Salivary Metabolic Hormones as Biomarkers of Childhood Obesity

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Abstract

Purpose

Obesity is a major threat to the health and development of children. While hormones in serum samples have been extensively investigated in childhood obesity research, our study attempts to explore salivary metabolic hormones as biomarkers of childhood obesity.

Methods

Obese (n=83) and non-obese (n=83) school children aged 6-11 years were recruited as cases and controls, respectively, for this case-control study. Salivary concentrations of the hormones, Ghrelin, Leptin, Adiponectin and Insulin were assessed and compared between obese and non-obese children to evaluate their role in childhood obesity.

Results

The mean ghrelin (1.69 ± 0.38) and adiponectin (95627.56 ± 64915.04) levels of the non-obese children were higher than the mean values of the obese, while the mean values of the hormones leptin (265.95 ± 83.16) and insulin (224.56 ± 184.86) were found to be higher among the obese. Higher levels of the hormones insulin (OR: 1.09; 95% CI: 1.05-1.12) and leptin (OR: 1.03; 95% CI: 1.02-1.04) posed higher risks for childhood obesity, followed by adiponectin, which was found to be lower among the cases (OR: 0.99; 95% CI: 0.99995-0.99998) which increased the risk of childhood obesity.

Conclusion

This study demonstrates the efficacy of saliva as a promising non-invasive tool in childhood obesity research and highlights the significance of appetite regulatory hormones as biomarkers of childhood obesity.

Level of evidence

Level III, Case-control study.

Introduction

Childhood obesity is a global public health burden. It is alarming to see the increasing rates of childhood obesity in developing countries such as India. This is a serious challenge because, on one hand obesity and on the other, stunting and underweight project a major developmental threat; thus there is a double burden of underweight and overweight among Indian children. Childhood subsists to be a sensitive period for neurological, endocrine, and metabolic development. Obesity evolving at a young age contributes to an increased risk of diabetes, hypertension, and cardiovascular disease in adulthood [1].

Obesity is a multifaceted problem with many contributing factors including genetics, hormone levels, overconsumption of food and a sedentary lifestyle. Appetite regulation is a complex process involving communication between the hypothalamus within the brain, various gastrointestinal organs (including stomach, pancreas and intestines) and adipose tissue. Satiation (the signal that causes one to stop eating) may be initiated by neural input from the stomach to the brain, signaling gastric distension after food consumption. This is followed by the release of various hormones that sense the digestion and absorption of nutrients and initiate satiety (the feeling of fullness that persists after eating). The gastrointestinal tract is the largest endocrine organ in the body and plays an important appetite regulatory role as a source of numerous regulatory peptide hormones. When the levels of the hormones inducing hunger increase, the demand for food intake increases, thereby contributing to excess energy storage and increased levels of fat, all of which sum up to the cause of obesity [2].

While most metabolic biomarker analyses in the field of obesity are performed using blood samples, studies on salivary biomarker analysis are scarce. The salivary proteome consists of thousands of proteins, including hormonal mediators of energy balance. Saliva is mainly secreted from three pairs of glands in the oral cavity – parotid, submandibular and sublingual, as well as from other minor accessory glands in the tongue, palate and buccal mucosa. Studies have shown that human salivary glands can produce and release ghrelin and have also observed a significant correlation with serum or plasma ghrelin levels [3]. Saliva could be a boon in pediatric populations considering the parental apprehension towards withdrawal of blood samples from children. Salivary biomarkers have also been reported to be an effective and sustainable alternative because of the ease of tissue access, cost-efficiency and the ability to collect them in multiple settings [4]. In comparison to the collection of blood, saliva is associated with lower infection rates, decreased cost, increased patient acceptance, and higher participant compliance. Saliva also offers insight into the gastrointestinal tract, which could be useful when examining obesity [1].

Accordingly, in our study, we used saliva as the medium for biomarker analysis among obese children. The biomarkers of interest were appetite regulatory hormones such as ghrelin, leptin, adiponectin and insulin. These hormones are crucial mediators of satiety, hunger signals and energy balance, where ghrelin stimulates appetite and leptin decreases it. Although there are several studies on the causes of obesity in both children and adults, little is known about the physiological role of appetite regulatory hormones in humans, especially children. Hence, there is ample scope for research in this area [2]. In this pretext, the framework for our study was developed to evaluate the significance of salivary metabolic hormones as biomarkers of childhood obesity.

