Cannabinoid receptor 2 as anti-obesity target: inflammation, fat storage and browning modulation

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Context: Obesity is associated with a low-grade inflammatory state, and adipocyte hyperplasia/hypertrophy. Obesity inhibits the "browning" of white adipose tissue. **Cannabinoid receptor 2 (CB2) agonists reduce food intake and induce anti-obesity effect in mice. A common missense CB2-variant, Q63R, causes CB2 reduced function.**

Objective: To evaluate the influence of CB2 receptor on the modulation of childhood obesity and of adipocyte activity and morphology.

Design: CB2-Q63R variant was analyzed in obese Italian **children**. **The effects of an inflammatory stimulus and those of drugs selectively acting on CB2 were investigated on *in-vitro* adipocytes obtained** from mesenchymal stem cells of **adult** healthy donors or from subcutaneous adipose biopsies of **adult** non-obese and obese subjects.

Setting: Department of Women, Child and General and Specialist Surgery of the Second University of Naples.

Patients or Other Participants: Five hundred and one obese Italian children **(age 11±2.75)**. Twelve healthy bone marrow donors **(age 36.5±15)**. Seventeen subjects, 7 lean **(age 42±10)** and 10 obese **(age 37.8±12)**, underwent subcutaneous adipose tissue biopsies.

Main Outcome Measures: Effects of CB2 stimulation on adipokine, perilipin and UCP-1 expression.

Results: The less-functional CB2-R63 variant was significantly associated with a high z-score BMI. CB2 blockade **with AM630 reverse agonist** increased inflammatory adipokine release and fat storage and reduced browning. CB2 stimulation **with JWH-133 agonist** reversed all of the obesity-related effects.

Conclusion: CB2 receptor is a novel pharmacological target that should be considered for obesity.

Obesity is associated with a low-grade inflammatory state in white adipose tissue (WAT) and with excessive increases in white adipocyte (ADP) size and number (1).

ADPs secrete a number of bioactive molecules, adipo-kines, and maintain energy balance by storing triacylglycerols in cytoplasmatic lipid droplets (LDs) under energy overload and mobilizing them during energy deprivation (2).

Obesity dysregulates both these processes. Increased

Abbreviations:

proinflammatory adipokines release attracts into WAT macrophages and other immune cells, which heighten the inflammatory state (3, 4). Development of LDs into the characteristic unilocular morphology, promoted by specific LD-associated proteins, among which perilipin-1 plays a crucial role (5, 6), leads to adipocyte hypertrophy and hyperplasia (7, 8).

Adipose tissue also includes "thermogenic" brown tissue. Brown adipocytes produce a large amount of heat through activation of uncoupling protein (UCP)-1. UCP-1 expression can be also induced in ADPs of the WAT, termed beige adipocytes. Indeed, beige ADPs are allowed to act as simply energy storage or to activate a thermogenic program in response to different stimuli recruiting UCP-1 (9). Obesity decreases beige ADP cell number (10).

The endocannabinoid (EC) system, which comprises two metabotropic receptors, CB1 and CB2, their endogenous ligands and enzymes for EC synthesis and metabolism (11), plays a crucial role in regulating energy metabolism (12–14). Genetic ablation of CB1 results in reduced body weight in mice (15). Selective blockade of CB1 decreases food intake and body weight in humans (16–19).

A role of CB2 receptor has also been revealed. A common CB2 variant, Q63R, causing reduced CB2 function, has been associated with eating disorders in humans (20). CB2 agonists reduce food intake in lean mice (20) and improve both body weight and obesity-associated inflammation in diet-induced obese mice (21). Moreover, CB2 genetic ablation results in adiposity (22).

To evaluate the influences of CB2 receptor on the modulation of childhood obesity we analyzed the CB2-Q63R variant in a cohort of 501 obese Italian children and adolescents. To evaluate the ADP activity and morphology we performed molecular, biochemical and morphological studies on in vitro ADPs, differentiated from mesenchymal stem cells (MSCs) of healthy adult donors or derived from subcutaneous adipose tissue of nonobese and obese adult subjects, treated with the CB2 agonist JWH-133 or with the reverse agonist AM630.

Materials and Methods

Ethical Statement

All of the procedures performed in this study were in accordance with the Helsinki Declaration of Principles, the Italian National Legislation and the Ethics Committee of the Second University of Naples, which formally approved the study. Written informed consent was obtained from parents and assent was acquired from children before any procedures were performed.

Patients

Five hundred and one Caucasian obese children and adolescents referred to the Department of Women, Children and General and Specialized Surgery of the SUN from January 2010 to August 2014 were enrolled.

Physical examination of each patient included assessments of body weight, height, z-score-BMI (23), waist circumference, systolic/diastolic blood pressure (BP), pubertal stage (24), as previously described (25).

Patients were defined as obese if the BMI exceeded the 95th percentile for age and sex, according to the Italian charts (26). Triglyceride, high-density lipoprotein (HDL) cholesterol, insulin, and glycemia levels were measured as previously described (27, 28). Insulin resistance was assessed using the homeostasis model assessment (HOMA-IR), with a cut-off value of 3 (29).

Subjects for cell cultures

MSCs that were differentiated into adipocytes were isolated from residual cells of 12 healthy subjects (8 males, 4 females; median age 36.5 ± 16 years) who donated bone marrow (BM) for transplantation at the Bambino Gesù Children's Hospital.

Subcutaneous adipose tissue biopsies were collected from lean (n = 7; 4 males, 3 females; median age 42 ± 10 years; BMI < 25), and obese subjects (n = 10; 4 males, 6 females; median age 37.8 ± 12 years; BMI > 30), undergoing surgery for umbilical or inguinal hernia at the Department of General Surgery of the University of Naples.

Genetic Analysis

Genomic DNA was extracted from peripheral whole blood cells using a DNA extraction kit (Promega, Madison, WI, USA). Detection of the CNR2 rs35761398 polymorphism, underlying the CB2-Q63R substitution, was performed using a TaqMan Assay (Real Master Mix Probe, 5 PRIME, Germany) as previously described (30).

