CB1R antagonist increases hepatic insulin clearance in fat-fed dogs likely via upregulation of liver adiponectin receptors

Morvarid Kabir¹, Malini S. Iyer¹, Joyce M. Richey², Orison O. Woolcott¹, Isaac Asare Bediako, Qiang Wu¹, Stella P. Kim¹, Darko Stefanovski¹, Cathryn M. Kolka¹, Isabel R. Hsu², Karyn J. Catalano², Jenny D. Chiu², Viorica Ionut¹, Richard N. Bergman¹

Cedars-Sinai Diabetes and Obesity Research Institute, Los Angeles, California¹; Department of Physiology and Biophysics, Keck School of Medicine, University of Southern California, Los Angeles, California².

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Address correspondence to:
Morvarid Kabir, Ph.D.
Diabetes and Obesity Research Institute
Cedars-Sinai Medical Center
8700 Beverly Boulevard, Thalians E105
Los Angeles, CA 90048, USA
Telephone: +1 (310) 9672790
Fax: +1 (310) 967-3869
Email: morvarid.kabir@cshs.org
Abstract

The improvement of hepatic insulin sensitivity by the cannabinoid receptor 1 antagonist rimonabant (RIM) has been recently been reported to be due to upregulation of adiponectin. Several studies demonstrated that improvement in insulin clearance accompanies the enhancement of hepatic insulin sensitivity. However, the effects of RIM on hepatic insulin clearance (HIC) have not been fully explored. The aim of this study was to explore the molecular mechanism(s) by which RIM affects HIC, specifically to determine whether upregulation of liver adiponectin receptors (ADRs) and other key genes regulated by adiponectin mediate the effects. To induce insulin resistance in the skeletal muscle and the liver, dogs were fed a hypercaloric high-fat diet (HFD) for 6 weeks. Thereafter, while still maintained on a HFD, animals received either RIM (HFD+RIM; n=11) or placebo (HFD+PL; n=9) for an additional 16 weeks. HIC calculated as the metabolic clearance rate (MCR), was estimated from the euglycemic hyperinsulinemic clamp. HFD+PL group showed a decrease in MCR. In contrast, HFD+RIM group increased MCR. Consistently, the expression of genes involved in HIC, CEACAM1 and IDE as well as gene expression of liver ADRs, were increased in HFD+RIM group, but not in the HFD+PL group. We also found a positive correlation between CEACAM1 and IDE with ADRs. Interestingly, expression of liver genes regulated by adiponectin and involved in lipid oxidation were increased in the HFD+RIM group. We conclude that in fat-fed dogs, RIM enhances HIC, which appears to be linked to an upregulation of the adiponectin pathway.

Keywords: CB1R antagonist, dogs, insulin resistance, insulin clearance, liver
Background
The metabolic syndrome is a cluster of abnormalities (abdominal obesity, dyslipidemia, hypertension and non-alcoholic steatohepatitis) that are related to insulin resistance and subsequent development of type 2 diabetes. The endocannabinoid system has garnered a great deal of attention as a potential therapeutic target in combating obesity as well as its associated metabolic abnormalities. As compared to their corresponding controls, patients with obesity and type 2 diabetes exhibit higher levels of endocannabinoids in the visceral fat and serum, respectively (32). Chronic treatment with the cannabinoid receptor antagonist rimonabant (RIM) leads to weight loss and improved insulin sensitivity in obese rodents (15; 43), canines (44) and humans (51). Moreover, several studies have recently shown that RIM has beneficial effects on the liver (14; 15). RIM has been shown to reverse steatohepatitis and related features of metabolic syndrome (15; 49). Thus, while commercial cannabinoids have been withdrawn from the market, it remains important to study the cannabinoid receptor system if additional non-centrally acting agonists become available (50). Also, the tremendous increase in cannabinoid intake in the U.S. and elsewhere (10) supports studies of the effects of these agonists and their antagonists.

In abdominally obese patients with atherogenic dyslipidemia, CB1 receptor antagonist RIM improves multiple cardiometabolic risk markers and induces significant reductions in both intra-abdominal and liver fat (12), likely due to an increase and upregulation of adiponectin (2; 27; 50). Adiponectin is produced in adipose tissues and released into the circulation, and is directly linked to insulin sensitivity (22; 47); specifically euglycemic
clamp studies performed in obese humans demonstrate plasma adiponectin levels are significantly correlated with insulin sensitivity (7). In addition, RIM treatment has been shown to increase adiponectin mRNA expression in adipose tissue of obese fa/fa rats, diet-induced obese mice (18), and cultured adipocytes (2).

High fat feeding in canines results in increased fat accumulation in both the visceral (VIS) and subcutaneous (SQ) fat depots, and impairs whole body and hepatic insulin sensitivity (27; 44), whereas RIM treatment decreases body weight and adiposity independent of changes in food intake (44).

Insulin clearance has been shown to decrease in individuals with type 2 diabetes (29) and obesity (39), and reduction in insulin clearance appears to precede insulin resistance and hyperinsulinemia (25). In the canine model, Ader et al (1) showed a positive correlation between hepatic insulin clearance and insulin sensitivity as measured by the euglycemic hyperinsulinemic clamp.