Materials And Methods

i) Study Design

A case-control study design was adopted in this study. Obese and non-obese school children of the age group (6-11 years) were the subjects of interest in this study. The levels of salivary metabolic hormones –

Ghrelin, Leptin, Adiponectin and Insulin in obese and non-obese subjects were assessed and compared to determine the significance of these hormones as biomarkers of childhood obesity.

ii) Setting

The study was conducted in an elementary school and a summer campsite in Chennai, India, from July 2017 to March 2018, as part of an extensive research in the area of salivary appetite regulatory hormones and an intervention program targeting these hormones in obese children. Permission was obtained from the concerned authorities of the elementary school and the summer campsite for conducting the study. Anthropometric assessment of all children aged between six and eleven years was carried out at the sample sites to categorize them into obese and non-obese groups. Children who gave written informed parental consent and assent to participate in the study alone were recruited for the study as per the sample size recruitment.

iii) Participants

A total of 1432 children from an elementary school (n=1378) and a summer campsite (n=54) were assessed for childhood obesity, using standard protocols. Of the 1432, 166 children (83 obese and 83 non-obese) were recruited for this study based on inclusion and exclusion criteria and consent to participate in the study.

Inclusion criteria

- Children aged between six and eleven years categorized as normal (control) and obese (case) using the WHO growth reference for school aged children (2007) and
- Children who gave written informed parental consent and assent to participate in the study.

Exclusion criteria

- Children who had an obvious underlying medical cause of obesity and
- Children who had undergone any medical or nutritional therapy for obesity in the past six months.

iv) Tools Used

a) Anthropometry

Anthropometric measurements such as height (cm) and body weight (Kg) of the subjects were assessed. The height-for-age ‘Z’ (HAZ) scores; weight-for-age ‘Z’ (WAZ) scores; and body mass index-for-age ‘Z’ (BAZ) scores of the subjects were calculated using WHO AnthroPlus v.1.0.4 (2009) and the subjects were categorized into obese and non-obese categories based on their BAZ scores.

b) Salivary Analysis

Collection of samples of Saliva

On the day of sample collection, the subjects (in fasting state) were made to rinse their mouth with water before sample collection. Saliva was collected using sublingual cotton roll technique (cotton roll placed under the tongue of the subject for a minute). Using a sterile forceps, the cotton roll was transferred to a 50 mL syringe and injected into a vacutainer tube, to collect approximately two milliliter of saliva from each subject. The samples collected in vacutainer tubes were arranged in a 96-vial storage rack placed in a freezer box. The principle investigator shifted the samples stored in the freezer box to the testing laboratory under safe conditions within 24 hours of sample collection. The samples were stored at -20°C and analyzed within a period of six months.

Multiplex Analysis of Salivary Markers

Magnetic Luminex® Assays were used to assess the concentration of the selected hormones in each sample. The assays (166 saliva samples) for three biomarkers leptin, adiponectin and insulin were performed on 50 µl of saliva sample using premixed 3-plex magnetic bead panels on a Bioplex 200 platform with no dilution. The assay procedure was carried out following the manufacturers' protocol. Saliva samples were thawed every time before assay procedures. The kit components were brought to room temperature and reagents were prepared as instructed (wash buffers, beads, standards, etc.). Assay plates (96-well) with assay buffer, standards, samples and beads were covered and incubated on a horizontal orbital microplate shaker (800±50 rpm) for two hours at room temperature. Plates were washed and detection antibody cocktail was added. The plates were covered and incubated for one hour at room temperature on the shaker set at 800 ± 50 rpm. Streptavidin-phycoerythrin fluorescent reporter was added to all wells and the covered plate was incubated for 30 minutes at room temperature on the shaker set at 800±50 rpm. Plates were washed thrice and beads were resuspended in wash buffer and incubated on the shaker for two minutes at 800 ± 50 rpm. The results were read within 90 minutes and evaluated using an analyzer.

