Analyses of IGFBP2 DNA methylation and mRNA expression in visceral and subcutaneous adipose tissues of obese subjects

Zhang, Xiuli; Gu, Harvest F.; Frystyk, Jan; Efendic, Suad; Brismar, Kerstin; Thorell, Anders

Published in:
Growth Hormone and IGF Research

DOI:
10.1016/j.ghir.2019.03.002

Publication date:
2019

Document version
Accepted manuscript

Document license
CC BY-NC-ND

Citation for published version (APA):
Accepted Manuscript

Analyses of IGFBP2 DNA methylation and mRNA expression in visceral and subcutaneous adipose tissues of obese subjects

Xiuli Zhang, Harvest F. Gu, Jan Frystyk, Suad Efendic, Kerstin Brismar, Anders Thorell

PII: S1096-6374(19)30014-0
DOI: https://doi.org/10.1016/j.ghir.2019.03.002
Reference: YGHIR 1274
To appear in: Growth Hormone & IGF Research

Received date: 8 October 2018
Revised date: 18 March 2019
Accepted date: 19 March 2019

Please cite this article as: X. Zhang, H.F. Gu, J. Frystyk, et al., Analyses of IGFBP2 DNA methylation and mRNA expression in visceral and subcutaneous adipose tissues of obese subjects, Growth Hormone & IGF Research, https://doi.org/10.1016/j.ghir.2019.03.002

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.
Analyses of IGFBP2 DNA methylation and mRNA expression in visceral and subcutaneous adipose tissues of obese subjects

Xiuli Zhang¹, Harvest F. Gu²*, Jan Frystyk³,⁴, Suad Efendic⁵, Kerstin Brismar⁵,⁶, Anders Thorell⁷,⁸

¹Department of Nephrology, The Second People's Hospital of Shenzhen, The First Affiliated Hospital of Shenzhen University, Guangdong 518000 China
²Center for Pathophysiology, School of Basic Medicine and Clinical Pharmacy, China Pharmaceutical University, Nanjing 210009 China
³Department of Endocrinology, Odense University Hospital & Department of Clinical Research, Faculty of Health Sciences, University of Southern Denmark, DK-5000 Odense, Denmark
⁴Department of Clinical Medicine Health, Aarhus University, DK-8000 Aarhus C, Denmark
⁵Rolf Luft Center for Diabetes Research and Endocrinology, Department of Molecular Medicine and Surgery, Karolinska Institutet, Stockholm 17176 Sweden
⁶Department of Endocrinology, Diabetes and Metabolism. Karolinska University Hospital, Stockholm, Sweden
⁷Department of Clinical Science, Danderyds Hospital, Karolinska Institutet, Danderyd, Stockholm 18288 Sweden
⁸Department of Surgery, Ersta Hospital, Karolinska Institutet, Stockholm 11691 Sweden

*Corresponding author: Harvest F. Gu, E-mail: feng.gu@cpu.edu.cn
Center for Pathophysiology, School of Basic Medicine and Clinical Pharmacy, China Pharmaceutical University, Nanjing 210009 China

Abstract: 223 words
Main text: 2854 words
Table: 1
Figures: 3
Abstract
Insulin-like growth factor binding-protein 2 (IGFBP-2) is secreted by differentiating white adipocytes. Clinical studies demonstrate that circulating IGFBP-2 levels associated inversely with body mass index (BMI) and insulin resistance. To explore possible epigenetic changes of the IGFBP2 gene in obesity, we analyzed DNA methylation and mRNA expression in adipocytes from different depots. Healthy lean controls (BMI=24.5±0.3 kg/m², n=19) and obese subjects (BMI>35 kg/m², n=24) were recruited. All subjects were Swedish Caucasian. Visceral abdominal adipose tissue (VAT) and subcutaneous adipose tissue (SAT) fragments were homogenized. Genomic DNA and total RNAs were extracted. Four CpG sites in the IGFBP2 gene promoter region were analyzed with bisulfite pyrosequencing. IGFBP2 gene expression at mRNA levels was determined with TaqMan real time RT-PCR. Serum samples were used for measurement of circulating IGFBP-2 and leptin levels. IGFBP2 DNA methylation levels in VAT were increased in obese subjects compared with controls (P<0.05). By contrast, IGFBP2 mRNA expression levels in VAT were lower in obesity subjects than in controls (P<0.05). In SAT, IGFBP2 DNA methylation and RNA expression levels were lower than in VAT, irrespective of obesity. Obese subjects demonstrated increased serum leptin levels (P<0.001) and reduced serum IGFBP-2 levels compared to controls (P<0.05). In conclusion, the current study demonstrates that IGFBP2 DNA methylation levels are increased in VAT from obese subjects. This suggests that IGFBP-2 is epigenetically regulated in abdominal obesity.

