RESEARCH PAPER

Gene expression in a rarely studied intraabdominal adipose depot, the round ligament, in severely obese women: A pilot study

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ABSTRACT

Gene expression (qPCR) was compared in round ligament (RL), omental (OME) and mesenteric (MES) ATs from 48 severely obese women (BMI, 54±11 kg/m²; 38±9 yrs). The mRNA levels of enzymes of lipid metabolism (LPL, HSL, and PDE-3B), cortisol production (11βHSD-1), adipogenesis (PPAR-γ1/2), thrombosis and inflammation (PAI-1, IL-6, TNF-α and adiponectin) were determined. AT-LPL mRNA was highest in RL. The highest PDE-3B and lowest PAI-1 mRNA levels were observed in RL and MES. The lowest IL-6 and TNF-α and the highest adiponectin and PPAR-g1/2 mRNA levels were found in RL AT. 11βHSD-1 was highest in RL and OME. A higher lipogenic and adipogenic, and lower pro-inflammatory and pro-thrombotic profiles of the RL suggest a lesser deleterious impact on obesity-related complications.

INTRODUCTION

The prevalence of class III obesity continues to dramatically increase in Canada.1 With respect to health outcomes more important than obesity per se is regional fat distribution. Abdominal obesity is closely associated with an increased cardiometabolic risk.2 AT plays an important role in these metabolic disorders through its lipid storage and mobilizing capacities as well as its secretory activity.3,4 In addition, AT is a major site for glucocorticoid metabolism through 11βHSD-1, a key enzyme in the local cortisol sol production,5 which activates a class of nuclear receptors, the PPAR-γ1/2 that control adipogenesis, modulate fat mass accretion and regulate insulin-glucose homeostasis.6 AT mass expansion is also dependent on the functional balance between lipid storage and mobilization, mainly through LPL and HSL activities,7 whose major regulatory hormones are catecholamines acting on both α2- and β-ARs, but also via PDE-3B through which insulin exerts its antilipolytic and lipogenic effects.8 Finally, AT is known to secrete a number of factors among which adipokines such as IL-6 and TNF-α (pro-inflammatory), adiponectin (anti-inflammatory), as well as PAI-1 and angiotensinogen (pro-thrombotic) may be involved in obesity-related metabolic complications.9,10

Because of its anatomical location which provides direct access to the hepatic portal circulation, the uncontrolled release of FAs and secretory factors from intraabdominal fat accumulation may contribute to metabolic disorders.4,7,11,12 However, functional heterogeneity among intraabdominal ATs exists. Rebuffé-Schivre et al.13 reported in severely obese women a higher lipolytic response to catecholamines and a lower antilipolytic sensitivity to insulin, in intraperitoneal (i.e., OME and MES) fat drained by the portal vein than in retroperitoneal AT. Indeed, Fried et al.14 showed that MES adipose cells were more lipolytically responsive to norepinephrine than OME adipocytes, results contradictory to those of Van Harmelen et al.15 LPL activity was lower in OME than in MES AT, in massively obese women.16,17 Although MES fat is more rarely studied because of its difficult access, another intraabdominal depot, the RL AT drained by collaterals of the portal vein,7,18 has received even less attention. Our group has previously shown that the RL AT displayed higher fat cell size and LPL activity, a lower lipolytic response to catecholamines vs. a greater effect of insulin on both antilipolysis and glucose transport, when compared to OME fat in severely obese women.19,20

To the best of our knowledge, whether the RL AT has secretory characteristics similar to or different from other intraabdominal fat depots drained by the portal vein has never been examined in the context of severe obesity. Therefore, the main objective of our study was...
to compare gene expression of selected metabolic biomarkers and secretory factors between RL, OME and MES adipose depots. We hypothesized that the RL AT would display characteristics that contribute to limit the deleterious impact of intraabdominal fat accumulation to obesity-related metabolic complications.

