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Altered pattern of cannabinoid type 1 receptor expression in adipose tissue of dysmetabolic and overweight patients

Riccardo Sarzani[a,*], Marica Bordicchia[a], Pierfrancesco Marcucci[a], Samuele Bedetta[a], Silvia Santini[a], Andrea Giovagnoli[a], Lorena Scappini[a], Daniele Minardi[b], Giovanni Muzzonigro[b], Paolo Dessì-Fulgheri[a], Alessandro Rappelli[a]

[a]*Department of Internal Medicine, University of Ancona, "Politecnica delle Marche," 60131 Ancona, Italy*
[b]*Department of Urology, University of Ancona, "Politecnica delle Marche," 60131 Ancona, Italy*

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Abstract

In overweight patients (OW), the increased peripheral activity of the endocannabinoid system in visceral adipose tissue (VAT) may be mediated by cannabinoid type 1 (CB1) receptor expression. We determined whether CB1 receptor splice variants and messenger RNA (mRNA) levels in perirenal and subcutaneous adipose tissues are associated with obesity and metabolic syndrome (MetS). Gene expression with multiple-primers real-time polymerase chain reaction (TaqMan; Applied Biosystem, Weiterstadt, Germany) was performed to study VAT and paired subcutaneous adipose tissue (SAT) mRNA from 36 consecutive patients undergoing nephrectomy. Cannabinoid type 1A and CB1E mRNAs variants with the longer version of exon 4 were expressed. The CB1 expression in perirenal VAT significantly correlated with body mass index (BMI). Paired subcutaneous/perirenal samples from normal-weight patients (BMI <25 kg/m^2) showed higher CB1 expression in SAT ($P = .002$), whereas in OW (BMI ≥25 kg/m^2), the higher CB1 expression was in VAT ($P = .038$). In unpaired samples, SAT of normal-weight patients had significantly higher CB1 mRNA levels compared with SAT of OW, whereas higher CB1 expression ($P = .009$) was found in VAT of OW (n = 25). Overweight patients with increased visceral CB1 expression had higher waist circumference ($P < .01$), insulin ($P < .01$), and homeostasis model assessment index ($P < .01$). In addition, patients with the MetS (n = 22) showed higher CB1 expression in perirenal adipose tissues ($P = .007$). Visceral adipose CB1 expression correlated with BMI. Overweight patients and those with MetS showed a CB1 expression pattern supporting a CB1-mediated overactivity of the endocannabinoid system in human VAT.

1. Introduction

A role of peripheral cannabinoid type 1 (CB1) receptor in obesity-related dysmetabolism in human is supported by the results of 4 large clinical trials (Rimonabant in Obesity studies) [1-4].

Blockade of peripheral CB1 receptor with rimonabant has been shown to improve multiple cardiometabolic risk factors, such as abdominal obesity, dyslipidemia, glycemic control, and insulin resistance in overweight/obese patients [1-4].

Nevertheless, the relevance of adipose tissue CB1 receptor in the pathogenesis of visceral obesity and associated metabolic disorders is still unclear. Indeed, the peripheral role of the endocannabinoid system (ECS) in humans has just begun to be investigated. The visceral adipose tissue (VAT) itself appears to be a major site of cannabinoid activity in human obesity [5-8]. Moreover, higher levels of endogenous cannabinoid 2-arachidonoyl-glycerol in plasma of obese subjects were correlated with visceral adiposity [5-7], suggesting that a hyperactivation of ECS could contribute to obesity and related disorders.

To verify the hypothesis that CB1 blockers produce positive metabolic effects acting on VAT, a better knowledge of CB1 expression and regulation in human adipose tissue is needed. Such knowledge has been hampered by the difficulties to obtain human abdominal adipose samples from suitable overweight patients. Indeed, evidences about CB1 receptor expression levels in human adipose tissue were obtained from subcutaneous adipose tissue (SAT) and VAT of subjects with obesity (body mass index [BMI],

* Corresponding author. Tel.: +39 071 889499; fax: +39 071 889232.
E-mail address: r.sarzani@univpm.it (R. Sarzani).

