunger signal. The lack of a similar fall in the low ghrelin level in obese subjects is intriguing. It may indicate that ghrelin secretion is already maximally suppressed in obese subjects, or a persistent orxgenic drive, failing to respond to food intake that predisposes to obesity (English et al., 2002).

5.7 Serum lipid profile of the study population.

The mean levels of triglycerides and low density lipoprotein cholesterol (LDL-C) were significantly increased in cases compared to controls. This finding is in accordance with that adressed by Bhatti et al. (2001); Thorpe et al. (2004) and Fox, (2011). The lipid disturbance observed in the current study could be attributed to the development of insulin resistance in obese women. Reaven et al. (2004) and Al-Jedi (2011) found that insulin resistance was significantly higher in obese women. Hyperinsulinemia is known to enhance hepatic very-low-density lipoprotein synthesis and thus may directly contribute to the

increased plasma triglyceride and LDL-C cholesterol levels (Hwang et al., 2006).

5.8 Ghrelin relations

5.8.1 Ghrelin levels in relation to sociodemoghraphic characters, physical activity, soft drink and life style of the study population

When related to sociodemographic characters of the study population, ghrelin showed lower levels in married, less educated, unemployed women as well as in women with family history of obesity compared to single, highly educated, employed women and those with no family history of obesity. However the differences were not significant. Similar trend in ghrelin levels were found in women who did not do exercise and drunk soft drink. In addition results showed that the less active life style, the lower the level of ghrelin i.e. sedentary life style has the lowest ghrelin level. This positive relationship was statistically significant. The contribution of the previous factors to obesity coincided with the lower levels of ghrelin in obese women observed in the present study and reported by other investigators (Zamrazilová et al., 2008; Beasley et al., 2009 and Balagopal et al., 2010).

5.8.2 Ghrelin levels in relation to lipid profile and body mass index of the study population

The Pearson correlation test showed negative correlations between ghrelin levels and cholesterol, triglycerides and LDL-C levels and a positive correlation with HDL-C. However, such correlations were not significant. Similar finding was observed by Guo et al. (2007) who found that TG and LDL-C were negatively correlated with fasting preprandial ghrelin levels. Concerning BMI, the Pearson correlation test releaved a negative signifigant correlation between BMI and ghrelin level i.e. the higher the BMI, the lower the level of ghrelin. Such finding supports the previous result that ghrelin was significantly lower in obese women with higher BMI. The negative correlation

between BMI and ghrelin level is in agreement with that decomented by other authors (Tscho¨p et al., 2001; Shiiya et al., 2002 and Beasley et al., 2009).

Chapter 6

Conclusions and Recommendations

6.1 Conclusions

* The mean BMI of controls and cases were 22.6±1.8 and 35.1±4.6 kg\m^2, respectively.

* Marriage, low educational level, unemployment, family history of obesity, drinking soft drink, not doing exercise and sedentary life style are found to be risk factors of obesity among women in Southern Gaza Strip.

* The mean level of serum ghrelin was significantly lower in obese women compared to healthy normal weight controls (1060.0±646.8 pg/ml vs. 1473.4±690.7pg/ml, P=0.005).

* The average levels of triglycerides and low density lipoprotein cholesterol were significantly increased in obese women (141.8±36.5 and 89.5±32.1mg/dl) compared to controls (114.8±48.2 and 75.9±31.4mg/dl) with P=0.004 and P=0.049, respectively.

* The sociodemographic characters, physical activity and soft drink were not significantly associated with ghrelin levels (P>0.05). However, analysis of variance of the study population life style revealed that the less active life style, the lower the level of ghrelin. This positive relationship was statistically significant (P=0.015).

* Ghrelin levels decreased with increased cholestorel, triglycreide and low density lipoprotein cholesterol levels, but increased with increased level of high density lipoprotein cholesterol. However, these negative and positive correlations were not statistically significant (P>0.05).

* The Pearson correlation test showed negative correlation between BMI and ghrelin levels (r=-0.279, P= 0.009).

6.2 Recommendations

* Assessment the status of ghrelin hormone in obesity could constitute a promising therapy of obesity. In this context, multiple measures of ghrelin before and after a meal are needed.

* Launching of health education programs on obesity particularly among married, unemployed and low level educated women as well as women with family history of obesity are highly recommended.

* Follow up a healthy diet in term of reducing soft drink intake.

