ASSOCIATION OF GHRELIN AND PEPTIDE YY WITH INSULIN RESISTANCE AND BONE DENSITY IN THE NEWFOUNDLAND POPULATION

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Association of Ghrelin and Peptide YY with Insulin Resistance and Bone Density in the Newfoundland Population

by

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Abstract

Ghrelin and Peptide YY (PYY) are two important gut hormones involved in the regulation of food intake and energy homeostasis. Recently, data mainly from *in vitro* and animal models have suggested that these hormones may play a role in the regulation of glucose homeostasis and bone density. However, previous human studies were performed on a small sample size, and their results are inconclusive. Moreover, important confounding factors were not controlled in these studies.

In the first project, I addressed the association of circulating ghrelin with insulin resistance. A total of 2082 subjects from the Complex Diseases in the Newfoundland population: Environment and Genetics (CODING) study, participated in this investigation. Partial correlation analyses revealed that circulating ghrelin had significant inverse associations with insulin level and insulin resistance indices in the entire cohort (insulin: $r = -0.09$, $p < 0.001$; HOMA-IR: $r = -0.08$, $p < 0.001$; HOMA-β: $r = -0.1$, $p < 0.001$; and QUICKI: $r = 0.08$, $p < 0.001$) and also in men and women separately. These correlations were independent of age, percentage of trunk fat, and HDL-cholesterol.

In the second project, I investigated the relationship between circulating ghrelin and PYY and bone density using the same cohort, by controlling major confounding factors: age, BMI, physical activity, alcohol consumption, and smoking. A total of 2257 adult subjects were recruited in this study. Our results suggest a beneficial effect of circulating ghrelin level on bone density indices (L2-L4 BMD, L2-L4 Z-score, femoral neck BMD, femoral neck Z-score, total hip BMD, and total hip Z-score) in women. This effect was

independent of the major confounding factors. However, we did not find evidence that PYY is significantly associated with the bone density parameters.

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List of Abbreviations

ACTH	Adrenoorticotropic Hormone
AGRP	Agouti-Related Protein
BMC	Bone Mineral Content
BMD	Bone Mineral Density
BMI	Body Mass Index
CODING	Complex Disease in the Newfoundland population: Environment and Genetics
DEXA	Dual-Energy X-ray Absorptiometry
ELISA	Enzyme Linked Immunosorbent Assay
GLUT4	Glucose Transporter 4
HDL	High Density Lipoprotein
HOMA-IR	Homeostasis Model Assessment of Insulin Resistance
HOMA-β	Homeostasis Model Assessment of β cell function
HPLC	High Performance Liquid Chromatography
LDL	Low Density Lipoprotein
NPY	Neuropeptide Y
OPG	Osteoprotogenin
PYY	Peptide YY
QUICKI	Quantitative Insulin sensitivity Check Index
RANKL	Receptor Activator of Nuclear Factor Kappa-β Ligand
RIA	Radioimmunoassay
TG	Triacylglycerol
VLDL	Very-Low Density Lipoprotein

Chapter 1

Introduction

Ghrelin

Ghrelin was first discovered as a hormone that can stimulate growth hormone secretion. It has since been recognized that ghrelin has significant effects on appetite and energy homeostasis. The name "ghrelin" comes from the root "ghre", which is a Proto-Indo-European word for "grow" [1]. Human ghrelin can be purified from the stomach after several steps for gel filtration, two-ion exchange high performance liquid chromatography (HPLC), ghrelin-specific radioimmunoassay (RIA), intracellular calcium influx assays, and purification using C18 HPLC [2].

Structure of ghrelin

The human ghrelin gene is on chromosome 3p25-26. This gene is composed of 5 exons, and ghrelin is encoded from exons 1 and 2 [3]. Ghrelin is produced as a preprohormone that will change to a 28 amino acid peptide. A unique feature of ghrelin is a fatty acid chain modification in which the n-octanoic acid residue is bound to the serine residue at position three. This modification changes ghrelin to an active form, acylated ghrelin (Figure 1.1) [3, 4].

Secretion of ghrelin

Most of the body's ghrelin is secreted from the stomach. Lower amounts are secreted in the pancreas, pituitary, kidney, and placenta. A limited region of the arcuate nucleus of the hypothalamus contains small amounts of ghrelin [5].

Ghrelin is mostly found in the gastric fundus. In this region, oxyntic glands in distinctive endocrine cells known as P/D1 cells produce ghrelin [6]. There are two types of ghrelin – secreting cells. The first type secretes ghrelin to the lumen of the stomach where it is exposed to stomach contents. The second type lies near the capillary network of the lamina propria and secretes ghrelin to the blood [7].

Physiological actions of ghrelin

<u>Hypothalamic-pituitary actions:</u> Intravenous injection of ghrelin stimulates growth hormone release both in rats and humans [1, 8]. Moreover, ghrelin has stimulatory effects on the secretion of adrenocorticotropic hormone (ACTH) and prolactin [9]. Excessive secretion of ACTH under pathologic conditions such as Cushing disease causes weight gain and truncal obesity [10]. High level of circulating prolactin can also lead to metabolic abnormalities associated with obesity and insulin resistance [11].

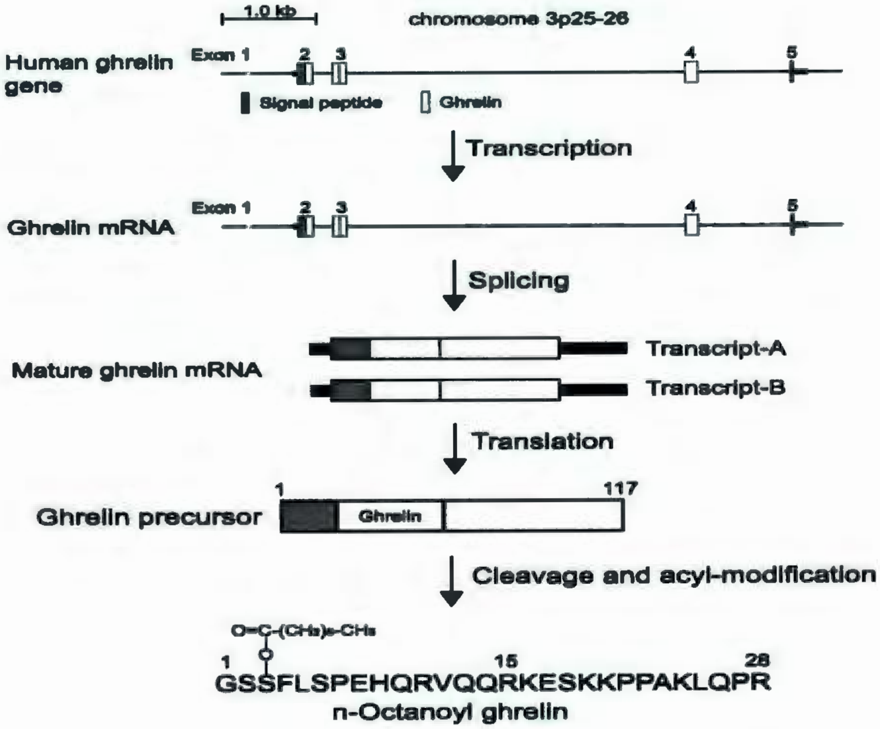

Figure 1.1- Structure of ghrelin [3]

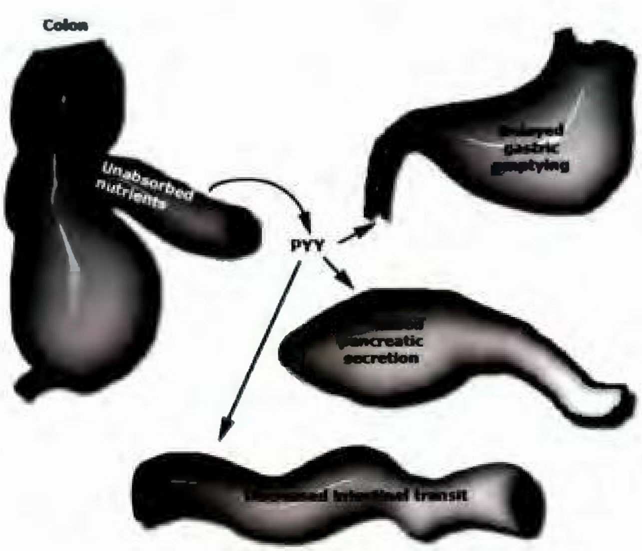

Figure 1.2- The role of PYY in the ileal brake [32]

Effects on food intake: Injection of ghrelin into the cerebral ventricles of rats increases appetite, stimulates food intake, and induces a positive energy balance that can cause weight gain [5]. Rapid increase of ghrelin before each meal and decrease of ghrelin after each meal indicate the effect of ghrelin on meal initiation. Interestingly, unlike other orexigenic hormones such as Neuropeptide Y (NPY), which acts only when injected to the brain, administration of ghrelin both peripherally and to the central nervous system causes the orexigenic effects [12].

Adiposity: Ghrelin secretion is up-regulated under conditions of negative energy balance such as anorexia nervosa [13]. The effect of positive energy balance on ghrelin is not clear. Some cross sectional studies reported decreased level of ghrelin in obese subjects compared with lean individuals [13, 14]. In our CODING (Complex Disease in the Newfoundland population: Environment and Genetics) study we did not see any significant difference in ghrelin concentration between various adiposity groups. However, in the overfeeding study, short term positive energy challenge caused a significant increase in the circulating ghrelin concentration [15].

Neuropeptide Y secretion: Central administration of ghrelin increases neuropeptide Y (NPY) and agouti-related protein (AGRP) mRNA expression in the arcuate nucleus of the hypothalamus [16]. Both NPY and AGRP are orexigenic hormones. NPY is a 36 amino acid peptide widely acts as a neurotransmitter [17]. It plays an important role in the regulation of body weight and energy homeostasis by increasing food intake [18], and accumulation of white fat storage and decreasing the brown fat thermogenesis [19, 20]. AGRP is a 132 amino acid neuropeptide which is co-expressed by NPY in the arcuate nucleus of hypothalamus. Overexpression of AGRP increases the food intake, decreases the energy expenditure, and as a result it causes weight gain [21, 22].

Effect of ghrelin on gastric motility: Ghrelin stimulates phase III-like contractions in the antrum and duodenum and increases gastric motility. Experimental animal studies showed that ghrelin can even increase the gastric motility in animals that have ileus because of vagotomy or post gastrointestinal operation. Therefore, the effect of ghrelin on gastric motility is mediated by both vagal and nonvagal pathways [23-25].

