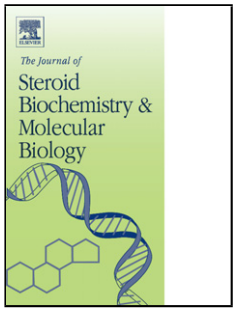

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PII: S0960-0760(18)30480-1
DOI: https://doi.org/10.1016/j.jsbmb.2018.11.004
Reference: SBMB 5250

To appear in: *Journal of Steroid Biochemistry & Molecular Biology*

Received date: 13 August 2018
Revised date: 14 October 2018
Accepted date: 5 November 2018

Please cite this article as: Yuzbashian E, Asghari G, Hedayati M, Zarkesh M, Mirmiran P, Khalaj A, Determinants of vitamin D receptor gene expression in visceral and subcutaneous adipose tissue in non-obese, obese, and morbidly obese subjects, *Journal of Steroid Biochemistry and Molecular Biology* (2018), https://doi.org/10.1016/j.jsbmb.2018.11.004

Determinants of vitamin D receptor gene expression in visceral and subcutaneous adipose tissue in non-obese, obese, and morbidly obese subjects

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Running title: Determinants of VDR gene expression in fat depots

Highlights

- The determinative factors on the visceral and subcutaneous adipose tissue VDR gene expression varied and depended on the degree of obesity.
- Serum 25(OH)D was the common factor to determine VDR gene expression visceral adipose tissue in all strata of BMI groups.
- BMI and HOMA-IR were the positive predictors of subcutaneous adipose tissue VDR gene expression

Abstract

We aimed to illustrate determinants of VDR gene expression in visceral and subcutaneous adipose tissue among individuals without diabetes. We gathered visceral and subcutaneous adipose tissues during an elective abdominal surgery form 33 morbidly obese (BMI >40 kg/m2), 23 obese (BMI=30–40 kg/m2), and 35 non-obese (BMI<30kg/m2) participants who were free of diabetes. Participants were classified according to their degree of obesity. Before the surgery, habitual dietary intake, physical activity, 25(OH)D, body mass index (BMI), waist circumference (WC), and HOMA-IR was gathered. Non-obese participants had significantly lower mean VDR gene expression in visceral adipose tissues than both the obese and morbidly obese ones and had also lower expression in subcutaneous adipose tissues than the morbidly obese participants. In multiple linear regression models, BMI and HOMA-IR were the independent positive predictors of VDR gene expression in subcutaneous fat. Among non-obese subjects, WC and 25(OH)D were the positive and negative independent predictors of visceral adipose tissue VDR gene

expression, respectively. Among obese participants, 25(OH)D was negatively, and BMI and HOMA-IR were positively associated with VDR mRNA levels in visceral adipose tissue. In morbidly obese participants, the independent positive predictors of VDR gene expression in visceral fat were BMI and HOMA-IR, and negative predictors were 25(OH)D and calcium intake. Our findings suggested that 25(OH)D concentrations are the fundamental elements to determine VDR gene expression in visceral fat which by increasing fat depots, the subsequent insulin resistance became another predictor of VDR gene expression.

Abbreviations

25(OH)D, 25-hydroxyvitamin D

BMI, body mass index

SBP, systolic blood pressure

DBP, diastolic blood pressure

VDR, vitamin D receptor

HOMA-IR, Homeostatic Model Assessment for Insulin Resistance

Keywords: VDR, 25(OH)D, dietary intake

Introduction

The active involvement of adipose tissue on energy balance and glucose homeostasis has been well established [1]. Adipose tissue is also the main site for vitamin D depots [2] as well as

expresses the vitamin D receptor (VDR) [3] and other enzymes involving in the metabolism of vitamin D [4]. Recent studies have demonstrated that vitamin D contributes to the adipogenic gene expression as well as involves in various physiological processes such as cell proliferation, differentiation, apoptosis, and angiogenesis. Besides, vitamin D can affect directly the expression of adipokines including leptin and adiponectin and reduce adipose tissue cytokine and inflammation release [5, 6]. The function of vitamin D in adipose tissue is mediated through the VDR which regulates many target proteins and genes [7] and is considered as a mechanistic basis for the connection between vitamin D deficiency and a number of disorders, including, diabetes (type 1 and type 2), metabolic syndrome, inflammatory bowel disease, CVD, and certain types of cancer [8-10].

