Parallel down-regulation of Retinol Binding Protein-4 (RBP4) and Adiponectin expression in Subcutaneous Adipose Tissue of Non-Morbidly Obese Subjects

Short Title: RBP4 and Adiponectin Down-regulation in Adipose Tissue

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ABSTRACT

_Context and Objective:_ Adipokines are involved in the etiopathology of obesity-related disorders. Since the role of adipokine Retinol-Binding Protein-4 (RBP4) in obesity remains uncertain and its relationship with other adipokines and inflammatory markers has not been examined in detail, we investigated the relationships of RBP4 mRNA expression and circulating protein levels with obesity, anthropometric and metabolic variables, as well as with obesity-related inflammatory markers adiponectin and C-reactive protein (CRP).

_Subjects and methods:_ 125 subjects participated, 36 lean (BMI: < 25Kg/m²) and 89 obese (overweight/obese; BMI: ≥25 <40) whose anthropometric and metabolic variables were assessed. mRNA expression was quantified by Real time PCR in subcutaneous adipose tissue (sub-AT) of 46 subjects.

_Results:_ There was a tendency for circulating RBP4 levels to positively correlate with waist circumference (β=0.29, p=0.08; R²=0.08) but there was no significant association with the obesity-related parameters analysed. RBP4 and adiponectin mRNA expression levels were similarly down-regulated in the sub-AT of obese subjects (0.5-fold), however, RBP4 down-regulation did not affect its circulating protein levels. The expression of RBP4 and adiponectin was positively correlated even after controlling for confounding factors (β=0.59, p< 0.0001; R²=0.40)

_Conclusions:_ In our population, RBP4 circulating levels were not significantly correlated with obesity-related parameters, although a tendency to correlate with waist circumference suggests a relationship with insulin resistance and other metabolic disorders. In addition, our results suggest that the production of RBP4 by other tissues such as liver, rather than sub-AT, may be involved in regulating RBP4 circulating levels.

**KEY WORDS:** RBP4, Adiponectin, C-reactive protein, Adipose Tissue, Obesity
INTRODUCTION

In addition to its main metabolic role of storing energy in the form of fat, adipose tissue (AT) is now considered an active endocrine organ that secretes a variety of bioactive peptides. These peptides are known as “adipokines” and they coordinate biological processes such as energy metabolism, immune and neuroendocrine functions (1-3).

Obesity is a condition that is associated with low-level chronic inflammation, insulin resistance (IR), hyperlipidaemia and other metabolic disorders (4). There is now growing evidence that adipokines are important in the etiopathology of obesity-related disorders, either through a traditional (circulating) hormonal effect or by a local action in the adipose tissue (5).

Since the circulating levels of a recently identified adipokine that acts as a carrier of retinol (vitamin A) in the blood, Retinol-Binding Protein-4 (RBP4), have been positively correlated with obesity and IR in an adipocyte-specific glucose transporter 4 knockout mouse (Glut4<sup>−/−</sup>) model (6), and, in this model, an insulin-sensitizing drug, reduced the elevated levels of RBP4 transcripts in AT as well as its systemic levels (6), it has been proposed that RBP4 may be behind the adipocyte-muscle connection that links obesity and insulin resistance (6). However, its role in human obesity and IR physiopathology is still unclear (7-14).

In the present study and, as an extension of our recent analysis of circulating RBP4 levels in 107 non-diabetic men with a variable degree of obesity (11), we have assessed circulating RBP4 levels and RBP4 expression in subcutaneous AT (sub-AT) in lean and obese patients. In order to gain a more complete understanding of how RBP4 may influence human obesity, we also assessed the levels of adiponectin and C-reactive protein (CRP), which are related to adiposity and its metabolic disorders and are differentially expressed in human AT. Indeed, while the circulating levels of CRP, an established inflammatory marker, are positively associated with obesity and IR (15), the circulating levels of adiponectin are inversely correlated with IR, obesity and CRP levels (16-19).

