

Original Article

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# Parallel Down-Regulation of FOXO1, PPAR $\gamma$ and Adiponectin mRNA Expression in Visceral Adipose Tissue of Class III Obese Individuals

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## Key Words

Adipose tissue · Obesity · Adiponectin · FOXO1

## Abstract

**Objective:** Adipose tissue is responsible for secretion of several cytokines that mediate systemic effects on obesity and insulin resistance. Subcutaneous abdominal adipose tissue (SAT) and visceral adipose tissue (VAT) are metabolically different and have differences in their gene expression profile. Our study evaluated the expression of adiponectin, FOXO1, PPAR $\gamma$ , and SIRT1 in VAT and SAT of non-obese and class III obese subjects. **Methods:** The adipose tissue samples were obtained by surgery. Reverse transcripts of studied genes were determined by quantitative real-time polymerase chain reaction (qRT-PCR). **Results:** Comparing the different lipid depots, adiponectin expression was lower only in VAT of obese individuals ( $p = 0.043$ ); FOXO1 and PPAR $\gamma$  levels were decreased in VAT of both groups. When non-obese and obese were compared, only adiponectin expression was lower in SAT and in VAT of obese subjects ( $p = 0.004$  and  $p = 0.002$ , respectively). No difference was found with regard to SIRT1 levels in VAT or SAT in both groups. FOXO1 expression in SAT of obese subjects had a negative correlation with age ( $r = -0.683$ ;  $p = 0.029$ ) and triglyceride serum levels ( $r = -0.794$ ;  $p = 0.006$ ). **Conclusion:** The decrease mRNA expression of this genes in VAT, responsible for central adiposity, may be associated with an increased risk of obesity and co-morbidities.

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## Introduction

The global epidemic of obesity is a major public health problem, which is driven largely by its co-morbidities including insulin resistance, type 2 diabetes, dyslipidemia, and cancer [1, 2]. The etiology of obesity is complex, with environmental and genetic influences [3]. The basic mechanism to its development is an imbalance between the energy ingested and expended by the body. The excess of energy ingested is stored as adipose tissue [4]. However, we know little about the participation of transcription factors in this modulation.

Adipose tissue depots are anatomically and metabolically different and have differences in their gene expression profile. The abdominal adipose tissue can be divided in subcutaneous (SAT) and visceral (VAT) adipose tissues [5]. Visceral adipocytes are generally believed to be hyperlipolytic by being highly responsive to catecholamine stimulation and more resistant to the antilipolytic effect of insulin, when compared to subcutaneous fat [6].

The peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily involved in the transcriptional regulation of lipid metabolism genes, energy balance, and inflammation. There are three isotypes: PPAR $\alpha$ , PPAR $\beta/\delta$  and PPAR $\gamma$  [9]. PPAR $\gamma$  is highly expressed in adipose tissue [7]. Its activation promotes lipid synthesis and storage in white adipose tissue as well as preadipocyte differentiation to mature adipocytes [8]. In spite of this, PPAR $\gamma$  activation is highly associated with increases of insulin sensitivity [9]. Similarly, adiponectin is also related to improvement of insulin resistance. Adiponectin is an abundant protein present in the blood and specifically derived from adipose tissue and may be a link between obesity and the development of insulin resistance [10]. Adiponectin levels are reduced in obese individuals, particularly among subjects with excess visceral adiposity [11].

FOXO1, a transcription factor member of the forkhead box-containing protein-0 superfamily, is predominant in insulin-responsive tissues, including adipose tissue [12]. FOXO1 is a negative regulator of insulin sensitivity in adipocytes [13]. In response to insulin, it is phosphorylated through the PI3K-dependent pathway, resulting in its nuclear exclusion and inhibition of target gene expression [14]. A study carried out investigating the effects of FOXO1 on PPAR $\gamma$  gene expression of human adipocytes observed that FOXO1 expression dose-dependently repressed PPAR $\gamma$  transcription [15].

Sirtuins (SIRT) are members of the mammalian NAD<sup>+</sup>-dependent histone deacetylase family [16]. There are seven mammalian sirtuins (SIRT 1–7), and SIRT1 has been the most studied. This protein has been implicated in the control of lipid and glucose metabolism by regulating many important transcription factors such as PPAR $\gamma$  and FOXO1 [17,18]. Bäckesjö et al. [19] showed that activation of SIRT1 blocked adipocyte development and increased the expression of osteoblast markers in the rat mesenchymal cell line C3H10T1/2. In spite of this, Qiao et al. [20] demonstrated that SIRT1 increased adiponectin transcription, a protein associated with insulin sensibility, by activating FOXO1 expression in 3T3-L1 adipocytes.