VDR gene expression in adipose tissue pertains to the weight status, so that, it has been reported that VDR mRNA levels in adipose tissue were higher among obese individuals than lean ones [11]. Besides, low concentration of 25-hydroxyvitamin D (25(OH)D) has an association with increased fat mass and body mass index (BMI) [12]. It should be noted that the adipose tissue VDR gene expression in response to the vitamin D supplementation depends on the BMI status [13]. Therefore, these findings indicate that body weight is an important factor which is independently caused distinction in VDR gene expression in adipose tissue.

Glucose hemostasis might also have a crucial role in the adipose tissue VDR mRNA levels [11]. Patients with diabetes had a dramatically higher expression of VDR from fat depots than healthy individuals [11]. In this regard, studies that have examined VDR expression levels according to type 2 diabetes induced by obesity failed to illustrate the impact of insulin levels changes and insulin resistance because most obese patients have diabetes with already altered glucose metabolism. This should be considered to distinguish whether different VDR gene expression is

associated with obesity by itself or whether it is a repercussion of insulin levels changes and insulin resistance.

Other important factors contribute to the 25(OH)D status which might directly or indirectly affect VDR gene expression in adipose tissue are dietary calcium intake, lipid profiles, anthropometrics, and physical activity [14, 15]. The contribution of these and other biological, dietary and lifestyle factors may vary among subjects with different weight status. Therefore, investigating individuals free of diabetes and based on their weight status would be illustrative to unravel the effect of related factors. Besides, factors determining the VDR gene expression changes in adipose tissue are not fully elucidated. To estimate VDR mRNA levels determinants, we needed to investigate multiple predictors together as predictive factors. In the current study, we aimed to assess determinants of VDR gene expression in visceral and subcutaneous adipose tissue among individuals without diabetes.

Materials and Methods

Participants

Current cross-sectional study comprised 91 participants aged ≥20, underwent minor abdominal surgery with minimal impact on dietary intakes at the Mostafa Khomeini Hospital and Khatam Al-Anbia Hospital, Tehran, Iran, and classified according to their BMI as 33 individual with morbidly obese (MO; BMI >40 kg/m^2), 23 individuals with obesity (BMI=30–40 kg/m^2), and 35 non-obese individuals (BMI<30kg/m2). The eligibility criteria considered to recruit participants were hospitalization less than 3 days, free of diagnosed diabetes mellitus or cancer, not using any lipid-lowering or anti-obesity medications, not pregnant or lactating, and not on any special diets for at least three months. After an overnight fasting, blood samples, anthropometric and

demographic indices, physical activity levels, and dietary intakes were obtained before surgery. During the surgery, approximately 100 mg of subcutaneous and visceral adipose tissues were collected. Samples were obtained by an expert general surgeon.

Ethics approval was obtained from the ethics committee of the Research Institute for Endocrine Sciences (RIES) of the Shahid Beheshti University of Medical Sciences (NO: IR.SBMU.ENDOCRINE.REC.1395.171) and the study was conducted in accordance with the Declaration of Helsinki and RIES institutional guidelines. Written informed consent was obtained from all participants.

Dietary Measurements

Regular dietary intake of each participant was assessed by an expert interviewer using a valid and reliable semi-quantitative food frequency questionnaire (FFQ) [16, 17]. We used the United States Department of Agriculture (USDA) food composition table (FCT) to analyze food and beverages. Total energy and calcium intake were collected. The reliability and validity of the FFQ, evaluated in a previous study against twelve 24-h dietary recalls and biomarkers, indicated that it provides reasonably valid measures of the average long-term dietary intakes [16, 17].