In order to clarify the role of RBP4 in human obesity, we have studied, in obese and non-obese subjects without diabetes, the relationship between circulating RBP4 levels and sub-AT RBP4 mRNA expression with adiposity parameters, insulin resistance indexes, and lipid parameters.
In addition, to integrate the role of RBP4 with that of other obesity associated molecules, we examined the relationship between sub-AT mRNA expression and circulating levels of RBP4, adiponectin and CRP. We also assessed RBP4, adiponectin and CRP expression in sub-AT fractions: mature adipocytes and non-fat cells of the stromal-vascular fraction (SVF).
SUBJECTS AND METHODS

Study population

Serum levels of RBP4 were measured in 125 subjects: 36 lean subjects, mean age 50.8±16.4 years (25 men and 11 women) with a BMI: < 25 kg/m$^2$ and 89 obese, mean age 57.8±14.3 years (58 men and 31 women) with a BMI between 25 and 39.9 kg/m$^2$. Forty-six subjects participated in the adipose tissue expression study: 22 lean subjects mean age 46.9±16.3 years (12 men and 10 women) and 24 obese, mean age 54.63±14.2 years (13 men / 11 women). Subjects were recruited at the Endocrinology Service of the University Hospital Joan XXIII and the Sant Pau i Santa Tecla Hospital (Tarragona, Spain). All subjects were of Caucasian origin and their body weight had fluctuated by no more than 2% for at least three months before the study. They showed no evidence of any metabolic disease other than obesity. Hypolipemians in all subjects and hormone therapy in menopausal women were discontinued four weeks before the study. Liver and renal diseases were specifically excluded through a biochemical work-up. Inclusion criteria were BMI < 40 kg/m$^2$, absence of any systemic disease, and absence of clinical symptoms and signs of infection during the previous month. This was ascertained using a structured questionnaire answered by the patient. Patients’ additional clinical data are given in Table 1 and Table 2. Although our obese population consisted of slightly obese (overweight; (BMI: 25 <30) and moderately obese subjects (BMI: 30 <40)), for simplicity reasons we will refer to them as the “obese population” throughout the manuscript.

Anthropometrical measurements and analytical methods

Body height and weight were measured with the patient standing in light clothes and without shoes. Body mass index was calculated as body weight divided by height squared (kg/m$^2$). The subjects’ waist was measured with a soft tape midway between the lowest rib and the iliac crest. Blood pressure was measured in the supine position on the right arm after 10 minutes of rest, and values used in the analysis are the average of three readings taken at 5 minutes intervals.
The same physician performed all examinations. Plasma and serum samples were stored at –80°C until analytical measurements were taken, except for glucose, which was determined immediately after blood was drawn. The serum glucose and lipid profile parameters were determined using standard clinical biochemistry methods. The level of LDL-cholesterol was estimated using the formula: total cholesterol - HDL-cholesterol - (Triglyceride ÷ 5). Fasting insulin was measured in duplicate using a monoclonal immunoradiometric assay (Medgenix Diagnostics, Fleunes, Belgium), with a sensitivity of 4.1μU/l. The degree of insulin resistance was measured using Homeostasis Model Assessments of Insulin Resistance Index (HOMA-IRI) with the equation HOMA-IRI = insulin (µU/ml) x glucose (mmol/l)/22.5 and with a Quantitative Insulin Sensitivity Check Index (QUICKI) calculated as 1/(log insulin (mU/l) + log glucose (mg/dl)). RBP4 in serum samples was measured by nephelometry (Dade Behring Inc., Marburg, Germany). The sensitivity of the method was 0.1ng/dl. CRP levels were determined by nephelometry with the high sensitivity CRP Kit (Dade Behring Inc., Marburg, Germany). Sensitivity was 0.17 mg/l. Plasma adiponectin were measured using the Human Adiponectin RIA Kit (LINCO Research, St. Charles, Missouri, USA) with a detection limit of 1ng/mL. All samples were measured in duplicate.