MSC-derived human adipocyte cultures

MSCs were obtained from mononuclear cells isolated from bone marrow aspirates as previously described (31). MSCs were harvested until they reached passage 2–3, split after they reached $\geq 80\%$ confluence for each passage using trypsin (Euroclone, Pero, MI, Italy) and replated for expansion. At passage (P) 2–3, MSCs were differentiated into ADPs. Briefly, to induce adipogenic differentiation, cells were cultured at a density of $8.000/cm^2$ in α-Minimal Essential Medium (Lonza, Verviers, Belgium) supplemented with 10% FBS, 10^{-7}M dexamethasone, 50 μg/ml ascorbic acid, 100 μg/ml insulin, [50 μM] IBMX, [0.5 mM] indometacin and [5 mM] β-glycerophosphate (Sigma, Milan, Italy), and then plated in 12-well Cell Culture Multiwell Plates (Corning Costar, Celbio, Milan, Italy) for molecular biological experiments and in 24/96-well Cell Culture Multiwell Plates (Corning Costar, Celbio, Milan, Italy) for the other procedures. Cultures were maintained for 14 days before any treatment.

Subcutaneous adipose tissue-derived human adipocyte cultures

Subcutaneous adipose tissues were collected in 10% paraformaldehyde in propylene tubes, mechanically fragmented and digested with collagenase type II (1 mg/ml; 37°C; 1 hour; Sigma-

Aldrich, St. Louis, MO, USA). Filtration (50–70 μ filter, FALCON) was performed following digestion to separate the mature adipocytes from the stromal vascular fraction. The filtered fraction, included preadipocytes, was centrifuged (1200 rpm; 10 minutes), and the pellet, resuspended in supplemented α-MEM, plated in a 12/24-well Cell Culture Multiwellplate (Corning Costar, Celbio, Milan, Italy) for isolation of mRNA and proteins or in 8-chamber slides (BD Falcon, Becton, Dickinson & Company, NJ, USA) for morphological analyses. Cells were cultured (37°C; 5% CO_2; medium replaced every three days) for approximately 14 days to allow for full adipogenic differentiation before any treatment.

Oil red O staining

Adipogenic-induced cells were stained for fat vacuoles by Oil Red O staining (Sigma-Aldrich, St.Louis, MO, USA). Medium was removed from each well, and cells were rinsed in PBS and fixed in 10% formalin (Sigma-Aldrich, St.Louis, MO, USA) for 1 hour at RT. The cells were then washed with PBS and incubated first with 60% isopropanol (Carlo Erba Reagents, Milan, Italy) for 5 minutes at RT and then with 60°C preheated Oil Red O working solution for 10 minutes at RT. After washing with water to remove unbound dye, the lipid droplets in differentiated cells were visualized using an optical microscope (Nikon Eclipse TS100, Nikon Instruments).

mRNA analysis

Total mRNA was extracted using RNA Tri-Reagent (Molecular Research Center Inc., Cincinnati, OH) and reverse transcribed using a RT High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA).

To quantify the expression levels of EC system components, adipokines and UCP-1, quantitative PCRs were performed. Specific amplification reactions for CB1 (*CNR1*, GeneID1268), CB2 (*CNR2*, GeneID1269), monoacylglycerol-lipase (*MAGL*, GeneID11343), diacylglycerol-lipase-α (*DAGLα*, GeneID747), n-acylphosphatidylethanolamine phospholipase D (*NAPE-PLD*, GeneID222236), fatty acid amide hydrolase (*FAAH*, GeneID2166), peroxisome proliferator-activated receptor gamma (*PPARγ*, GeneID5468), leptin (*LEP* GeneID3952), adiponectin (*ADPN* GeneID9370), interleukin 6 (*IL-6*, GeneID3569), interleukin 4 (*IL-4*, GeneID3565), tumor necrosis factor (TNF) (*TNFα,* GeneID7124) and uncoupling protein-1 (*UCP-1*, GeneID7350) were carried out. Their expression levels were compared with that of the 18S (GeneID100008588) or β-actin (GeneID60) housekeeping gene. The assays were performed in triplicate. The primers, selected using Primer3 software, and the PCR conditions are available on request. Gene expression profiling was performed using the comparative cycle threshold method of relative quantification to the housekeeping gene.

Western blot

Total lysates were obtained via RIPA buffer lysis and analyzed for IL-6, perilipin and UCP-1 expression by immunoblotting. Fifty micrograms of denatured proteins were loaded onto a 10% polyacrylamide gel and transferred onto a PVDF membrane (Millipore, Milan, Italy). Membrane strips were alternatively incubated overnight at 4°C with the following horseradish peroxidase-conjugated antibodies: rabbit polyclonal anti-IL-6 (1:1000; ab6672, Abcam, Cambridge, UK); rabbit polyclonal antiperilipin A (1:500; ab3526, Abcam, Cambridge, UK), and

anti-UCP-1 (1:500; orb13739, Biorbyt, Cambridge, UK), and then with a goat-antirabbit secondary antibody (1:2000; AP307P, Merck Millipore, Life Science-Merck KGaA, Darmstadt, Germany) for 1 hour, and reactive bands were visualized on X-ray film (Fuji Corporation, Tokyo, Japan). **Images were captured, stored, and analyzed with the Quantity One software (Bio-Rad, California, USA).** Mild stripping was performed at 60°C for 10 minutes if the same membrane was used to determine the expression of more than one protein of interest. A monoclonal anti-β-actin antibody (1:1000 or 1:2000; Sigma, Milan, Italy) was used as a housekeeping protein to check for identical protein loading.

Drugs and treatments

LPS, AM630, and JWH-133 (Tocris, Avonmouth, UK) were dissolved in PBS containing DMSO (Sigma-Aldrich, St.Louis, MO, USA). The final DMSO concentration in the cultures was 0.01%. Cultured ADPs were treated with AM630 [10 μM], JWH-133 [100 nM], or 1 mg/ml LPS, alone or in combination after a dose-response curve (not shown). Untreated cultured cells were maintained in incubation medium during the relative treatment time, with or without vehicle (0.01%DMSO). Drugs were added to the complete cell medium when differentiation was completed. RNA extraction and biochemical analysis were performed at 24 hours after the treatments.