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35 ± 7 kg/m^2) [5] or severe obesity (BMI, 40 ± 5 kg/m^2) [9]. Morbid obesity generally begins at young age, is characterized by a predominant subcutaneous adiposity, and is often considered as a distinct entity when compared with the common overweight and obesity of middle-aged subjects. Indeed, the cardiometabolic risk is increased even in patients with a slight overweight (BMI ≥25 kg/m^2) especially when central obesity and/or metabolic syndrome (MetS) is present [10-12]. Therefore, the main aim of the present work was to evaluate the association of adiposity and MetS with CB1 gene expression using unselected patients with a wide range of BMI. Increased CB1 gene expression is followed by increased CB1-mediated endocannabinoid effects [13,14] and, therefore, is functionally relevant. The CB1 expression in human VAT and SAT was studied to gain insights on CB1 alternative splicing and expression pattern in different fat depots. The CB1 expression patterns were then related to the clinical parameters of the studied population that compared normal-weight to obese patients.

2. Materials and methods

2.1. Patients and human tissue samples

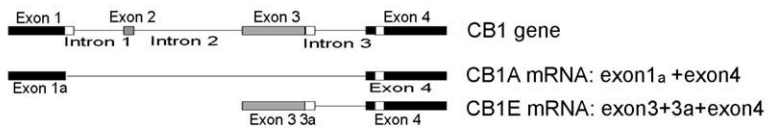
Thirty-six consecutive patients treated with nephrectomy for intracapsular renal carcinoma (without any evidence of local or metastatic cancer spread, T1/T2, N0, M0) were recruited at the "Ospedali Riuniti" University Hospital of Ancona (Italy) between February 2006 and February 2007.

All patients were classified into 2 different groups: normal weight (NW; n = 11, BMI <25 kg/m^2) and overweight (OW; n = 25, BMI ≥25 kg/m^2). All the women (n = 9) were in menopause to lessen metabolic differences and to increase sample's homogeneity. To gain further homogeneity of the studied population, all patients were free from hormone replacement and diabetes therapies. *Metabolic syndrome* was defined following the criteria of the International Diabetes Federation [15,16]. Samples of perirenal adipose tissue, kidney cortex, and medulla (from the intact portion of the removed kidney, far from the localized intracapsular carcinoma) were collected from all patients. From the same set of patients, 13 paired samples of visceral retroperitoneal (perirenal) and SAT from lateral abdominal wall were taken. After removal, samples were snap-frozen in liquid nitrogen and stored at −80°C until RNA extraction. The local Ethics Committee approved the study protocol, and all patients gave written informed consent for the collection of tissue samples and clinical data.

2.2. Gene expression study

Total RNA was extracted from tissue samples after homogenization in guanidine thiocyanate buffer and by CsCl gradient modified as previously reported [17]. Ribonucleic acid was solubilized in autoclaved RNase-free water (Sigma-Aldrich, Steinheim, Switzerland) and stored at −80°C. Ribonucleic acid (1.5 μg) was reverse-transcribed with High-Capacity cDNA Reverse Transcription Kits with RNase Inhibitor (Applied Biosystems, Warrington, Cheshire, United Kingdom). Single-strand complementary DNA was used to evaluate the splicing variants expressed in adipose tissue. The CB1 gene is formed by 4 exons and 3 introns (Fig. 1A) [18,19], and only the exon 4 contains the coding region. For the CB1 receptor, 3 different functional proteins were identified and called *CB1*, *CB1a,* and *CB1b* [19,20], which represent CB1 full length, CB1 long variant (variant 1; gi1520864), and CB1 short variant (variant 2; gi1520847). The full-length protein was described as the more abundant with respect to the low levels of alternative splice CB1a and CB1b that are generated by the use of different splice donor/acceptor sites.

Moreover, exon 4 together with the other codons of the CB1 gene can produce, by alternative splicing, at least 5 distinct messenger RNAs (mRNAs) called *CB1-A*, *CB1-B*, *CB1-C*, *CB1-D*, and *CB1-E* [18], which have been never studied in adipose tissue. The primers sets were designed to analyze the 5 different splicing products and to distinguish the mRNA sequences (full length, variant 1, and variant 2) in



A

	Forward primer sequences	Reverse primer sequences
Primer 1	5'-CCCTGCAGAGCTCTCCGTAGTC-3'	5'-GTCCATGAAGTTCTCCCCACACTG-3"
Primer 2	5'-GGCAGACGAGAGTTGCATACATGTC-3'	5'-GTCCATGAAGTTCTCCCCACACTG-3'