Effect of ghrelin on immune function: Ghrelin's receptors are present on human T cells and monocytes, and ghrelin may function as anti-inflammatory hormone [26, 27].

Peptide YY

Peptide YY (PYY) is a member of the NPY family of peptides. It is released mainly from enteroendocrine L-cells that are more abundant in the distal part of the gastrointestinal tract. Enteric neurons of the stomach and pancreatic endocrine cells can also secrete small amount of this hormone [28].

Structure of PYY

PYY is a 36 amino acid peptide with a tyrosin residue at both C and N terminal. There are two forms of PYY. The form that was originally isolated from the gut has 36 amino acids (PYY1-36). However, PYY can also be found in the shortened form that does not have the first two amino acids (PYY3-36). Most of the circulating PYY is in the form of PYY 3-36 [29].

Receptors of PYY

The NPY family of peptides has several receptor subtypes. These receptors are called Y receptors and all are G protein-coupled receptors. Currently, five functional Y receptors have been identified [30]. PYY1-36 binds to Y1, Y2 and Y5 receptors. However, truncated PYY (PYY3-36) mostly binds to the Y2 receptor [31].

Physiologic actions of PYY

PYY inhibits gastric acid secretion. Moreover, it can slow down gastric emptying, motility, and delivery of food to the intestine. This action of PYY is called the "ileal brake" (Figure 1.2) [32]. PYY is an important hormone in the regulation of food intake and energy homeostasis. Most of the previous studies have shown that PYY has anorectic effects. $PYY^{-/-}$ mice are hyperphagic, and they show a significant increase in body weight and adiposity on a regular chow diet [33]. Moreover, peripheral infusion of PYY in rodents, and obese humans, inhibits food intake [34, 35].

Metabolic syndrome

Metabolic syndrome has become one of the important public health challenges worldwide. Metabolic syndrome is the result of the co-occurrence of interrelated risk factors of metabolic origin mainly because of obesity and the consequent insulin resistance that appears. Metabolic syndrome directly promotes the development of atherosclerotic cardiovascular disease and increases risk for developing type 2 diabetes mellitus [36].

Definition of metabolic syndrome

There are several definitions for metabolic syndrome. Although there might be some differences between them, the commonality between all is the presence of obesity or insulin resistance. Table 1.1 shows different methods used to define metabolic syndrome.

Table 1.1 –Definitions of the metabolic syndrome

Method	**Definition**
National Cholesterol Education Program/ATP III [37]	presence of any three of the following five traits: • Abdominal obesity [waist circumference in men ≥102 cm (40 in) and in women ≥88 cm (35 in)] • Serum triglycerides ≥1.7 mmol/L • Serum HDL cholesterol <1 mmol/Lin men and <1.3 mmol/L in women • Blood pressure ≥130/85 mmHg • Fasting plasma glucose ≥5.6 mmol/L or drug treatment
International Diabetes Federation [38]	Increased waist circumference plus any of two of the following: • Triglycerides >1.7 mmol/L • HDL cholesterol <1.03 mmol/L in men or <1.29 mmol/L in women • Blood pressure >130/85 • Fasting plasma glucose > 5.6 mmol/L
Wildman *et al.*[39]	Two or more criteria: • Blood Pressure ≥ 130/85 mm Hg or antihypertensive medication use • Triglyceride level ≥ 150 mg/dL • HDL-C <1.03 mmol/L in men or <1.29 mmol/L in women • Fasting glucose level ≥ 5.6 mmol/L • Insulin resistance (HOMA-IR > 5.13) [ie. The 90^{th} percentile] • hsCRP level > 0.1 mg/L[ie. The 90^{th} percentile]

Underlying causes of metabolic syndrome

The cause of metabolic syndrome is still unclear. Insulin resistance, which is a hallmark of obesity, is one of the important factors involved in pathogenesis of metabolic syndrome [40]. Several metabolic pathways are involved in the link between insulin resistance and hyperinsulinemia to metabolic risk factors. Glucose transporter 4 (GLUT4), the key protein for transporting glucose into the cells, is regulated by insulin level. Insulin resistance can also affect enzymes that are involved in gluconeogenesis and causes the increase in endogenous glucose production [41, 42]. Moreover, insulin resistance state leads to the dysregulation of chylomicron release from the gut and production of very low density lipoproteins (VLDLs). As a result, increase in triglyceride and decrease in the HDL level are observed. Detrimental effect of insulin resistance on cardiovascular function might be due to discrepancy between nitric oxide production and secretion of endothelin-1. This imbalance causes vasoconstriction, impaired blood flow, and increase risk for cardiovascular diseases [43].

Previous studies on twins revealed that both genetic and environmental factors such as physical activity, dietary habits, age, medication use, and alcohol intake are involved in the pathogenesis of metabolic syndrome [44-46].

Definition and quantification of insulin resistance

Insulin resistance is a state in which there is subnormal β cell function and insulin secretion. This dysfunction leads to postprandial hyperglycemia, and consequently, an exaggerated insulin response that down regulates insulin receptors. Chronic consequences of insulin resistance include the development of type 2 diabetes, cardiovascular disease, and other malignancies associated with obesity [44].

The euglycemic hyperinsulinemic clamp technique is the gold standard for the measurement of insulin resistance [47]. In this method, two catheters are inserted in the veins and plasma insulin concentration is increased acutely to 100 μU/L and is kept at the same level by continuous infusion of insulin. The glucose concentration is measured once the insulin infusion starts, and it is maintained at 90 mg/dl by a variable infusion of 20% glucose. After this steady state is reached, the infusion of glucose reflects the uptake of glucose by the tissues and therefore it is an index for insulin sensitivity [48].

Since the euglycemic clamp is an invasive method, scientists created equations based on the fasting levels of insulin and glucose that can be used for the measurement of insulin sensitivity. Previous studies have shown that there is a good correlation between these equations and the results of the euglycemic clamp. The equations are mentioned in Table 1.2 [49, 50].

Table 1.2- Equations used for measurement of insulin resistance

Name	**Equation**
Homeostasis Model Assessment of Insulin Resistance (HOMA-IR)	HOMA-IR = Fasting Insulin [mU/L]× Fasting Glucose [mmol/L] / 22.5
Homeostasis Model Assessment of β cell Function (HOMA-β)	HOMA-β = 20 × Fasting Insulin [mU/L]/ Fasting Glucose [mmol/L]-3.5
Quantitative Insulin Sensitivity Check Index (QUICKI)	QUICKI = 1 / (log Fasting Insulin + log Fasting Glucose)

As mentioned previously, the two important appetite regulatory gut hormones, ghrelin and PYY, are involved in regulation of energy homeostasis and adiposity. Therefore, investigating the role of these hormones in the development of insulin resistance may provide valuable insight into underlying mechanisms responsible for the metabolic syndrome.

Effect of ghrelin on insulin resistance

Initial animal and human studies revealed that ghrelin inhibits insulin secretion and stimulates glucagon secretion from pancreatic islets, and infusion of exogenous ghrelin decreases glucose stimulated insulin secretion [51-54]. Ghrelin knockout mice showed improvement in insulin sensitivity and glucose homeostasis [55]. However, other studies do not support the proposed effect that ghrelin can have in the development of diabetes [56-58]. An important factor is that most studies are done on animals or small group of humans and they have not adequately controlled for percentage of body fat or other confounding factors for insulin resistance in their analyses.

Effect of PYY on insulin resistance

Animal investigations have shown that PYY is also involved in the regulation of glucose homeostasis. Studies on rodents revealed that PYY inhibits glucose stimulated insulin secretion from pancreatic islets [59]. Moreover, PYY knockout mice had significantly higher fasting or glucose-stimulated serum insulin concentrations compared to wild-types [60]. However, some studies revealed that PYY infusion can decrease body weight, improve insulin sensitivity and glucose concentration, and therefore its effect on decreasing insulin level is secondary to reducing food intake and body weight [61, 62]. Although there are some animal studies examining the effect of PYY on insulin resistance, there is still a lack of population based studies, specifically the large ones with the measurement of body composition for controlling adiposity in the analysis.

Osteoporosis Definition

Osteoporosis is one of the most common metabolic bone diseases worldwide. National Institute of Health consensus defined osteoporosis as a "disease characterized by low bone mass and micro architectural deterioration of bone tissue, leading to enhanced bone fragility and a consequent increase in fracture risk" [63]. According to the World Health Organization, osteoporosis is defined as a bone mineral density value more than 2.5 standard deviations below the mean for the normal young Caucasian.

Prevalence of osteoporosis

It is estimated that approximately 44 million Americans have low bone mass, and 10 million of this population have osteoporosis. For the women and men at the age of 50 years, the approximate likelihood of osteoporotic fractures in their remaining life is 50 % and 25-30 % respectively. The annual cost for treating osteoporosis is 17.9 billion dollars and it will increase to three times more than that in 2040 [64].

In Canada, almost 1 in 3 women and 1 in 5 men will experience an osteoporotic fracture during their lifetime [65]. The annual cost of osteoporotic fractures is 2.3 - 4.1 billion dollars, 1.3% of Canadian health care budget. The number of hospitalizations as a result of osteoporosis in Canada is more than the number for stroke or heart attack. Therefore, early diagnosis and understanding of the potential factors that cause osteoporosis, and the prevention of osteoporotic fractures, are of great value [66].

Osteoporosis diagnosis and bone density measurement

Osteoporosis is a silent disease, which was previously diagnosed only after fracture occurred [67]. Due to the increasing prevalence of osteoporosis, there is a huge demand for the development of facilities that can help the early diagnosis of osteoporosis. Measurement of bone mineral density (BMD) is the best means for the prediction of osteoporosis. For each standard deviation decrease in BMD, there is a two fold increase in fracture risk. Currently, dual energy X-ray absorptiometry (DXA) of the lumbar spine and proximal femur is the gold standard for bone density measurement. The DXA system consists of a padded table that patient lies on, a movable C-arm with an X-ray tube below the patient that produces two different energy levels, and a detector above the patient. In this technique, penetration of two X-ray sources through soft tissue and bone are compared, and with subtraction of soft tissue, an estimate of skeletal BMD is measured. Radiation exposure to the patient in DXA technology is almost 3–4 mrem/site. Background radiation is approximately 300 mrem/year. Therefore, the radiation acquired through DXA is negligible [68].