Adiponectin is known to antagonize excess lipid storage and protect against inflammation and steatosis in the liver (6). Consistently, plasma adiponectin levels were lower and adiponectin receptor2 (ADR2) staining weaker in obese, insulin resistant subjects with nonalcoholic steatohepatitis (NASH) (23). Interestingly, the carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM 1) has been linked not only to insulin clearance but also to liver fat accumulation and NASH (34). In fact, null mutation of CEACAM1 (Cc1-/-) leads to impairment of insulin clearance and hyperinsulinemia (41),
and induces the transcription of lipogenic enzymes in liver, resulting in increased hepatic lipid production and output (9; 36).

Many of the peripheral effects of RIM have been studied, and the beneficial metabolic effects have been associated with an upregulation of adiponectin; however, the effects of RIM on hepatic insulin clearance are not well understood. The aim of this study was to explore the molecular mechanism(s) by which RIM affects hepatic insulin clearance in fat-fed dogs, specifically to determine whether this is associated with upregulation of liver adiponectin receptors (ADRs) and other key genes regulated by adiponectin.

Materials and Methods

Animals and diet. Briefly, twenty male mongrel dogs (body weight: 29 ± 0.9 kg) were housed in the vivarium of the Keck School of Medicine, University of Southern California (USC), under controlled kennel conditions (12:12-h light-dark cycle). Experimental protocols were approved by the Institutional Animal Care and Use Committee of USC. Data depicting metabolic changes were previously published (44).

Upon arrival, dogs underwent a period of acclimation for three weeks, during which they were fed a standard diet. After 3 weeks animals were fed a hypercaloric high-fat diet (HFD) which consisted of ~5527 kcal/d (28% carbohydrates, 53% lipids and 19% proteins), representing an approximate 20% increase in calories from fat. Previous studies have demonstrated that 6 weeks of fat feeding promotes a consistent increase of fat in the abdominal trunk and subcutaneous depot rendering dogs insulin resistant (24).
Thus, the HFD was maintained for a minimum period of 6 weeks (Average: 9 ± 2 wks) prior to RIM treatment.

Treatment. After the initial establishment of insulin resistance with 6 weeks of HFD, dogs were randomly assigned to two groups while being maintained on the HFD for an additional 16 weeks: one group received rimonabant, HFD+RIM (n = 11); the other group received placebo, HFD+PL (n=9). Hence, in total each animal was on 22 weeks of HFD by the end of the study. RIM (Sanofi-Aventis, Paris, France) was encapsulated (AMC Pharmacy, Burbank, CA) and administered orally at 1.25 mg/kg/day. Placebo-treated animals received inert capsules (44).

In vivo metabolic assessment and tissue biopsies. Data about food intake, body weight and some biochemical parameters have been published elsewhere (27; 44). All animals underwent metabolic assessments in random order at 3 time periods: prior to fat feeding (week -6 or pre-fat); after 6 weeks of an initial fat feeding period to induce insulin resistance (week 0 or HFD), and after an additional 16 weeks of fat-feeding concomitant with either placebo (HFD+PL) or rimonabant (HFD+RIM). At each of the three designated time points (pre-fat, HFD, HFD+PL and (HFD+RIM), biopsies were taken from SQ and VIS fat depots as well as liver, where we excised approximately 500 mg of tissue and placed it in RNase-free conditions. Biopsies were conducted rapidly under general inhalant anesthesia and tissues were frozen in liquid nitrogen. All studies and plasma measurements were performed after an overnight fast (~12 hours).

Insulin sensitivity was assessed by the euglycemic hyperinsulinemic clamp (27). Hepatic insulin clearance was measured as the metabolic insulin clearance (MCR), estimated as
the ratio of insulin infusion rate to steady state plasma insulin levels during the
euglycemic hyperinsulinemic clamp (27).

Total RNA isolation and real-time PCR (RT-PCR). RNA was extracted from biopsied
tissues using the Tri-Reagent® Kit (Molecular Research Center, Cincinnati, OH). First-
strand cDNA was synthesized, according to the manufacturer’s protocol, from 1μg of total
RNA obtained from the liver and adipose tissue biopsies using Superscript II (Invitrogen,
Carlsbad, CA). The RT-PCR was performed using a Light-Cycler 4.8 instrument (Roche
Applied Science, Indianapolis, IN). The cDNA was amplified using the ‘universal probe
system’ on a Roche microplate with a final volume of 10 µl reaction mix containing 2.5 µl,
100-fold diluted cDNA, 7µl LightCycler TaqMan Master Mix buffer (Roche Probes Master
kit, Roche Applied Science, Indianapolis, IN), 1 µM specific forward-reverse primers and
0.5µl specific universal probes. Primers and universal probes are shown in Table 3.
Quantification of 18S rRNA was used for sample normalization using SYBER Green I kit.
PCR was performed according to the manufacturer’s protocol (Roche Applied science,
Indianapolis, USA). The specificity of amplification was determined by melting curve
analysis. The results were analyzed by relative quantification, ΔΔC method.