Anthropometric and Laboratory Measurements

Weight was measured in light clothing to the precision of 0.1 kg on a SECA digital weighing scale (Seca 707; Seca Corporation, Hanover, Maryland; range 0.1-200 kg) and height was measured without shoes to the nearest 0.1 cm. Body mass index (BMI) was calculated as weight (kg) divided by square of height (m^2). Physical activity during interviews was assessed, using the long forms of the reliable and validated Persian version of the International Physical Activity

Questionnaire (IPAQ) [18]. In order to measure energy expenditure, the concept of metabolic equivalents (MET) was used.

Blood samples were collected from all participants who had fasted a 10–12 h overnight. Fasting plasma glucose (FPG) was measured using an enzymatic colorimetric method with glucose oxidase. Inter- and intra-assay coefficients of variation (CV) were both 1.0% for FPG. Triglyceride (TG) levels were determined using the enzymatic colorimetric method with glycerol phosphate oxidase. Inter- and intra-assay CV for TGs were 0.4 and 2.1%, respectively. Total cholesterol (TC) was assayed with the cholesterol esterase and cholesterol oxidase method; inter- and intra-assay CVs were 0.5 and 1.7, respectively. Measurements of FPG, TC, and TGs were performed using commercial kits (Pars Azmoon Inc., Tehran, Iran). Insulin was measured using the enzyme-linked immunosorbent assay (ELISA) with Mercodia kits (Uppsala, Sweden). Inter- and intra-assay CVs of insulin were 1.7 and 2.3, respectively. Insulin resistance was calculated as the following definition:

Homeostatic model assessment of insulin resistance (HOMA-IR) = [fasting insulin (μU/ml) × fasting glucose (mmol/l)]/22.5.

25(OH)D concentrations were determined by the ElectroChemiLuminescence immunoassay (ECLIA) method, using Roche Diagnostics kits. Intra- and inter-assay CVs were <7.5 for 25(OH) D.

Quantitative Real-time Polymerase Chain Reaction Analysis of Gene Expression

Total RNA was extracted from fresh snap-frozen visceral and subcutaneous fat tissues using TRIzol reagent (Invitrogen U.S. Cat. No. 15596-026) according to the manufacturer's protocol. RNA quantity and purity was assessed by NanoDrop spectrophotometer (Thermo Fisher

Scientific, Waltham, USA) and the ratio of absorption (260/280 nm) of all preparations was in an acceptable range.

Total RNA was treated with DNaseI in order to remove traces of genomic DNA before complementary DNA (cDNA) synthesis. The cDNA synthesis kit (Thermo Scientific, USA) was used according to the manufacturer's instructions.

The amplification condition of VDR templates was optimized by quantitative reverse transcriptase real-time PCR (qRT-PCR) using the Rotor-Gene 6000 (Corbett Research, Sydney, Australia) with annealing at 60°C. Real-time quantification was monitored by measuring the fluorescence activity. Human GAPDH gene was used as internal control to normalize mRNA levels. All experiments were repeated twice. Primers were designed from the sequences of the human cDNA as following: VDR Forward: 5′- ATA CCA GGA TTC AGA GAC C -3′; VDR Reverse: 5′- GAC TCA TTG GAG CGC AA -3′; GAPDH Forward: 5′-CTG CTC CTC CTG TTC GAC AGT-3′; GAPDH Reverse: 5′-CCG TTG ACT CCG ACC TTC AC-3′. PCR amplification was performed in 20 µL volumes, using the SYBR Green master mix (BioFACT, Korea). The relative expression of VDR in each sample was calculated based on its threshold cycle (Ct), normalized to the Ct of the reference gene. All qPCR laboratory procedures were performed according to the MIQE guidelines [19].

Statistical analysis

All data were analyzed using the Statistical Package for the Social Sciences program (SPSS) (version 15.0; SPSS Inc, Chicago IL) and *P*-values <0.05 were considered statistically significant. Continuous variables were described as mean±standard deviation. Comparisons between the study groups were made with ANOVA and LSD post hoc tests. The relationship between VDR gene expression and anthropometric and metabolic variables were examined by

calculating Pearson correlation coefficients. Multiple linear regression models were used to examine the predictors of VDR gene expression among weight-based categorized participants after considering covariates including age, BMI, 25(OH) D concentration, WC, HOMA IR, calcium intake, and physical activity.