B

Fig. 1. (A) Schematic diagram of CB1 gene and of the 2 mRNA splicing variants present in adipose tissue (figure modified from Zhang et al [18]). (B) Oligonucleotides used for CB1 variants analysis. Primer set 1 was used to amplify 4 variants (A, B, C, and D) with exon 1a and exon 4; primer set 2 was used to analyze CB1E variant. Exons 1, 1a, 2, 3, and 4 are listed under GenBank accession numbers AY505113, AY505115, AY505116, and NM016083 respectively.

adipose tissue. Primers sets shown in Fig. 1B were used for the simultaneous amplification of all 5 known CB1 transcripts. Polymerase chain reaction (PCR) products were purified (QIAquick Gel Extraction Kit; Qiagen, Hilden, Germany) and sequenced (ABI PRISM 310 Genetic Analyzer; Applied Biosystems, Foster City, CA). To directly compare the relative abundance of the CB1 mRNA splice variants, the PCR products in the linear range of amplification of the 2 identified mRNAs were quantified with computer software for densitometric semiquantitative analysis (Image 1.2; National Institutes of Health, Bethesda, MD).

Real-time gene expression of human CB1 receptor was analyzed with TaqMan Gene Expression Assay (Hs00275634_m1; Applied Biosystems, Weiterstadt, Germany) characterized by a mix of primers/probe designed on exon 4 coding sequence and analyzed using an ABI 7300 for real-time PCR (Applied Biosystems, Darmstadt, Germany) with the standard curve method. Differences in starting total RNA and different efficiency of complementary DNA synthesis among samples were normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression as housekeeping control gene, and results were reported as arbitrary units. As a further semiquantitative internal control, 18S ribosomal RNA was also amplified to normalize the results and to verify possible differences of GAPDH expression in OW patients. There were no differences in the expression results when normalized to GAPDH or 18S ribosomal RNA controls, and we verified GAPDH expression stability in the whole population and in OW patients. Results were therefore reported as normalized to GAPDH.

2.3. Statistical analysis

Expression differences between paired SAT and VAT were assessed using paired Student *t* test, and differences between more than 2 groups were analyzed by analysis of variance and post hoc Bonferroni-Holm test. When the patients were divided into 2 groups by BMI cutoff or MetS criteria, the differences were analyzed by independent-sample *t* test. The correlation between perirenal CB1 expression and BMI across the entire population studied was analyzed by Pearson correlation. Data are presented as box plots (with median, 95% confidence intervals, and extremes). Statistical analysis was performed with SPSS 11.0 statistical software (SPSS, Chicago, IL), and a level of P less than .05 was considered significant.

3. Results

The anthropometric and metabolic characteristics evaluated before surgery are reported in Table 1. The average age of our patients (66.5 ± 11.6 years) corresponds to the average age of the highest MetS prevalence in many populations.

The analysis of CB1 mRNAs in different fat depots did not show any difference regarding CB1 gene splice variants. Sequence analysis revealed that alternative splicing in both SAT and VAT produced mainly 2 different mRNA variants: CB1-A and CB1-E. Densitometric analysis showed that the CB1-A variant was about 6-fold more abundant than CB1-E in both subcutaneous and perirenal adipose tissues. Moreover, sequence analysis showed the presence of CB1 full-length receptor with only the longer version of exon 4 (variant 1) encoding the isoform “a” of CB1 receptor. Thereafter, comparative semiquantitative CB1 gene expression analysis among tissue samples was based on real-time PCR of exon 4.

Comparing CB1 expression in kidney vs VAT and SAT, the mRNA levels in adipose tissue were about 90-fold higher than those in kidney ($P < .001$, n = 13; data not shown).