Statistics

The continuous variables are summarized as the mean±standard deviation (SD), and the categorical variables are presented as absolute and relative frequencies. The molecular and biochemical data are presented as the mean±SD. ANOVA, followed by the unpaired Student–Newman–Keuls post hoc test, was used to evaluate differences in the mean values between the groups and according to the number of experiments performed. The χ^2 test was used for categorical variables.

Multivariate analysis, including all independent variables with biological relevance to the aim of the study, was performed using a general linear model.

All *p*-values were normalized for age, sex, pubertal age and z-score BMI and were considered to be statistically significant if they were less than 0.05.

All statistical analyses were performed using Statgraphics Centurion XV.II (Adalta, Arezzo, Italy; Statpoint Technologies Inc., VA, USA).

Results

Genetic Study

The clinical and genetic characteristics of the 501 obese Italian children and adolescents included in this study are reported in Table S1.

The allele frequencies were in Hardy-Weinberg equilibrium (P = .87; Table S2).

The clinical features of the patients with respect to the CB2-Q63R genotype are shown in Table 1.

The CB2-R63 variant was found to be associated with an increased z-score BMI (P = .0068), and in a subgroup

Table 1.   Biochemical characteristics in 501 Italian obese children and adolescents according to CB2 Q63R variant

CB2 Q63R variant	QQ	QR	RR	p
Patients, n (%)	76 (15.2)	260 (51.9)	165 (32.8)	*p*
Males, n (%)	39 (7.78)	144 (28.74)	75 (14.97)	0.14
Females, n (%)	37 (7.39)	116 (23.15)	90 (17.96)	
Age, years (mean±SD)	10.60 ± 3.15	10.97 ± 1.70	11.35 ± 2.14	0.12
z-score BMI (mean±SD)	2.83 ± 0.73	2.95 ± 0.39	3.10 ± 0.49	**0.007**
Weight/Height (mean±SD)	0.64 ± 0.05	0.63 ± 0.03	0.63 ± 0.03	0.35
SD diastolic pressure, (mean±SD)	0.17 ± 1.0	0.25 ± 0.57	0.35 ± 0.71	0.31
SD systolic pressure, (mean±SD)	1.22 ± 1.4	1.05 ± 0.78	1.24 ± 0.97	0.24
Total cholesterol, mg/dl (mean±SD)	156.32 ± 35.4	159.62 ± 19.2	159.41 ± 24.2	0.70
Triglycerides, mg/dl (mean±SD)	89.04 ± 58.59	100.4 ± 31.68	95.75 ± 40	0.21
Glicemia t0, (mean±SD)	78.43 ± 9.55	79.43 ± 5	80.25 ± 6.54	0.29
Glicemia t120, (mean±SD)	110.8 ± 25.19	110.3 ± 13.6	113.5 ± 17.09	0.30
Insulin t0, (mean±SD)	29.36 ± 21.74	29.10 ± 11.71	28.54 ± 15.01	0.94
HOMA, (mean±SD)	5.79 ± 4.53	5.72 ± 2.42	5.71 ± 3.10	0.99
WBISI, (mean±SD)	2.66 ± 2.35	2.71 ± 1.30	2.73 ± 1.68	0.97
IL-6, pg/ml (mean±SD)[1]	2.77 ± 2.39	2.62 ± 1.40	3.44 ± 1.88	**0.03**

SD:standar deviation; BMI: body mass index; AST: aspartate-aminotransferase; ALT: alanine aminotransferase; t0: blood drawn after fast; t120: blood drawn after glycemic load; [1] *n* = 37; *p*-value less than 0.05 are shown in bold; all *p*-values are normalized for age, sex and z-score BMI

of 37 patients, with a high plasma IL-6 level (*P* = .03). No other association was found.

The genotype distributions of MSC donors and subjects who underwent biopsy are reported in Table S3.

Molecular Study

1) Adipocytes express EC receptors and enzymes

Adipocytes, differentiated in vitro from MSCs or from WAT biopsies, expressed cannabinoid receptors and enzymes for the synthesis and breakdown of anandamide and 2-arachidonoylglycerol (Figure S1). Leptin, adiponectin and PPARγ expression indicated the cultured cells were ADPs.

2) Adipokine expression is modulated by adiposity and CB2 stimulation

Adipokine expression in lean- and obese-derived ADPs was analyzed. The effects of an inflammatory stimulus exerted through lipopolysaccharide (LPS) treatment on lean-derived ADPs and those of selective CB2 agonism on obese-derived ADPs were investigated.

Adipokine expression significantly differed among the lean- and obese-derived ADPs. In both obese-derived and LPS-treated lean-derived ADPs, higher levels of PPARγ and leptin were observed compared with the lean-derived ADPs, while the adiponectin levels were significantly decreased. Obese-derived ADPs treated with JWH-133 had lower levels of PPARγ and leptin compared with obese-derived ADPs (Figure 1A).

Levels of the proinflammatory cytokines IL-6 and TNFα were significantly increased in obese-derived ADPs and LPS-treated lean-derived ADPs, while that of IL-4 was significantly decreased. Obese-derived ADPs treated with

JWH-133 exhibited lower levels of IL-6 and TNFα and a higher level of IL-4 compared with obese-derived cells (Figure 1B).

Biochemical Study

3) IL-6 levels are increased by adiposity and decreased by CB2 stimulation

The plasma IL-6 levels in a subgroup of 37 obese patients (8 QQ; 19 QR; 10 RR) were analyzed by ELISA. The association of the Q63R variant with z-score BMI was still significant (*P* = .03) and the R63-allele was associated with a high plasma IL-6 level (*P* = .008, not normalized) (Figure S2A). A general linear model, with the normalization of confounding factors, such as age, sex, z-score BMI and a well-known polymorphism affecting IL-6 receptor function (A358D; Table S4), revealed that CB2-R63 allele was the main risk factor (model *P* = .04; CB2-Q63R single contribution *P* = .004) (Figure S2B).