The DXA system also gives us the values for T and Z scores. The T score values that are used for the diagnosis of osteoporosis compares the patient's bone mineral density with the mean bone density in young adult white population. Z-score is used to compare the patient's BMD to a population of peers of the same ethnicity and sex [69].

Pathogenesis of osteoporosis

Age and estrogen deficiency

Osteoporosis is the consequence of imbalanced bone remodeling. In a normal situation, there is a balance between bone resorption, in which osteoclasts resorb bone by acidification and proteolytic digestion, and bone formation, in which osteoblasts secrete osteoid into the osteoid cavity. Most cases of osteoporosis are primary osteoporosis, which are idiopathic, and result from cellular and molecular mechanisms related to estrogen deficiency or aging. Although primary osteoporosis can be seen in both sexes, the prevalence in women is two to three times more than men. The reason is that in women there are two stages of decrease of bone density: 1) the rapid stage after menopause that continues for 4 to 8 years, and causes 5 to 10 percent of cortical bone loss and 20 to 30 percent of trabecular bone loss, and 2) the slow continuous stage that continues throughout the life and causes 20 to 25 percent of cortical and trabecular bone loss in both sexes. However, men undergo only the slow continuous stage. Therefore, women lose more bone compared to men [70].

Cellular and molecular mechanisms

Cellular and molecular mechanisms are also involved in the pathogenesis of osteoporosis. For example, decrease in the estrogen level after menopause results in an increase in cytokines like receptor activator of nuclear factor-κB ligand (RANKL), and decrease in osteoprotegerin (OPG) that accelerates bone resorption, breakdown of collagen and matrix, and an inability of osteoblasts to compensate that [71, 72].

In spite of the higher prevalence for primary causes of osteoporosis, understanding the potential factors that can cause secondary osteoporosis is of great importance. Some of the secondary causes of osteoporosis are summarized in Table 1.3. However, there are still more factors that can affect bone density which have not been fully discovered and evaluated in human studies. Previous studies have shown that pathological conditions such as subtotal gastrectomy that affect the gut peptide levels can cause secondary osteoporosis [73]. Nevertheless, the effect of gut hormones on bone density in general population is still largely unknown.

Table 1.3- Secondary causes of osteoporosis [74]

Endocrine Diseases
Female hypogonadism
Hyperprolactinemia
Hypothalamic amenorrhea
Anorexia nervosa
Premature and primary ovarian Failure
Male hypogonadism
Primary gonadal failure
Secondary gonadal failure
Delayed puberty
Hyperthyroidism
Hyperparathyroidism
Hypercotisolism
Growth hormone deficiency
Vitamin D deficiency
Idiopathic hypercalciuria
Diabetes mellitus
Gastrointestinal Diseases
Subtotal gastrectomy
Malabsorption syndromes
Chronic obstructive jaundice
Primary biliary cirrhosis and other cirrhosis
Alactasia
Bone Marrow Disorders
Multiple myeloma
Lymphoma
Leukemia
Hemolytic anemias
Systemic mastocytosis
Disseminated carcinoma
Connective Tissue Diseases
Osteogenesis imperfect
Ehlers-Danlos syndrome
Marfan syndrome
Homocystinuria
Drugs
Miscellaneous Causes
Immobilization
Rheumatoid arthritis
Renal tubular acidosis

Effect of ghrelin on bone density

Ghrelin has been shown to increase growth hormone secretion and regulate body weight and energy homeostasis; however, there has been debate as to whether circulating ghrelin level is correlated with bone mineral density. Moreover, it is not clear whether any possible correlations with bone mineral density in small studies with limited statistical power, are true direct correlations or secondary to the direct effect of ghrelin on body weight and growth hormone secretion.

In vivo and animal studies have shown that osteoblasts express ghrelin receptor and ghrelin increases osteoblasts proliferation and differentiation. To assess whether this effect is dependent on growth hormone axis, previous studies have examined the effect of ghrelin administration on GH deficient rats. It was discovered that even in the GH-deficiency state, ghrelin could increase bone density. Also, *in vivo* studies on animals using concentrations of ghrelin that did not change the body weight and food intake, showed that the effect of ghrelin on bone density is independent of body weight [75].

Human studies related to the effect of ghrelin on bone density are limited and the results are inconsistent. Some studies reported a positive correlation between ghrelin and BMD [76, 77]. However, other studies have noted no correlation at all [78-80].

Effect of PYY on bone density

PYY can influence the regulation of body weight and energy homeostasis. Body weight is a key factor in determining bone density. Previous studies have shown that hypothalamic Y2 receptors regulate bone formation and PYY may affect bone density directly through this receptor as well [81]. The initial investigations regarding the effect of PYY on bone density were conducted on rodents but the results were inconsistent. Y2 receptor deficient mice had increased trabecular bone volume. However, PYY deficient mice had a decrease of trabecular bone mass and developed osteopenia [81, 82]. Very few cross sectional studies have been performed in humans. All of the studies available in the literature were conducted on small sample size and on metabolic conditions that affect PYY such as anorexia nervosa, or in athletic subjects [83, 84]. No study has been performed in the general population. Therefore, there is a necessity to explore the association of circulating ghrelin and PYY concentration and bone mineral density, with considering major confounding factors in a large population.

In conclusion, insulin resistance and osteoporosis are both complex diseases with high prevalence, morbidity, and mortality. Therefore, understanding the factors that can be involved in the pathogenesis of these diseases is of critical importance. The findings from previous *in vitro* and animal studies indicate that gut hormones might be involved in the regulation of blood glucose and bone density. The gut produces more than 20 hormones with different functions. However, ghrelin and PYY were selected for this project based on their physiological function, anatomical location, previous evidence that suggested they might be linked to diabetes and osteoporosis, and more importantly lack of population based association studies related to the effect of these hormones on insulin resistance or bone density. Therefore, the objectives of this project are: 1) To investigate the association between circulating fasting ghrelin and insulin resistance adjusting for age, gender, percentage of body fat, and HDL- cholesterol in the general population of the

Canadian province of Newfoundland and Labrador. 2) To examine the effect of fasting circulating ghrelin and PYY on bone density in the general population, taking into consideration age, BMI, physical activity, smoking, and alcohol consumption as the most important confounding factors for bone density.

Chapter 2

Serum acylated ghrelin is negatively correlated with the insulin resistance in the CODING study

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Abstract

Objective: Ghrelin is a 28-amino acid orexigenic peptide synthesized mainly in the stomach. Acute administration of ghrelin has been found to decrease insulin secretion. However, little data is available regarding whether ghrelin contributes to the long-term regulation of insulin resistance at the population level. The aim of this study is to investigate the association between circulating ghrelin and insulin resistance in a large population based study.

Design: A total of 2082 CODING study (Complex Diseases in the Newfoundland population: Environment and Genetics) subjects were assessed. Subjects were of at least third generation Newfoundland descent, between the ages of 20 and 79 years, and had no serious metabolic, cardiovascular, or endocrine diseases. Ghrelin was measured with an Enzyme Immunoassay method. Insulin and fasting glucose were measured by Immulite 2500 autoanalyzer and Lx20 clinical chemistry analyzer, respectively. Homeostatic Model Assessment of β cell function (HOMA-β) and Insulin Resistance (HOMA-IR) and Quantitative Insulin-sensitivity Check Index (QUICKI) were used for measurement of insulin resistance.

Results: Partial correlation analyses showed a significant negative correlation between circulating ghrelin and insulin level and insulin resistance in the entire cohort and also in men and women separately. The aforementioned correlation was independent of age, percentage of trunk fat and HDL-cholesterol. According to menopausal status, only pre-menopausal women revealed negative correlations.

Conclusion: Our results suggest that except for postmenopausal women, high circulating ghrelin level is associated with lower insulin resistance in the general population.

Introduction

Diabetes mellitus is one of the most common chronic diseases worldwide. The estimated number of diabetic patients in 2010 was 366 million globally and it is predicted that this number will increase to 552 million by 2030 [1]. Type 2 diabetes is the most common type of diabetes. As a complex disease, both genetic and environmental factors are involved in development of this disease [2]. Pathogenesis of type 2 diabetes is complicated by several factors and insulin resistance, insulin deficiency, or both may contribute to this disease [3].

The gastrointestinal tract (gut) is the largest endocrine organ of the body. The gut produces hormones that have important roles in controlling body weight and energy homeostasis through the gut-brain axis [4, 5]. Previous studies showed that gastric bypass surgery results in a significant improvement of type 2 diabetes, and gut hormones play a role in this remission [6].

Ghrelin is a 28 amino acid orexigenic peptide synthesized mainly in stomach [7]. Circulating levels increase during fasting and decrease rapidly after a meal, so ghrelin has a role in acute changes in energy balance and satiety [8]. Ghrelin is a pleiotropic hormone that can influence different metabolic functions such as increasing food intake, inducing positive energy balance, promoting enlargement of adipocytes and also releasing growth hormone [9, 10]. Function of ghrelin on energy metabolism has been thought to be mediated by the central mechanisms, such as activation of the ghrelin receptor in the hypothalamic neuropeptide Y and agouti-related protein (NPY/AgRP) neurons [11]. Recently accumulating data suggest that ghrelin has central and peripheral effects on glucose regulation and insulin level [12, 13].

In rats ghrelin inhibits insulin secretion and stimulates glucagon secretion from pancreatic islets [14]. Infusion of exogenous ghrelin in healthy humans decreases glucose stimulated insulin secretion [12, 15, 16]. Moreover, it was shown that fasting ghrelin in type 2 diabetic patients is lower than in those who do not have diabetes [17]. Adolescent obese polycystic ovarian syndrome (which is characterized by insulin resistance) patients had lower ghrelin level compared with lean subjects and ghrelin was negatively correlated with Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) [18].

On the other hand, no association was found between insulin sensitivity measured with euglycemic hyperinsulinemic clamp and ghrelin level in men [19]. In a prospective follow up study no significant difference was found between the ghrelin levels of subjects who had normal glucose tolerance and those who developed impaired fasting glucose, impaired glucose tolerance and type 2 diabetes mellitus [20].

Due to the controversy of the data regarding the effect of ghrelin on insulin resistance we designed the present study to investigate the association between ghrelin and insulin resistance in a large population based study: the CODING study (The Complex Diseases in the Newfoundland population: Environment and Genetics study).

Method

Study population

A total of 2082 subjects (1582 women and 500 men) were enrolled from the ongoing nutrigenomics CODING study [21]. Volunteers were 1) between the ages of 20 and 79 years old; 2) of at least third generation Newfoundland descent 3) without any serious metabolic, cardiovascular, or endocrine diseases and 4) females were not pregnant at the time of the study.