Ghrelin Assay

As inclusion of ghrelin in the premix used for the other markers was not possible, a separate test kit (RayBio® Ghrelin Enzyme Immunoassay (EIA) Kit: EIA-GHR, EIAM-GHR, EIAR-GHR) was used to assess ghrelin in 100 µl of each saliva sample. The assay employed an in vitro quantitative technique for detecting ghrelin peptides based on competitive enzyme immunoassay principle. In this assay, a biotinylated ghrelin peptide was spiked into the samples and standards. The samples and standards were then added to the plate, where the biotinylated ghrelin peptide competed with endogenous (unlabeled) ghrelin for binding to the anti-ghrelin antibody. After a wash step, any bound biotinylated ghrelin then interacted with horseradish peroxidase (HRP)-streptavidin, which catalyzed a color development reaction. The intensity of the colorimetric signal was directly proportional to the amount of captured biotinylated Ghrelin peptide and inversely proportional to the amount of endogenous Ghrelin in the standard and samples. A standard curve of kown concentration of Ghrelin peptide was established and the concentration of Ghrelin peptide in the samples was assessed.

v) Sample size

166 (Obese, Cases = 83; Non-obese, Controls = 83) was the calculated sample size, assuming μ1 = 48.0 and μ2 = 63.1; Difference of means = 24.6 [5] and a Power of 80%. The sample size was calculated using Piface by Russell V. Lenth. Version 1.76 – 29 June 2011.

vi) Statistical analysis

All descriptive statistics are expressed using mean and standard deviation. Independent sample t-test was used to test the mean difference between the control and case groups. Univariate binary logistic regression was used to calculate the risk factor analysis. Odds ratio was calculated to evaluate the odds of salivary metabolic hormones as biomarkers among the obese and non-obese groups and to imply an association between the salivary hormones and childhood obesity. The statistical analyses were done using SPSS version 23.0 and any p - value less than 0.05 was considered as statistically significant.

Results

Of the 1432 children assessed for enrollment in the study, 758 (256 obese and 502 non-obese) fulfilled the inclusion criteria, out of which 592 subjects were excluded from the study due to dissent to participate and randomized selection. Finally, a total of 166 subjects (83 obese and 83 non-obese) participated in the study and there were no drop outs, as the study involved a one-time diagnostic observation. Study report flowchart according to the STROBE (Strengthening the Reporting of Observational Studies in Epidemiology) is presented in Figure 1. The mean baseline characteristics of the subjects are mentioned in table 1. The results of the study show that the mean BAZ of the subjects belonging to the control group was found to be – 0.13 ± 0.93 and that of the cases was found to be 2.11 ± 0.28 (p value <0.001).

Figure 2 represents the mean values of salivary metabolic hormones among the subjects, pictorially. The mean ghrelin (1.69 ± 0.38) pg/ml and adiponectin (95627.56 ± 64915.04) pg/ml levels of the non-obese group were higher than the mean values of the obese, while the mean values of the hormones leptin (265.95 ± 83.16) pg/ml and insulin (224.56 ± 184.86) pg/ml were found to be higher among the obese group when compared to the non-obese controls. The statistical evaluation of mean difference of salivary metabolic hormones between the obese and non-obese groups revealed that there was a significant difference in the concentration of all the salivary biomarkers (P <0.001).

The odds ratio is a measure of relative risk and is usually calculated by dividing the odds of exposure among the cases by the odds of exposure among the controls [6]. In this study, the exposure was considered to be salivary metabolic hormone concentrations, in order to investigate the odds of these hormones as risk factors for childhood obesity. The results of the univariate binary logistic regression analysis highlighted the significance of salivary metabolic hormones as biomarkers of childhood obesity. It reveals that, higher levels of the hormones insulin (OR: 1.09; 95% CI: 1.05-1.12) and leptin (OR: 1.03; 95% CI: 1.02-1.04) pose higher risks for childhood obesity, followed by adiponectin, which was found to be lower among the cases (OR:0.99; 95%CI:0.99995-0.99998) increasing the risk for childhood obesity (Table 2).

Discussion

The gut–brain axis harbors a pivotal role in the regulation of food intake and the maintenance of body weight. A complex array of signals from peripheral and central nervous systems, likely under epigenetic programming influences psychological and social factors to determine energy balance and body weight homeostasis [7]. The cluster of hormones that regulate appetite and food intake is wide-ranging. When the levels of the hormones inducing hunger increase, the demand for food intake increases, thereby contributing to excess energy storage and increased levels of fat, all of which sum up to the cause of obesity. Belfort-DeAguiar and Seo (2018) concluded that, obese individuals have elevated insulin and leptin levels and decreased ghrelin levels in comparison to normal weight individuals [8]. They have affirmed that insulin and leptin levels parallel body weight status, and insulin and leptin resistance play an indicative role in the pathogenesis of obesity. Thanakun et al., (2014) and Li et al., (2010) found significant correlation between salivary and serum ghrelin and adiponectin levels, suggesting salivary ghrelin could be a possible alternative to serum ghrelin as a biomarker in predicting the risk of childhood obesity [9,10].