Key words:
Adipose tissues, DNA methylation, IGFBP2, mRNA expression, obesity

E-mail addresses:
Zhangxiuli54321@sina.com
Feng.gu@cpu.edu.cn
Jan.Frystyk@rsyd.dk
Enes.Efendic@erstadiakoni.se
Kerstin.brismar@ki.se
Anders.Thorell@erstadiakoni.se
List of abbreviation:
BMI – body mass index
DBP – diastolic blood pressure
GBD – global burden of disease
IGFBP-2 – insulin-like growth factor binding protein 2
IGT – impaired glucose tolerance
IR – insulin resistance
LEP – leptin
SBP – systolic blood pressure
SAT – subcutaneous adipose tissue
T2D – type 2 diabetes
VAT – visceral adipose tissue
WHR – waist-to-hip circumference ratio
1. Introduction

Obesity has become a major and fast growing public health problem in the world. According to the recent report from the Global Burden of Disease (GBD) study, the prevalence of obesity has more than doubled since 1980. The global prevalence of obesity is now 5% in children and 12% in adults [1]. In particular, abdominal obesity (also known as central obesity) has a strong negative impact on health [1, 2]. The risk of obesity is largely influenced by the family background as indicated by heritability rates of up to 40% for body mass index (BMI) [3]. Although well investigated, the identified genetic variants up to now explain less than 5% of the observed heritability of obesity [3, 4]. One of the reasons for this “missing information” on heritability could be that epigenetic factors are involved in the complex interplay between genes and environment in obesity.

In recent years, we have carried out epigenetic studies of the insulin-like growth factors (IGF-I and IGF-II) and their binding proteins (IGFBPs) in diabetes [5-7]. IGFBP-2 is the second most abundant circulating IGFBP and expressed by the liver and white adipose tissue [8-10]. IGFBP-2 exerts its effects via binding to IGF-I and IGF-II, inhibiting their effects, but has also IGF independent effects via its RGD sequence, which can bind to the integrin receptor [11, 12]. IGF-I and IGF-II have been suggested to be associated with lipid metabolism and body weight regulation [13, 14]. Previous observations have shown that circulating IGFBP-2 levels are decreased in subjects with obesity and negatively associated with hyperinsulinemia and insulin resistance (IR) [15, 16]. Furthermore, high IGFBP-2 levels are positively associated with a better metabolic risk profile and reduced risk of type 2 diabetes (T2D), but paradoxically also with increased mortality in elderly men [17]. Nevertheless, available data suggest that IGFBP-2 may have a protective effect against the development of obesity, IR and T2D [18, 19]. Furthermore, experimental studies have demonstrated that the liver IGFBP2 is a leptin (LEP)-regulated gene [20, 21].

Genetic association studies have demonstrated that the IGFBP2 gene polymorphisms rs4402960 and rs1470579 are associated with higher BMI and IR in T2D [22-24]. Biological experiments with animal models of obesity and T2D such as ob/ob and db/db mice have indicated that igfbp2 mRNA expression levels in visceral white adipose tissue are lower in ob/ob, db/db and high fat-fed mice compared with their respective lean and chow-fed littermates [25]. Recently, Kammel et al. reported that methylation of IGFBP2 in DNA samples extracted from whole blood cells was increased in obese men with impaired glucose
tolerance and in fatty liver tissues of mice receiving high fat diet [26]. The latter study, however, was limited by the lack of human adipocyte tissue specific DNA methylation analysis. Therefore, in the current study, we collected visceral adipose tissue (VAT) and subcutaneous adipose tissues (SAT) from Swedish individuals, including healthy controls and obese subjects. We analyzed IGFBP2 DNA methylation and mRNA expression changes in VAT and SAT in order to explore the possible epigenetic effects of IGFBP2 in obesity.