Western blotting was performed to determine the intracellular IL-6 level in ADP lysates. Obese-derived ADPs contained significantly less intracellular IL-6 compared with lean-derived cells. LPS administration to lean-derived ADPs significantly decreased the intracellular IL-6, while incubation of obese-derived ADPs with the CB2 agonist JWH-133 induced a significant increase in this level (Figure S2C).

4) Lipid droplet number and size are increased by adiposity and reduced by CB2 stimulation

4.1 Oil red staining

4.1a MSC-derived ADPs

LD staining of MSC-derived ADPs with oil-red (Figure 2A) or with a perilipin fluorescent antibody (Figure S3A)

revealed increases in their number and size following application of an inflammatory stimulus, such as LPS. These increases were prevented by concomitant CB2 stimulation with JWH-133 [100 nM]. Increases in LD number and size were observed after treatment with the CB2 reverse agonist AM630 [10 μM].

4.1b Biopsy-derived ADPs

The same experiments were conducted on human subcutaneous WAT biopsy-derived ADPs. ADPs from the obese individuals showed more numerous and larger LDs compared with those acquired from lean individuals. LPS treatment increased the LD number and size in lean subject-derived ADPs, whereas CB2 modulation with the JWH-133 agonist [100 nM] or the AM630 reverse agonist [10 μM] in obese-derived ADPs induced a reduction and increase in the LD size and number, respectively (Figure 2B and S3B).

### 5) Perilipin expression is increased by adiposity and decreased by CB2 stimulation

Lysates of obese-derived ADPs showed significantly higher perilipin levels compared with those of lean-derived ADPs (Figure 3A). Perilipin expression was altered when JWH-133 [100 nM] or AM630 [10 μM] was applied to obese-derived ADPs, including a decrease after CB2 stimulation and an increase after CB2 blockade, respectively. The effect induced by JWH-133 was prevented by pretreatment with AM630 (Figure 3B).

6) UCP-1 expression is decreased by adiposity and increased by CB2 stimulation

6.1 Molecular expression

ADPs from the obese subjects exhibited a significantly lower UCP-1 mRNA level compared with those from the lean subjects. LPS treatment significantly reduced UCP-1 expression in lean-derived ADPs. Treatment of obese-derived ADPs with the CB2 agonist JWH-133 significantly enhanced UCP-1 expression (Figure 4A). This effect was dose-dependent and prevented by AM630 pretreatment (Figure S4A). UCP-1 expression was significantly decreased by LPS and CB2 blockade in MSC-derived ADPs. Moreover, CB2 stimulation significantly enhanced UCP-1 expression and counteracted the LPS-induced decrease (Figure 4B).
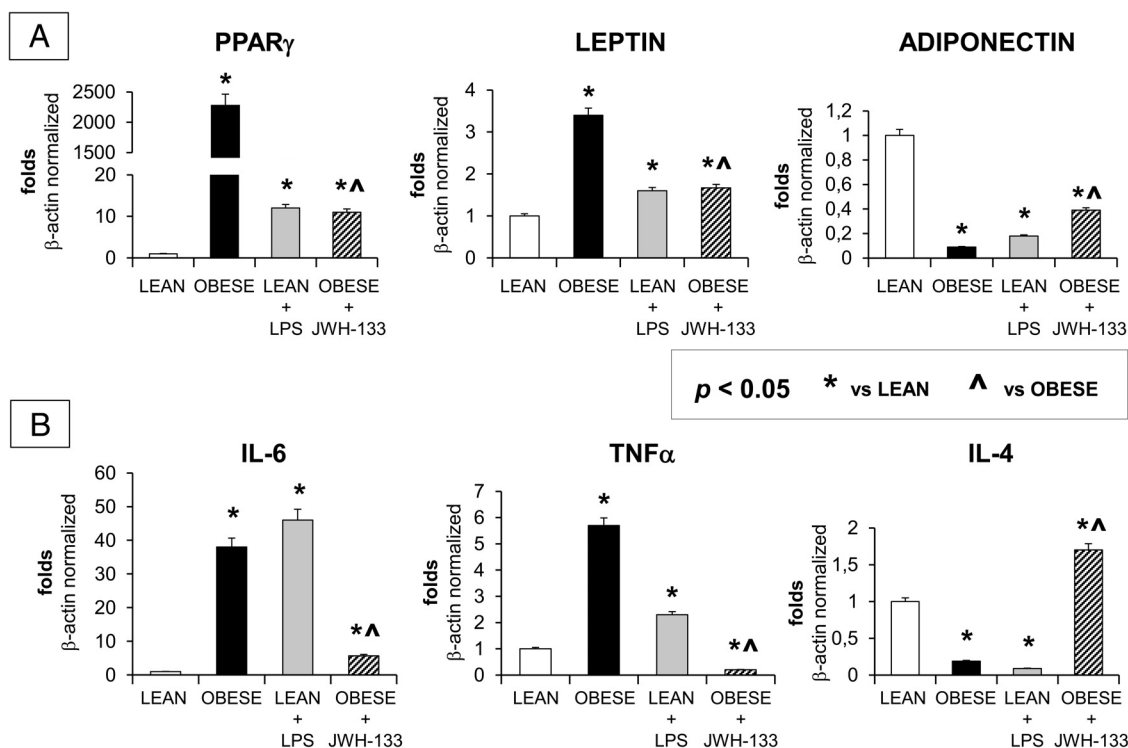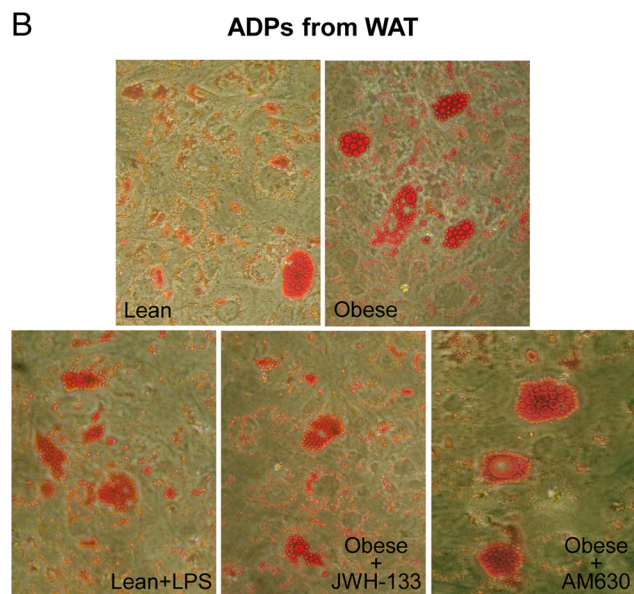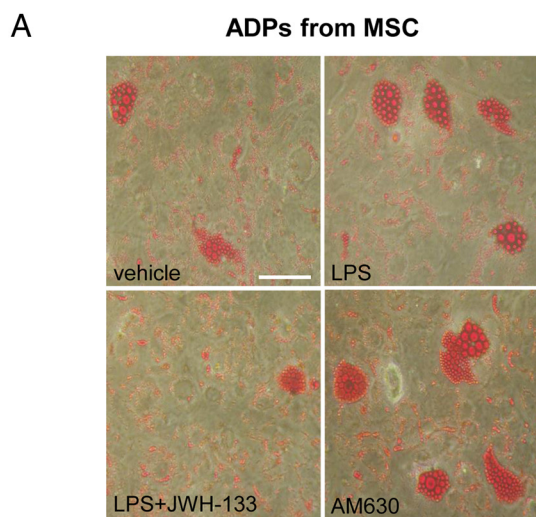