In our study we observed a significant difference in the concentration of salivary metabolic hormones among the obese and non-obese subjects. Several studies on serum analysis of appetite hormones highlight that obese children show significant lower adiponectin and ghrelin concentrations and higher insulin and leptin levels [11-13]. The findings of the study conducted by Goodson et al., (2014) on metabolic disease risk in children using salivary biomarkers were consonant with the results of ours, presenting lower levels of ghrelin and adiponectin in the obese group when compared to the normal subjects and higher levels of insulin and leptin in the obese group [14].

The mechanism behind the downregulation of ghrelin in obese children could be attributed to their elevated leptin or insulin levels, as studies show that fasting ghrelin levels negatively correlate with fasting insulin and leptin levels. This state represents an adaptation towards positive energy balance and increased weight gain in these children [15]. Decreased concentration of adiponectin among the obese children in our study is similar to the findings of many studies carried out in the serum samples of obese children [16-18]. Adiponectin may be one of the signals linking inflammation and obesity. Soliman et al., (2012) suggest that in a majority of obese individuals, serum leptin concentrations are increased and leptin administration shows only very limited effects due to leptin resistance [19]. Leptin resistance is associated with insulin resistance and abdominal obesity. Increased appetite is associated with altered levels of appetite regulatory hormones and thus, these hormones are identified as potential neuroendocrine markers and mediators in childhood obesity, as insisted by Hagen et al., (2015) [20].

Strengths and Limits

This study is one of its kind to explore the probability of using saliva as a promising tool of analysis in the fields of endocrinology and metabolomics, particularly in children, considering the scarce evidence available in this research area in India.

Due to the difficulty in obtaining permission to conduct the study in many schools, the study was conducted only at two sites resulting in sampling bias. The study did not perform a matched case-control study.

What is already known in this subject?

Role of circulating appetite regulatory hormones is well established in obesity. The levels of these hormones vary among obese and non-obese individuals.

What this study adds?

Role of salivary metabolic hormones in obesity is less explored in India, particularly in children. This study avouches the efficacy of saliva, a non-invasive tool in understanding the causal mechanisms of childhood obesity.

Conclusion

Despite the need for robust research in childhood obesity, the practical challenges and risks in including children being vulnerable groups, as subjects, limit the scope of clinical research in this field. While difficulty in obtaining permissions from authorities and parents for using invasive techniques like blood withdrawal are on one hand, on the other, convincing children to such invasive methods pose a great challenge. Such difficulties form one of the major reasons for dropouts from the study. Hence, the conquest for reliable, sustainable and effective non-invasive tools of clinical research is of dire need. Saliva seems to be a promising non-invasive tool to widen the scope of clinical research in childhood obesity. This study was an attempt to understand appetite regulating salivary metabolic hormones as biomarkers of childhood obesity. The present study has thrown light on the possibility of considering salivary samples in the evaluation of metabolic hormones, which may further enable us to intervene and prevent or treat the onset or incidence of childhood obesity.

Declarations

Funding

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Conflicts of interest

The authors declare that there is no conflict of interest.

Availability of data and material

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Code availability

Not applicable.

Authors' Contributions

Shiny Lizia M. conducted the study, analysed the data and wrote the manuscript. Dr.Hemamalini A.J. and Dr. Latha Ravichandran corrected and revised the article critically for important intellectual content and approved the version to be published.

Ethics approval

All procedures performed in this study were in accordance with the ethical standards of the institutional and national research committee (Indian Council of Medical Research) and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The study was approved by the Institutional Ethics Committee of Sri Ramachandra Institute of Higher Education and Research (IEC-NI/15/FEB/45/07).

Consent to participate

Written informed consent was obtained from the parents of all individual subjects included in the study.

Consent for publication

Not applicable.