**Blood and adipose tissue samples**

Blood samples were obtained from the subjects after an overnight fast. For the expression study, samples were obtained on the same day from the patients with abdominal elective surgical procedures (abdominal hernia surgery and cholecystectomy). Blood samples were collected before the surgical procedure. Adipose tissue samples were obtained from the subcutaneous abdominal region (periumbilical region) and were immediately frozen in liquid nitrogen. Each hospital’s ethics committee approved the study and informed consent was obtained from each subject.
Isolation of adipocytes and stromal-vascular cells

Subcutaneous adipose tissue samples from eight subjects, 4 men and 5 women, (age: 52.5±13.4 years and BMI: 28±1.6 kg/m²) were immediately transported to the laboratory in M199 media (Gibco, Invitrogen Corporation, Ca, USA) supplemented with 4% bovine serum albumin and 5.5 mM D-glucose, and digested with 0.15 mg/g adipose tissue of collagenase type I (Sigma, Inc. St. Louis, USA) for 1 h at 37°C. Adipose cells were separated from undigested material (adipose tissue matrix) by filtration through 200 µm mesh fabric. Mature adipocytes were separated from non-fat cells (stromal-vascular fraction) by centrifugation for 10 min at 1500xg. Isolated adipocytes and stromal-vascular fraction cells were washed twice in 1x PBS for five minutes and stored at -80ºC.

RNA analysis

Selection of primers

Primer sets were generated using genomic sequences obtained from Genbank of the National Centre for Biotechnology Information (NCBI) and the Primer3 (v.0.4.0) software. Primers were checked for potential homology to sequences other than the designated target in BLAST (NCBI) searches. The primers, expected cDNA length and annealing temperatures are provided in Table 3.

Verification of primer specificity

To verify primer specificity, each primer set was used in a standard polymerase change reaction (PCR) to amplify cDNA generated by RT-PCR (Promega Corp., Madison, USA) using random hexamer priming of the total RNA isolated from normal sub-AT. Thermal cycling profile consisted of a pre-incubation step at 95ºC for 10 min, followed by 35 cycles of denaturation (95 ºC, 1 min), annealing (55-66, 1 min) and extension (72ºC, 1 min). Products were run on an agarose gel and stained with ethidium bromide to confirm that only one band was amplified and that no primers dimers had formed. An aliquot of the PCR products was then purified using the QIAquick PCR Purification Kit (QIAGEN Science, Maryland, USA) and sequenced to confirm
target specificity. Aliquots of purified PCR products were used to construct standard curves for real-time PCR (see below).

**RNA extraction and quantification**

Total RNA was extracted from frozen sub-AT samples and adipocyte fractions using RNeasy® Lipid Tissue Midi Kit and from stromal-vascular fractions using RNeasy® Mini Kit, (QIAGEN Science, Maryland, USA, both). We verified RNA quality with a gel electrophoresis and optical density measurements. RT was performed with one µg of total RNA using random hexamers (Promega Corp., Madison, USA.). Real-time quantitative PCR for RBP4, adiponectin and CRP was performed using Lightcycler technology with a LightCycler® FastStart DNA Master SYBR Green I Kit (Roche Diagnostics, Basel, Switzerland), following the indications of the manufacturers’ manual. To prepare the standard curves, purified PCR products encoding RBP4, adiponectin, CRP and β-actin (housekeeping gene) were cloned independently in the pCRII-TOPO (TOPO TA cloning kit; Invitrogen Corporation, California, USA), digested with a restriction enzyme and checked by sequentiation. Standard curves and samples were run in duplicate in each real time PCR reaction. mRNA expression values were calculated from the standard curve by Lightcycler Quantification software using the crossing point value and the second derivative maximum method. Gene expressions were expressed relative to the expression of the β-actin housekeeping gene (Ratio=mRNA gene / mRNA housekeeping gene). All four PCRs showed PCR efficiencies close to 2.0. To avoid detection of non-specific PCR products, the purity of each amplified product was confirmed using a melting curve analysis.