2. Patients and methods
2.1. Subjects and adipose tissue sample collection
Forty-three subjects were enrolled in the present study. All were of Swedish Caucasians origin. The subjects were divided into two groups. One group included 24 obese subjects without diabetes (women/men=14/10, BMI 40.6±0.5 kg/m²). Another group consisted of 19 non-diabetic, non-obese subjects who served as controls (8/11, BMI 24.5±0.3 kg/m²). Subjects in the obese group were scheduled for bariatric surgery (laparoscopic gastric bypass) due to morbid obesity, whereas control subjects were planned to undergo either laparoscopic cholecystectomy for symptomatic gall stone disease or fundoplication (for gastroesophageal reflux disease).

All participants gave informed consent and the regional ethics committee in Stockholm approved the experimental protocol, which was in accordance with the Declaration of Helsinki II. Adipose tissues were collected during surgery from all studied subjects. Briefly, on a separate day before the planned surgical procedure, patients reported to the clinical research center at 8:00h after a 12h overnight fast. After collection of anthropometric data (height, weight, waist and hip circumference) and a 30-min period of bed-rest, all control subjects and obese patients without known diabetes underwent a 2h lasting 75g oral glucose tolerance test (OGTT) in order to exclude any undiagnosed T2D. Three obese subjects were in fact diagnosed with T2D and they were therefore excluded from the study.

On the day of surgery, after induction of anesthesia, subcutaneous and visceral adipose tissue specimens (100-200 mg) were surgically collected from all subjects. The subcutaneous specimens were obtained through an incision immediately below the umbilicus and approximately 15 cm below the right costal arch in patients undergoing cholecystectomy and gastric bypass, respectively. Visceral adipose tissue was collected from an identical location in the lower right aspect of the greater omentum in all subjects. Each tissue sample was
divided in two pieces. One piece was then immediately frozen in liquid nitrogen. The second piece was used for RNA analysis and treated with RNAlater buffer (Qiagen, Hilden, Germany) for RNA stabilization. All tissue samples were then stored at -80°C until use. Clinical and laboratory parameters of all participants are summarized in Table 1.

2.2. Serum IGFBP-2 and leptin analyses

IGFBP-2 levels in serum were measured using an in-house TR-IFMA as described previously [27]. Leptin (LEP) levels in serum were determined using a human LEP radioimmunoassay (Millipore, Missouri, USA), which utilized 125I-labeled human leptin and a human LEP antiserum by the double antibody-PEG technique [28, 29].

2.3. DNA extraction and bisulfite pyrosequencing

Extraction of genomic DNA from the tissue samples was performed by the Gentra Puregene tissue kit (Qiagen), which enables purification of high molecular weight DNA (100-200 kb) suitable for archiving. DNA concentrations were quantified using NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Wilmington, USA). The scalable purification procedure gently removes contaminants and inhibitors and large-volume samples can be purified for genetic and epigenetic analyses.

DNA samples were first treated with sodium bisulfite using EpiTect bisulfite kit (Qiagen). This kit gives complete conversion of unmethylated cytosine to uracil and subsequent purification in less than 6h. This highly sensitive technique utilizes an innovative protection against DNA degradation and ensures high conversion rates of over 99%.