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Tables

Table 1

Mean Baseline Characteristics of the Subjects

Characteristics	Groups (N=166)		p-value
	Obese (Case) (Mean ± S.D.) (n=83)	Non-obese (Control) (Mean ± S.D.) (n=83)	
Age	8.67 ± 1.09	8.61± 1.03	0.716
Height	133.76 ± 6.23	135.07 ± 7.14	0.211
Weight	38.74 ± 5.59	29.62 ± 5.73	0.000**
WAZ	1.72 ± 0.40	0.07 ± 0.94	0.000**
HAZ	0.12 ± 0.46	0.40 ± 0.75	0.024*
BAZ	2.11 ± 0.28	– 0.13 ± 0.93	0.000**
**P<0.01; *P<0.05			

Table 2

Salivary Metabolic Hormones as Biomarkers of Childhood Obesity (N=166)

Variables	Odds Ratio	p-value
Age	1.06 (0.79-1.41)	0.714
Gender - Female	1 (0.55-1.84)	1.000
Height	0.97 (0.93-1.02)	0.211
Weight	1.33 (1.22-1.45)	0.000**
WAZ	132.99 (20.86-847.86)	0.000**
HAZ	0.47 (0.28-0.80)	0.005*
Ghrelin	0.001 (0-0.01)	0.000**
Leptin	1.03 (1.02-1.04)	0.000**
Adiponectin	0.99 (0.99995-0.99998)	0.000**
Insulin	1.085 (1.054-1.117)	0.000**
**P<0.01; *P<0.05		