**Statistical analysis**

The statistical analysis was performed with the SPSS/PC+ statistical package (v.12 for Windows; Chicago, Illinois, USA). We previously calculated the sample size necessary to obtain a statistical power of 90%. Assuming a circulating RBP4 value of 30±1.0 µg/ml in lean subjects, we need 21 obese patients to obtain a difference of 5 points with a risk α=0.05. In the study with 125 subjects, variables with skewed distribution assessed using the Kolmogorov-
Smirnov test of normality were log-transformed before the analysis, and they were analysed by parametric tests using a two-tailed independent t-test for comparison between groups, and two-tailed Pearson’s correlation coefficient and linear regression analysis for relations between variables. For the expression study, the U Mann-Whitney test and Spearman’s correlation were used. The validity of the regression model and its assumptions was assessed using the plot of residuals vs. predicted. Multivariable linear regression analysis with the classical method was performed to analyse the association between RBP4 systemic concentrations and waist circumference, adjusting for possible confounding factors. Several models were carried out, including age, gender, insulin sensitivity (HOMA-IRI or fasting insulin) and lipid profile (HDL-c and TGL) and we chose the model with the best goodness-of-fit. In the same manner, multiple regression analysis was performed to analyse the association between RBP4 and adiponectin mRNA expression, adjusting for age, gender and waist circumference (or BMI). The level of significance chosen was p < 0.05.
RESULTS

Relationship between RBP4 and metabolic and anthropometric parameters

As expected, the metabolic variables and parameters of insulin resistance analysed were significantly different between lean and obese patients (Table 1). However, while we did not find differences in RBP4 serum levels between lean and obese patients, serum adiponectin levels were significantly lower and CRP levels were significantly higher in the obese patients (Table 1).

In all subjects, there was a tendency for circulating RBP4 levels to be positively correlated with waist circumference \(r=0.24, p=0.06\) and fasting insulin \(r=0.23, p=0.06\), while there was no significant relationship between circulating RBP4 levels and BMI \(r=0.04, p=0.69\), fasting glucose \(r=0.13, p=0.26\), blood pressure (SBP: \(r=0.08, p=0.67\); DBP: \(r=0.10, p=0.50\)), or the insulin resistance measured by either the HOMA-IRI \(r=0.13 p=0.32\) or QUICKI indexes \(r=-0.06, p=0.66\). In addition, RBP4 was not correlated with the lipid profile parameters: total cholesterol \(r=0.33, p=0.60\), triglycerides \(r=0.11, p=0.42\), HDLc \(r=0.13, p=0.30\) and LDLc \(r=0.21, p=0.14\).

When the 38 subjects with the lowest and highest RBP4 serum levels were selected (10.9±0.45 versus 40.6±0.7µg/ml; \(p<0.0001\)), we found no difference in either the HOMA index of insulin resistance (1.0±0.7 versus 1.1±0.8, \(p=0.75\)) or the QUICKI index (0.39±0.05 versus 0.38±0.04; \(p=0.93\)) between each group.

In the multiple regression analysis, in a model including waist circumference and, as confounding factors, age, gender and HOMA-IRI, we found that waist circumference may independently explain the RBP4 systemic levels \(\beta=0.29, p=0.08; R^2 =0.08\), although the circulating RBP4 levels were not significantly correlated with any parameter.

Likewise, we analysed the association between sub-AT RBP4 mRNA expression and the same parameters. The metabolic characteristics of the subpopulation from which sub-AT tissue samples were obtained (Table 2) were similar to those of the whole population (Table 1). We found that RBP4 mRNA expression was not correlated with the parameters described above (\(p>0.09\) for all) with the exception of waist circumference, which was inversely correlated with
RBP4 mRNA levels ($r=-0.33$, $p=0.03$). Due to this significant relation, we performed the correlation and the multiple regression analysis in lean and obese subjects separately. This association of mRNA RBP4 expression levels with waist circumference only remained in the obese group, ($r=-0.56$, $p=0.01$), even after a multivariable regression analysis in a model with age, gender and BMI as confounding factors ($\beta = -0.86$, $p=0.01$; $R^2 = 0.37$).

**Circulating RBP4, adiponectin and CRP and their expression in subcutaneous adipose tissue**

When the mRNA expression of RBP4, adiponectin and CRP was analysed in sub-AT from lean and obese subjects, we observed a significant down-regulation of RBP4 and adiponectin (0.5-fold) and a significant increase in CRP expression (2.7-fold) in the obese population (Fig. 1A).

A comparison of the relative levels of the different molecules in the adipose tissue (Fig. 1A) showed a similar level of RBP4 and adiponectin expression ($1.3 \times 10^0$ and $1.6 \times 10^0$ respectively), whereas CRP expression ($8 \times 10^{-5}$) was weak in comparison to both RBP4 and adiponectin.