The IGFBP2 gene (HGNC: 5471) is located in chromosome 2q35. For the analysis of DNA methylation changes in the IGFBP2 gene, we used PyroMark CpG assay (ENSG00000115457, Qiagen) and pyrosequencing protocol. There are four CpG sites in this gene as indicated with the bold letters and underline (TTAGCGGGACGGCGGAAAGKCAGGCCGGGGA). The PyroMark PCR kit (Qiagen) and PyroMark PCR master mix including HotStarTaq DNA Polymerase and optimized PyroMark Reaction Buffer containing 3 mM MgCl2 and dNTPs, 10x CoralLoad Concentrate, 5x Q-Solution, 25 mM MgCl2, and RNase-free water. The PCR amplicon length is 85 bp and covers the sequence within human chromosome 2:216,632,828-216,664,436 (version GRCh38). Methylation levels of these CpG sites were detected by using PyroMark Gold 96
Reagent kit (Qiagen) and PyroMark Q96 ID Pyrosequencing System (Biotage, Uppsala, Sweden). Pyrosequencing methylation analysis of CpG sites is a sensitive and accurate method [30, 31]. PyroQ-CpG software (Biotage) was used for methylation data analysis. Unmethylated bisulfite converted and unconverted DNAs (Qiagen) were used for control of conversion efficiency of the bisulfite treatment and accuracy in methylation analyses.

2.4. RNA extraction and TaqMan real time RT-PCR

The homogenization of fat tissues was prepared according to the protocol developed in our laboratory [32]. First 100 mg of fat tissues from RNAlater buffer was placed into a 2 ml microcentrifuge tube containing 0.5 ml of 2 mm diameter Zirconia beads (BioSpec, Bartlesville, USA) and 0.6 ml of RLT buffer (Qiagen). Subsequently, tubes were shaken four times at 3000 rpm for 20 s each. The tubes were kept on ice and the supernatant was collected quickly. Finally, total RNAs were extracted with RNeasy mini kit (Qiagen). The quantity and quality of all RNA samples were evaluated by using NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific) and 1% agarose gel electrophoresis (Bio-Rad, Hercules, USA). According to the manufacturer’s protocol, the first strand cDNA was synthesized from 1 µg mRNAs extracted from fat tissues of each subject employing random hexamer oligonucleotide in a final volume of 20 µl using QuantiTech reverse transcription kit (Qiagen). We used TaqMan real time RT-PCR protocol to analyze the IGFBP2 gene expression at mRNA levels with a specific TaqMan assay (Hs01040719_m1, Applied Biosystems, Foster, USA). The amplicon length of PCR is 54 bp (Chr.2: 216633404-216664436, version GRCh38). The probe covers the exon boundary 3-4 and labeled with 6’-carboxyl-fluorescein (FAM) as reporter dye and TAMRA as quencher dye. Amplification was performed using the 5’-nuclease TaqMan method with two-step PCR protocol (95 °C for 10 min, followed by 36 cycles of 95 °C for 15 s and 60 °C for 1 min) in ABI 7300 system (Applied Biosystems). The same protocol was used for the LEP gene expression analysis with a TaqMan assay (Hs00174877_m1, Applied Biosystems). TaqMan assay of GAPDH (Hs03929097_g1, Applied Biosystems) was used as an endogenous control. The experimental replication was done for two or three times.

2.5. Statistical analyses

Data presented in the Table and Figures are presented as means ± SE. Continuous variable among the groups of controls and obese subjects were compared by using an unpaired t-test.
Linear regression analyses with and without adjustments for confounding factors, including age and gender, were performed to examine the correlation between IGFBP-2 and LEP. \( P \)-values <0.05 were considered statistically significant. All data were analyzed by using PASW statistics program (SPSS 20.0, Chicago, USA).

3. Results

3.1. IGFBP2 DNA methylation

DNA methylation analyses of four CpG sites in the IGFBP2 gene in VAT and SAT from control and obese subjects are shown in Figures 1A and B, respectively. The DNA methylation levels of the IGFBP2 gene in VAT from obese subjects were significantly increased compared with controls \( (P=0.010) \) (Figure 1A). In contrast, no statistically significant difference of DNA methylation levels in SAT was found between control and obese subjects (Figure 1B). IGFBP2 DNA methylation levels in SAT was significantly lower compared to that in VAT \( (P<0.001) \).

3.2. IGFBP2 and LEP mRNA expressions

IGFBP2 gene expression at mRNA levels in VAT and SAT are presented in Figure 2A and B. IGFBP2 mRNA expression levels in VAT of obese subjects were significantly decreased compared with control subjects \( (P=0.028, \) Figure 2A). In SAT, IGFBP2 mRNA expression was lower than in VAT \( (P<0.001) \) but we observed no difference between lean and obese subjects (Figure 2B).