A significant correlation was found between CB1 expression levels in perirenal adipose tissue and BMI values (Fig. 2; $r = 0.361$, $P = .042$ and $r = 0.435$, $P = .016$

Table 1
Clinical and biochemical characteristics of the patients studied (N = 36)

Characteristics	No MetS	MetS	NW (BMI <25)	OW (BMI ≥25)
Age (y)	65.18 ± 14.344	64.91 ± 12.25	64.91 ± 14.82	65.72 ± 11.56
BMI (kg/m^2)	24.57 ± 3.73	27.68 ± 3.75	22.94 ± 2.31	28.41 ± 3.08
Waist circumference (cm)	92 ± 11.382	103.55 ± 10.71	91.80 ± 11.43	103.11 ± 10.27*
SBP (mm Hg)	83 ± 11.437	87.59 ± 22.21	84.36 ± 11.90	86.57 ± 21.76
DBP (mm Hg)	78.1 ± 5.9	84.5 ± 12.5	85.5 ± 12.9	80.5 ± 10.5
Fasting glycemia (mg/dL)	131.7 ± 14.8	146.0 ± 23.4	148.8 ± 17.5	138.8 ± 23.3
Fasting insulinemia (μU/mL)	3.82 ± 2.401	5.97 ± 4.298	3.00 ± 1.34	6.38 ± 4.24*
HOMA index	0.79 ± 0.582	1.28 ± 0.92	0.62 ± 0.27	1.37 ± 0.93*
Total cholesterol (mg/dL)	165.91 ± 49.28	194.05 ± 53.16	168.55 ± 52.17	192.73 ± 52.53
HDL cholesterol (mg/dL)	39.45 ± 8.583	39.18 ± 13.33	41.55 ± 10.85	38.14 ± 12.36
Triglycerides (mg/dL)	101 ± 71.21	136.14 ± 53.79	109.82 ± 72.06	131.73 ± 55.67
LDL cholesterol (mg/dL)	107.16 ± 41.79	127.65 ± 44.11	105.95 ± 44.79	128.26 ± 42.36
Non-HDL cholesterol (mg/dL)	126.45 ± 47.17	154.86 ± 46.30	127.00 ± 47.91	154.59 ± 46.11

For 3 patients, the data set was incomplete to classify or not for MetS. Data are mean and SD. SBP indicates systolic blood pressure; DBP, diastolic blood pressure; HOMA, homeostasis model assessment; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

* Significant differences of anthropometric and metabolic parameter between OW vs NW ($P < .01$).

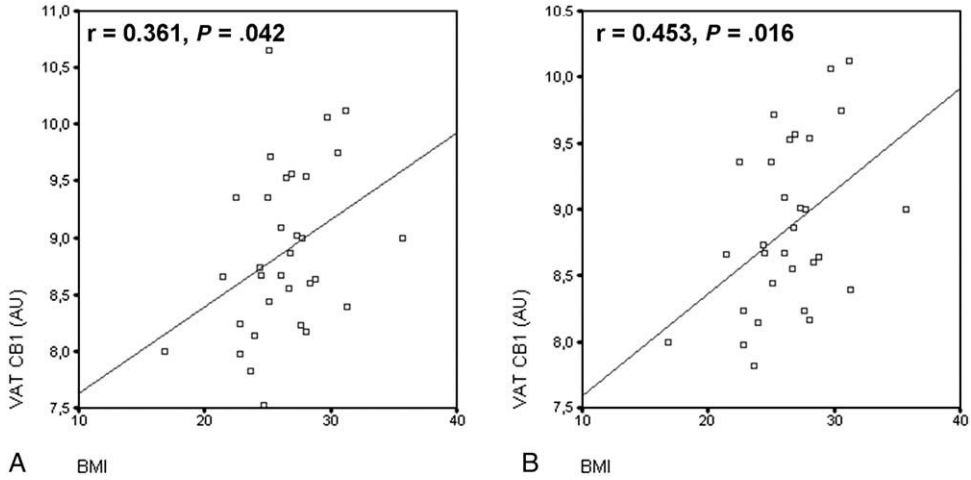


Fig. 2. (A) Correlation between all BMI values and CB1 gene expression in VAT of the entire population ($r = 0.361$, $P = .042$, n = 36). (B) When 2 patients with the highest and lowest CB1 expression level were excluded, the Pearson correlation test resulted in $r = 0.435$, $P = .016$. Data are presented as the median, 95% intervals of confidence, and range; circles represent out-of-range values.

excluding the highest and lowest levels of CB1 expression). No association was found between CB1 expression level and waist measurements. Thus, adipose tissue CB1 expression analysis was conducted by dividing the population according to (*a*) BMI values (NW/OW), (*b*) type of adipose tissue (SAT/VAT), and (*c*) presence or absence of MetS.