Serum adiponectin levels were lower in the obese patients, whereas there was no difference in RBP4 protein levels between lean and obese subjects. Furthermore, the circulating CRP protein level was elevated in the obese patients (Fig. 1B).

In addition, the expression of RBP4 adiponectin or CRP levels in the sub-AT was not correlated with their circulating levels: $r=0.13$, $p=0.45$ for RBP4; $r=0.27$, $p=0.45$ for adiponectin; and $r=0.083$, $p=0.71$ for CRP.

Men and women expressed similar amounts of RBP4 mRNA in AT (ratio=$0.88\pm0.80\times10^0$ versus $1.0\pm0.78\times10^0$; $p= 0.72$) and had similar circulating levels ($30.4\pm0.8 \mu g/ml$ versus $30.3\pm1.4 \mu g/ml$; $p= 0.77$).

**RBP4, adiponectin and CRP expression in cell fractions of adipose tissue**

RBP4 mRNA expression was analysed in the sub-AT fractions and was approximately 500-fold higher in the isolated mature adipocytes than in the non-fat cells of the SVF ($p=0.008$, Table 4). This pattern of RBP4 expression was similar to that of adiponectin, which was also
preferentially expressed in the adipocyte fraction (Table 4). CRP expression was weak in both fractions (Table 4).

**Relationships between RBP4, adiponectin and CRP**

The circulating levels of RBP4 were not correlated with adiponectin or CRP levels (p>0.32 both). The circulating levels of adiponectin were negatively correlated with the CRP levels in the whole population (r =-0.41, p=0.009) and in obese patients (r =-0.50, p=0.005).

Interestingly, RBP4 expression in the sub-AT was positively correlated with adiponectin expression in the whole population (r=0.28, p<0.0001, Fig. 2). This association was evident in the analysis of non-obese and obese subjects separately, yet it was only significant in the obese group (r=0.35, p=0.11 in lean, and r=0.46, p=0.04 in obese).

To ascertain how important the influence of adiponectin expression was on RBP4 mRNA expression, we used a multiple regression analysis model with the possible confounding factors of gender, age, and waist circumference. The relationship between the expression of these two adipokines remained significant even after these adjustments, (β=0.59, p< 0.0001; R² = 0.40).
DISCUSSION

Our analysis between the circulating RBP4 protein levels and parameters of obesity revealed a trend for RBP4 to be positively correlated with waist circumference even after adjusting for the effects of age, gender and HOMA-IRI. On the other hand, RBP4 was not associated with other parameters of adiposity, insulin resistance indexes, blood pressure, glucose or lipid metabolism. The association of RBP4 with waist circumference suggests that this adipokine could be related to abdominal adipose tissue mass (subcutaneous and visceral AT), which has been strongly associated with metabolic disorders, including IR (20).

The lack of relationship between circulating RBP4 levels and indexes of insulin resistance was reported by us in a previous study (11), and is in agreement with several scientific reports (12-14, 21, 22), but not with others (7-10). The reason for such discrepancies among similar human studies remains unclear although it has been suggested they may reflect methodological differences in the determination of RBP4 or IR parameters. In a recent article by Graham et al, (23) the nephelometric assay of RBP4, which we have used in this study, had a good correlation with RBP4 quantitative Western blotting, which is the most reliable method for determining RBP4 protein levels in blood (23). Furthermore, the inclusion in the study population of subjects with diabetes (9), or morbid obesity (10), the differences in ethnicity (8), or the different age of the obese population (7), could affect the regulation of RBP4 and possibly contribute to such discrepancies. In our case RBP4 was not related to IR in the same population in which as expected (18,19), an association of adiponectin with IR and the lipid profile was evident (data not shown).

Likewise, the statistical analysis of RBP4 mRNA expression in sub-AT showed that RBP4 did not associate with BMI, insulin resistance indexes, blood pressure, glucose, and lipid metabolism. However, we did find a significant negative correlation of RBP4 with waist circumference in this tissue, which was maintained even after adjusting for the effects of age, gender and BMI only in obese subjects. This finding is consistent with the down-regulation of RBP4 mRNA expression in the obese population and points to other tissues as principal sources of serum RBP4, including visceral AT, muscle and liver (see below).
We show that, in human sub-AT, RBP4 mRNA expression levels are comparable to the mRNA levels of adiponectin, one of most abundant adipokines (24). Interestingly, in cultures of human sub-AT explants, the rate of RBP4 protein observed was comparable to that of adiponectin (12). In addition, we found that both RBP4 and adiponectin were predominantly expressed in the mature adipocyte fraction of sub-AT, in agreement with other scientific reports (12-14).