Figure 1. Adipokine expression is modulated by adiposity and CB2 stimulation. Adipokine release significantly differed among the lean- and obese-derived ADPs. **A,** Obese-derived **ADPs** and LPS-treated lean-derived ADPs show higher levels of the **specific** markers PPARγ and leptin compared with the lean-derived ADPs, and lower levels of adiponectin. Obese-derived ADPs treated with JWH-133 [100 nM] show lower levels of PPARγ and leptin and a higher level of adiponectin compared with obese-derived ADPs. **B,** Obese-derived ADPs and LPS-treated lean-derived ADPs show higher levels of the proinflammatory cytokines IL-6 and TNFα compared with the lean-derived ADPs, and lower levels of IL-4. Obese-derived ADPs treated with JWH-133 [100 nM] show lower levels of IL-6 and TNFα and a higher level of IL-4 compared with obese-derived cells. Data have been revealed from in vitro ADPs by real time PCR starting from 100 ng of total mRNA for the RT reaction and have been normalized for the housekeeping gene β-actin. Data are represented as mean$\pm$SD from three different assays performed in duplicate. A t test has been used to evaluate differences in the levels of adipokine expression among groups. A p value less than 0.05 was considered statistically significant.

6.2 Protein expression

According to the molecular data, the UCP-1 protein level in obese-derived ADPs was significantly lower compared with that in lean-derived ADPs, and it was significantly increased after CB2 stimulation. Lean-derived ADPs exposed to LPS, and both lean- and obese-derived ADPs treated with the CB2 reverse agonist AM630, had a significantly lower UCP-1 level compared with lean-derived ADPs and untreated (or vehicle-treated) cells. Conversely, theUCP-1 levels were increased in both lean- and obese-derived ADPs treated with the CB2 agonist JWH-133. Blockade of CB2 receptor with AM630 in obese-derived ADPs exacerbated the obesity-induced effect of UCP-1 suppression (Figure 4C). Stimulation of CB2 receptor with JWH-133 in LPS-treated lean-derived ADPs significantly counteracted the effect of the inflammatory stimulus (Figure S4B).

7) IL-6, perilipin and UCP-1 levels are associated with CB2 Q63R variant

According to the pharmacological findings, ADPs from the obese RR subjects showed a significantly lower intracellular IL-6 level and a high level of perilipin compared with those in ADPs from the obese QQ subjects (Table S3; Figure 5A).

Finally, obese-derived ADPs from patients homozygous for the less functional CB2-R63 variant exhibited a significantly lower UCP-1 level compared with those from QQ patients (Figure 5B, C). In addition, IL-4 levels were significantly lower in ADPs from the RR compared with the QQ obese subjects (Figure 5C).

Discussion

Despite improvements in the understanding of the pathogenesis of obesity, only a limited number of drugs can be used to effectively treat this condition. One of the most effective, a diaryl-pyrazole (Rimonabant), a potent and selective CB1 receptor, was withdrawn from the market due to psychiatric side effects. Indeed, CB1 receptor signaling enhances the rewarding effects of food seeking and stimulates the growth of adipocytes, whereas CB2 receptor **stimulation** promotes antiobesity effects **by reducing food intake and weight gain without an adverse impact on mood** (20, 21) and inhibiting activated macrophages and T-cells 22.

Recent evidence indicates that CB2 receptors, even if at much lower levels compared with CB1, are also expressed in the brain and involved in neuropsychiatric functions. Interestingly, it has been shown that only chronic activation of CB2 increases excitatory synaptic transmission, whereas its short-term activation has little effect on synaptic activity (32). A therapeutic use of CB2 as antiobesity target might presume a related chronic neuronal activation, that, in turn, increasing excitatory synaptic transmission, should facilitate the peripheral antiobesity effects without exerting remarkable psychotropic activity. Nevertheless, selective CB2 agonists that cannot cross-over the emato-encephalic barrier could be designed.