3.1. Results in paired SAT/VAT samples in NW and OW

When paired SAT/VAT samples from NW (BMI <25 kg/m^2) were compared, CB1 expression was about 2-fold higher in SAT (Fig. 3A; $P = .002$, n = 7), whereas in OW (BMI ≥25 kg/m^2), paired SAT/VAT samples showed an inverse expression pattern, with mRNA level 1.5-fold higher in perirenal tissue (Fig. 3B; $P = .038$, n = 6). The SAT/VAT expression ratio showed a significantly lower ratio in OW patients (3.5 in NW vs 0.35 in OW, $P < .001$).

3.2 Results in unpaired SAT/VAT samples of NW vs OW

The CB1 expression evaluated in SAT was higher in NW (Fig. 4A, $P = .037$), whereas in VAT, it was higher in OW (Fig. 4B, $P = .009$), confirming the existence of a distinct expression pattern depending on total adiposity. Taken together, these data indicate that a distinct CB1 expression pattern depending on total adiposity and on adipose depot exists.

The OW patients with altered CB1 expression pattern had significantly increased waist circumference ($P = .008$), insulin ($P = .002$), and homeostasis model assessment index ($P = .002$) (Table 1).

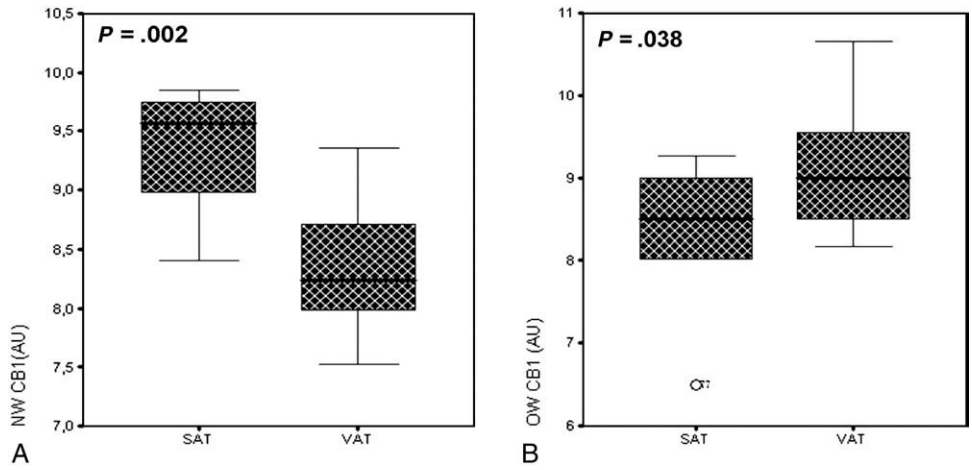


Fig. 3. (A) The CB1 mRNA expression in NW patients (n = 11). The CB1 mRNA levels were higher in SAT compared with perirenal VAT (paired *t* test, $P = .002$, n = 7 per group). (B) The CB1 mRNA expression in OW (n = 25). The CB1 mRNA levels were higher in VAT compared with SAT (paired *t* test, $P = .038$, n = x per group). Data are presented as the median, 95% intervals of confidence, and range; circles represent out-of-range values. Group comparison by paired Student *t* test.