The mRNA expression of RBP4 and adiponectin diminished approximately 50% in obese patients. However, down-regulation of RBP4 and adiponectin in the sub-AT was not followed by a down-regulation of the systemic RBP4 levels. This discrepancy between adiponectin and RBP4 could be due to the different tissues that express and secrete these two adipokines.

Adiponectin is only expressed in AT, in classic and/or ectopic fat deposits (25), and it is established as a hormone released by the AT into the circulation. By contrast, RBP4 is expressed in the AT and other tissues such as liver, skeletal muscle and kidney (26). The contribution of AT and these other tissues to the systemic levels of RBP4 is not known in humans. It has been suggested that hepatocytes contribute to a large proportion of systemic RBP4 protein concentration (27), as it happens in rodents (28). Circulating RBP4 has also been related to the amount of fat accumulation in non-adipose tissues such as skeletal muscle and the liver (29). Our observation of a decrease in RBP4 expression in the sub-AT while RBP4 circulating levels remained similar was also observed by Janke et al., in obese women compared with lean (14). In both studies the subjects were non-diabetic and non-morbidly obese, it is possible that the inclusion of morbidly obese subjects could differentially affect the expression of RBP4 in sub-AT (10).

RBP4 and adiponectin mRNA expression was positively correlated and, interestingly, this correlation was also observed in the skeletal muscle of non-diabetic subjects by Ribel-Madsen et al. (30). The mechanisms that underlie the association between these two adipokines in sub-AT may be related to the reported autocrine action of adiponectin on primary adipocytes described previously (31).

On the other hand, we did not observe any relationship between RBP4 and CRP. Circulating CRP was inversely correlated with adiponectin levels in obese patients, as reported previously.
The failure to identify an association between circulating levels of RBP4 and CRP suggests that RBP4 is not directly associated with obesity-related low-grade inflammation. One limitation of our expression study is that we only assessed adipokine expression in sub-AT and thus, we cannot translate these relationships to visceral AT where changes in systemic RBP4 levels have recently been associated with adipokine expression (33). Indeed in this tissue single nucleotide polymorphisms of RBP4 have also been related to changes in RBP4 expression (34).

In summary, in this study we have simultaneously examined RBP4, adiponectin and CRP expression in the sub-AT and we have compared their circulating levels in obese patients with those in lean subjects. Although the association of serum RBP4 with waist circumference may suggests a relationship with insulin resistance and other metabolic disorders, RBP4 circulating levels or mRNA expression were not significantly correlated with obesity-related parameters in our population. RBP4 was expressed at levels comparable to those of adiponectin, which is the most highly expressed adipokine in AT. Our results suggest that the production of RBP4 by other tissues such as liver, rather than sub-AT, may be involved in the fluctuations of its circulating levels.
DECLARATION OF INTEREST:

The authors declare that no financial or other potential conflict of interests exists in relation to this study.

ACKNOWLEDGMENTS

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REFERENCES


10. 


FIGURE TITLES & LEGENDS

Figure 1
Subcutaneous adipose tissue mRNA expression and circulating levels of RBP4, adiponectin and CRP. RBP4, adiponectin and CRP mRNA expression in subcutaneous adipose tissue (A) and protein circulating levels (B) of lean compared with obese subjects. Values are means ± SE. Ratio expresses mRNA gene / mRNA β-actin gene. Differences between groups were analysed using U Mann-Whitney test. * Denotes p < 0.05

Figure 2
Relationship between RBP4 and adiponectin expression levels in subcutaneous adipose tissue in whole population. Values of $R^2$ and p are from nonadjusted data.
Figure 1

A

\[ \text{Ratio } \times 10^0 \]

\[ \begin{align*}
&\text{lean} \quad \boxed{\text{black}} \\
&\text{obese} \quad \boxed{\text{white}}
\end{align*} \]

\[ * \text{ p = 0.04} \]

\[ \text{RBP4} \]

\[ \text{µg/mL} \]