To elucidate the link between IGFBP2 epigenetics and the regulation by leptin, we analyzed LEP mRNA expression. We found that LEP mRNA expression in VAT of obese subjects were higher compared with controls \( (P<0.05, \) Figure 3). There was no significant correlation in term of mRNA expression between IGFBP2 and LEP. In SAT, the LEP mRNA expression levels were below the level of detection irrespective of group.

3.3. Serum IGFBP-2 and leptin levels

Serum LEP levels were increased in obese subjects compared to controls \( (P<0.001) \), while serum IGFBP-2 levels were lower in obese subjects \( (P=0.014) \) (Table 1). There was a significant negative correlation between serum IGFBP-2 and LEP levels in the group of lean
control subjects ($R^2$=-0.352, $P$=0.002). This correlation, however, was absent in the group of obese subjects ($R^2$=0.049, $P$=0.410).

4. Discussion

In the current study, we analyzed IGFBP2 DNA methylation and mRNA expression levels in VAT and SAT from Swedish Caucasians obese non-diabetic subjects vs. lean subjects. We found that IGFBP2 DNA methylation and mRNA expression levels were higher in VAT than in SAT. By contrast serum levels of IGFBP-2 were lower in obese subjects, suggesting that IGFBP-2 is epigenetically regulated in obesity. Finally, we found that the IGFBP2 DNA methylation and mRNA expression levels were higher in VAT than SAT.

Accumulating evidence demonstrate that an intra-abdominal localization of fat for a given BMI is associated with IR in obesity, resulting in metabolic derangements, while subcutaneous fat accumulation has a more benign effect on the cardio-metabolic risk [2]. One way to better understand the pathophysiology of VAT is to explore difference in the gene activity and regulation between visceral and subcutaneous fat tissues. IGFBP2 is highly expressed in white adipose tissues and may in this location play a role in the development of obesity. Although several clinical observations have reported that circulating IGFBP-2 levels are decreased in obese subjects, data on IGFBP2 gene expression and epigenetic effects in obesity are sparse. Previously, Li and Picard performed an experimental study of IGFBP2 gene activity in animal models of obesity (ob/ob mice) and demonstrated that IGFBP2 mRNA expression was decreased in visceral white adipose tissue of ob/ob and high fat diet-fed mice compared with tissues derived from matched lean and chow diet-fed littermates. Moreover, the IGFBP2 mRNA expression levels were also decreased in visceral fat tissues of 12 and 24 months old mice compared with those of their 4 months old counterparts [25]. In SAT from these animals, the IGFBP2 mRNA expression levels were lower compared with VAT and there was no difference in IGFBP2 mRNA expression between groups [25]. This is in agreement with our present study. Recently, Kammel et al. not only replicated the findings regarding the IGFBP2 mRNA expression but also conducted DNA methylation analysis of IGFBP2 in liver tissue of high fat diet-fed mice and also in whole blood cells from obese subjects and individuals with and without impaired glucose tolerance (IGT). They reported that IGFBP2 DNA methylation levels were increased in liver of high fat diet-fed mice as well as in whole blood cells in subjects with obesity [26].
IGFBP2 DNA methylation and mRNA expression levels in VAT and SAT in obese subjects and lean controls (Table 1). Our results, in corroboration with previous and recent studies [25, 26], implicate that in VAT from obese subjects, IGFBP2 undergoes an epigenetic regulation with increased DNA methylation and decreased mRNA expression. This may explain the lower serum levels of IGFBP-2. Increased insulin secretion due to obesity may trigger the DNA methylation [33]. In humans, there are approximately 45,000 CpG islands per haploid genome compared with only 37,000 in mice [34]. Therefore, it is not always possible to design an epigenetic study for a particular gene using animal models. However, the IGFBP2 gene in human contains 66% G-Cs and has relatively high homology in DNA sequences with animals such as mouse and rat [35]. Therefore, studies of igfbp2 epigenetic effects with animal models of obesity and T2D are expected to correlate well with human findings, as indicated here. Due to the limitation of sample size, the question concerning the difference of IGFBP2 DNA methylation levels between males and females is remained for further investigation with large cohorts. Interestingly, Wittenbecher C et al. have very recently conducted a prospective association study of circulating IGFBP-2 concentrations and of IGFBP2 DNA methylation changes, respectively with the risk of T2D. In the promoter region of the IGFBP2 gene, DNA methylation levels of 5 CpGs were increased in T2D compared with controls. The data were generated with Illumina array and blood samples, while DNA methylation changes of 2 CpGs in intronic regions were decreased [35]. In the current study, we have described adipocyte tissue specific DNA methylation changes of IGFBP2 gene in obese and lean subjects. Our data are consistent with what has been presented for the promoter CpGs [35] as reported but not with intronic CpGs by Wittenbecher C et al. [36].