Results and discussion

Patients’ characteristics

As shown in Table 1, severely obese women whose mean BMI was greater than 50 kg/m², were characterized by a high degree of abdominal fat reflected by a mean waist circumference of 140 cm. Despite their massive obesity, their lipid-lipoprotein profile was not markedly deteriorated, although resting systolic and diastolic BP values approached the thresholds for hypertension defined by the Eighth Joint National Committee. The large range of fasting glycemia, insulinemia and HOMA-insulin resistance index indicated that our sample included patients ranging from glucose tolerant to diabetic, according to the Diagnosis and Classification of Diabetes Mellitus WHO guidelines.22

Our data show clear differences in the characteristics of the 3 ATs examined, highlighting important inter-depot potential to contribute to metabolic perturbations.

Regional variation in AT gene expression of FA metabolism and antilipolysis/lipolysis biomarkers

As illustrated in Figure 1 (panel A), LPL mRNA levels were the highest in the RL depot (0.001<\(p<\)0.05), although HSL expression did not show any regional variation (panel B). The highest PDE-3B expression was observed in both RL and MES fat, when compared to the OME AT (0.001<\(p<\)0.05) (panel C). The greater lipogenic capacity of the RL AT, reflected by its higher LPL expression, is concordant with the increased LPL activity of this depot compared to OME fat previously reported by our group. The similar lipolytic capacity of the RL and OME fats, attested by a comparable HSL expression, is in accordance with the lack of regional variation in lipolysis stimulated by dibutylryl-cyclic AMP (which selectively acts at the level of protein kinase-HSL complex) also previously observed by our group. Moreover, the higher PDE-3B expression we found in the RL and MES fat, compared to the OME AT, probably reflects a greater insulin-induced antilipolysis in the former depots. Two decades ago, we reported that RL adipose cells displayed a higher antilipolytic effect of insulin than OME adipocytes. In this regard, trends for a higher theophylline (inhibitor of PDE)-stimulated lipolysis in RL than in OME adipocytes were reported 2 decades ago. As both insulin and LPL favor triacylglycerol accumulation in adipocytes, the highest LPL expression combined with the greatest PDE-3B one of the RL AT may suggest that this depot is quite efficient in lipid storage. In agreement with this hypothesis, RL adipocytes were already found to be more sensitive to the antilipolytic action of insulin and its stimulatory effect on glucose transport, when compared to OME fat cells.

The lack of differences in LPL mRNA levels between OME and MES depots in the present study suggests that enzyme activity is similar in these intraperitoneal ATs. This contrasts with the data of Fried and Kral, who documented a higher LPL activity in the MES than in the OME AT of massively obese women, although others did not find any regional variation in severely obese individuals. Reasons for such discrepancies could be partly due to differences in the subjects’ characteristics and methods used to measure LPL activity. The lack of differences in HSL mRNA levels between OME and MES fat is concordant with the similar expression and enzyme activity found in these depots by Nagashima et al. suggesting that enzymes such as the adipose triglyceride lipase and carboxylesterase 1 could play a more important role in regulating regional variation in lipolysis.

The highest a2-AR mRNA levels were observed in both the RL and OME depots compared to the MES (\(p<\)0.05), while the lowest a2-AR mRNA levels were found in the RL fat (0.001<\(p<\)0.05) (Fig. 1, panels D and E, respectively). The lower a2-AR mRNA levels of the RL than of the OME fat that we report here is in line with the reduced \(\beta\)-adrenergic lipolytic sensitivity and \(\beta\)-AR density we have previously shown in this tissue.
The comparable β2-AR expression observed in the MES and OME fat depots is concordant with their similar β-(and more particularly β2-) AR function reported by Van Harmelen et al.\textsuperscript{15} The high α2-AR expression in the RL and OME fat also fits well with their enhanced α2-adrenergic antilipolytic sensitivity and epinephrine responsiveness, but not with the greater α2-AR density of the former depot we have already reported.\textsuperscript{19} However, the lower α2-AR expression in the MES than in the OME AT in our study does not fully agree with the similarly reduced α2-AR function of both depots observed by Van Harmelen et al.\textsuperscript{15} Such discrepancies could be due to the low number of subjects, their physical and metabolic characteristics, and to the fact that data at molecular (the present study) and cellular levels were compared.\textsuperscript{15,19} We could, however, hypothesize that the α2-AR component of the RL and OME fat depots may represent a protective mechanism against obesity-related metabolic complications, by impairing the uncontrolled release of FAs to the portal vein.