Results

The mean age of the total participants was 41.0 years (Non-obese, obese, and morbid obese was 44.3, 42.3, and 36.7 years respectively). There was no significant difference of 25(OH)D levels between groups. Non-obese participants had significantly lower mean VDR gene expression in visceral adipose tissues than both the obese (0.55 vs. 3.83, $p<0.001$) and morbidly obese (0.55 vs. 5.01, $p<001$) ones. Besides, Non-obese subjects had also lower VDR gene expression in subcutaneous adipose tissues than the morbidly obese (2.10 vs. 3.92. $p=0.049$) participants (Figure 1). There was a significant correlation between BMI and VDR gene expression in visceral (r=0.589, $p<0.001$) and subcutaneous (r=0.315, $p=0.002$) adipose tissue in the whole sample (Figure 2).

Anthropometric and clinical characteristics of non-obese participants and their correlation with VDR gene expression in visceral and subcutaneous adipose tissues are detailed in Table 1. Among non-obese participants, serum vitamin D had a significant negative correlation with visceral (r=-0.433) VDR gene expression. VDR gene expression in subcutaneous adipose tissue was not correlated with any considered variables.

VDR mRNA levels in visceral adipose tissues were positively correlated with BMI (r=0.477), fasting insulin (r=0.585), HOMA-IR (r=0.571), and physical activity (r=0.551) and negatively correlated with serum vitamin D (r=-0.415). In addition, in visceral adipose tissues, VDR mRNA

levels had positive correlation with BMI (r=0.489), fasting insulin (r=0.591), HOMA-IR (r=0.624), and physical activity (r=0.536) (Table 2).

Among morbidly obese participants, in both visceral and subcutaneous fat depots, VDR gene expression was positively correlated with BMI, fasting insulin, HOMA-IR. Besides, in visceral adipose tissue, VDR gene expression was negatively correlated with serum vitamin D (r=-0.380) and calcium intake (r=-0.359) and positively correlated with physical activity (r=0.351) (Table 3).

In multiple linear regression models, among non-obese individuals, WC (β=0.145, P=0.024) and serum vitamin D (β=-0.105, p=0.004) contributed independently to visceral adipose tissue VDR gene expression after controlling for age, BMI, HOMA-IR, calcium intake and physical activity. In addition, serum vitamin D (β=-0.103, p=0.036) was the only determinant of visceral adipose tissue VDR gene expression; however, BMI and HOMA-IR were independently positively associated with VDR gene expression in subcutaneous adipose tissue among obese participants. Furthermore, among morbidly obese participants, BMI, HOMA-IR, serum vitamin D, and calcium intake were the independent determinative factors for VDR mRNA levels in visceral fat depots, and BMI and HOMA-IR were determinants of VDR mRNA levels in subcutaneous fat (Table 4).

Discussion

To best of our knowledge, current investigation is the first cross-sectional study to assess the predictors of VDR gene expression in adipose tissue when non-diabetic participants in wide ranges of BMI values taking into account whether the subjects were non-obese, obese, and morbidly obese. This approach, avoided the interaction of obesity and diabetes with VDR gene

expression, showed that the determinants of VDR gene expression in individuals were related to their amount of excess weight but not glycemic status. Interestingly, serum 25(OH)D was the common factor to determine VDR gene expression visceral adipose tissue in all strata of BMI groups. In line to our finding, an experimental study showed that higher 25(OH)D among obese rats down-regulates VDR gene expression in visceral adipose tissue [20]. However, Nguyen et al investigating among premenopausal women with BMI>35 kg/m^2 showed that there was no significant association between serum 25(OH)D and VDR mRNA expression in human visceral adipose tissue [15]. In the that study, most of the population were vitamin D sufficient, while in the current study, we recruited a population with a broad spectrum of 25(OH)D concentration. Our finding is explained by the fact that down-regulation of VDR gene expression is a response to higher vitamin D concentration. However, contrary to our finding, there were positive associations between 25(OH)D concentration and expression of VDR in vascular endothelial cells [21] and swine epicardial adipose tissue [22]. This discrepancy might be because of the tissue-specific influence of 25(OH)D concentration on the expression of VDR.