SAT and VAT are metabolically different, but there are few studies about the levels of FOXO1, adiponectin, PPAR $\gamma$ , and SIRT1 expression in these tissues. Furthermore, in the present study, we examined expressions of these genes in SAT and VAT of non-obese and class III obese subjects.

## Material and Methods

### Samples

Samples of SAT and VAT were obtained from 13 subjects with class III obesity (BMI  $\geq$  40kg/m<sup>2</sup>), who underwent Roux-en-Y gastric bypass at the Centro de Obesidade e Síndrome Metabólica of Hospital São Lucas of Pontifícia Universidade Católica of Rio Grande do Sul (COM/PUCRS, Brazil). The extensive

**Table 1.** Anthropometric and biological parameters of subjects with obesity class III (n = 13)

Characteristics	Values
Age, years	33 (28–38)
BMI, kg/m <sup>2</sup>	49.6 (43.8–55.3)
Waist circumference, cm	131.1 (121.7–140.5)
Fasting glucose, mg/dl	128 (72–184)
Fasting insulin, $\mu$ U/ml	26.4 (18.6–34.2)
HOMA-IR, mmol/l $\times$ $\mu$ U/ml	8.2 (4.7–11.7)
Total cholesterol, mg/dl	203 (167–239)
HDL-cholesterol, mg/dl	47.4 (38.7–56)
LDL-cholesterol, mg/dl	116.6 (97.8–135.3)
Triglycerides, mg/dl	171 (101.5–226.5)

<sup>a</sup>Values shown are mean values (95% confidence interval).

clinical and laboratory data routinely collected for each obese individual are given in table 1. To evaluate insulin resistance, we used a mathematical model of Homeostasis Assessment Model Insulin Resistance (HOMA-IR). It is calculated as follows: HOMA-IR = Fasting plasma insulin level (mg/dl)  $\times$  (Fasting plasma glucose level ( $\mu$ U/ml)  $\times$  0.05551/22.5 [21]. Similar samples of VAT and SAT were obtained from 10 non-obese individuals who underwent elective surgery; their age range was 22–55 years, with BMI of 17.9–29.0 kg/m<sup>2</sup>, fasting glucose of 85–105 mg/dl, and serum triglycerides of 87–120 mg/dl. Patients' weights had been stable for at least 1 year. None of the subjects were taking any medications affecting adipocyte metabolism.

The study was approved by Ethics Committee of Universidade Federal do Rio Grande do Sul (n<sup>o</sup> 2007/936). All subjects were informed about the aim of the study and signed the informed consent form.

#### Analysis of mRNA Expression

Adipose tissue samples were collected and immediately immersed in TRIzol reagent (Invitrogen, Darmstadt, Germany). Approximately 2  $\mu$ g of total RNA were added to each cDNA synthesis reaction using the M-MLV Reverse Transcriptase (Invitrogen). Reactions were performed at 42 °C for 1 h using the primer T23V (5' TTT TTT TTT TTT TTT TTT TTT TTV 3'). The cDNA samples were stored at –20 °C. The sequences of the primer pairs used in PCR reactions are listed in table 2. Applied-Biosystems 7500 real-time cyler (Applied Biosystems, Carlsbad, CA, USA) was used to carry out the quantitative real-time PCR (qRT-PCR). Reaction settings were composed of an initial denaturation step of 5 min at 95 °C followed by 40 cycles of 15 s at 95 °C, 10 s at 60 °C, 15 s at 72 °C and 35 s at 60 °C; samples were held for 15 s at 95 °C for annealing and then heated for 1 min at 60 °C with a ramp of 0.1 °C/s to acquire data to produce the denaturing curve of the amplified products. qRT-PCRs were made in a final volume of 20  $\mu$ l, composed of 10  $\mu$ l of each reverse transcription sample diluted 40–100 times, 2  $\mu$ l 10 times PCR buffer, 1.2  $\mu$ l of 50 mmol/l MgCl<sub>2</sub>, 0.1  $\mu$ l of 5 mmol/l dNTPs, 0.4  $\mu$ l of 10  $\mu$ mol/l primer pairs, 4.25  $\mu$ l of water, 2.0  $\mu$ l of SYBR green (1:10,000; Molecular Probes®, Invitrogen), and 0.05  $\mu$ l of Platinum Taq DNA polymerase (5 U/ $\mu$ l) (Invitrogen). Each sample was run in quadruplicate.