Serum Measurement

Venous blood samples were drawn from all volunteers following a 12 hour fasting period. Blood was collected into tubes with EDTA for plasma preparation and serum separator tubes (SST) with clot activator. SST tubes were centrifuged at 3500 rpm for 10 min and EDTA tubes were centrifuged at 1300g for 15 min to separate serum and plasma respectively. Plasma and serum samples were stored in a −80 °C freezer.

Serum ghrelin was measured with an enzyme immunoassay method (Human Acylated Ghrelin Enzyme Immunoassay Kit of Spibio-bertin pharma) with the specifity of 100 % , intra-assay coefficient of variation (CV) of 5.7 % and inter-assay CV of 17 %. Insulin and fasting glucose were measured by Immulite 2500 immunoassay analyzer and Lx20 clinical chemistry analyzer (Beckman Coulter Inc.CA, USA) respectively.

Insulin resistance measurement

Although euglycemic hyperinsulinemic clamp is considered as the gold standard for measurement of insulin resistance, previous studies have shown that there is a high correlation between insulin resistance measured with HOMA and QUICKI and one that is measured by euglycemic clamp ($R = 0.88$, $p < 0.0001$ and $r = 0.69$, $p < 0.05$) [22, 23].

Homeostatic Model Assessment of β-cell function (HOMA-β) and Insulin resistance (HOMA-IR) were calculated from fasting glucose and insulin levels using the equations [22]:

$$\text{HOMA-}\beta = 20 \times \text{fasting insulin[mU/L]} / (\text{fasting glucose [mmol/L]} - 3.5)$$

$$\text{HOMA-IR} = (\text{fasting insulin[mU/L]} \times \text{fasting glucose [mmol /L]}) / 22.5$$

Three volunteers excluded from the study because of having fasting glucose lower than 3.5 mmol/L that result in negative values for HOMA-β.

Quantitative insulin-sensitivity check index (QUICKI) was the other insulin sensitivity index that was used for measurement of insulin sensitivity. It is determined by this mathematical equation [24]:

$$\text{QUICKI} = 1/[\log \text{fasting insulin(mU/L)} + \log (\text{fasting plasma glucose(mmol/L)} \times 18.0182)]$$

Subjects were divided into diabetic and non-diabetic groups (based on the history and glucose level). According to the 2006 WHO criteria fasting glucose 7.0 mmol/L was considered as diabetes [25]. 80 volunteers were in diabetic group and 2002 volunteers were in non-diabetic group.

Anthropometric and body composition measurements

Anthropometric measurements were taken with participants dressed in light clothing and without shoes. Standing height was measured to the nearest 0.1 cm using a stadiometer. Body weight was measured to the nearest 0.1 kg using a calibrated balance scale (Health O Meter, Bridgeview, IL). BMI was calculated from weight and height in kilograms per square meter. Waist circumference was measured midway between the lowest rib and iliac crest and hip circumference was measured at the widest point over the greater trochanters using a flexible tape measure and they were taken to the nearest millimeter.

Dual-energy X-Ray absorptiometry (DXA) Lunar Prodigy (GE Medical Systems, Madison, WI) was used for measurement of body composition.

Medication use and menopausal status

Volunteers were divided to medication users and non-medication users. Medication users were those who reported using prescribed medications or multivitamins regularly.

All of the female volunteers filled out a menstrual cycle and menopausal status questionnaire and they were categorized as premenopausal or postmenopausal based on this questionnaire.

Statistical analysis

SPSS version 18.0 was used for all of the statistical analyses. The summary statistics for continuous variables with normal distribution were expressed as mean and standard deviation. Non-normally distributed variables were expressed as median, minimum and maximum. The level of statistical significance was set at P value < 0.05. Logarithmic transformation was used for the variables that did not have normal distribution (ghrelin, insulin, HOMA-IR, HOMA-β, QUICKI and Triglyceride). Analysis was performed on the entire cohort and also on men and women separately. Women were also divided into pre-menopausal and post-menopausal groups and the analysis was conducted within these two groups. Pearson correlation analyses were used to examine the relationship between various potential factors that may have an effect on ghrelin or insulin sensitivity. Partial correlation analyses (controlling for age, percentage of trunk fat and HDL-cholesterol) were also performed. General linear model (multivariative analyses) was used to compare insulin resistance among the ghrelin groups, in which ghrelin groups were set by ghrelin tertiles and the covariates were age, trunk fat percentage and HDL-cholesterol. All analyses were repeated excluding diabetic patients for controlling the effect of blood glucose.

Ethical Considerations

The current study was approved by the Human Investigation Committee of the Faculty of Medicine of Memorial University, St. John's, Newfoundland and Labrador, Canada and all of the volunteers signed informed consent to participate in the study.

Results

Physical and biochemical parameters

Mean and standard deviation of the parameters that were normally distributed are summarized in Table 2.1. Ghrelin, insulin, HOMA-IR, HOMA-β, QUICKI and Triglyceride were not normally distributed. Median and range of these values are shown in Table 2.2.

Table 2.1- Biochemical and body composition characteristics [1]

	Entire Cohort (n=2063-2085) Mean(SD)	Women (n=1571-1584) Mean (SD)	Men (n=492-501) Mean (SD)
Age (y)	42.92 (12.8)	43.75 (12.1)	40.29 (14.3)
Weight (kg)	73.64 (15.8)	69.93 (14.2)	85.41 (15.1)
Height (cm)	165.51(8.4)	162.26 (5.9)	175.81 (6.6)
BMI (kg/m^2)	26.82 (5.1)	26.57 (5.2)	27.61(4.5)
Waist (cm)	92.13 (14.7)	90.49 (14.6)	97.36 (13.8)
Hip (cm)	101.23 (11.8)	101.65 (12.2)	99.90 (10.0)
Body fat (%)	35.01 (9.1)	37.95 (7.4)	25.66 (7.5)
Trunk fat (%)	37.23 (9.3)	39.30 (8.5)	30.66 (8.8)
Android fat (%)	42.54 (10.9)	44.27 (10.3)	37.03 (10.8)
Gynoid fat (%)	41.17 (9.6)	44.98 (6.4)	29.09 (7.8)
Glucose (mmol/L)	5.11 (0.8)	5.07 (0.8)	5.26 (0.8)
Total Cholesterol(mmol/L)	5.17(1.1)	5.21 (1.0)	5.04 (1.1)
HDL-Cholesterol(mmol/L)	1.46 (0.4)	1.54 (0.4)	1.21 (0.3)
LDL-Cholesterol(mmol/L)	3.14 (0.9)	3.12 (0.9)	3.18 (0.9)

[1]All values are means ± Standard Deviations (SDs).

Table 2.2- Biochemical characteristics of data not normally distributed

Variables	Entire Cohort		Female		Male	
	Median	Min-Max	Median	Min-Max	Median	Min-Max
Ghrelin (ng/L)	194.05	0.74-2339.95	193.44	0.74-2329.09	195.97	2.12-2339.95
Insulin (pmol/L)	55.90	14.40-272	55.55	14.40-272	56.95	14.40-270
HOMA-IR [1]	1.78	0.41-16.01	1.77	0.41-16.01	1.87	0.41-10.92
HOMA-β[2]	109.11	12.96-3801.3	111.16	12.96-3801.3	102.52	13.84-1382.29
QUICKI[3]	0.35	0.26-0.45	0.35	0.26-0.45	0.35	0.27-0.45
Triglyceride (mmol/L)	1.01	0.23-5.88	0.98	0.23-5.88	1.16	0.31-5.54

[1]HOMA-IR: Homeostatic Model Assessment of Insulin Resistance

[2]HOMA-β: Homeostatic Model Assessment of β cell function

[3]QUICKI: Quantitative Insulin-sensitivity Check Index

Association between circulating ghrelin level and insulin resistance

Partial correlation analyses after controlling for confounding factors (age, percentage of trunk fat and HDL-cholesterol) showed negative correlation between circulating ghrelin level and insulin, HOMA-IR, HOMA-β and positive correlation between circulating ghrelin level and QUICKI. This association was not gender specific and it was reported in both male and female subjects (Table 2.3).

Table 2.3- Partial correlation of ghrelin with insulin resistance indices after controlling for age, Percentage of trunk fat and HDL-cholesterol

	Entire Cohort		Female		Male	
	r	**P**	**r**	**P**	**r**	**P**
Glucose	0.00	0.99	0.01	0.75	-0.03	0.52
Insulin	-0.09	0.00*	-0.07	0.00*	-0.11	0.02*
HOMA-IR	-0.08	0.00*	-0.06	0.01*	-0.10	0.02*
HOMA-β	-0.10	0.00*	-0.09	0.00*	-0.09	0.04*
QUICKI	0.08	0.00*	0.06	0.01*	0.10	0.02*

*P value < 0.05

Influence of menopause on the relationship between ghrelin and insulin resistance

To explore the influence of menopause on the association between ghrelin and insulin resistance, females were divided into pre-menopausal and post-menopausal groups and partial correlation analysis was performed after controlling for age, trunk fat percentage and HDL-cholesterol. In pre-menopausal women, there was a significant negative relationship between circulating ghrelin level and insulin, HOMA-IR, HOMA-β and positive correlation between circulating ghrelin and QUICKI whereas in post-menopausal women, there were no significant associations between ghrelin and insulin resistance factors (Table 2.4).

Table 2.4- Partial correlation analyses of ghrelin with physical and biochemical characteristics regarding menopausal status

	pre-menopausal women		post-menopausal women	
	r	**P**	**r**	**P**
Glucose (mmol/l)	0.00	0.98	0.01	0.81
Insulin (pmol/L)	-0.08	0.02*	-0.06	0.14
HOMA-IR	-0.07	0.03*	-0.05	0.25
HOMA-β	-0.09	0.01*	-0.07	0.08
QUICKI	0.07	0.04*	0.05	0.26

*P value < 0.05

Comparison of insulin resistance in low, medium and high ghrelin groups

After dividing volunteers into three groups based on the ghrelin tertile, general linear model (multivariative analysis) was used for the comparison of insulin resistance between low, medium and high ghrelin groups. For insulin, HOMA-IR, HOMA- β and QUICKI there was no significant difference between low and medium ghrelin level or medium and high ghrelin levels but significant difference was evident between low and high ghrelin level (p value = 0.003, 0.01, 0.00 and 0.02 respectively). Figure 2.1 shows the mean and 95% confidence interval for mean of insulin, HOMA-IR, HOMA-β and QUICKI in high, medium and low ghrelin groups.