Figure 2. Lipid droplet size and number are increased by adiposity and reduced by CB2 receptor stimulation. LD staining of in vitro ADPs with oil red. **A,** LPS increases number and size of MSC-derived ADPs. These increases are prevented by concomitant CB2 stimulation with JWH-133 [100 nM]. Increases in LD number and size are also observed if the CB2 reverse agonist AM630 [10 μM] is applied. **B,** Obese-derived ADPs show more numerous and larger LDs compared with those from lean individuals. LPS increases the LD number and size in lean subject-derived ADPs, whereas in obese-derived ADPs, CB2 stimulation with JWH-133 [100 nM] or blockade with AM630 [10 μM] induces a reduction and increase in the LD size and number, respectively. Panels are representative of data from two different assays performed in triplicate. Scale bar 50 μm.

Differential functioning of CB2 has been demonstrated in relation to the presence of a glutamine or an arginine at position 63 of the N-terminal domain. Experiments on the proliferation and activity of T-cells from QQ and RR subjects have demonstrated that the R-allele is less functional than the Q-allele (33), and association studies have indicated that the R63 allele is a risk factor for inflammatory and auto-immune diseases (34, 35).

To further expand upon the previous findings, we genotyped 501 Italian obese children for the CB2 Q63R variant, searching for associations with clinical features. **Moreover, we attempted to identify a role of CB2 receptor in the modulation of ADP function, to provide support for its use as a safe antiobesity target. Thus,** we exposed ADP cultures obtained from differentiation of healthy **adult** donor-derived MSCs and of **adult** lean and obese fat tissues to CB2 **stimulant or blocker** agents.

Our data revealed a significant association between the less-functional variant CB2-R63 and a high z-score BMI. **However, this association between the CB2 Q63R variant and childhood BMI percentile has been only demonstrated in obese children referred to a tertiary obesity service and, therefore this is not a population study demonstrating the frequency of CB2 Q63R variant in childhood.**

Indeed, the genetic distribution of the polymorphism overlapped with the control frequencies, but the RR patients exhibited increased weights compared with the QQ obese subjects. Thus, CB2 receptor might have a pivotal role in responses to environmental stimuli in obese children.