Leptin is, like IGFBP-2, secreted by white adipocytes and plays a major role in the regulation of body weight. Leptin acts through the leptin receptor, which regulates a signaling pathway that can inhibit food intake and/or regulate energy expenditure to maintain adipose tissue mass. IGFBP-2 is considered a leptin-regulated gene because low dose leptin can increase IGFBP-2 expression and this has been reported to correlate with leptin’s antidiabetic effect [20]. Serum IGFBP2 levels are lower in leptin-deficient human subjects compared with controls and increase after leptin treatment [20, 21]. In order to explore the correlation between IGFBP-2 and leptin, we analyzed LEP mRNA expression by the same TaqMan protocol that was used for detection of IGFBP2 mRNA expression levels. We could demonstrate that LEP mRNA expression levels in VAT from obese subjects were significantly higher than in VAT from lean subjects. Furthermore, we found that serum leptin
levels were increased while IGFBP-2 levels were decreased in obese subjects compared to the controls. The inverse correlation between serum levels of leptin and IGFBP-2 in the lean controls is probably explained by a common regulation by insulin and does not reflect the positive leptin regulation of liver derived IGFBP-2 found in mice models and humans with leptin deficiency. The lack of correlation in the obese subjects may be explained by both leptin resistance and the epigenetic changes of IGFBP-2 found in these subjects.

5. Conclusions
The current study provides the first evidence that the decreased IGFBP2 mRNA expression in VAT from obese subjects are associated with an increased DNA methylation level. This is in agreement with lower serum IGFBP-2 levels in obese subjects. In summary, these observations suggest that IGFBP-2 is epigenetically regulated in abdominal obesity.
Acknowledgements

The authors wish to thank Ms. Anette Bratt for excellent management of adipocyte tissue samples and Ms. Elisabeth Norén-Krog, Susanne Sørensen and Anette Landstrom for laboratory assistance.

Funding

The study was supported by Karolinska Institutet fund and The Erling-Persson Family Foundation.

Duality of Interest

Dr. Jan Frystyk, who is co-authoring this paper, also serves as Editor-in-Chief of Growth Hormone and IGF Research. However, this has not influenced on the handling of the paper, which has been subjected to the Journal's usual procedures. Thus, the peer review process has been handled independently of Dr. Jan Frystyk, who has been blinded to the review process. The authors otherwise have nothing to disclose.

Author Contributions

HFG, AT and SE designed the study; AT collected adipocyte tissues from the subjects; XZ, HFG, KB and JF collected and analyzed experimental data; All authors contributed to data interpretation, discussion and revision of the paper; HFG and XZ prepared and all edited the manuscript.
References