Comparison of ghrelin level between diabetic and non-diabetic subjects

Repeating the analysis after excluding the volunteers who report a history of diabetes and volunteers who had fasting blood glucose more than 7 mmol/L, no significant changes in the results were present.

Association between fasting ghrelin and age and HDL-cholesterol

Pearson correlation analyses showed no significant relationship between circulating ghrelin level and body composition characteristics, either in the total cohort or in the males and females separately. There was a positive correlation between fasting ghrelin level and age in the entire cohort (r = 0.08 and p = 0.00) and in females (r = 0.12 and p = 0.00) but not in males (r = -0.02, p = 0.72). No association was found between circulating ghrelin level and HDL-cholesterol.

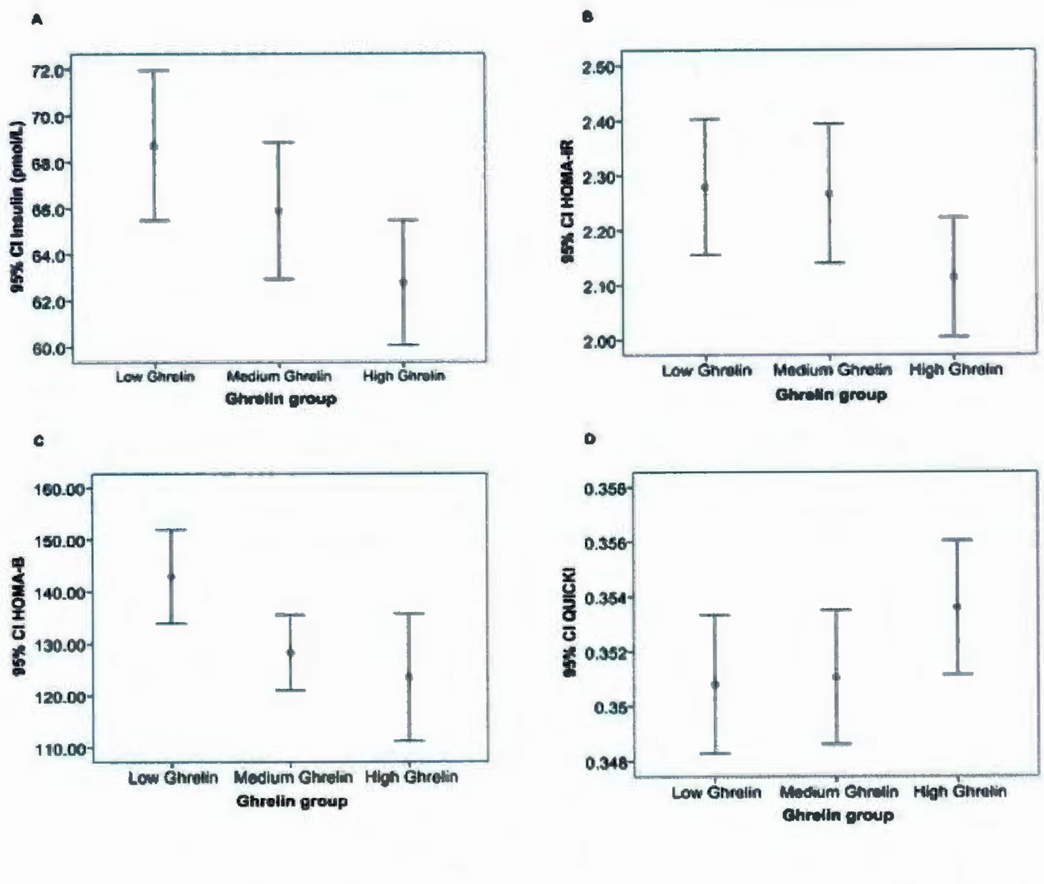

Figure 2.1- Error bars show the mean and 95% confidence interval for mean of insulin(A), HOMA-IR (B), HOMA-β (C) and QUICKI (D) values in high, medium and low ghrelin groups

Discussion

The major finding in the present study is that circulating fasting ghrelin level is negatively correlated with insulin resistance and beta cell function in our CODING study. These findings were consistent using both HOMA and QUICKI as indices of insulin resistance. More importantly, our results indicate that the association of ghrelin with insulin resistance and secretion is independent of age, body composition and circulating HDL cholesterol. To our knowledge this is the largest study to evaluate the effect of ghrelin on insulin resistance, with the most comprehensive controls for major confounding factors, in the general population.

A number of experimental studies have evaluated the effect of ghrelin on insulin secretion in humans. Broglio *et al.* and Tong *et al.* showed that acute administration of ghrelin induced inhibitory effects on insulin secretion. These effects seem to be dose dependent and non-growth hormone mediated [12, 15]. In patients with metabolic syndrome, ghrelin was inversely correlated with insulin level and insulin resistance measured by HOMA-IR [26]. In a cross sectional study, low ghrelin level has been shown to be associated with type 2 diabetes and insulin resistance in middle-aged subjects and these associations remained significant after adjustment for sex, BMI and age [17]. Our findings provide further evidence that ghrelin level is negatively correlated with insulin level and resistance.

In pancreatic β-cells, ghrelin can inhibit glucose-induced insulin release via $G\alpha_{i2}$-mediated activation of voltage dependent K^+ channels and diminish action potential in β-cells [27]. Moreover, high ghrelin level may down-regulate growth hormone or its receptors and decrease insulin secretion secondarily [28]. Ghrelin receptors have been identified in the pancreas and ghrelin is produced partly in islet ε cells of the pancreas. Therefore the inhibitory effect of ghrelin on pancreatic β-cells might partly be due to paracrine mechanisms [15, 29]. Lower insulin resistance might be a compensatory response to decreased insulin level to maintain blood glucose within a normal range.

In contrast, there are some studies that did not report any association between ghrelin level and insulin resistance. A group in Sweden did not observe association between ghrelin level and insulin sensitivity measured by euglycemic hyperinsulinemic clamp in 104 subjects after adjustment for fat free mass [19]. Measurement of insulin resistance with euglycemic hyperinsulinemic clamp was certainly good. However, the sample size was very small compared with our sample size. On top of that the older age in this study could hinder the detecting of signals because of increased use of medications and other common chronic diseases. In a longitudinal study with 5.1 year follow up on 201 subjects, glucose tolerance and baseline fasting ghrelin level were measured. They found that fasting ghrelin levels failed to predict the development of glucose intolerance or type 2 diabetes [20]. The major concern for this study is that ghrelin is only one of the many factors affecting the development of insulin resistance and type 2 diabetes, so the possible effect of ghrelin on insulin resistance cannot be excluded.

Furthermore, there are studies that found a positive association between ghrelin level and insulin resistance. Vestegard *et al.* reported increased insulin resistance in 6 healthy men and eight hypopituitary men on stable replacement therapy with growth hormone and hydrocortisone after acute administration of ghrelin [16]. Similarly, acute administration of ghrelin in 10 patients who had the total gasterectomy and truncal vagotomy, reduced insulin mediated glucose disposal rate [30]. Due to the small sample size the results of these studies were only suggestive.

Our results, together with the results from others, indicate that there is a very wide range of ghrelin concentration in the population. The large standard deviation of circulating ghrelin would require a very large sample size to achieve the statistical power to detect the effect of ghrelin on insulin resistance and other phenotypes. This factor has to be considered since most of the previous studies seemed to be under power because of the small sample size which could lead to false positive or false negative results.

As the only known orexigenic gut hormone, the role of ghrelin in the development of human obesity is still unclear. Data from animal studies indicate that ghrelin induces the accumulation of adipose tissue [31]. However, cross sectional studies in humans reported negative correlation between ghrelin and adiposity [32]. In our current study we did not find significant association between fasting ghrelin levels and any adiposity phenotype. Detailed information will be available in another paper from our laboratory.

Effect of age on circulating ghrelin is still unclear. Paik and Thoshinai reported an inverse correlation between ghrelin and age [33, 34] whereas Cummings *et al.* reported a positive correlation between ghrelin level and age and they suggest that rising ghrelin levels could play a role in the effect of aging on increasing body fat [35]. In the present CODING study a positive correlation was observed between circulating ghrelin and age. As indicated in the analysis, the effect of age on ghrelin has been properly controlled in the present study.

In postmenopausal women, insulin resistance increases likely due to the reduced levels of sex hormones and physical activity and increased body fat [36-38]. Several studies explored the effect of menopause on ghrelin level. Purnell *et al.* reported that menopausal status or hormone replacement therapy do not have any effect on the ghrelin levels [39]; while Soni *et al.* found that estrogen hormone therapy can decrease ghrelin level and discontinuing it can increase ghrelin level in post-menopausal women [40]. In our study we did not see any significant difference in ghrelin level between premenopausal and postmenopausal women. However, we found a significant association between ghrelin and insulin resistance only in premenopausal women and not in postmenopausal women. The factors involved in the absence of relationship between circulating ghrelin and insulin sensitivity in postmenopausal women could be complicated by many factors including reduced level of sex hormones and possibly use of medications.

Previous studies have reported that ghrelin can bind to HDL particles in the blood [41] and HDL cholesterol level might have some effect on the measurement of ghrelin [39]. However, in our CODING study no significant association between circulating ghrelin and HDL-cholesterol was found.

To control the potential effect of diabetes status on the results of our study all analyses were repeated after volunteers who had blood glucose more than 7 mmol/L and reported to have diabetes were excluded. All findings remained significant.

In summary, the relationship between fasting ghrelin level and insulin resistance was systematically evaluated in the large CODING study with more than 2000 adult subjects from the Newfoundland population. To our knowledge this is the largest general population based study on the relationship between fasting ghrelin level and insulin resistance. Major confounding factors including age, gender, menopausal status and HDL-cholesterol level have been carefully analyzed and properly controlled. With the strong statistical power we provide reliable evidence that ghrelin may be a factor that help to reduce insulin resistance at the population level. This association is absent in the postmenopausal women. However, because of the nature and limitations of a cross sectional study, longitudinal study is warranted to fill the knowledge gap.

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Chapter 3

Beneficial association of serum ghrelin and peptide YY with bone mineral density in the Newfoundland population

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Abstract

Background: Ghrelin and peptide YY (PYY) are appetite regulating hormones secreted from the gastrointestinal tract (gut). Aside from their known effect on energy homeostasis, accumulating data indicates that these gut hormones also affect bone metabolism. However, data regarding the influence of ghrelin and PYY on bone density in humans is very limited, and the results are inconclusive. This study was designed to investigate the potential association between circulating ghrelin and PYY with bone density indices in the general population.