#### Data Analysis

Gene expression was quantified using the 2<sup>- $\Delta$   $\Delta$  C<sub>t</sub></sup> (threshold cycle) method [22]. For each sample, a  $\Delta$ C<sub>T</sub> value was obtained by subtracting the beta-2-microglobulin (B2M) C<sub>T</sub> value from the C<sub>T</sub> value of the interest gene. To calculate the difference between SAT and VAT in obese and non-obese, the  $\Delta$ C<sub>T</sub> mean value obtained for the SAT of non-obese was used to calculate the  $\Delta$   $\Delta$  C<sub>t</sub> of each gene (2<sup>- $\Delta$   $\Delta$  C<sub>t</sub></sup>).

#### Statistics

To compare adiponectin, FOXO1, PPAR $\gamma$ , and SIRT1 mRNA expression between the two depots (SAT and VAT), we used the non-parametric Wilcoxon test. To compare the expression pattern between non-obese and obese we used the non-parametric Mann-Whitney U test. Data are expressed as median (25th–

**Table 2.** Oligonucleotides used in qRT-PCR reactions

Gene	Sequence
B2M	Forward 5'-TGCTGTCTCCATGTTTGATGTATCT-3' Reverse 5'-TCTCTGCTCCCCACCTCTAAGT-3'
Adiponectin	Forward 5'-TGGTGAGAAGGGTGAGAA-3' Reverse 5'-AGATCTTGGTAAAGCGAATG-3'
FOXO1	Forward 5'-TGGACATGCTCAGCAGACATC-3' Reverse 5'-TTGGGTCAGGCGGTTCA-3'
PPAR $\gamma$	Forward 5'-AGGCCATTTTCTCAAAC-3' Reverse 5'-AGAAATGCTGGAGAAGTCAACA-3'
SIRT1	Forward 5'-GAGTGGCAAAGGAGCAGA-3' Reverse 5'-TCTGGCATGTCCCACTATC-3'

75th percentile). We used Spearman correlation test ( $r_s$ ) to correlate the characteristics of patients with class III obesity and adiponectin, FOXO1, PPAR $\gamma$  and SIRT1 mRNA expression in both tissues, VAT and SAT. All variables were examined for normality using the Kolmogorov-Smirnov test. P values < 0.05 (two-tailed) were considered to be statistically significant. SPSS 14.0 (SPSS Inc., Chicago, IL, USA) was used for the analyses.

## Results

The relative expression of adiponectin, FOXO1, PPAR $\gamma$ , and SIRT1 mRNA in VAT and SAT of non-obese and obese subjects were obtained by RT-PCR. Table 3 reflects the mRNA expression profile of adiponectin, FOXO1, PPAR $\gamma$ , and SIRT1 in VAT and SAT of non-obese and class III obese individuals. In non-obese subjects, FOXO1 ( $p = 0.046$ ) and PPAR $\gamma$  ( $p = 0.028$ ) expressions were lower in VAT when compared to SAT. SIRT ( $p = 0.116$ ) and adiponectin ( $p = 0.080$ ) mRNA expression showed no difference between the tissues in non-obese individuals. When analyzing the expression patterns in obese individuals, we found a lower expression of adiponectin ( $p = 0.043$ ), FOXO1 ( $p = 0.038$ ), and PPAR $\gamma$  ( $p = 0.028$ ) in VAT compared to SAT. However, SIRT mRNA expression showed no difference between fat depots ( $p = 0.465$ ).

When we compared the expression patterns between non-obese and class III obese individuals, we found lower adiponectin mRNA levels in the obese than in the non-obese group in both adipose tissues, SAT ( $p = 0.004$ ) and VAT ( $p = 0.002$ ). However, in SAT and VAT, the mRNA expression of FOXO1, PPAR $\gamma$ , and SIRT1 showed no difference between non-obese and obese subjects.

We also performed correlation analysis between anthropometric and biochemical parameters of class III obese subjects with adiponectin, FOXO1, SIRT1, and PPAR $\gamma$  mRNA expression in both tissues, VAT and SAT. We found that levels of FOXO1 mRNA expressed in SAT (fig. 1) has an inverse correlation with age ( $r = -0.683$ ;  $p = 0.029$ ) and triglycerides serum levels ( $r = -0.794$ ;  $p = 0.006$ ).