**Regional variation in AT gene expression of inflammatory and prothrombotic factors**

As shown in Figure 2, TNF-α expression was lower in the RL than in the other depots (p < 0.001) (panel A). The lowest IL-6 (0.01 < p < 0.05) and the highest adiponectin (p < 0.001) mRNA levels were observed in the RL AT (panels B and C, respectively). Moreover, PAI-I expression was lower in both the RL and MES than in the OME fat (p < 0.001), while angiotensinogen expression did not show any regional variation (Fig. 2, panels D and E, respectively). The lower TNF-α and IL-6 and the higher adiponectin mRNA levels found in the RL AT attest to its low pro-inflammatory vs. high anti-inflammatory characteristics, when compared to the OME and MES ATs. Of these 2 latter depots, OME is known to express the more deleterious inflammatory profile,\textsuperscript{3,4} although MES has been little studied in this regard. Although being similar to MES AT, the reduced expression of PAI-I in the RL compared to OME fat clearly attests to a low pro-thrombotic potential. However, whether the RL AT plays a more protective role against inflammation and thrombosis than OME and/or MES fat needs to be confirmed by studies on moderately obese and/or overweight individuals.

As shown in Figure 3 (panels A and B), the highest expression of PPAR-γ1/2 was found in the RL AT (0.001 < p < 0.05). PPAR-γ2 mRNA levels were approximately 2-fold higher than PPAR-γ1 mRNA levels, irrespective of the adipose region (p<0.01). Also, 11βHSD-1 mRNA levels were higher in both the RL and OME depots than in the MES fat (p<0.001) (Fig.3, panel C). The increased 11βHSD-1 expression of the RL AT is
concordant with this tissue’s higher PPAR-γ1/2 expression, compared to the MES fat, as cortisol is known to activate these nuclear receptors that control adipogenesis. However, the fact that OME showed a local cortisol production similar to the RL AT does not fit with its lower PPAR-γ1/2 mRNA levels, thus suggesting that other factors such as TNF-α and IL-6, whose expression is higher in OME than in RL fat, may inhibit its adipogenic potencies. Also, the increased adipogenesis of the RL AT, compared to the other sites, suggests that this depot could have an embryological origin different from the OME and/or MES fat. In one study performed on human depots, pre-adipocytes of MES AT showed an expression profile closer to that of subcutaneous abdominal rather than OME fat. In this regard, although the lateral plate mesoderm was recently considered a major source of visceral fat, the common developmental origin of 6 different depots examined (perirenal, gonadal, epicardial, retroperitoneal, OME and MES) is still a matter of debate. Although the Wilms’ tumor gene, Wt1, was shown to be a determinant of mesenchymal progenitors in the development of visceral fat, further studies are clearly needed to examine whether RL AT derives from the Wt1-progenitors similar to those of OME and MES fat depots. Table 2 is a graphic representation which summarizes the regional variation in the AT gene expression profile.

**Figure 2.** Regional variation in AT mRNA levels of inflammatory TNFα, IL-6 and Acrp30 (panels A, B and C, respectively) and of prothrombotic PAI-1 and AGT factors (panels D and E, respectively). Acrp30: Adiponectin; AGT: Angiotensinogen; Statistical significance at \( p < 0.001; \) \( p < 0.01 \) and \( p < 0.05 \).