Methods: A total of 2257 adult subjects from the CODING (Complex Diseases in the Newfoundland Population: Environment and Genetics) Study participated in this investigation. Acylated ghrelin and total PYY were measured in fasting serum with the Enzyme- Linked Immunosorbent Assay (ELISA) method. Bone mineral density was measured by dual-energy X-ray absorptiometry at the spine, femoral neck, and total hip. Multiple regression analyses adjusting for age, BMI, physical activity, smoking, and alcohol consumption were employed to analyze the association between serum ghrelin and PYY with bone mineral density parameters.

Results: Significant positive associations of ghrelin level with L2-L4 BMD, L2-L4 Z-score, femoral neck BMD, femoral neck Z-score, total hip BMD, and total hip Z-score were found in women. No significant correlations between ghrelin and bone density indices were revealed in men. After dividing the female group into pre-menopausal and post-menopausal, ghrelin was positively correlated with femoral neck Z-score, and total hip Z-score in pre-menopausal women and L2-L4 BMD, and Z-score in post-menopausal group. Moreover, no significant association was discovered between serum PYY and bone density at any site.

***Conclusion*:** Our results suggest a beneficial association of circulating ghrelin level with bone density in women at the population level. This association is independent of major confounding factors including body composition, physical activity, age, alcohol consumption and smoking. Effect of menopause on this association seemed to be site specific. However, PYY does not seem to be associated with bone density parameters.

***Key words*:** Ghrelin, Peptide YY, Osteoporosis, Bone Density

Background

Osteoporosis is a global problem. According to the International Osteoporosis Foundation (IOF) data, the annual treatment cost for osteoporosis fractures of people in the workplace in the USA, Canada and Europe is almost 48 billion USD [1]. Therefore, understanding the potential factors that cause osteoporosis is of great value. Most cases of osteoporosis are idiopathic because of estrogen deprivation and aging [2]. However, many other factors are involved in the pathogenesis of osteoporosis. In populations aged 50 years and over, secondary causes of osteoporosis such as endocrine, gastrointestinal, and connective tissue diseases, have been found in 41.4% of women and 51.3% of men [3]. In addition, the gastrointestinal hormones, ghrelin and PYY, which aid in energy homeostasis and weight management, have been found to be involved in the regulation of bone density. Ghrelin is a 28 amino acid appetite stimulant peptide secreted primarily from the stomach, and PYY is an appetite suppressant hormone secreted from the enteroendocrine cells of the ileum and colon [4-6].

The initial investigations regarding the effect of ghrelin on bone density, that were conducted on rodents and *in vitro* situations, have shown that ghrelin increases osteoblast replication, osteoblast specific gene expression, differentiation of osteoblast markers, and bone mineral density (BMD) [7-9]. Human studies regarding the effect of ghrelin on bone density are very limited and the results are inconsistent. In a study with 137 elderly men, ghrelin was positively correlated with femoral neck BMD [10]. In another study, eleven months after gasterectomized surgery, a significant decrease in circulating ghrelin and bone mineral density was found [11]. However, no association was found between serum ghrelin concentration with femoral neck BMD or lumbar spine BMD in 81 Korean men [12]. A study by Makovey *et al.* also did not find any significant correlation between ghrelin concentration and bone mass parameters in 79 pairs of opposite sex twins [13]. Similarly, Weiss *et al.* did not find any association between ghrelin and BMD in older men or women after adjusting for age and BMI [14].

Results from animal studies on the effect of PYY on bone density are also inconsistent. The hypothalamic Y2 receptor serves as the receptor of PYY. Y2 receptor deficient mice have increased trabecular bone volume, and rate of bone mineralization and formation [15]. However, PYY deficient mice developed decrease of trabecular bone mass and osteopenia [16]. Human studies on the effect of PYY on bone density are extremely limited in terms of a general population level, as previous studies have only been performed on special groups such as anorexic patients or women experiencing exercise [17-20].

Emerging data suggest the functionally related gut hormones, ghrelin and PYY, are linked to bone metabolism and BMD. However, data from humans is limited and the results are contradictory and subject to statistical errors due to small sample size. Moreover, BMD is a complex physiological measure and many factors can exert a significant effect on it. Therefore, it is important to evaluate whether the possible associations between these two important gut hormones and bone mineral density are independent of major confounding factors. The objectives of the current study were: 1) to determine if ghrelin and PYY are associated with bone density parameters in a large population-based cohort; 2) to evaluate whether this possible association is different in men and women, and also in pre- and post-menopausal women; and 3) to explore whether the possible associations between ghrelin or PYY and bone mineral density are independent of age, BMI, physical activity, alcohol consumption, and smoking.

Methods

Study population

A total of 2,257 subjects from the CODING (Complex Diseases in the Newfoundland population: Environment and Genetics) study, including 551 men and 1706 women were recruited in the present study through advertisement in public media and word of mouth by previous volunteers. All volunteers were at least third-generation Newfoundlander, between the ages of 20 and 79 years old, without any serious metabolic, cardiovascular, or endocrine diseases, and women were not pregnant at the time of the study.

Ethical considerations

This study was approved by the Health Research Ethics Authority of the Faculty of Medicine of Memorial University, St. John's, Newfoundland, Canada. Informed assent and consent were obtained from all of the volunteers.

Anthropometric and body composition measurements

Anthropometric measurements were performed with participants dressed in a standardized hospital gown. Standing height was measured to the nearest 0.1 cm using a fixed stadiometer. Subjects were weighed to the nearest 0.1 kg using a platform manual scale balance (Health O Meter, Bridgeview, IL). BMI was calculated from weight and height in kilograms per square meter [(weight-kg) / $(\text{height-m})^2$].

Body composition and bone mineral density measurements

The measurements of bone mineral mass were carried out by dual-energy X-ray absorptiometry (DXA) Lunar Prodigy (GE Medical Systems, Madison, WI) equipped with encore software v12.3. Volunteers were scanned by the same technician in standardized clothing (hospital gown) with no removable metal objects, while lying flat on their backs with arms at their sides. In all subjects, BMD was measured at the sites of lumbar spine, femoral neck, and total hip. Moreover, Z-score and T-score were measured for these areas.

According to the World Health Organization (WHO), T-score ≥ -1 is considered normal, T-score < -1 and > -2.5 is considered osteopenia, and T-score ≤ -2.5 is considered osteoporosis [21].

Physical activity

The Baecke questionnaire was used for evaluation of the subject's physical activity based on the work, sports, and leisure activity [22].

Blood analysis

Venous blood samples were obtained from all volunteers in the morning after an overnight fast (12 hours). Serum samples were isolated from blood and stored at -80 °C until assayed.

Serum acylated ghrelin was measured with an Enzyme - Linked Immunosorbent Assay (ELISA) method (Human Acylated Ghrelin Enzyme Immunoassay Kit of Spibio-bertin pharma). All samples used for the measurement of acylated ghrelin were thawed for the first time on the day of analysis, and while running ELSIA kits, all work was completed on ice. Intra- and inter-assay coefficients of variation (CV) were 5.7% and 17% respectively.

Serum total PYY concentration was measured with the ELISA kit from Millipore (Millipore Corporation Pharamaceuticals, Billerica, MA, USA). The intra-assay CV was 4.8 % - 5.4% and inter-assay CV was 5.1% [23].

Statistical analysis

Statistical analyses were performed using SPSS, version 20.0 (SPSS Inc, Chicago). All tests were two-sided and p value < 0.05 was considered to be statistically significant. Evaluation of data normality was performed with the Kolmograv- Smirnov test. Demographic and physical characteristics values were expressed as mean (standard deviation). Logarithmic transformation was performed for ghrelin, PYY and bone density parameters, except Z-score (because of the negative values) that were not normally distributed. These values were reported as median, minimum and maximum in the results. Analyses were performed on the entire cohort and, as well, on men and women separately. Women were further subdivided according to their menopausal status and the analyses were conducted between pre- and post-menopausal groups. Pearson correlation was used to determine the relationship between ghrelin and PYY and bone mineral density indices. Stepwise multiple regression analyses were used to identify predictors of bone density indices. Gut hormones and other identified confounders of bone density such as age, BMI, physical activity, smoking, and alcohol consumption, were considered independent variables. Percentage of body fat as the more accurate measure for body composition was also replaced with BMI to see whether the effect of body fat percentage differed from BMI. The results were similar. Therefore, in order to remain consistent with previous literature, BMI was entered into the model.

Results

Subject characteristics

Mean and standard deviation of demographic and physical characteristics of the subjects are presented in Table 3.1. Ghrelin, PYY, and bone density parameters are described as median, minimum and maximum in Table 3.2. According to the WHO criteria (based on the L2-L4 T-score), 80.8% of volunteers had normal bone density, 16.9% were osteopenic, and 2.2 % were osteoporotic. According to the femoral neck T-score, 76.6% were normal, 22.6% and 0.7% met the criteria of osteopenia and osteoporosis respectively, and based on total hip T-score 83.5%, 16.2%, and 0.3% were normal, osteopenic, and osteoporotic respectively.

Table 3.1- Demographic and physical characteristics of volunteers

	Entire cohort (n = 2257)	Female (n = 1706)	Male (n = 551)
	Mean (SD)	**Mean (SD)**	**Mean (SD)**
Age (yr)	43.1 (12.3)	44 (11.7)	40.3 (13.7)
Weight (kg)	73.4 (15.8)	69.5 (14.1)	85.4 (14.7)
Height (cm)	165.6 (8.5)	162.3 (5.9)	176.1 (6.5)
BMI (kg/m^2)	26.7 (5.1)	26.4 (5.2)	27.5 (4.5)
Percent body fat (%)	34.5 (9.4)	37.5 (7.6)	25.1 (7.8)
Percent trunk fat (%)	36.6 (9.7)	38.8 (8.8)	30 (9.3)
Percent android fat (%)	41.9 (11.3)	43.7 (10.6)	36.1 (11.4)
Percent gynoid fat (%)	40.8 (9.8)	44.7 (6.6)	28.6 (8)
Total fat mass (kg)	25.5 (10.3)	26.6 (10.2)	22.1 (9.8)
Total lean mass (kg)	44.6 (10.6)	39.7 (53.9)	59.9 (7.9)

Pearson correlation of ghrelin and PYY with bone density measures

Pearson correlation analyses showed positive correlations between ghrelin and L2-L4 Z-score, femoral neck Z-score, and total hip Z-score in the entire cohort ($r = 0.05$, $p = 0.03$, $r = 0.07$, $p = 0.004$, and $r = 0.05$, $p = 0.03$ respectively), and in females ($r = 0.08$, $p = 0.006$, $r = 0.09$, $p = 0.003$, and $r = 0.07$, $p = 0.006$ respectively).