Except for non-obese group, BMI and HOMA-IR were the positive predictors of subcutaneous adipose tissue VDR gene expression independent of age, WC, 25(OH)D concentration, calcium intake, and physical activity. Besides, VDR gene expression in visceral adipose tissue in both obese and morbidly obese participants was associated with BMI and insulin resistance. Consistent with ours, adipose tissue VDR gene expression was positively associated with HOMA-IR among non-African American obese women [15]. In addition, overweight and obese individuals with insulin resistance or diabetes indicated higher VDR gene expression in visceral adipose tissue than those with normoglycemia [11]. Furthermore, our study illustrated higher BMI had a positive correlation with VDR gene expression in both adipose tissues. In this

regards, evidence ascertains that obesity is accompanied by lower 25(OH)D levels [12, 23]. Clemente-Postigo et al reported that VDR gene expression in visceral fat was significantly higher in morbidly obese compared to the other groups with a lower BMI, and correlated positively with BMI [11]. Altogether, these findings highlighted the pivotal role of insulin resistance on the VDR gene expression in adipose tissue which might be as a consequence of obesity. The positive association of VDR gene expression in adipocyte to insulin resistance among patients with excess weight might be a response to insulin action improvement mediated by vitamin D directly through the presence of VDRs in adipose tissue by stimulation of peroxisome proliferator activator receptor-δ (PPAR- δ) [24] and a β-Oxidation-related transcription factor [25].

Among morbidly obese participants, findings demonstrated that in addition to the positive association of insulin resistance and excess weight, the negative association of serum 25(OH)D and calcium intake were predictors of VDR mRNA levels in visceral adipose tissue. The negative impact of calcium-enriched diet on VDR gene expression of visceral fat depots had been shown in an experimental study on rats [14]. Higher intake of calcium leads to lower VDR mRNA levels in fat depots, could be the result of a decreased glucocorticoid action in adipocyte since this hormone stimulates VDR gene expression [26].

Some limitations of this investigation need to be mentioned. Due to the cross-sectional nature of the study design, causal inferences cannot be made. The assessment of insulin sensitivity was based on HOMA-IR, calculated from fasting blood glucose and insulin concentrations; however, the gold standard for investigating and quantifying insulin resistance is hyperinsulinemic–euglycemic clamp.

The strengths of the current study include, this being the first study to provide data on the determinants of VDR gene expression in adipose tissue among participants with a wide range of BMI. Besides, in the current study VDR mRNA level was directly measured in both visceral and subcutaneous adipose tissue. Furthermore, all covariates were gathered during an interview instead of self-report which it can increase the credibility of the data.

Serum 25(OH)D was the main determinants of VDR gene expression in visceral adipose tissue beyond the degree of obesity. In addition to 25(OH)D concentrations, in morbidly obese participants, VDR gene expression in visceral adipose tissue was positively associated with BMI and HOMA-IR and negatively associated with calcium intake and 25(OH)D concentrations. Our findings suggested that 25(OH)D concentrations are the fundamental elements to determine VDR gene expression in visceral fat which by increasing fat depots, the subsequent insulin resistance became another predictor of VDR gene expression. Further studies will be required to unravel the physiological consequences of the different adipose tissue response of VDR gene expression depending on the degree of obesity and its relevance in clinical practice, as well as to confirm the role of VDR in glucose hemostasis.

Acknowledgment

We would like to acknowledge Ms. Niloofar Shiva for a critical edition of English grammar and syntax of the manuscript.

Support and Financial Disclosure Declaration

On behalf of all authors, the corresponding authors hereby declare that there is no conflict of interest

Figure 1- Vitamin D receptor (VDR) mRNA expression in visceral and subcutaneous adipose tissues in non-obese, obese, and morbidly obese. Results are expressed as mean±SEM; One-way ANOVA with post-hoc LSD test was used to compare group means.