Table 1

Clinical and laboratory parameters of healthy controls and obese subjects

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Obese</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (Female %)</td>
<td>19 (42%)</td>
<td>24 (58%)</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>54±2</td>
<td>41±1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.5±0.3</td>
<td>40.6±0.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Waist (cm)</td>
<td>92.2±3.7</td>
<td>126.5±1.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>WHR</td>
<td>0.94±0.01</td>
<td>1.04±0.02</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>0 min PG (mmol/l)</td>
<td>5.4±0.1</td>
<td>5.5±0.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>120 min PG (mmol/l)</td>
<td>6.5±0.3</td>
<td>7.3±0.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HbA1c (mmol/mol)</td>
<td>36.1±0.5</td>
<td>36.5±0.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>117±2</td>
<td>132±2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>77±2</td>
<td>83±1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TChol (mmol/l)</td>
<td>3.4±0.1</td>
<td>4.6±0.1</td>
<td>0.178</td>
</tr>
<tr>
<td>HDL-Chol (mmol/l)</td>
<td>1.3±0.1</td>
<td>1.1±0.2</td>
<td>0.432</td>
</tr>
<tr>
<td>LDL-Chol (mmol/l)</td>
<td>1.8±0.1</td>
<td>2.7±0.1</td>
<td>0.139</td>
</tr>
<tr>
<td>TG (mmol/l)</td>
<td>0.8±0.1</td>
<td>1.6±0.2</td>
<td>0.034</td>
</tr>
<tr>
<td>LEP (µg/l)</td>
<td>16.3±2.1</td>
<td>40.3±2.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IGFBP-2 (µg/l)</td>
<td>201±17</td>
<td>147±11</td>
<td>0.014</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE. BMI, body mass index; WHR, waist-hip ratio; PG, plasma glucose; HbA1c, glycated haemoglobin; SBP, systolic blood pressure; DBP, diastolic blood pressure; TChol, total cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TG, triglycerides; LEP, leptin; IGFBP-2, insulin like growth factor binding protein 2.
Legend to Figures:

Figure 1 *IGFBP2* DNA methylation levels in visceral (VAT) and subcutaneous (SAT) adipose tissues from control and obese subjects

A. The mean DNA methylation levels of *IGFBP2* in VAT of obese subjects (OB) were increased compared with non-diabetic, non-obese control subjects (Cont) (*P*=0.034). B. *IGFBP2* DNA methylation levels in SAT were reduced compared to that in VAT. *IGFBP2* DNA methylation levels at CpG sites 1-4 in the groups of Cont (light) and OB (grey) were also analyzed and showed beside Figure 1A and B.

Figure 2 *IGFBP2* mRNA expression levels in A visceral (VAT) and B subcutaneous (SAT) adipose tissues from control and obese subjects

A. *IGFBP2* mRNA expression levels in VAT of obese subjects (OB) were decreased compared to control subjects (Cont) (*P*=0.016). B. In SAT, *IGFBP2* expression was very low, and no statistically significance between groups was seen. *GAPDH* was used as the endogenous control.

Figure 3 *LEP* mRNA expression levels in visceral (VAT) adipose tissues from control and obese subjects

*LEP* mRNA expression in VAT from obese subjects (OB) without type 2 diabetes (T2D) were higher compared to control subjects (Cont) (*P*<0.001). *GAPDH* was used as the endogenous control.
- IGFBP-2 is secreted by differentiating white adipocytes. Clinical studies demonstrate that circulating IGFBP-2 levels associated inversely with BMI and insulin resistance.

- By using the bisulfite pyrosequencing protocol, IGFBP2 DNA methylation levels in visceral abdominal adipose tissue were increased in obese subjects compared with lean controls, while IGFBP2 mRNA expression levels in the fat tissues were lower in obese subjects than in lean controls.

- Obese subjects demonstrated increased serum leptin levels and reduced serum IGFBP-2 levels compared to lean controls.

- This study demonstrates that IGFBP2 DNA methylation levels are increased in VAT from obese subjects and suggests that IGFBP-2 is epigenetically regulated in abdominal obesity.
Figure 1

(A) IGFBP2 DNA methylation in VAT (%)

(B) IGFBP2 DNA methylation in SAT (%)

Cont vs OB: * indicates statistical significance, ns indicates no significance.
Figure 2

A

IGFBP2 mRNA expression in VAT (Arbitrary unit)

Cont | OB

*  

B

IGFBP2 mRNA expression in SAT (Arbitrary unit)

Cont | OB

ns
Figure 3

**LEP mRNA expression in VAT (Arbitrary unit)**

- **Cont**
- **OB** with significant difference indicated by ****