Western blot. Protein lysates were prepared by homogenizing 100 mg of liver tissue in
Cell Lysis Buffer (Cell Signaling Technology, Danvers, MA) supplemented with protease
inhibitor cocktail and phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). The
homogenate was centrifuged at 13,000 RPM for 30 min at 4°C. The supernatant was
collected and total protein concentration in the supernatant was determined using BCA
protein assay kit (Thermo Fischer Scientific, Canoga Park, CA). Equal amounts of proteins were separated on a 10% Mini-Protean®-TGX™ precast SDS-PAGE gel (BIO-RAD Laboratories, Irvine, CA) by electrophoresis, transferred onto a nitrocellulose membrane (BIO-RAD Laboratories, Irvine, CA) using a Trans-Blot SD Semi Dry Transfer Cell (BIO-RAD Laboratories, Irvine, CA). For AMPK and AMPK-P as well as Beta-actin (Cell Signaling Technology, Danvers, MA) the membranes were blocked with 5% nonfat dry milk in 1X Tris-buffered saline with Tween 20 (TBST) for 1 h at room temperature and then incubated overnight at 4°C with 1:500 diluted polyclonal primary antibodies. For CB1 (Abcam Cambridge, MA) the membranes were blocked 2 h at room temperature in 5% BSA in TBST then incubated overnight at 4°C with 1:200 diluted polyclonal primary antibodies. For CEACAM1 and CEACAM-P (kindly provided by Dr. Sonia Najjar) the membranes were blocked overnight in Na-orthovanadate, 3% BSA and Phosphate Buffered Saline with the detergent Tween 20 (PBST) then incubated overnight at 4°C with 1:500 diluted polyclonal primary antibodies. The membranes were then washed and incubated with 1:5000 diluted Alexa Fluor 680 anti-rabbit secondary antibody (Life technology) for 1 h at room temperature. Signals were detected and quantitated with the Odyssey Infrared Imaging System (Li-COR, Lincoln, NE) and accompanying Li-COR software. The band densities of AMPK, p-AMPK, CEACAM1, CEACAM1-P and CB1R were normalized to that of β-actin.

Liver Histology and triglyceride measurement. Livers were fixed with 10% neutral formalin, embedded in paraffin, cut with a microtome in 5 μm thick paraffin sections, placed on glass slides and stained with hematoxylin and eosin (H&E). We also used Oil Red O (Sigma-Aldrich, St. Louis, MO) staining to quantify liver lipid content. Liver sections
were flash-frozen and embedded in frozen medium (Optimal Cutting Temperature Compound, Ted Pella). The 5-μm sections were cut and fixed to microscope slides, allowed to air dry overnight at room temperature and stained with fresh Oil Red O for 15 minutes, rinsed in water, and then counterstained with hematoxylin. Ten randomly selected sections were photographed and examined. Liver fat accumulation was graded based on semi-quantitative estimation of the percentage of lipid-laden hepatocytes. Triglyceride (TG) content in liver tissue was determined as previously described. Briefly, total lipids from 60 mg of liver tissues were extracted with 30 vol (vol/wt) of acidified chloroform-methanol (2:1). After removal of the upper phase and conversion of the infranatant to a uniform phase with methanol, total TG content was determined enzymatically with a commercially available assay (Sigma-Aldrich, St. Louis, MO) using triolein as the calibration standard (48).

**Biochemical parameters.** Glucose was measured using a YSI 2300 analyzer (Yellow Springs Instruments, Yellow Springs, OH). Insulin from plasma samples was determined by ELISA (Linco Research, St. Charles, MO), using dog plasma for standards (27). Free fatty acids were measured utilizing a colorimetric assay based on the acylation of coenzyme A from Wako (NEFA C; Wako Pure Chemical Industries, Richmond, VA). Canine adiponectin, leptin and glucagon concentrations were measured using ELISA kits (Millipore, Temecula, CA, USA). Serum Alanine transaminase (ALT) and Aspartate transaminase (AST) levels were measured by Quest laboratory only in pre-fat, HFD+PL and HFD+RIM periods (Irvine, CA). Plasma IL-6 and TNFα were measured using canine ELISA kits (R&D systems, Minneapolis, MN, USA).
**Statistical Analyses.** All experimental data presented in this study are expressed as means ± SE unless otherwise indicated. For the comparison of changes from baseline (HFD) to W16 between the two groups (HFD+PL vs HFD+RIM), two way ANOVA adjusted by baseline was used. When the data were not normally distributed non-parametric median score one-way analysis was used. Changes from pre-fat to HFD, before randomization were compared to ‘zero’ (null hypothesis) using Wilcoxon signed ranked test. Spearman correlation was used to test the association between CEACAM-1, IDE, adiponectin receptors, MCR and plasma adiponectin. As multiple statistical tests were performed simultaneously on a single data set the Bonferroni correction, as conservative method, was applied to adjust the $P$ values (critical $P$ value (alpha) was divided by the number of comparisons made). All statistical analyses were performed with SAS version 9.2 (SAS Institute Inc., Cary, NC).

**Results**

**Metabolic profile.**

**Effects of treatments:** The effects of treatments are presented in Table1. Animals in HFD+PL (W16) group increased their body weight by ~2 kg (HFD: 31.83 ± 1.45 vs HFD+PL: 33.90 ± 1.86) whereas HFD+RIM (W16) treated dogs lost ~0.90 kg compared to HFD (HFD: 31.70 ± 1.34 vs HFD+RIM: 30.80±1.14) ($P < 0.0002$). At the end of 16 weeks of treatment period, fasting glucose, insulin and FFA remained unchanged between the groups. Glucagon decreased by 13 pg/ml in HFD+PL group compared to RIM group. However, the statistical test showed non significant difference, when taking
account of the multiplicity of tests using a Bonferroni adjustment. There was no change in fasting adiponectin levels by HFD, whereas adiponectin levels increased by 80% with RIM treatment \( (P < 0.0002) \) (27). High fat diet increased leptin levels in HFD+PL compared to HFD+RIM group \( (P < 0.029) \). At the end of 16 weeks of treatment ALT and AST remained unchanged (Table 1).