Figure 2- Correlation between vitamin D receptor (VDR) gene expression in visceral and subcutaneous adipose tissues and body mass index (BMI). (A) Correlation of VDR gene expression in visceral fat with BMI, and (B) Correlation of VDR gene expression in subcutaneous fat with BMI with subcutaneous.

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Table 1- Anthropometric and metabolic parameters of non-obese participants and their correlation coefficients to vitamin D receptor gene expression in visceral and subcutaneous adipose tissue

		Visceral		Subcutaneous	
	Mean±SD	r	P value*	r	P value*
Age (years)	44.26±15.7	-0.102	0.558	0.099	0.571
Body mass index (kg/m^2)	24.5±2.9	0.092	0.600	0.171	0.326
Waist circumference (cm)	87.7±10.9	0.148	0.397	0.087	0.618
Fasting glucose (mg/dl)	86.4±10.4	-0.142	0.416	0.037	0.831
Fasting insulin (μU/ml)	7.22±7.50	0.301	0.076	0.275	0.831
HOMA.IR	1.56±1.51	0.299	0.081	0.303	0.077
Total cholesterol (mg/dl)	166.8±46.4	0.076	0.663	0.249	0.149
Fasting triglycerides (mg/dl)	79.5±51.6	-0.026	0.882	0.059	0.737
Serum vitamin D	15.16±15.7	-0.433	0.009	-0.197	0.256
Calcium intake (g/d)	811.4±409.9	0.009	0.960	0.264	0.125
Physical activity (h/week)	36.5±60.4	-0.042	0.809	0.041	0.816

* Pearson correlation

Table 2- Anthropometric and metabolic parameters of obese participants and their correlation coefficients to vitamin D receptor gene expression in visceral and subcutaneous adipose tissue

		Visceral		Subcutaneous	
	Mean±SD	r	P value*	r	P value*
Age (years)	42.3±14.0	-0.144	0.513	-0.168	0.442
Body mass index (kg/m^2)	34.6±4.0	0.477	0.021	0.489	0.018
Waist circumference (cm)	105.4±13.3	0.119	0.588	0.248	0.255
Fasting glucose (mg/dl)	87.1±9.8	0.105	0.635	0.255	0.241
Fasting insulin (μU/ml)	12.8±15.0	0.585	0.003	0.591	0.003
HOMA.IR	3.3±4.1	0.571	0.004	0.624	0.001
Total cholesterol (mg/dl)	211.8±41.9	0.103	0.640	0.033	0.882
Fasting triglycerides (mg/dl)	101.3±54.9	0.100	0.650	0.186	0.394
Serum vitamin D	13.8±10.2	-0.415	0.049	-0.340	0.113
Calcium intake (g/d)	1013.4±522.5	0.118	0.591	0.132	0.549
Physical activity (h/week)	33.6±42.4	0.551	0.006	0.536	0.008

* Pearson correlation

Table 3 - Anthropometric and metabolic parameters of morbid obese participants and their correlation coefficients to vitamin D receptor gene expression in visceral and subcutaneous adipose tissue

		Visceral		Subcutaneous	
	Mean±SD	r	P value*	r	P value*
Age (years)	36.7±11.2	-0.074	0.683	0.018	0.922
Body mass index (kg/m^2)	47.3±4.3	0.515	0.002	0.461	0.007
Waist circumference (cm)	133.2±12.4	0.409	0.018	0.276	0.119
Fasting glucose (mg/dl)	88.0±12.4	0.258	0.155	0.270	0.129
Fasting insulin (μU/ml)	15.1±13.3	0.474	0.005	0.434	0.012
HOMA.IR	4.19±4.94	0.493	0.004	0.438	0.011
Total cholesterol (mg/dl)	181.8±31.4	-0.008	0.963	-0.046	0.798
Fasting triglycerides (mg/dl)	86.6±53.9	-0.050	0.780	-0.161	0.798
Serum vitamin D	13.9±10.0	-0.380	0.029	-0.228	0.202
Calcium intake (g/d)	1021.2±411.1	-0.359	0.040	-0.160	0.373
Physical activity (h/week)	37.5±46.2	0.351	0.045	0.256	0.151