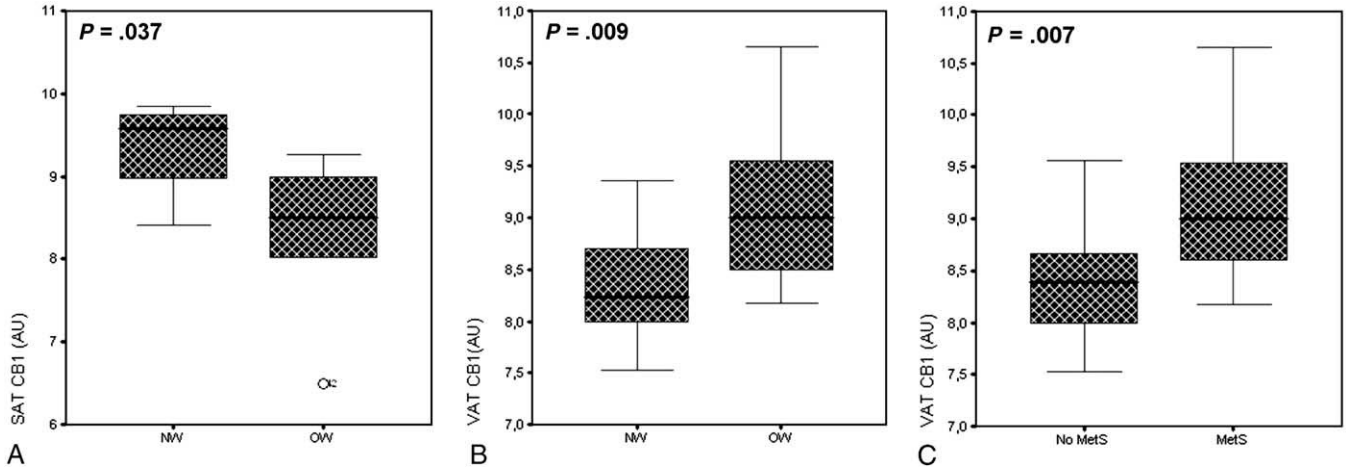


Fig. 4. (A) Comparison of subcutaneous CB1 gene expression between NW (n = 7) and OW (n = 6), with lower expression in OW (independent *t* test, *P* = .037). (B) Comparison of perirenal CB1 expression between NW (n = 11) and OW (n = 25) patients, with higher CB1 expression in OW (independent *t* test, *P* = .009). (C) Comparison of visceral retroperitoneal CB1 expression between patients with (n = 22) or without (n = 11) MetS, with a higher expression in patients with MetS (independent *t* test, *P* = .007). Data are presented as the median, 95% intervals of confidence, and range; circles represent out-of-range values. Group comparison by unpaired Student *t* test.

3.3. Results in patients with or without MetS

Patients were also subdivided by the presence (n = 11) or the absence (n = 22) of MetS. The patients with MetS were defined by International Diabetes Federation criteria, although BMI was not significantly different between patients with or without MetS. When CB1 expression was analyzed in patients with MetS vs controls (Table 1), a significant overexpression of CB1 was found in VAT of MetS patients (Fig. 4C, *P* = .007).

4. Discussion

The CB1 receptor mRNA is about 90-fold more abundant in adipose tissue than kidney, indicating the key role of the receptor in adipose tissue. The CB1 mRNA variants in human adipose tissue and the changes of CB1 mRNA levels regulate CB1 receptor synthesis and, thus, peripheral effects of endocannabinoids [21]. The main finding of this study is that increasing BMI, a well-established index of total adiposity, deeply affects CB1 gene expression pattern in SAT and VAT. The increased CB1 expression in retroperitoneal adipose tissue in relation to BMI and in patients with MetS appears especially important. The relationship between perirenal-visceral CB1 gene expression and BMI taken together with the results of the Rimonabant in Obesity studies suggests that increasing BMI might be CB1 driven and amenable with CB1 blockade.

The CB1 gene is formed by 4 exons and 3 introns that are expressed in hippocampus producing several transcripts [20]. Exon 4 coding sequences themselves can originate CB1 full-length protein but also, at least, 2 different-length CB1 receptor isoforms [19,20]. Published data revealed that, given the low abundance of the exon 4 shorter version, it could be argued that it does not contribute to the cannabinoid pharmacology [20]. Indeed, in adipose tissue, we found that the longer version of exon 4 and CB1-A was about 6-fold more abundant than the CB1-E variant, both in perirenal and subcutaneous adipose tissue.

Semiquantitative analysis of CB1 expression in paired SAT/VAT samples showed a different CB1 expression pattern on the basis of BMI. Normal-weight patients revealed higher expression in SAT, whereas in OW patients, the higher CB1 expression was in perirenal fat depot. Furthermore, among all patients studied, a significant correlation between CB1 expression in VAT and BMI was found. This correlation is likely to be of functional relevance because a significant overexpression of CB1 was also found in perirenal fat depot of patients with MetS. This CB1 higher expression in VAT and the very likely corresponding increase of CB1 receptors and receptor-mediated effects support an important role of CB1 overexpression in the pathogenesis of intraabdominal obesity and the associated metabolic disorder. A marker of visceral adiposity is waist circumference, but we did not observe significant associations among CB1 gene expression and waist values in our patients.