**Effects of 6 weeks fat feeding:** The effect of fat feeding is presented in Table 2. As expected fat feeding increased body weight by ~2 kg \( (P < 0.0001) \), insulin by 55% \( (P < 0.0006) \) and leptin by 77% \( (P < 0.0001) \). Glucose, glucagon, FFA and adiponectin remained unchanged by 6 weeks of fat feeding.

**In vivo hepatic insulin clearance and liver expression of CEACAM-1 and IDE.** MCR decreased from the Pre-fat to HFD period by 12.6 % \( (20.44 \pm 0.89 \text{ to } 17.87 \pm 1.12 \text{ ml·kg}^{-1}·\text{min}^{-1}, P <0.05) \). MCR are not normally distributed, non-parametric median score one-way analysis showed that the median change of MCR from HFD to HFD+PL was -4.70 ml·kg\(^{-1}\)·min\(^{-1}\) and -1.97 ml·kg\(^{-1}\)·min\(^{-1}\) in HFD+RIM group \( (P < 0.05) \), showing that RIM ameliorated the impact of additional fat feeding on hepatic insulin clearance (Figure 1a). In addition, steady-state plasma insulin during the clamp increased in HFD compared to pre-fat period \( (228 \pm 1 \text{ vs } 287 \pm 33 \text{ pM, } P < 0.05) \). At the end of the study, steady-state plasma insulin during the clamp were not normally distributed when compared to baseline. Non-parametric median score one-way analysis showed that the median change in HFD+PL was 69.47 pM \( (\text{HFD: } 307 \pm 59 \text{ pM vs HFD+PL: } 324 \pm 19 \text{ pM}) \) compared to 28.84 pM in HFD+RIM group \( (\text{HFD: } 271\pm36 \text{ pM vs HFD+RIM: } 279 \pm 13 \text{ pM}) \)
(P < 0.05). Hence, steady state plasma insulin was decreased with RIM treatment, consistent with increased insulin clearance. To demonstrate changes in hepatic insulin clearance at the molecular level, we studied two genes involved in hepatic insulin clearance; CEACAM-1 and the insulin-degrading enzyme IDE (Figure 1, b, c). CEACAM-1 gene expression did not change from Pre-fat to HFD period; however, CEACAM-1 gene expression decreased after 22 weeks of fat feeding (P < 0.05). Interestingly RIM treatment increased expression of this gene compared to the HFD+PL period. Since insulin induces phosphorylation of CEACAM-1 which, in turn, facilitates its endocytosis and degradation (40), we measured phosphorylated CEACAM-1 ratio to total CEACAM-1 protein expression. Similar to gene expression data, we showed that the ratio of CEACAM1-P to the total CEACAM-1 decreased significantly compared to the pre-fat period (P < 0.05), RIM increased the expression of CEACAM1-P compared to the PL group (Figure 1b). IDE expression decreased significantly by 27% during that period (P < 0.05). IDE decreased by 65% with additional fat feeding, in HFD+PL group. However, additional fat feeding with RIM treatment prevented decrements in IDE. Hence, for the first time we demonstrate that RIM increases the expression of enzymes associated with liver insulin degradation.

*Adiponectin gene expression in the fat depot, liver adiponectin receptors and CB1R receptor gene and protein expressions.* Six weeks of fat feeding did not modify adiponectin gene expression in either SQ or VIS fat depots. However, RIM treatment increased adiponectin gene expression in the SQ by 67%, (P < 0.05) and by 3 fold, (P < 0.001) in the VIS fat depots compared to pre-treatment or HFD period (Figure 2a). To
study the downstream effects of adiponectin, we evaluated mRNA levels of the adiponectin receptors, ADR1 and ADR2 in the liver. ADR1 decreased significantly following prolonged fat feeding in the HFD+PL in contrast to the HFD+RIM group ($P < 0.05$) where ADR1 was increased by 2.28 fold. Similarly ADR2 showed a tendency to decrease in HFD+PL group ($P < 0.07$) as compared to the HFD period, whereas RIM treatment significantly increased it 1.86 fold ($P < 0.05$) (Figure 2b). Gene expression of liver CB1R increased in HFD group by 4 fold compared to the pre-fat group, ($P < 0.01$). There was no significant difference in CB1R expression between HFD+PL and HFD+RIM compared to the HFD. However, there was a tendency for CB1R protein expression to be increased in HFD+PL group ($P = 0.09$) compared to the pre-fat period. CB1R protein expression of liver CB1R remained unchanged between HFD+PL and HFD+RIM (Figure 2c).

**Insulin clearance correlation with adiponectin.** Both types of adiponectin receptors, in the liver were strongly correlated with CEACAM-1 (ADR1 and CEACAM-1, $R = 0.69, P < 0.001$; ADR2 and CEACAM-1, $R = 0.68, P < 0.01$) (Figure 3a). Likewise, ADR1 showed a positive correlation with IDE ($R = 0.71, P < 0.005$), and a similar tendency was observed for ADR2 and IDE ($R = 0.40, P = 0.08$) (Figure b). MCR and plasma adiponectin showed a tendency to be correlated ($R = 0.43, P = 0.052$). These findings, together, suggest that the increased hepatic insulin clearance by RIM is linked to an upregulation of the adiponectin.