* Pearson correlation

Table 4- Multiple linear regression analysis using vitamin D receptor gene expression in visceral and subcutaneous adipose tissue as dependent variable in non-obese, obese, and morbidly obese participants

	Visceral		Subcutaneous	
	β	P	β	P
Non-obese				
Age	0.025	0.474	0.048	0.322
Body mass index	0.398	0.084	0.426	0.170
Waist circumference	0.145	0.024	0.092	0.274
HOMA IR	0.461	0.182	0.899	0.061
Serum vitamin D	-0.105	0.004	-0.048	0.301
Calcium intake	0.088	0.500	0.365	0.057
Physical activity	-0.003	0.698	0.005	0.642
Obese				
Age	0.015	0.682	0.031	0.430
Body mass index	0.292	0.052	0.358	0.028
Waist circumference	-0.012	0.774	-0.023	0.600
HOMA IR	0.285	0.052	0.382	0.018
Serum vitamin D	-0.103	0.036	-0.083	0.100
Calcium intake	0.028	0.756	0.059	0.538
Physical activity	0.020	0.143	0.018	0.221
Morbid Obese				
Age	0.023	0.611	0.043	0.537
Body mass index	0.309	0.007	0.363	0.037
Waist circumference	0.014	0.757	0.005	0.944
HOMA IR	0.362	0.004	0.379	0.034
Serum vitamin D	-0.114	0.042	-0.093	0.274
Calcium intake	-0.307	0.008	-0.177	0.300
Physical activity	-0.008	0.554	-0.014	0.493

Figure 1-

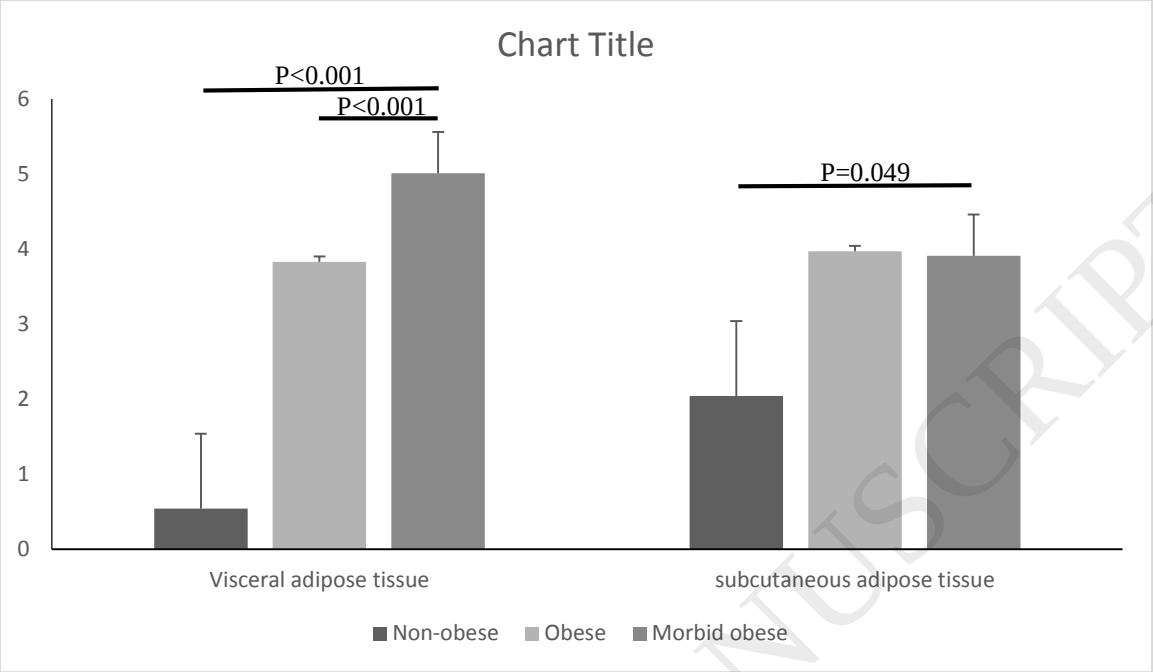

Figure 2-