B

\[ \text{Ratio } \times 10^0 \]

\[ \begin{align*}
&\text{Adiponectin} \\
&\text{CRP}
\end{align*} \]

\[ * \text{ p = 0.006} \]

\[ p = 0.05 \]

\[ * \text{ p = 0.03} \]

\[ \text{µg/mL} \]
Figure 2

![Graph showing mRNA expression of adiponectin and RBP4](image)

- mRNA adiponectin expression
- mRNA RBP4 expression

R^2 = 0.28

p < 0.0001
Table 1. Metabolic characteristics of lean and obese subjects

<table>
<thead>
<tr>
<th></th>
<th>Lean BMI &lt;25 (n=36)</th>
<th>Obese BMI ≥25 &lt; 40 (n=89)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>50.8 ± 16.4</td>
<td>57.8 ± 14.3</td>
<td>0.01*</td>
</tr>
<tr>
<td>Sex (n, men/women )</td>
<td>25/11</td>
<td>58/31</td>
<td>0.65</td>
</tr>
<tr>
<td>BMI (kg/m^2)</td>
<td>22.8 ± 1.8</td>
<td>29.5 ± 3.5</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Waist (cm)</td>
<td>82.4 ± 9.6</td>
<td>99.9 ± 11.4</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Fasting glucose (mM)</td>
<td>5.1 ± 0.7</td>
<td>5.5 ± 0.6</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Fasting insulin(µU/l)</td>
<td>4.4 ± 3.9</td>
<td>5.8 ± 3.8</td>
<td>0.02*</td>
</tr>
<tr>
<td>HOMA (µU/mL x mM)</td>
<td>0.8 ± 0.06</td>
<td>1.5 ± 1.2</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>QUICKI</td>
<td>0.4 ± 0.04</td>
<td>0.3 ± 0.05</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>122.4 ± 17.2</td>
<td>132.9 ± 22.0</td>
<td>0.004*</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>70.9 ± 10.9</td>
<td>76.3 ± 10.1</td>
<td>0.003*</td>
</tr>
<tr>
<td>Total cholesterol (mM)</td>
<td>4.9 ± 1.0</td>
<td>5.5 ± 0.8</td>
<td>0.009*</td>
</tr>
<tr>
<td>Triglycerides (mM)</td>
<td>1.1 ± 0.6</td>
<td>1.5 ± 0.7</td>
<td>0.03*</td>
</tr>
<tr>
<td>LDL cholesterol (mM)</td>
<td>3.2 ± 0.9</td>
<td>3.9 ± 0.7</td>
<td>0.009*</td>
</tr>
<tr>
<td>HDL cholesterol (mM)</td>
<td>1.4 ± 0.2</td>
<td>1.2 ± 0.1</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>RBP4 (µg/ml) (nephelometry)</td>
<td>30.2 ± 1.0</td>
<td>30.4 ± 3.4</td>
<td>0.47</td>
</tr>
<tr>
<td>Adiponectin (µg/ml)</td>
<td>15.4 ± 5.8</td>
<td>11.5 ± 5.0</td>
<td>0.001*</td>
</tr>
<tr>
<td>CRP (mg/dl)</td>
<td>1.3 ± 1.3</td>
<td>3.0 ± 3.8</td>
<td>0.002*</td>
</tr>
</tbody>
</table>

Data are mean ± S.D.; BMI: body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; LDL: low-density lipoprotein; HDL: High-density lipoprotein. Differences between groups were analysed by two-tailed Student’s t Test. *Denotes statistical differences between the groups (p< 0.05)
Table 2. Metabolic characteristics of lean and obese subjects for the expression study