**Genes regulated by adiponectin: Fatty acid oxidation, thermogenesis and inflammation gene expressions.** ADR1 is strongly related to the activation of the adenosine monophosphate–activated protein kinase (AMPK) pathway. ADR2 is involved
in the activation of the peroxisome proliferator–activated receptor α (PPARα), which 
stimulates energy dissipation by increasing fatty acid oxidation (20). We quantified 
phosphorylated versus total AMPK protein ratio by western blot analysis. AMPK-P to total 
AMPK ratio decreased by 52% \( (P < 0.05) \) in HFD+PL compared to pre-fat group, while 
RIM treatment partially improved (by 32%) this ratio (Figure 4a) indicating an activation of 
adiponectin-R1-AMPK pathway.

RIM increased hepatic PPARα by 8 fold as compared to HFD+PL group \( (P < 0.005) \). We 
also measured hepatic genes downstream of PPARα that are involved in fatty acid 
oxidation. RIM increased the expression of carnitine palmitoyltransferase 1 (CPT1) by 7 
fold compared to HFD+PL group \( (P < 0.001) \) (Figure 4b). As mentioned above, HFD+RIM 
treated dogs had lower body weight compared to placebo treated dogs despite the same 
amount of food intake, which suggests that RIM is improving glucose homeostasis by 
augmenting energy expenditure. Therefore, we determined if the levels of the Uncoupling 
proteins (UCP’s) were changed by RIM treatment. UCPs are the inner mitochondrial 
membrane ion carriers that dissipate the proton gradient to generate energy during 
oxidative phosphorylation (45). HFD led to a significant reduction in mRNA levels of 
(UCP2) compared to pre-fat period \( (-45\% ; P < 0.01) \). This reduction in UCP2 expression 
was more pronounced in the HFD+PL group \( (-71\% ; P < 0.05) \) compared to HFD group. 
UCP2 expression remained unchanged compared to the pre-fat period in RIM+PL group. 
We found UCP-3 to have a similar trend; UCP3 decreased significantly in HFD+PL \( (-65\% ; P < 0.05) \), while UCP3 remained unchanged compared to the pre-fat period (Figure 4b). 
This data demonstrated that RIM restored UCP2 and 3 expression to pre-treatment
levels. We also studied three key enzymes involved in mitochondrial fatty acid oxidation (short, medium, and long chain acyl-CoA dehydrogenase, SCAD, MCAD, LCAD). Twenty-two weeks of fat feeding decreased SCAD expression by 69% ($P < 0.005$) in HFD+PL group, while RIM treatment increased SCAD by 40% ($P < 0.01$) when compared to the pre-fat period. A similar trend was observed for MCAD and LCAD (Figure 4c). These experiments suggest fatty acid oxidation as well as thermogenesis increased by RIM treatment which is consistent with reductions in body weight following RIM treatment without changes in food intake.

We also measured expression of two major pro-inflammatory cytokines namely, IL-6 and TNF$\alpha$, in the liver (Figure 5 a). IL-6 expression was significantly increased by fat feeding (2 fold, $P < 0.005$). Further exposure to fat feeding did not increase IL-6 gene expression in the HFD+PL group. However, RIM treatment significantly decreased IL-6 expression compared to the pre-fat condition (3 fold $P < 0.005$). A similar trend was observed for TNF$\alpha$ gene expression; TNF$\alpha$ increased by 4 fold by 6 weeks of fat feeding ($P < 0.001$). TNF$\alpha$ returned back to the pre-fat period in HFD+PL group while RIM treatment significantly decreased TNF$\alpha$ expression compared to the pre-fat condition (5 fold, $P < 0.001$). These data suggest that RIM may decrease liver inflammation. In addition, measurement of IL-6 and TNF$\alpha$ in the plasma (Figure 5b) found a significant increase in IL-6 and TNF$\alpha$ levels with HFD compared to the pre-fat period ($P < 0.001$). Plasma IL-6 had a tendency to be decreased by HFD+RIM compared to the HFD+RIM group ($P = 0.052$), however RIM decreased significantly TNF$\alpha$ compared to the HFD+PL group ($P < 0.05$). Taken together, these data suggest that RIM may decrease liver inflammation.
Liver histopathology and the hepatic triglyceride content. As revealed by H&E and red oil O staining, dogs fed a HFD for 22 weeks (HFD+PL) had a mild accumulation of fat in the liver which was reversed by RIM treatment. (Figure 6 a, b). Although liver TG content was not significantly different between the groups, we observed the same trend as we did in the histology measurements (Figure 6c).

Lipogenic enzyme gene expressions. Fatty acid synthase (FAS) tended to decrease after RIM treatment ($P < 0.08$). Interestingly sterol regulatory element-binding transcription factor 1c (SREBP1c) decreased significantly by RIM treatment ($P < 0.01$) (Figure 7), further suggesting RIM treatment may decrease lipogenesis.

Discussion
Clinical and animal studies have previously demonstrated RIM reduces body weight, improves symptoms associated with the metabolic syndrome, and decreases cardiovascular risk factors (11; 43; 44). Several studies have demonstrated beneficial effects of RIM on the liver (15; 53), suggestive of an important role for adiponectin, as demonstrated by the fact that RIM increases plasma adiponectin in animals and humans (15; 27; 50). Our data suggests that the endocannabinoid system plays a fundamental role in the regulation of body weight. More recently, promising new pharmacology suggest that non-brain penetrating CB1R antagonists improve insulin sensitivity (8; 50).