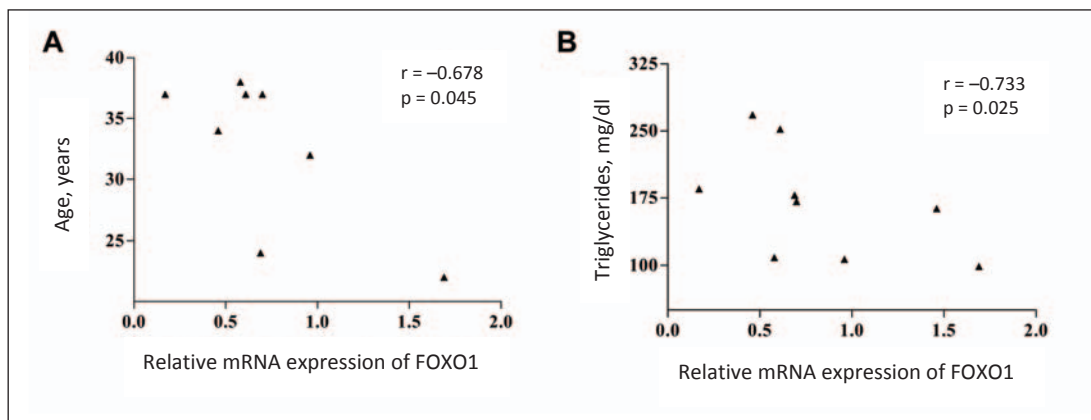
**Table 3.** Adiponectin, FOXO1, PPARγ, and SIRT1 mRNA expression in intra-abdominal adipose tissue of non-obese and class III obese subjects<sup>a</sup>

	SAT	VAT
Adiponectin		
Non-obese	1 (0.877–1.523)	2.421 (1.324–2.617)
Obese	0.293 (0.270–0.328) <sup>b</sup>	0.075 (0.027–0.600) <sup>b,c</sup>
FOXO1		
Non-obese	1 (0.821–1.181)	0.422 (0.315–0.621) <sup>c</sup>
Obese	0.701 (0.650–0.908)	0.310 (0.275–0.378) <sup>c</sup>
PPARγ		
Non-obese	1 (0.617–1.364)	0.232 (0.221–0.279) <sup>c</sup>
Obese	0.731 (0.507–1.298)	0.147 (0.077–0.265) <sup>c</sup>
SIRT1		
Non-obese	1 (0.404–1.169)	0.348 (0.175–0.524)
Obese	0.647 (0.420–1.285)	0.705 (0.288–0.933)

<sup>a</sup>The relative mRNA expression values are shown as median values (25th and 75th percentile). Relative expressions were normalized against β-2-microglobulin (ΔCt) and calibrated to the mean value of SAT ( $2^{-\Delta\Delta C_t}$ ) of non-obese group.

<sup>b</sup>Statistical difference between non-obese and obese subjects performing the Mann–Whitney U test.  $P < 0.05$  was considered statistically significant.

<sup>c</sup>Statistical difference between SAT and VAT using Wilcoxon test.



**Fig. 1.** Correlation between the relative mRNA expression of FOXO1 in subcutaneous adipose tissue of **A** class III obese subjects and age and **B** serum triglycerides levels.

## Discussion

The regional distribution of adipose tissue is associated with profound physiological changes in obesity. Human VAT and SAT display differences in metabolic and biochemical properties as well as differences in gene expression profiles [23]. In the present study, we evaluated the mRNA expression levels of adiponectin, FOXO1, PPARγ, and SIRT1 in VAT and SAT of class III obese and non-obese subjects.

In our study, the obese subjects had lower expression of adiponectin in VAT when compared to SAT. This profile was not observed in non-obese subjects who showed no difference between fat depots. Incubated samples of VAT from obese subjects exhibited significantly lower expression of adiponectin than those of SAT [24]. Moreover, adiponectin released by visceral adipocytes of obese women is significantly reduced when compared to subcutaneous adipocytes [25].

A decreased mRNA expression of adiponectin in adipose tissue has been reported in obesity [26]. We found that adiponectin mRNA expression in VAT was 32-fold higher in the non-obese when compared to obese subjects. The same tendency was observed in SAT, where adiponectin levels were 3.4-fold higher in the non-obese. These results agree with a previous study [24] that showed higher adiponectin mRNA expression in VAT in lean than in obese subjects. Furthermore, the plasma concentrations of adiponectin in obese subjects were significantly lower than in non-obese [27]. Still the mechanism underlying the decreased adiponectin production in obesity remains unknown. Nevertheless, plasma adiponectin levels in obese subjects were found to be inversely correlated to circulating levels of IL-6 and TNF- $\alpha$ , inflammatory cytokines associated with obesity [28, 29]. Studies *in vitro* have shown that TNF- $\alpha$  has an inhibitory effect on adiponectin mRNA levels in adipose tissue [28, 30]. Adiponectin levels also are negatively correlated to adipocyte size (hypertrophy of adipocytes). Hypertrophy adipocytes in VAT are associated with many of the most common metabolic diseases, including insulin resistance and obesity [4]. Therefore, we hypothesized that adiponectin-reduced levels in VAT can be triggered by a pro-inflammatory state common in obesity. In addition, in our recent work, we found that resveratrol can stimulate adiponectin mRNA expression in visceral adipose cells of obese individuals, reverting the low expression [18]. However, more studies are necessary to confirm the relation of adiponectin with a pro-inflammatory state in visceral adiposity. In this way, the down-regulation of adiponectin mRNA expression in VAT of obese individuals could be related to insulin resistance development in obesity.