According to the well-established association of obesity with systemic chronic inflammation and adipocyte hypertrophy/hyperplasia, we found the R63 variant was also associated with a high level of proinflammatory IL-6 and selective CB2 blockade in lean-derived ADPs resulted in increases in IL-6 expression and LD size, similar to what occurs in the obese state. Alternatively, CB2 stimulation with a selective agonist in obese-derived ADPs caused the adipokine profile to become similar to that in lean-derived ADPs and reduced the LD number and size. Moreover, CB2 stimulation appeared to hinder IL-6 release, as demonstrated by the higher intracellular IL-6 level in obese-derived ADPs treated with JWH-133 compared with untreated cells. The effects on LD features may occur via the CB2-induced modulation of PPARγ, which mediates perilipin expression and LD morphology. Indeed, i) both the PPARγ and perilipin expression levels were greatly increased in obese-derived ADPs compared with lean-derived ADPs; ii) LDs of obese-derived ADPs were increased
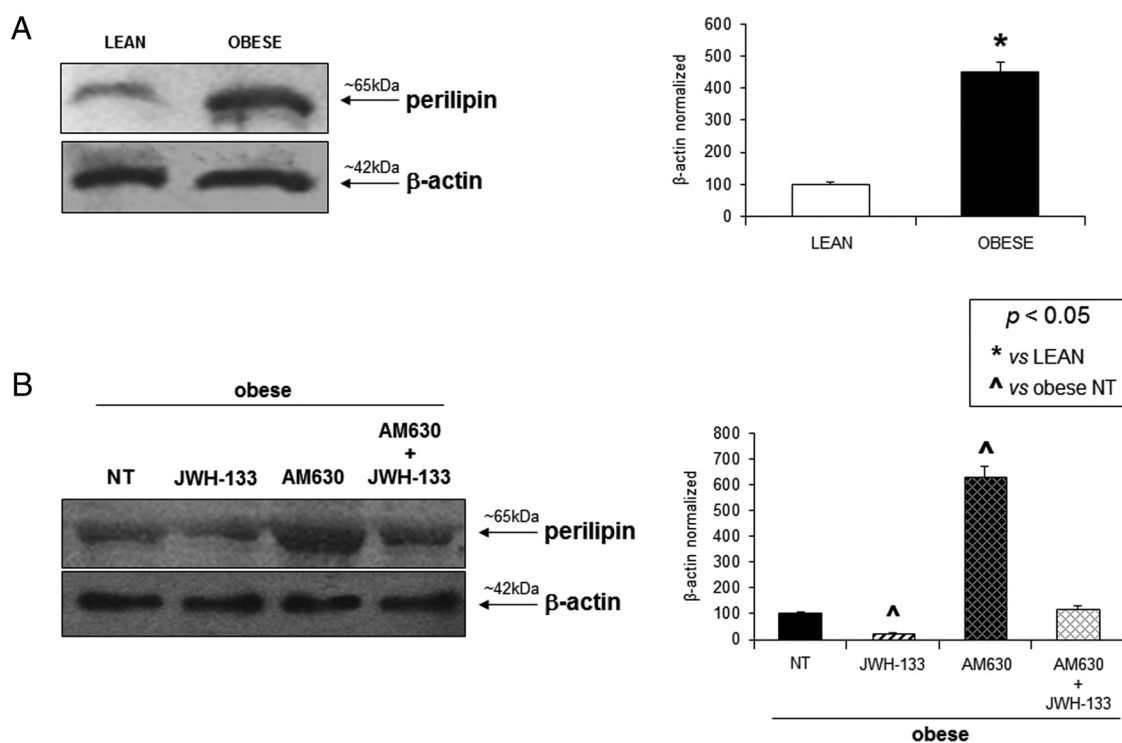
Figure 3. Perilipin expression is increased by adiposity and decreased by CB2 stimulation. **A,** Western blot panel and the relative quantification show significantly higher perilipin levels in obese-derived ADPs compared with those of lean-derived ADPs. **B,** Western blot panel and the relative quantification show that application of JWH-133 [100 nM] or of AM630 [10 μM] to obese-derived ADPs induces a decrease after CB2 stimulation and an increase after CB2 blockade, respectively. The effect induced by JWH-133 is prevented by pretreatment with AM630. Data have been revealed from in vitro ADPs by western blot from 50 μg of total lysates and have been normalized for the housekeeping protein β-actin. Data are represented as a mean$\pm$SD from three different assays. ANOVA followed by Tukey post hoc test has been used for statistical analysis. A p-value less than 0.05 was considered statistically significant.
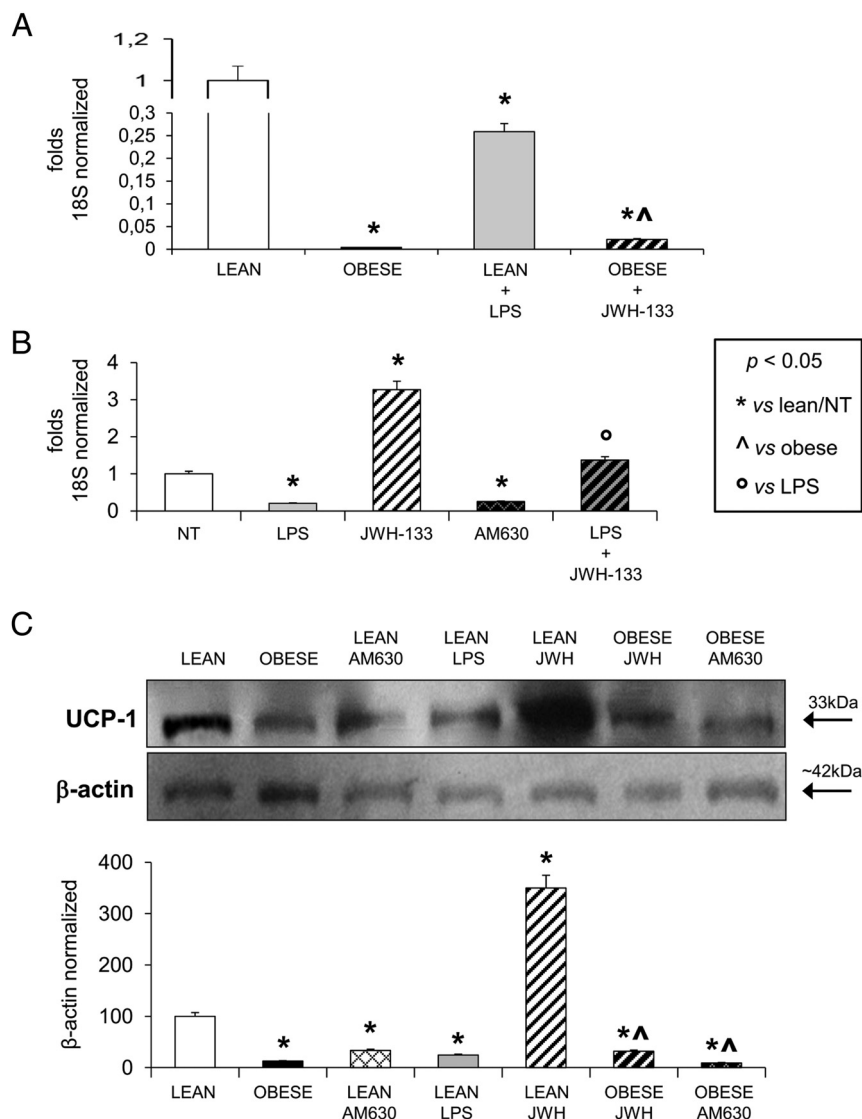
Figure 4. UCP-1 expression is decreased by adiposity and increased by CB2 stimulation. **A,** Obese-derived ADPs exhibit a significantly lower UCP-1 mRNA level compared with those from the lean subjects. Treatment with LPS significantly reduced UCP-1 expression in lean-derived ADPs. Treatment of obese-derived ADPs with the CB2 agonist JWH-133 [100 nM] significantly enhanced UCP-1 expression. **B,** LPS treatment or CB2 blockade with AM630 [10 μM] significantly decreases UCP-1 expression in MSC-derived ADPs. CB2 stimulation with JWH-133 [100 nM] significantly enhances UCP-1 expression and counteracts the LPS-induced decrease. **C,** Western blot panel and the relative quantification show significantly lower UCP-1 protein level in obese-derived ADPs compared with that in lean-derived ADPs. **Lean-derived ADPs exposed to LPS show a significantly lower UCP-1 level compared with lean-derived ADPs. Both lean- and obese-derived ADPs treated with the CB2 reverse agonist AM630 [10 μM] show a significantly lower UCP-1 level compared with the relative untreated cells.** Both lean- and obese-derived ADPs treated with the CB2 agonist JWH-133 [100 nM] show increased levels of UCP-1 **compared with the relative untreated cells.** Blockade of CB2 receptor with AM630 [10 μM] in obese-derived ADPs exacerbates the obesity-related effect of UCP-1 suppression. Data have been revealed from in vitro ADPs by western blot from 50 μg of total lysates and have been normalized for the housekeeping protein β-actin. Data are represented as mean$\pm$SD from three different assays. ANOVA followed by Tukey post hoc test has been used for statistical analysis. A p-value less than 0.05 was considered statistically significant.

whereas its selective stimulation caused substantial reductions in both the PPARγ and perilipin levels.

We also observed that in lean-derived ADPs, an inflammatory stimulus, such as LPS, induced the "obese" phenotype in terms of either the pro-inflammatory adipokine profile or the LD features. Interestingly, coapplication of the CB2 agonist not only inhibited the fusion of LDs to create larger ones but also led to the appearance of very small LDs, suggestive of the induction of lipolytic stimuli. These data confirm that CB2 receptor is an important anti-inflammatory target and suggest that it is a novel molecular determinant of LD features and in turn, ADP functioning.