The liver plays an important role in hyperinsulinemic compensation by modulating hepatic insulin clearance. We have previously shown that in our obese insulin resistant dogs, hepatic insulin clearance reduces significantly to maintain hyperinsulinemia (25). In the
present study, we explored the mechanisms by which RIM enhances hepatic insulin clearance.

We have previously demonstrated that plasma adiponectin levels doubled with RIM treatment (27). Consistent with these findings, adiponectin gene expression increased in the SQ and in the VIS fat depots by RIM treatment. According to the “portal theory”, the visceral adipose tissue directly releases its metabolites into the portal vein which is then delivered to the liver (5; 19). Given that adiponectin is an insulin sensitizer (3; 47) and is directly delivered to the liver via the portal circulation, it is plausible that adiponectin secreted by the visceral fat due to RIM treatment improved the observed increase in hepatic insulin sensitivity.

Thus, we measured ADR1 and ADR2 gene expressions in the liver, both receptors demonstrating decreases after prolonged fat diet exposure in the HFD+PL group. Consistent with our data, it has been demonstrated that expression levels of ADR1 and ADR2 were decreased by approximately 65% or 55%, respectively, in the livers of db/db mice as compared with wild-type mice (21). Interestingly, despite continued fat-feeding, RIM increased the expressions of ADR1 and ADR2 as compared to HFD+PL group, suggesting a direct effect of RIM on adiponectin receptors in the liver. Moreover, CB1R expression increased with 6 weeks of fat feeding with no further change in RIM group. We also measured CB1R protein expression in pre-fat, HFD+PL and HFD+RIM groups, although there was no significant change between the groups, HFD+PL showed a tendency to increase compared to the pre-fat period. We did not observe a difference in
CB1R protein expression between HFD+PL and HFD+RIM. These results suggest that the beneficial effects of RIM on the liver might be mediated primarily via the adiponectin pathway.

In the present study, we estimated hepatic insulin clearance using MCR, a surrogate method based on the euglycemic hyperinsulinemic clamp, while controlling insulin infusion rates (26; 27). We demonstrated that 6 weeks of fat feeding as well as prolonged fat-feeding (22 weeks in HFD+PL) decreased MCR. Interestingly, we demonstrated that the MCR increased in the HFD+RIM compared to the HFD+PL group. There is a tendency for fasting plasma insulin to be decreased in RIM compared to HFD+PL group. In addition, plasma insulin concentration during the clamp steady state decreased in the HFD+RIM group, further suggestive of an increase in insulin clearance following RIM treatment. We have previously reported no changes in plasma insulin during high glucose challenge in dogs that received RIM treatment. However, we did observe a tendency for lower plasma C-peptide concentrations (52). The MCR findings are supported by a 2.5 fold increase in the expressions of genes involved in insulin clearance, namely, CEACAM1 and IDE. CEACAM1 is an important factor involved in hepatic receptor mediated endocytosis and degradation of insulin (41). Loss of CEACAM1 causes insulin resistance, hepatic steatosis, and inflammation (35). We found that 22 weeks of fat-feeding decreased the gene expression of CEACAM1. Similarly, CEACAM1-P ratio to total CEACAM1 decreased in HFD+PL group compared to the pre-fat period. Interestingly, RIM increased the gene expression of CEACAM1 by 2.5 times and CECAM1-P/CEACAM1 protein expression by 40% compared to HFD+PL. IDE showed
similar results. We also found a positive correlation between gene expression of CEACAM1 and adiponectin receptors. Similar results were observed with a correlational analysis between IDE and adiponectin receptors as well as MCR and plasma adiponectin. Our findings suggest that RIM increases hepatic insulin clearance possibly via upregulation of adiponectin receptors in insulin resistant canine model. However, it has been shown in the mouse model, CB1 receptor could be important exerting direct effect on the liver, inducing hyperinsulinemia and reducing insulin clearance. Liu et al. demonstrated that the HFD induced hepatic insulin resistance in wild-type mice, but not in CB1−/− mice or mice with hepatocyte-specific deletion of CB1. Additionally, they demonstrated that CB1−/− mice with overexpression of CB1 specifically in hepatocytes became hyperinsulinemic as a result of reduced insulin clearance due to down-regulation of IDE (31).

We further studied genes regulated by adiponectin. Adiponectin when bound to ADR1 and ADR2 activates two different pathways in the liver. ADR1 leads to phosphorylation activation of AMPK which in turn stimulates fatty acid oxidation, while ADR2 is involved in the activation of PPARα2 which propagates energy dissipation (20). Also, studies by Yamauchi T et al., indicate that the insulin sensitizing effect of adiponectin on the liver is mediated by an increase in the hepatic fatty acid oxidation (54; 55). Hence, we studied the factors regulating fatty-acid oxidation and energy dissipation in the liver. We found that with 22 weeks of fat feeding in HFD+PL group, AMPK phosphorylation decreased significantly. However, RIM treatment partially restored AMPK activation. Our finding is consistent with Heiker JT et al., who showed that increased serum adiponectin results in
an increase in AMPK activation via ADRs (16). Our results are also consistent with
glucagon-mediated activation of AMPK in the liver (4; 28). We demonstrated that fasting
glucagon had a tendency to be decreased in HFD+PL compared to HFD+RIM group,
which is consistent with the results obtained for AMPK activation.