For PYY, there was no significant association with any of the bone density parameters either in the entire cohort or in the males and females separately.

Multiple regression analyses of ghrelin and PYY with bone density indices adjusting for BMI, age, physical activity, smoking and alcohol consumption

Stepwise multiple regression analyses were performed to clarify the determinants of BMD and Z-score in males and females separately. In females, there were significant positive associations between ghrelin and L2-L4 BMD and Z-score, femoral neck BMD and Z-score, and total hip BMD and Z-score (Table 3.3). [For Z-score, age was not included in the model because Z-score is the number of standard deviations above or below what is normally expected for someone of their age, sex, and ethnic or racial origin]

For PYY after entering the variables into the model, no significant association was found between PYY and BMD or Z-score values.

Table 3.2- Descriptive statistics for ghrelin, PYY, and bone density indices

Variables	Entire Cohort		Female		Male	
	Median	Min-Max	Median	Min-Max	Median	Min-Max
Ghrelin (pg/ml)	194.7	0.74- 2329.09	193.44	0.74-2329.09	196.73	2.12-2289.26
PYY (pg/ml)	95	3.67- 368.53	92.52	3.67-368.53	103.67	8.37-364.66
Spine BMD (g/cm^2)	1.21	0.76- 1.85	1.20	0.76- 1.85	1.26	0.81-1.78
Left Hip BMD (g/cm^2)	0.98	0.52-1.83	0.96	0.52- 1.65	1.04	0.67- 1.83
Total hip BMD (g/cm^2)	1.02	0.61- 1.68	0.99	0.61- 1.58	1.1	0.76- 1.68
L2-L4 Z score (%)	0.2	-4.1- 6.1	0.2	-4.1- 6.1	0.1	-3.4- 4.8
Femur Neck Z score (%)	0.1	-2.5- 5.7	0.2	-2.5- 5	0.1	-2- 5.7
Total hip Z-score (%)	0.2	-3.12- 4.4	0.21	-3.12- 4.12	0.18	-2.63- 4.4

Table 3.3- Regression analyses of ghrelin with BMD and Z-scores in women and men [1]

	Female					Male				
	Variables	β*	(95% CI)†	P	R^2	Variables	β	(95% CI)	P	R^2
L2-L4 BMD	Age	-0.003	(-0.003, -0.002)	<0.001	0.1	BMI	0.006	(0.003, 0.009)	<0.001	0.045
	BMI	0.005	(0.004, 0.007)	<0.001		Age	-0.001	(-0.002, 0.000)	0.012	
	Smoking	-0.043	(-0.065, -0.021)	<0.001						
	Ghrelin	0.009	(0.002, 0.017)	0.015						
Femoral Neck BMD	Age	-0.004	(-0.005, -0.004)	<0.001	0.238	Age	-.006	(-0.006, -0.005)	<0.001	0.361
	BMI	0.008	(0.006, 0.009)	<0.001		BMI	.012	(0.010, 0.015)	<0.001	
	PA [2]	0.011	(0.007, 0.016)	<0.001		PA	.015	(0.007, 0.023)	<0.001	
	Smoking	-0.044	(-0.065, -0.023)	<0.001		Smoking	-.047	(-0.083, -0.010)	0.012	
	Ghrelin	0.008	(0.000, 0.015)	0.04		Alcohol	.001	(0.000, 0.002)	0.020	
Total Hip BMD	BMI	0.011	(0.009, 0.012)	<0.001	0.234	BMI	.013	(0.011, 0.016)	<0.001	0.247
	Age	-0.003	(-0.003, -0.002)	<0.001		Age	-.002	(-0.003, -0.001)	<0.001	
	PA	0.009	(0.005, 0.014)	<0.001		PA	.013	(0.005, 0.020)	0.002	
	Smoking	-0.036	(-0.057, -0.016)	<0.001		Alcohol	.001	(0.000, 0.002)	0.019	
	Ghrelin	0.009	(0.002, 0.016)	0.009		Smoking	-.037	(-0.072, -0.002)	0.036	
L2-L4 Z-score	Smoking	-0.458	(-0.675, -0.241)	<0.001	0.022					
	Ghrelin	0.102	(0.027, 0.177)	0.007						
Femoral Neck Z-score	Smoking	-0.338	(-0.502, -0.173)	<0.001	0.04	PA	0.158	(0.092, 0.224)	<0.001	0.085
	PA	0.078	(0.044, 0.113)	<0.001		BMI	0.055	(0.031, 0.079)	<0.001	
	Ghrelin	0.073	(0.015, 0.131)	0.013						
	BMI	0.013	(0.003, 0.024)	0.015						
Total Hip Z-score	BMI	0.043	(0.033, 0.053)	<0.001	0.076	BMI	0.08	(0.058, 0.102)	<0.001	0.137
	Smoking	-0.286	(-0.447, -0.125)	0.001		PA	0.122	(0.059, 0.185)	<0.001	
	PA	0.059	(0.026, 0.093)	0.001		Alcohol	0.007	(0.000, 0.015)	0.042	
	Ghrelin	0.081	(0.024, 0.137)	0.005						

[1]Regression model adjusted for age, BMI, alcohol consumption, physical activity, and smoking [2] Physical Activity * Unstandardized β coefficients †95% Confidence Interval

Influence of menopause on the relationship between ghrelin and bone density

To evaluate the influence of menopause on the association between ghrelin and bone density, multiple regression analyses were performed in females after they were divided into pre- and post-menopausal groups. Significant associations were seen between ghrelin and femoral neck and total hip Z-scores in pre-menopausal women. In post-menopausal group ghrelin was positively associated with L2-L4 BMD, and Z-score (Table 3.4).

Table 3.4- Regression analyses of ghrelin with BMD and Z-scores in women based on menopausal status [1]

	Pre-menopausal (N= 971)					Post-menopausal (N= 653)				
	Variables	β*	(95% CI)†	P	R^2	Variables	β	(95% CI)	P	R^2
L2-L4 BMD	BMI	0.005	(0.003, 0.006)	<0.001	0.05	BMI	0.006	(0.003, 0.008)	<0.001	0.088
	Smoking	-0.032	(-0.059, -0.004)	0.023		Smoking	-0.055	(-0.092, -0.017)	0.005	
						Age	-0.002	(-0.004, -0.001)	0.001	
						Ghrelin	0.014	(0.001, 0.027)	0.037	
Femoral Neck BMD	BMI	0.008	(0.006, 0.010)	<0.001	0.139	Age	-0.004	(-0.006, -0.003)	<0.001	0.195
	Age	-0.003	(-0.004, -0.002)	<0.001		BMI	0.007	(0.005, 0.009)	<0.001	
	PA [2]	0.013	(0.007, 0.019)	<0.001		Smoking	-0.044	(-0.077, -0.012)	.007	
	Smoking	-0.039	(-0.067, -0.010)	0.007		PA	0.008	(0.001, 0.015)	.019	
Total Hip BMD	BMI	0.01	(0.008, 0.011)	<0.001	0.164	BMI	0.011	(0.009, 0.013)	<0.001	0.263
	PA	0.014	(0.008, 0.019)	<0.001		Age	-0.003	(-0.004, -0.002)	<0.001	
	Smoking	-0.034	(-0.063, -0.006)	0.017		Smoking	-0.041	(-0.071, -0.011)	0.008	
L2-L4 Z-score	Smoking	-0.394	(-0.673, -0.116)	0.006	0.012	Smoking	-0.596	(-0.964, -0.229)	0.002	0.034
						Ghrelin	0.135	(0.006, 0.263)	0.04	
Femoral Neck Z-score	PA	0.106	(0.058, 0.154)	<0.001	0.051	Smoking	-0.421	(-0.658, -0.185)	.001	0.025
	Smoking	-0.304	(-0.538, -0.071)	0.011						
	BMI	0.018	(0.003, 0.033)	0.018						
	Ghrelin	0.096	(0.017, 0.175)	0.018						
Total Hip Z-score	BMI	0.043	(0.028, 0.058)	<0.001	0.083	BMI	0.043	(0.029, 0.057)	<0.001	0.084
	PA	0.092	(0.044, 0.140)	<0.001		Smoking	-0.32	(-0.552, -0.089)	0.007	
	Smoking	-0.315	(-0.546, -0.085)	0.007						
	Ghrelin	0.107	(0.029, 0.185)	0.008						

[1] Regression model adjusted for age, BMI, alcohol consumption, physical activity, and smoking [2] Physical Activity * Unstandardized β coefficients † 95% Confidence Interval

Discussion

In the present study, we examined the associations between the levels of circulating ghrelin and PYY, with bone mineral density indices controlling for major confounding factors in the Newfoundland population. The most important finding from our study is that circulating fasting ghrelin level is significantly and positively correlated with femoral neck, total hip, and lumbar spine bone mineral densities and Z-scores in females. More importantly, we demonstrated that the association of ghrelin with bone mineral density is independent of age, body composition, alcohol consumption, physical activity, and smoking. We found out that serum PYY is not significantly correlated with any of the bone density measures in this study. To our knowledge this is the largest human study that simultaneously evaluated the relationship of the two gut hormones, ghrelin and PYY, with bone mineral density with comprehensive control of major confounding factors.

Similarly, a study with the sample size of 137 men aged 55 years or older revealed a positive correlation between ghrelin and femoral neck BMD [10]. The average ages in both women and men in our study are younger, and the results are very reliable with such a large sample size.

Data from both cultured cell based and animal experiments supported the significant association between ghrelin and BMD. An animal study has shown that ghrelin receptors are present in osteoblasts and ghrelin can increase osteoblast proliferation and differentiation markers [9]. Moreover, gasterectomy in mice, in which ghrelin secretion is significantly reduced, can cause decreased bone density [24].