**Regional variation in AT gene expression of selected biomarkers and secretory factors between diabetic and non diabetic patients**

In order to evaluate the potential impact of the inclusion of diabetic subjects on the reported depot differences, we re-ran the ANOVA analyses after excluding these 13 subjects. In only 2 instances did we lose a significant difference reported: for LPL, the difference between LR and MES depots was lost \( (p=0.097) \), and for IL6, difference between OME and LR was lost \( (p=0.053) \). To explain these observations, it could be argued that the loss of statistical power was an important contributor. Overall, we conclude that the inclusion of the diabetic subjects had no or very limited impact on differences in gene expression.

**Correlational studies**

Based on the wide range of plasma adipokine levels observed in our patients (Table 1), we wondered whether these concentrations could be partly explained by AT gene expression, irrespective of the anatomic location of fat. No significant relationship was, however, observed between plasma TNF-α, IL-6, adiponectin or PAI-1 and their respective AT mRNA levels in the 3 depots examined \((-0.18<r<0.22)\) (data not shown). Finally, the lack
of relationships between plasma and AT mRNA levels of the adipokines investigated should be interpreted with caution as measuring their circulating levels cannot inform us on the changes to the expression or secretory profile of any given specific intraperitoneal and portal-vein drained adipose depot.

Our study does have some limitations. First, we were not able to directly measure body composition, as patients who weigh more than 130 kg cannot be subjected to dual energy X-ray absorptiometry at our research center due to equipment limitations. Second, as visceral fat accumulation could not be assessed by computed tomography, we could not measure or estimate the size of the intraabdominal fat depots examined. Third, as AT contains adipocytes and stromal cells including pre-adipocytes, monocytes and lymphocytes (i.e., the stromal vascular fraction), depot-specific heterogeneity in the proportion of each cell type could modulate the differences in gene expression we observed.

In summary, our study is the first to compare the gene expression profile of some metabolic biomarkers and secretory factors of the RL AT to those of better documented intra-abdominal fat depots, the OME and MES. The higher lipogenic, adipogenic and anti-inflammatory characteristics, as well as the lower pro-inflammatory and pro-thrombotic profiles of the RL AT, could suggest a lesser deleterious impact on some obesity-related metabolic complications in severely obese women.

Materials and methods

Patients

This study included 48 Caucasian premenopausal severely obese women, who were candidates for biliopancreatic diversion with duodenal switch surgery at the Quebec Heart and Lung University Institute Hospital. Subjects with hepatotoxic medication, history of hepatitis, prior weight-loss surgery, smoking and/or consuming more than 100 g of alcohol per week were excluded. Thirteen women were diabetic according to the Diagnosis and Classification of Diabetes Mellitus WHO guidelines. Although diabetic subjects were treated pharmacologically for their condition, none was treated with glitazones.

The experimental design was approved by our local hospital and university ethics committees (Laval University Ethics Committee approval CERUL # 2004-108), and all participants provided their written informed consent.

Patients were weighed in a light gown on an electronic Toledo scale adapted for body weights greater than 136 kg, and BMI was calculated as the ratio of weight (kg) to height squared (m²). Body fat distribution was assessed using waist circumference measured in a standard position at the widest circumference of the umbilical level. Resting BP measurements were performed at the Quebec Heart and Lung University Institute Hospital, the day before bariatric surgery by the nursing staff using Life Sign (Welch Allyn Co., Skaneatles, NY) with a large cuff while subjects were recumbent.

Blood sampling was performed in the morning after a 12-hour-overnight fast. Plasma cholesterol, high-density lipoprotein-cholesterol, low-density lipoprotein-cholesterol, triacylglycerol and fasting glucose concentrations were evaluated by routine methods in the clinical laboratories of the Quebec Heart and Lung University Institute Hospital.
Table 2. Summary of regional variation in AT gene expression of selected biomarkers and secretory factors.

| Family                      | Gene                   | RL | MES | OME |
|-----------------------------|------------------------|----|-----|-----|
| FA Metabolism               | LPL                    |    |     |     |
|                             | HSL                    |    |     |     |
|                             | PDE-3B                 |    |     |     |
|                             | α2-AR                  |    |     |     |
|                             | β2-AR                  |    |     |     |
| Antilipolysis/lipolysis     | TNF-α                  |    |     |     |
|                             | IL-6                   |    |     |     |
| Inflammation                | Acrp30                 |    |     |     |
| Vascular homeostasis        | PAI-1                  |    |     |     |
| Adipogenesis and local cortisol production | PPAR-γ1             |    |     |     |
|                             | PPAR-γ2                |    |     |     |

For abbreviations, see the list provided in the front page. Expression level is indicated by shade intensity, black being highest and white lowest. Depots sharing the same shade for a given gene have similar (not statistically different) mRNA levels.