PPARα is the transcription factor that regulates the expression of several genes involved
in mitochondrial, peroxisomal and microsomal fatty acid oxidation enzymes in the liver
(17). We found an 8 fold increase in PPARα with RIM treatment as compared to Pre-fat,
HFD and HFD+PL groups. CPT1 is a key regulatory enzyme in the mitochondria,
responsible for transferring fatty acids from the cytosol to the mitochondria before beta-
oxidation (46). We found a 7 fold increase in CPT-1 gene expression, indicating an
increased flux of FFA to the mitochondria with RIM treatment. These results are
consistent with a recent study showing that RIM treatment increased fatty acid oxidation
via upregulation of CPT1 and PPARα only in adiponectin +/+ and not in adiponectin -/-
mice (49).

Uncoupling proteins (UCPs) are the inner mitochondrial membrane ions carriers that
dissipate the proton gradient to generate energy during oxidative phosphorylation (45).
Zhou M et al., showed that adiponectin treatment restores mitochondrial function,
depletes lipid accumulation, and up-regulates the mRNA and protein expression of UCP2
in the liver of adiponectin KO mice. In addition, it has been shown that hepato-protective
functions of adiponectin were significantly attenuated in UCP2 KO mice (56). In the
present study, we demonstrated that UCP2 and UCP3 gene expressions were decreased
by 22 weeks of fat feeding in the HFD+PL group. We showed for the first time that RIM
treatment restores the expression of these two genes to the pre-treatment period. We
also evaluated the mRNA expressions of SCAD, MCAD and LCAD as markers of enzymes mitochondrial β-oxidation. Fat feeding significantly decreased SCAD in the HFD+PL group as compared to the pre-fat period. We observed similar trends in the expressions of MCAD and LCAD with fat-feeding. RIM, however, restored SCAD, MCAD and LCAD levels to the pre-fat period. Thus, our results are consistent with other studies (13; 49) showing that RIM increased fatty acid oxidation in fat fed model of insulin resistance.

The anti-inflammatory property of adiponectin has been extensively studied in the past decade (38). Human and animal studies have demonstrated that plasma adiponectin levels are inversely correlated with plasma pro-inflammatory cytokines (37; 38). In our study, we found that gene expression of hepatic IL-6 and TNFα as well as plasma concentrations increased significantly after 6 weeks of fat feeding. Based on our results, it appears that high fat-feeding causes an initial robust inflammatory response in the liver that is only partially sustained with further chronic high-fat feeding, suggesting an adaptation of the hepatic inflammatory cytokines with chronic fat-feeding in the HFD+PL group. However, we demonstrated that plasma cytokines levels remained elevated with longer fat feeding. Interestingly, RIM treatment decreased gene expression as well as plasma concentrations of these cytokines to those found in HFD group.

We also found mild fat accumulation and TG accumulation in the liver after fat feeding. This is in contrast to the findings in the rodent model and humans, where fatty liver is a commonality (30; 42). Interestingly, histology of the liver showed that RIM reversed the
diet-induced mild fat accumulation in the liver to the pre-fat period. In addition, we found that factors involved in lipid accumulation and *de novo* lipogenesis, namely, SREBP1c decreased significantly by RIM treatment. Fatty acid synthase gene expression demonstrated the same tendency. These results are consistent with other studies showing that reversal of HFD induced hepatic steatosis and fibrosis by RIM requires adiponectin (49). Although this study demonstrated that some hepatoprotective effects of RIM such as improvement of hepatic steatosis and fatty acid oxidation are adiponectin-dependent, other metabolic effects such as decrease improvement of hepatic insulin resistance by RIM are independent of adiponectin. By contrast, a study in diet-induced obesity mouse showed that RIM treatment decreased hepatic insulin resistance via adiponectin-dependent mechanism (33). There are a number of factors that may account for the variability in adiponectin dependence, such as the animal model and the duration of the treatment. In our study, we did not showed direct effect of adiponectin improving insulin sensitivity. However, we demonstrated that adiponectin receptors decreased by HFD and improved by RIM treatment. We also showed strong correlation between insulin clearance and adiponectin receptors. Hence, some effects of CB1 antagonist such as an increase of insulin clearance, fatty acid oxidation and decrease of inflammation markers and lipid in the liver may be mediated by adiponectin. Further studies are needed to clarify the direct effect of adiponectin on insulin sensitivity.

In conclusion, the findings of the present study demonstrate that RIM increases hepatic insulin clearance, most likely by targeting CEACAM-1 and IDE, which in turn is associated with upregulation of adiponectin receptors. We also confirmed the hepatoprotective effect
of RIM, namely upregulation of genes linked to fatty acid oxidation, improved inflammation and fat accumulation in the liver, mainly via upregulation of adiponectin and its receptors. Given the metabolic benefits of RIM, the development of new non-brain penetrating CB1R antagonists may provide similar effects while preventing the undesirable central nervous system effects of RIM.
Acknowledgements

We express our extreme gratitude to Dr. Sonia Najjar to provide us CEACAM-1 and CEACAM1-P antibodies.

We would like to thank Erlinda Kirkman for her excellent veterinarian expertise in facilitating these studies. We also would like to thank our lead laboratory animal technician Edward Zuñiga and Edgardo Paredes.