* Regular physical activity and active life style are more likely to be useful in reducing the risk of obesity.

Chapter 7

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Annex 1

Questionnaire

This questionnaire is designed to study obesity and factors that help in its prevalence among women from Southern of Gaza.

I am researcher / Alya H El-smiri (Master student / The Islamic University) will be very grateful if you help me in completing this study which focuses on the role of a novel hormone ghrelin in obesity in women from Southern Gaza strip.

Name: …….. Age: ……..

Address ……

Tel. …… **Mobile:** ……

Weight ……kg Height……m BMI…….

1. Marital status:

   ☐ Single ☐ Married

2. Previous pregnancy:

   ☐ Yes ☐ No

3. Level of education:

   ☐ University/diploma ☐ Secondary school

   ☐ Preparatory school and less

4. Occupation:

   ☐ Employed ☐ Unemployed

5. Family members (including parents):

   ☐ <5 ☐ 5-10 ☐ >10

6. Family income:

   ☐ < 1000 NIS ☐ 1000-2000 NIS ☐ >2000 NIS

7. Is there any family history of obesity?

☐ Yes ☐ No

8. Do you drink soft drinks?

☐ Yes ☐ No

9. Do you eat sweet snacks (biscuits, cake and chocolate)?

☐ Yes ☐ No

10. Do you eat potato chips?

☐ Yes ☐ No

11. How many meals do you have daily?

☐ One ☐ 2-3 ☐ more than 3

12. Do you have food while watching television/ computer?

☐ Yes ☐ No

13. Do you exercise?

☐ Yes ☐ No

14. Do you walk regularly?

☐ Yes ☐ No

15. How would you describe your current lifestyle?

☐ Sedentary ☐ lightly active

☐ Moderately active ☐ Highly active

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

Thank you for your cooperation

Researcher / Alya H El-smiri

إستبانة

أختي الكريمة / أنا الباحثة علياء السميري (طالبة ماجستير / الجامعة الإسلامية) أرجو مساعدتي في إتمام هذه الدراسة والتي صممت لدراسة السمنة والعوامل المؤدية إلى إنتشارها مع التركيز على دراسة حالة هرمون الجريلين في النساء البدينات في جنوب قطاع غزة ، وذلك من خلال تعبئة هذا الإستبيان وتبرعك لنا بعينة دم لإجراء بعض الفحوصات والتجارب عليها .

الاسم : ..... العمر : .....

العنوان : .....

رقم الهاتف :..... رقم الجوال :.....

الوزن :..... كجم الطول :..... متر .

(BMI) : ..... كجم / م^2

1. الحالة الإجتماعية

☐ آنسة ☐ متزوجة

2. حدوث الحمل

☐ نعم ☐ لا

3. مستوى التعليم

☐ دبلوم / جامعة ☐ تعليم ثانوي ☐ إعدادي أو أقل

4. الوظيفة

☐ عاملة ☐ غير عاملة

5. عدد أفراد العائلة (بما يشمل الآباء)

☐ أقل من 5 ☐ 5-10 ☐ أكثر من 10

6. دخل العائلة

☐ أقل من 1000 شيقل ☐ 1000-2000 شيقل ☐ أكثر من 2000 شيقل

7. هل يوجد أحد في العائلة يعاني من السمنة

☐ نعم ☐ لا

8. هل تتناولين المشروبات الغازية

☐ نعم ☐ لا

9. هل تتناولين الوجبات الخفيفة (بسكويت ، شيكولاتة ، كعك)

☐ نعم ☐ لا

10. هل تتناولين الشيبسي

☐ نعم ☐ لا

11. كم عدد الوجبات التي تتناولينها في اليوم

☐ واحدة ☐ من 2-3 ☐ أكثر من 3

12. هل تتناولين الطعام أثناء مشاهدة التلفاز / الحاسوب

☐ نعم ☐ لا

13. هل تمارسين التمارين الرياضية

☐ نعم ☐ لا

14. هل تمارسين رياضة المشي بشكل منتظم

☐ نعم ☐ لا

15. كيف تصفين نمط حياتك الحالية

☐ كثيرة الجلوس ☐ نشاط خفيف ☐ نشاط متوسط ☐ نشاط عال

أنا موافقة على تعبئة هذا الإستبان الذي يتعلق بصحتي

التوقيع /..... التاريخ / .....

شكراً لكم على حسن تعاونكم

الباحثة / علياء السميري