Analyses of FOXO1 and PPAR $\gamma$  mRNA levels showed minor expression in VAT compared to SAT in both groups (non-obese and obese). The FOXO1 has been shown to be a mediator of the effect of insulin, glucose homeostasis and adipocytes differentiation [13, 31]. Although the FOXO1 isoform is known to play a key role in adipogenesis, its physiologic role in adipose tissue remains unclear [32]. A study that evaluated FOXO1 expression in adult porcine model also found that FOXO1 mRNA expression was higher in SAT than in VAT [33]. FOXO1 haploinsufficiency seems to protect against insulin resistance and these effect may be explained by enhance of PPAR $\gamma$  [32]. With respect to PPAR $\gamma$ , Giusti et al. [34] detected higher mRNA levels in SAT compared to VAT in obese women (BMI > 35 kg/m<sup>2</sup>). In SAT, PPAR $\gamma$  agonists such as thiazolidinediones promote adipocyte differentiation (hyperplasia), causing an insulin sensitivity improvement [35]. On the other side, adipocyte hyperplasia in SAT is related to insulin resistance [4]. In our study, obese and non-obese individuals presented lower levels of FOXO1 and PPAR $\gamma$  in VAT compared to SAT, and this profile was accompanied by unaltered SIRT1 mRNA expression. Although FOXO could reduce the expression of PPAR $\gamma$  through interaction with other proteins, it also seems to bind directly to PPAR $\gamma$ , to disrupt its DNA binding ability and to interfere with promoter DNA occupancy of this receptor [32]. Thus, the patterns of expression of FOXO1 and PPAR $\gamma$  may predispose to reduced action of PPAR via direct repression by FOXO1, promoting equal susceptibility to insulin sensitivity disorders. Because of this expression patterns, we believe that obesity-related co-morbidities could be related to low levels of adiponectin rather than to the the expression of PPAR $\gamma$  and FOXO1.

The pattern of FOXO1 mRNA expression in SAT has a significant inverse correlation with age of obese subjects, and this result was not observed in non-obese subjects. A study that compared FOXO1 mRNA expression in SAT of 1-day-old and 180-day-old pigs observed

that FOXO1 expression was higher in the youngest group [33]. The FOXO factors interact with several other transcription factors (NF- $\kappa$ B and p53) and, thus, can regulate the cellular survival responses, including metabolic stress resistance and cell-cycle responses and, ultimately, longevity [36, 37]. In this way, obesity seems to interfere in longevity, and this situation could be mediated by FOXO1 expression. The FOXO1 mRNA expression in obese SAT also showed inverse correlation with serum triglycerides. Zhang et al. [38] found modestly lower triglyceride levels in transgenic mice that express an active form of FOXO1 in the liver. This study also showed that expression of lipoprotein lipase is increased in the liver of FOXO1 transgenic mice, which can enhance the breakdown of triglycerides and metabolism of fatty acids [38]. Nonetheless, from the clinical point of view further studies on the molecular mechanism of FOXO1 action in adipose tissue are important.

This study evaluated the mRNA expression of some genes, but it will be necessary to know more about protein translation in the future. This work deals with transcriptional control of different adipose tissues in obese and non-obese individuals. The results reveal an equal pattern of SIRT1 and a different pattern of adiponectin, FOXO1 and PPAR $\gamma$  mRNA expression in SAT and VAT. As seen in other research models, our data suggest also metabolic differences among the different adipose depots. The decreased mRNA expression of these genes in VAT, responsible for central adiposity, may be associated with an increased risk of obesity and co-morbidities.

In conclusion, the patterns of gene expression in adipose tissue of non-obese and obese individuals will contribute to understand the pathogenesis of obesity and related disorders. The knowledge of these genes' modulation may be important to implement therapeutic research in a specific manner.

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### Disclosure Statement

There is no financial interest to report.

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