Evidence indicates that interplay between the metabolic and immune systems is critical to the obesity pathogenesis. A specific lymphocyte subset in the WAT that arises due to macrophage infiltration as a consequence of altered adipokine release, accounts for both tissue inflammation and the fates of ADP precursors. Critical roles of Th2 cells and cytokines in driving the development of adipocyte precursors into beige rather than white ADPs, in addition to regulating the basal level of UCP-1 in WAT, have been highlighted. This Th2-mediated beige fat biogenesis occur via activation of IL4/IL-4Rα signaling because administration of IL-4 has been shown to increase beige fat mass and reduce obesity in thermoneutral mice (36–39). **Noticeably, CB2 receptor stimulation in immune cells facilitates type 2 T-cell (Th2) polarization** (40). Moreover, the expression of GATA3, a transcription factor selectively expressed in Th2 cells, drives IL-4 production, which increases in a CB2-dependent manner [41].

Accordingly, we found dramatic reductions in both the IL-4 and UCP-1 levels in obese-derived ADPs compared with lean-derived ADPs, and in number as well as in size compared with those of lean-derived ADPs; and iii) the CB2 blockade in obese-derived ADPs resulted in a marked increase in the perilipin level,
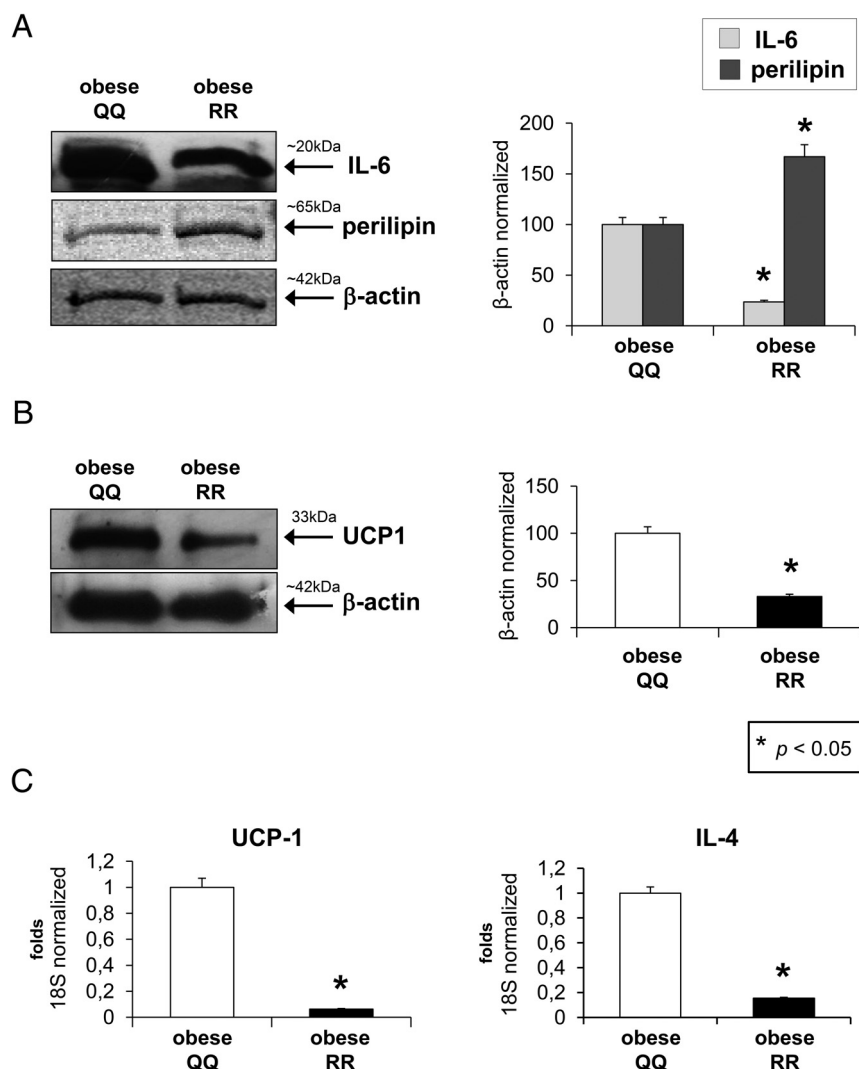
Figure 5. IL-6, perilipin and UCP-1 levels are associated with CB2-Q63R variant. All subjects underwent tissue biopsies were genotyped for the CB2 Q63R variant (Table S3) **(A)** ADPs from the obese RR subjects show a significantly lower intracellular IL-6 level and a high level of perilipin compared with those in ADPs from the obese QQ subjects. **B,** Obese-derived ADPs from the obese RR subjects show significantly lower levels of UCP-1 protein and **(C)** mRNA compared with those from QQ patients. Moreover, IL-4 levels are significantly decreased in ADPs from the RR obese subjects compared with those from the QQ obese subjects. **(RR obese subjects: n = 4; QQ obese subjects: n = 1; Table S3)** Data are represented as a mean±SD from three different assays. ANOVA followed by Tukey post hoc test has been used for statistical analysis. A *p*-value less than 0.05 was considered statistically significant.

these reductions were significantly counteracted by CB2 receptor stimulation. Interestingly, the obesity effects on IL-4 and UCP1 expression are modulated by the CB2 Q63R functional variant, resulting to be attenuated in ADPs derived from QQ obese subjects and exacerbated in ADPs from RR patients. **Of course, to strengthen this first evidence of a browning effect mediated by CB2 receptor stimulation, it could be very interesting to demonstrate the induction of the thermogenesis process in cultured adipocytes after treatment with the CB2 agonist JWH-133 (42)** .

Collectively, these data **suggest** that CB2 receptor **might** represent a pharmacological target for reducing the

obesity-related **inflammatory state**, as well as **the excessive fat storage in LDs of the WAT**, through IL-6 and perilipin modulation. **Finally, these data** suggest CB2 receptor may be a novel target for inducing browning, possibly through up-regulation of IL-4 and **induction** of UCP-1 signaling.

Acknowledgments

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