Plasma insulin concentrations were determined by a high-sensitivity ELISA (Cedarlane Laboratories Ltd, Hornby, Ontario, Canada). Insulin resistance based on the HOMA index was evaluated according to the following equation: insulin (μU/mL) x glucose (mmol/L)/22.5. Plasma TNF-α and IL-6 concentrations were measured by a high-sensitivity ELISA (R&D Systems Inc., Minneapolis, MN). The run-to-run coefficients of variation were less than 10% for IL-6 and TNF-α. Plasma adiponectin concentrations were determined by ELISA (B-Bridge International, Inc., San José, CA), and intra- and inter-assay coefficients of variation were 3.3 and 7.4%, respectively. Finally, another blood sample was collected in plastic tubes containing trisodium citrate anticoagulant with great care in order to minimize platelet activation. Plasma PAI-1 antigen levels were measured by ELISA using Asserachrom PAI-1 kits from Diagnostica Stago (Asnières, France), and both intra- and inter-assay coefficients of variation were less than 5%.

**Adipose tissue removal**

AT samples were recovered following an overnight (12-hour) fast. General balanced anesthesia was induced by a short-acting barbiturate and maintained by fentanyl and a mixture of oxygen and nitrous oxide. Patients did not receive drugs acting on the autonomic nervous system or modifying catecholamine levels. All adipose depots under consideration are drained by the portal vein or its collaterals, and classified as intraabdominal, and more particularly as intraperitoneal fat. After surgical excision, AT samples of approximately 200 mg were obtained from the OME and MES fat, as well as from the RL AT surrounding the liver. From these samples, 100 mg were used for quantification of the 13 selected AT gene mRNA levels. All measurements were performed in duplicate.

**RNA preparation and quantification of AT gene mRNA levels**

Briefly, about 5 to 10 μg of total RNA was extracted from AT samples of 100 mg, using RNeasy Mini kits (Qiagen, Mississauga, Ontario, Canada). This technique integrates phenol-guanidine lysis and silica gel-membrane purification of total RNA. The isolated total RNA concentration was quantified by absorbance at 260 nm, and its integrity was verified using agarose gels (1%) stained with ethidium bromide. The 260/280 nm absorption of all preparations ranged between 1.9 and 2.1. For each depot, 1 μg of total RNA was reverse transcribed into cDNA, using SuperScript II Reverse Transcriptase kit (Invitrogen Canada, Burlington, Ontario), and 50 ng of cDNA were used for qPCR. AT mRNA levels were quantified by fluorescent qPCR on a Rotor Gene 3000 (Corbett Research, Montreal Biotech, Kirkland, Québec, Canada), using the SYBR Green Jump Start TAQ ready mix (Sigma-Aldrich, Oakville, Ontario, Canada).

The 13 genes examined were classified into 5 “families”: FA metabolism (LPL, HSL and PDE-3B), antilipolysis/lipolysis (α2-ARs, β2-ARs), inflammation (TNF-α, IL-6 and adiponectin), vascular homeostasis (PAI-1, angiotensinogen), adipogenesis and local cortisol production (PPAR-γ1, 11βHSD-1). Primer forward and reverse sequences for genes of interest as well as the corresponding number of cycles for PCR are shown in Table 3. Conditions for PCR consisted in an initial denaturation step at 95°C for 2 min, followed by the specified number of cycles composed of 20 sec at 95°C, 20 sec annealing at 60°C or 64°C depending on the gene, and 20 sec elongation at 72°C. Reactions were then heated between 72 and 99°C, raising each step by 1°C for 5 sec to obtain the melting curve and to confirm a single product of PCR. The mRNA levels were calculated using the standard curve method (Rotor Gene 3000) and normalized to GAPDH considered as the housekeeping gene.