<table>
<thead>
<tr>
<th></th>
<th>Lean BMI &lt;25 (n=22)</th>
<th>Obese BMI ≥ 25 &lt; 40 (n= 24)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>47.0 ± 16.3</td>
<td>55.0 ± 14.2</td>
<td>0.11</td>
</tr>
<tr>
<td>Sex (n, men/women)</td>
<td>12/10</td>
<td>13/11</td>
<td>0.76</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.0 ± 1.4</td>
<td>30.0 ± 3.9</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Waist (cm)</td>
<td>81.1 ± 9.2</td>
<td>100.3 ± 9.9</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Fasting glucose (mM)</td>
<td>5.1 ± 0.4</td>
<td>5.7 ± 0.9</td>
<td>0.05</td>
</tr>
<tr>
<td>Fasting insulin(µU/l)</td>
<td>3.3 ± 2.6</td>
<td>5.5 ± 2.8</td>
<td>0.04*</td>
</tr>
<tr>
<td>HOMA (µU/mL x mM)</td>
<td>0.7 ± 0.4</td>
<td>1.4 ± 0.7</td>
<td>0.03*</td>
</tr>
<tr>
<td>QUICKI</td>
<td>0.4 ± 0.04</td>
<td>0.3 ± 0.05</td>
<td>0.03*</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>126.3 ± 15.9</td>
<td>133.1 ± 15.6</td>
<td>0.27</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>72.9 ± 10.2</td>
<td>81.9 ± 7.8</td>
<td>0.007*</td>
</tr>
<tr>
<td>Total cholesterol (mM)</td>
<td>4.6 ± 0.1</td>
<td>5.7 ± 1.0</td>
<td>0.02*</td>
</tr>
<tr>
<td>Triglycerides (mM)</td>
<td>1.3 ± 0.8</td>
<td>1.6 ± 1.3</td>
<td>0.05</td>
</tr>
<tr>
<td>LDL cholesterol (mM)</td>
<td>3.1 ± 0.6</td>
<td>4.1 ± 1.0</td>
<td>0.04*</td>
</tr>
<tr>
<td>HDL cholesterol (mM)</td>
<td>1.3 ± 0.3</td>
<td>1.2 ± 0.2</td>
<td>0.53</td>
</tr>
</tbody>
</table>

Data are mean ± S.D.; BMI: body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; LDL: low-density lipoprotein; HDL: High-density lipoprotein. Differences between groups were analyzed by U Mann-Whitney Test. *Denotes statistical differences between the groups (p < 0.05)
Table 3. Primers sequences, cDNA length, and annealing temperatures used for expression assessment of RBP4, adiponectin, CRP and housekeeping gene (β-actin) by Real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence Sense Anti-sense</th>
<th>cDNA length (bp)</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBP4</td>
<td>gcctttttgtcaggacaaacaagggcttgcaccagcatgctt</td>
<td>132</td>
<td>60.7</td>
</tr>
<tr>
<td>Adiponectin</td>
<td>gctgggagctgttactgc</td>
<td>233</td>
<td>55.8</td>
</tr>
<tr>
<td>CRP</td>
<td>gggacttctagcccttgacacgtcttggctgccccagcatacga</td>
<td>321</td>
<td>55</td>
</tr>
<tr>
<td>β-actin</td>
<td>ggacttgagcaagagatggactcctgctgtggctacag</td>
<td>234</td>
<td>66</td>
</tr>
</tbody>
</table>
Table 4. Relative expression of RBP4, adiponectin and CRP in isolated adipocytes and in the stromal-vascular fraction (SVF) of human subcutaneous adipose tissue.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Adipocyte</th>
<th>SVF</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBP4</td>
<td>2.5x10^{-1} ± 6.2x10^{-4}</td>
<td>5.6x10^{-4} ± 2.6x10^{-4}</td>
<td>0.008*</td>
</tr>
<tr>
<td>Adiponectin</td>
<td>5.6x10^{-1} ± 1.0x10^{-1}</td>
<td>3.6x10^{-6} ± 1.8x10^{-6}</td>
<td>0.005*</td>
</tr>
<tr>
<td>CRP</td>
<td>4.0x10^{-6} ± 3.7x10^{-8}</td>
<td>2.6x10^{-5} ± 1.1x10^{-5}</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Subcutaneous adipose tissue biopsies were obtained from eight subjects and the two fractions were separated by collagenase digest. Expression by Real-time PCR was expressed as ratio: mRNA of gene/mRNA β-actin gene and the results were the means ± S.D. of values. *Indicates statistically significant difference (p < 0.05) using U Mann-Whitney test.