The mechanism by which ghrelin increases bone mineral density has yet to be completely understood. Ghrelin is a natural ligand for the growth hormone secretagogue receptor, and growth hormone increases bone density. Therefore, ghrelin may also affect bone density through the growth hormone related pathway. Moreover, previous studies have shown that osteoblasts express the ghrelin receptor. Ghrelin stimulates both osteoblast cell proliferation and differentiation [9]. However, Delhanty *et al.* did not find expression of GHS-R1a (Growth hormone secretagogue Receptor-1a) in osteoblasts. They found out that the effect of ghrelin on bone density is through ERK and PI3K, and MAPK pathway [25]. A recent study on wild type and ghrelin receptor deficient mice has shown that ghrelin can inhibit osteoclastogenesis and this effect is age dependent. With aging inhibitory effect of ghrelin on osteoclasts increases [26].

Data from some studies do not support the association between ghrelin and bone density. In ghrelin knockout mice, bone mineral density and bone mineral content between ghrelin -/- mice and wild type mice were similar [27]. There was no significant association between ghrelin and BMD in a study consisting of 80 male adults. In this study, the effect of alcohol, smoking, and physical activity was not controlled [12]. Also, in a study with 977 old adults, no significant association was found between ghrelin and BMD in either sex after controlling for age and BMI [14]. Ghrelin has extremely high standard deviation, and therefore studies on ghrelin need a very large sample size to have reasonable statistical power. Otherwise it is likely to have type II error. Caution should be taken on either negative or positive results from the studies with small sample size.

In our study, the positive association between ghrelin and BMD was found only in females. The reason for the sex difference in the association between ghrelin and BMD is unknown, and could be due to the small number of male subjects, differences in sex hormones, or other unknown factors.

Bone density is a complex physiological marker. Many factors can potentially be involved in the regulation of BMD. In the present study, one of the important goals was to clarify if the significant positive association of ghrelin with bone mineral density is secondary to any confounding factor. We were able to demonstrate that the positive association is indeed independent of the major confounding factors available in the study.

Physical activity and age are important in determining bone density [28, 29]. In our study, ghrelin was positively correlated with age. Previous primary studies have shown that bone density decreases at most sites after age of fifty due to trabecularization of cortical bones [30, 31]. Physical activity, especially weight-bearing sport-specific activity, is positively associated with femoral neck bone density after adjustment for age, sex, ethnicity, smoking, menopausal status, lean body mass, and total body fat [32]. After adjusting for age and physical activity, the association of ghrelin with bone density indices remained significant.

Alcohol consumption in adolescence can also cause reduction in bone density [33]. In our study, almost 77% of the volunteers reported the drink of alcohol (irrespective of the dosage of the alcohol they drink). Therefore alcohol drinking was adjusted in the analyses as well, and it did not affect the significant results.

Previous studies on the effect of menopause on ghrelin are contradictory. In a study on 57 females, the level of ghrelin was lower in peri-menopausal and post-menopausal women compared to pre-menopausal group, and ghrelin seemed to be positively correlated with bone density [34]. However, another study did not reveal any difference between ghrelin levels of pre- and post-menopausal women [35]. To eliminate the potential influence of menopausal status, the females were divided into two groups based on the menopausal status. We found positive associations between ghrelin and femoral neck, and total hip Z-scores in pre-menopausal women. In post-menopausal group, ghrelin was associated positively with L2-L4 BMD and Z-score. Although this difference might be consequence of changes in sex hormones caused by menopause, factors such as physical activity, body composition, and the smaller number of women in post-menopausal group might also be the reason for the difference observed between these groups.

In this study, fasting PYY was not significantly associated with bone density in the entire cohort or in males or females. Previous studies evaluated the association between PYY and bone density in metabolic diseases that affect PYY such as anorexia nervosa or in special groups such as athletes with high physical activity. In two studies on anorexia nervosa patients, PYY was negatively associated with bone density [19, 36]. However, these studies were done in patients that had lower body weight and usually lower BMD because of the anorexia nervosa [37]. Considering many important factors including smoking, alcohol consumption, and physical activity, it would be difficult to interpret the effect of PYY on bone density in this special group and in small study.

In another study on 47 adolescent girls (aged 12-18 years) in 3 groups of amenorrheic athletes, eumenorrheic athletes, and non-athletic controls, PYY was a negative predictor of lumbar Z-score. In their study, although they controlled for lean mass, other confounding factors such as physical activity, alcohol consumption, smoking were not entered in the regression model [20]. The subjects of this study were only adolescents.

The amount of physical activity and also age may exert significant influence on BMD and appetite, which in turn could affect PYY.

We did not find any significant association between PYY and bone density indices. Our data suggest that PYY is not likely an important player in determining BMD. The effect of PYY on bone density, that was reported in previous studies, might be through Y2 receptors to reduce NPY [15]. Also, effect of PYY on bone density might be secondary to its effects on body composition and BMI.

Our study had certain limitations. We had a cross-sectional study and correlation data collected does not prove causality. Therefore, interventional study of ghrelin administration to osteoporotic patients might be necessary to further evaluate this finding. Also, vitamin D and bone density markers were not measured in our study. Another limitation of our study was that the random design of the study evidently resulted in the recruitment of volunteers, where the number of males was less than females. Despite these limitations we are confident that considering the effect of two important gut hormones simultaneously with controlling most of the confounding factors in a big population base study made our results unique and reliable.

Conclusions

The present study investigated the relationship of two gut hormones, ghrelin and PYY, with BMD in the Newfoundland population. To our knowledge, this is the first study that simultaneously investigates the association of ghrelin and PYY with BMD. It is also the largest population based study with the most number of confounding factors adjusted in analysis. With such a large sample size, the present study had significantly higher power than all reported studies to detect the potential statistical signals. The significant positive associations of circulating ghrelin with BMD in women suggest that high levels of ghrelin might have beneficial effects on bone density in the female population. The beneficial effect is independent of body composition, physical activity, age, and smoking. The clinical significance of ghrelin on BMD warranted future studies. In our study, PYY was not a significant player in determining bone density.

Competing interests

The authors declare that they have no competing interests.

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Chapter 4

Summary

Diabetes and osteoporosis are common endocrine disorders with high global burden [1, 2]. Exact mechanisms for developing both diseases are unknown. Interaction of genetics, endocrine, and environmental factors affect both glucose homeostasis and bone density measures [3-5]. Most recently, data mainly from animal models have suggested that gut hormones, especially ghrelin and PYY, may have a role in bone density and glucose homeostasis [6-9]. Ghrelin is an orexigenic gut hormone released from gastric fundus. Since its initial discovery as a growth hormone secretagogue receptor, ghrelin has been shown to be involved in different functions such as regulation of food intake and energy homeostasis [10]. However, the role of ghrelin on the regulation of insulin resistance and bone density is still unclear in humans. Very few cross-sectional studies were performed in this area. None of them have reasonable sample size, and results from the literature are controversial [11-14]. Furthermore, previous investigations have not adequately controlled for confounding factors that might cause the association between ghrelin and insulin resistance or bone density.

Peptide YY (PYY) is another gut hormone with an anorexigenic effect, secreted from enteroendocrine L-cells of the distal part of gastrointestinal tract [15]. PYY mostly acts through Y2 receptor [16]. Separate studies on Y2 receptor knockout mice and PYY knockout mice revealed that PYY is also involved in the regulation of bone metabolism [9, 17]. However, there is a necessity to explore the association between PYY and bone density in a large population based study.

Therefore, the objectives of this project were: 1) To investigate the association between circulating fasting ghrelin and insulin resistance adjusting for age, gender, percentage of body fat, and HDL- cholesterol in the general population of the Canadian province of Newfoundland and Labrador. 2) To examine the effect of fasting circulating ghrelin and PYY on bone density in the general population, taking into consideration age, BMI, physical activity, smoking, and alcohol consumption as the most important confounding factors for bone density.

In order to address the first objective, 2082 subjects from the Complex Diseases in the Newfoundland population: Environment and Genetics (CODING) study were recruited in our investigation. Serum ghrelin was measured with the ELISA Kit of Spibio-bertin pharma. Serum insulin was measured with the Immulite Immunoassay analyzer. Insulin resistance was determined with the homeostasis model assessment of insulin resistance, β cell function (HOMA-IR, HOMA-β), and quantitative insulin sensitivity check index (QUICKI). Dual-energy X-ray absorptiometry (DXA) was used for the body composition and bone mineral density measurements.

Associations between circulating fasting ghrelin and insulin level, as well as insulin resistance indices, were evaluated with partial correlation analyses adjusting for age, percentage of trunk fat, and HDL-cholesterol. These analyses revealed a negative correlation between ghrelin and insulin resistance indices. The negative effect was not gender specific. Most importantly, this relationship was independent of major confounding factors.

To further explore this unique finding, we stratified our female subjects into pre-menopausal and post- menopausal groups to determine whether menopausal status can affect this association. Interestingly, ghrelin was negatively correlated with insulin resistance only in the pre-menopausal group. These findings suggest that in post-menopausal women other factors such as reduction in sex hormones, increase in body fat, decrease in physical activity, and possible use of medications are more important in determining insulin resistance.

To our knowledge, this is the largest general population based study of its kind with the most comprehensive control of major confounding factors in the analyses. Our results, with a strong statistical power, suggest that ghrelin can attenuate insulin resistance.

For the second objective, data from 2257 volunteers from the CODING study were used. DXA scan was performed for measurement of bone density at the spine, femoral neck, and total hip. Stepwise multiple regression analyses adjusting for age, BMI, physical activity, smoking, and alcohol consumption were employed to explore the relationship between ghrelin and bone mineral density parameters.

Significant positive associations were revealed between ghrelin level and L2-L4 BMD, L2-L4 Z-score, femoral neck BMD, femoral neck Z-score, total hip BMD, and total hip Z-score in women. After stratifying the female group to pre-menopausal and post-menopausal, the favorable association was significant for femoral neck and total hip Z-scores in pre-menopausal group, and L2-L4 BMD and Z-score in post-menopausal group.

Circulating PYY was not significantly associated with bone density either in entire cohort or in men and women separately.

Our results indicate that ghrelin has beneficial effect on femoral and lumbar spine bone mineral densities and Z-scores in females. Additionally, we demonstrated that this protective effect is independent of age, body composition, alcohol consumption, physical activity, and smoking.

It is important to identify our limitations in this project. We performed an observational study and correlation data collected do not prove causality. In order to get a better understanding of the effect of ghrelin on insulin resistance or bone metabolism, longitudinal studies or interventional studies of ghrelin administration in diabetic or osteoporotic patients are necessary. Another limitation of this project is that there are over 20 gut hormones, but we selected only ghrelin and PYY. Therefore, future studies on the role of other gut hormones and interaction between different hormones are warranted.

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Chapter 1 Introduction

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Chapter 3

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Chapter 4

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