**Statistical analyses**

Results are presented as means ± standard deviation (SD) in tables and as means ± standard error (SE) in figures. Regional variation was analyzed by an analysis of variance (ANOVA) and post-hoc analysis using Bonferroni correction was used to compare expression of genes between the 3 fat depots. Finally, associations between variables of interest were quantified by linear regression analysis using Pearson’s product moment correlation coefficients. All analyses were conducted using the SAS software (9.1.6 for Windows) and statistical significance was considered attained if p<0.05.
Table 3. Primer sequences and PCR conditions for genes of interest.

| Gene            | Annealing T | Ct | Primers                                                                 |
|-----------------|-------------|----|-------------------------------------------------------------------------|
| PPAR-γ1         | 60 C        | 35 | F 5'-AAAAAGACGGCAAGACAAAGCCTTCCATTACGGAGAGATCC-3'                        |
| PPAR-γ2         | 60 C        | 35 | F 5'-GGGATCCCTCTTACTGATATCAG-TATAACGAGATCC-3'                            |
| PAI-1           | 60 C        | 40 | F 5'-AGCTCCTAACCGGCTGAGAGAAGATGAGTGGCAGGCCG-3'                           |
| IL-6            | 64 C        | 40 | F 5'-GAAGATCCCTGCCCTTCCTCCTACTCCCTCCTACA-3'                              |
| TNF-α           | 64 C        | 35 | F 5'-AAGCTGTAGCCCATGGTCTGCTA-3'                                        |
| Acrp30          | 64 C        | 30 | F 5'-CTGTTGCTGGACAGCTGCTA-3'                                            |
| AGT             | 64 C        | 40 | F 5'-CTGAGCCTCACAGAAGTAGATG-3'                                          |
| 11βHSD-1        | 64 C        | 35 | F 5'-TTGAGAAGGAGGAGACATGAGAAG-3'                                        |
| HSL             | 64 C        | 40 | F 5'-CCCCACACTCTCTATGGCTA-3'                                            |
| LPL             | 64 C        | 30 | F 5'-GTGGTACACTCCTGCTCACC-3'                                            |
| PDE-3B          | 64 C        | 40 | F 5'-GTCGCTGCTAGTCCTCACC-3'                                             |
| α2-AR           | 64 C        | 30 | F 5'-GTTGAGGAGGAGGAGACATGAGAAG-3'                                       |
| δ2-AR           | 64 C        | 35 | F 5'-ACAGCATTGGCAAGTTGAGAAG-3'                                          |
| GAPDH           | 64 C        | 35 | F 5'-ACAGCCATTGGCAAGTGGAGAAG-3'                                         |

F: forward; R: reverse; T: Temperature; Ct: Cycle threshold.

For other abbreviations, see the list provided in the front page.

**Abbreviations**

AT: adipose tissue
ARs: adrenoceptors
BMI: body mass index
BP: blood pressure
ELISA: enzyme-linked immunoabsorbent assay
FA: fatty acid
GAPDH: GlycerAldehyde Phosphate Dehydrogenase
HOMA: HOmeostasis Model Assessment
HSL: Hormone-Sensitive Lipase
11βHSD-1: 11betaHydroxySteroid Dehydrogenase type-1
IL-6: InterLeukin-6
LPL: Lipoprotein Lipase
MES: mesenteric
OME: omental
PAI-1: Plasminogen Activator Inhibitor-1
PPAR-γ: Peroxisome Proliferator-Activated Receptors-gamma
PDE-3B: Phosphodiesterase-3B
qPCR: quantitative Polymerase Chain Reaction
RL: round ligament
TNF-α: Tumor Necrosis Factor-α
WHO: World Health Organization

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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