Only very recently, CB1 expression in human adipose tissue has been studied [7-11]. The main data were originated from the analysis of mRNA in SAT and VAT obtained from different obese populations. In these 2 studies, the patients were obese (BMI, 35 ± 7 kg/m^2; mean age, 58 ± 11 years) [10] or with severe obesity (BMI, 40 ± 5 kg/m^2; mean age, 40 ± 20 years) [12] and quite young in relation to MetS prevalence. Bluher et al [5] showed that higher CB1 levels were found in VAT of lean controls. On the contrary, Lofgren et al [9] did not find any difference in adipose tissue CB1 gene expression but compared severely obese men and women with BMI of 40 ± 5 kg/m^2 vs lean women only and found no relationship between either SAT/VAT CB1 expression or clinical or metabolic variables. Our study

design allowed collecting consecutive patients with a complete BMI range (mean BMI, 26.7 ± 4.1 kg/m^2) and with a mean age (66.5 ± 11.6 years) corresponding to the highest prevalence of MetS. Clinical parameters were obtained before surgery, avoiding well-known postsurgery aspects including weight loss. Nevertheless, the comparison between our data, obtained using perirenal (retroperitoneal) fat depot, and previous studies using intraperitoneal adipose tissue is a possible source of bias. One of the most popular hypotheses on the pathophysiologic relevance of "visceral" adiposity is based on its venous drainage via vena porta directly to the liver. On the contrary, perirenal adipose tissue is localized in the retroperitoneal space and drained into systemic venous system. However, different studies underline the similarity between perirenal characteristics (anatomical, glucose uptake, lipid metabolism/fatty acid composition, adiponectin production, and clinical metabolic significance) and intraabdominal adipose tissue [22-25]. Thus, perirenal adipose tissue could be considered as an indicator of visceral obesity and cardiovascular risk factors in the MetS and is a depot that is believed to be particularly active in metabolism [24].

Unfortunately, because of technical limitations, we were not able to evaluate ECS levels in adipose tissue. Published data suggest activation of the ECS in obesity, both in brain and peripheral organs [7,13]. A marked increase of circulating endocannabinoids (2-arachidonoyil glycerol, anandamide) was also described in obesity [8,13,26]. Moreover, there were evidences that endocannabinoids increased food intake and promoted weight gain through activation of CB1 receptors in both central and peripheral tissues. These data suggest that a loop among increased local endocannabinoids production, ECS activity mediated, and increased CB1 expression could exist. These findings also suggest that hyperactivation of ECS is a possible factor contributing to obesity and related disorders and that ECS may be a therapeutic target of body weight and obesity.

The reduced SAT/VAT ratio of CB1 mRNA levels in OW coupled with similar changes of CB1-mediated effects might shift lipogenesis toward the visceral compartment and may explain the somewhat specific effect of the CB1 blocker rimonabant in reverting central obesity and related metabolic complications.

Recently, Pagano et al [27] found that ECS is linked with the regulation of glucose metabolism in human adipocytes; and this effect is mediated by increased influx of extracellular calcium and by P13 kinase activity. Moreover, this study showed that dysregulation of ECS in adipose tissue is depot specific and suggested a role for ECS in the regulation of adipose tissue in physiology and pathology.

A possible limitation of this study is that the patients studied had intrarenal, intracapsular carcinoma. A malignant growth might trigger systemic low-grade inflammation affecting glucose and lipid metabolism. The histologic analysis of our adipose samples did not show any evidence of inflammation. Moreover, our patients were "normalized" for the presence of intracapsular renal cancer because all of them were affected by the same disease. We believe that our results are related only to the individual anthropometric and metabolic features of these patients and cannot result from the presence of renal cancer.

In conclusion, CB1 receptor gene expression appeared to have a regulated pattern with a reduced expression in SAT and an increased expression in VAT in OW patients and in patients with MetS. Thus, our results suggest that an increased CB1 expression in VAT coupled with a reduced expression of SAT in OW patients is likely to be a primary mediator in the etiopathogenesis of visceral obesity and its related metabolic complications, indirectly supporting therapeutically the use of CB1 blockers in OW patients and in patients with MetS.

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