Figures

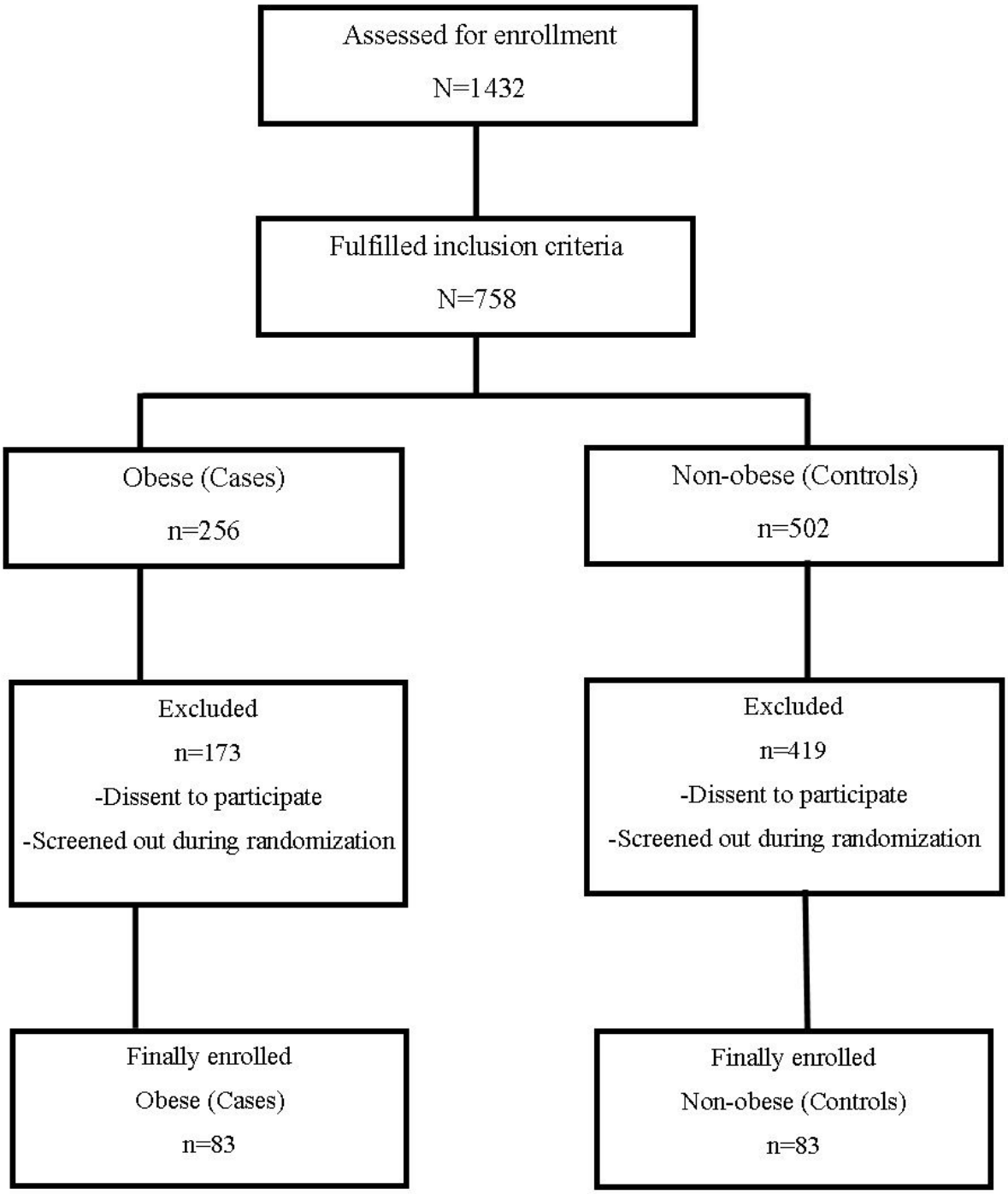

Figure 1

STROBE Flowchart

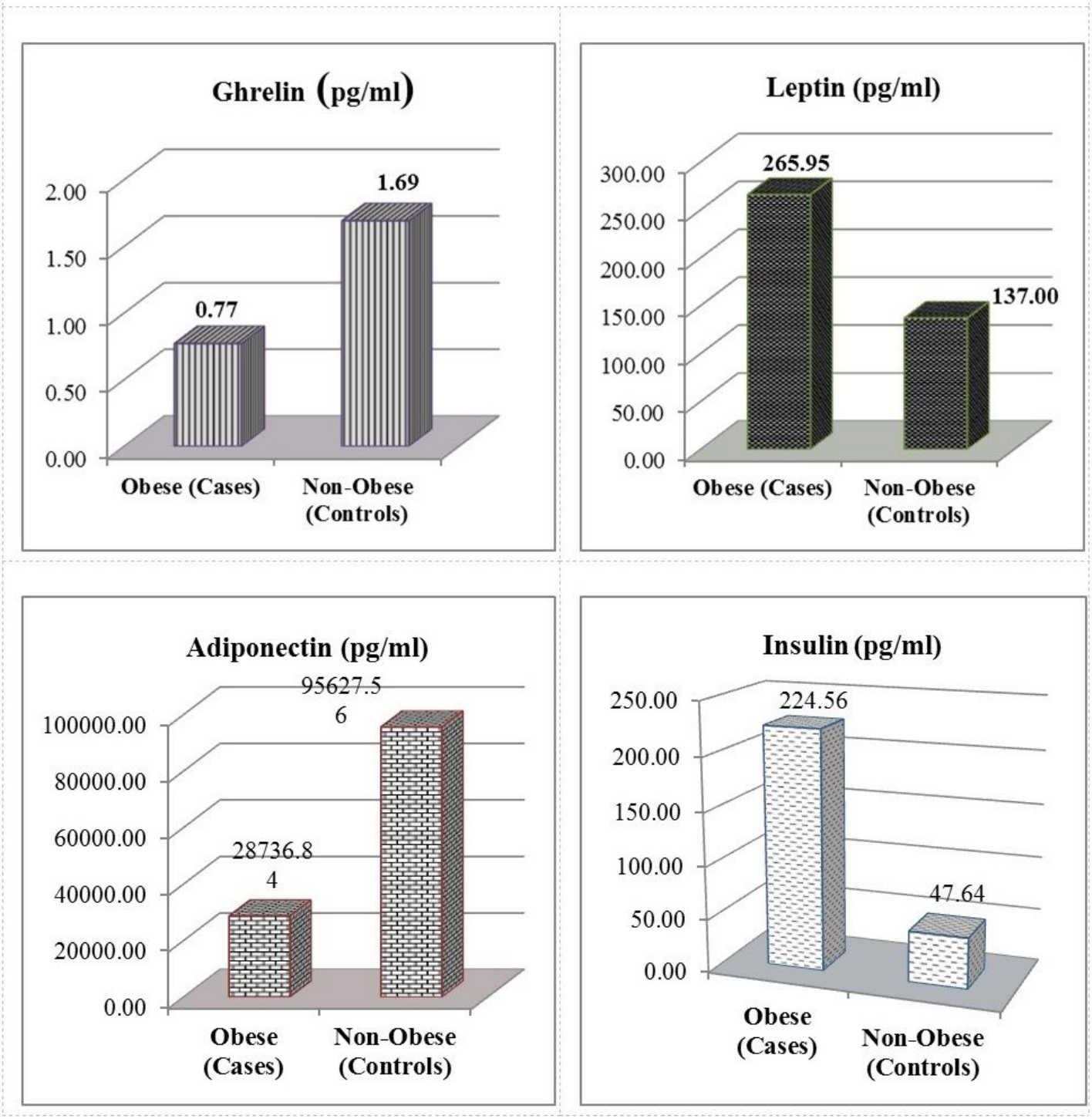

Figure 2

Mean Concentrations of Salivary Metabolic Hormones among the Subjects