M.K., R.N.B., and J.M.R. did the conception and design of the research; M.K., M.S.I., I. A.Q.W., performed molecular biology experiments; O.O.W., performed physiology experiments, edited and revised manuscript, S.P.K., D.S., C.K., I.R.H., K.J.C., J.D.C, V.I., and J.M.R. performed physiology experiments; M.K., D.S. and M.S.I. analyzed the data; M.K. and M.S.I. interpreted the results of the experiments; M.K. prepared the figures; M.K. drafted the manuscript; M.K., M.S.I., O.O.W, S.P.K, C.K., R.N.B., and J.M.R. revised the manuscript. M.K. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

This work has been supported by Sanofi-aventis and grant from the National Institutes of Health (NIH DK029867).

Disclosure

This work has been supported by Sanofi-aventis.
Table 1: Body weight and fasting plasma parameters in high fat (HFD (W0)), in HFD + Placebo (W16) and HFD + Rimonabant (W16) (n=20).

<table>
<thead>
<tr>
<th>Mean ± SEM</th>
<th>Baseline (W0)</th>
<th>Endpoint (W16)</th>
<th>Change from baseline to endpoint (W16 – W0)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HFD + Rim</td>
<td>HFD + PL</td>
<td>HFD + Rim</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>31.70±1.34</td>
<td>31.83±1.45</td>
<td>30.80±1.14</td>
</tr>
<tr>
<td></td>
<td>-0.90±0.35</td>
<td>2.07±0.52</td>
<td>P=0.0002 (a)</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>89.18±2.59</td>
<td>81.78±2.50</td>
<td>88.00±2.70</td>
</tr>
<tr>
<td></td>
<td>0.00±2.02</td>
<td>0.78±3.28</td>
<td>P=0.477 (b)</td>
</tr>
<tr>
<td>Insulin (pM)</td>
<td>111.46±11.96</td>
<td>88.57±12.06</td>
<td>94.79±11.55</td>
</tr>
<tr>
<td></td>
<td>-19.98±10.49</td>
<td>6.95±28.87</td>
<td>P=0.065 (b)</td>
</tr>
<tr>
<td>Glucagon (pg/ml)</td>
<td>38.68±5.69</td>
<td>44.68±6.71</td>
<td>40.52±3.60</td>
</tr>
<tr>
<td></td>
<td>1.69±5.29</td>
<td>-13.42±6.44</td>
<td>P= 0.033* (a)</td>
</tr>
<tr>
<td>FFA (mM)</td>
<td>0.78±0.07</td>
<td>0.79±0.06</td>
<td>0.73±0.07</td>
</tr>
<tr>
<td></td>
<td>-0.02±0.11</td>
<td>-0.01±0.14</td>
<td>P=0.894 (a)</td>
</tr>
<tr>
<td>Adiponectin (μg/ml)</td>
<td>10.50±0.97</td>
<td>10.10±1.07</td>
<td>18.90±1.83</td>
</tr>
<tr>
<td></td>
<td>8.39±1.29</td>
<td>0.99±0.59</td>
<td>P=0.0002 (a)</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>2.03±0.38</td>
<td>1.37±0.29</td>
<td>1.85±0.41</td>
</tr>
<tr>
<td></td>
<td>-0.53±0.33</td>
<td>1.33±0.57</td>
<td>P=0.029 (a)</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>40.11±4.25**</td>
<td>54.56±15.30</td>
<td>30.00±3.06</td>
</tr>
<tr>
<td></td>
<td>-9.38±3.63</td>
<td>12.00±20.00</td>
<td>P=0.186 (a)</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>34.89±2.31**</td>
<td>39.78±3.81</td>
<td>32.25±4.62</td>
</tr>
<tr>
<td></td>
<td>-2.75±3.85</td>
<td>-3.67±4.14</td>
<td>P=0.617 (a)</td>
</tr>
</tbody>
</table>

No significant difference on baseline

a) 2 ways ANOVA adjusted by baseline

b) Non-parametric median score test

* Only a trend is observed, taking account of the multiplicity of tests, using a Bonferroni adjustment (9 tests) the critical P-value would be 0.0055 (0.05/9)

**Missing data on W0, W6 is taken
Table 2: Body weight and fasting plasma parameters from W-6 (Pref-fat) to high fat (HFD W0) (n=20).

<table>
<thead>
<tr>
<th>N</th>
<th>Mean ± Sd</th>
<th>Pre-fat (W-6)</th>
<th>HFD (W0)</th>
<th>Delta (W0 – W-6)</th>
<th>Test vs 0*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (kg)</td>
<td>29.62 ± 0.83</td>
<td>31.76 ± 0.96</td>
<td>2.14 ± 0.26</td>
<td>P&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>84.60 ±1.88</td>
<td>85.85 ±1.96</td>
<td>1.25 ±2.53</td>
<td>P=0.628</td>
<td></td>
</tr>
<tr>
<td>Insulin (pM)</td>
<td>65.19 ± 5.70</td>
<td>101.16 ± 8.71</td>
<td>35.97 ±8.61</td>
<td>P=0.0006</td>
<td></td>
</tr>
<tr>
<td>Glucagon (pg/ml)</td>
<td>51.99±6.30</td>
<td>41.52±4.30</td>
<td>-11.62±8.86</td>
<td>P=0.182</td>
<td></td>
</tr>
<tr>
<td>FFA (mM)</td>
<td>0.75 ± 0.08</td>
<td>0.79 ± 0.05</td>
<td>0.03±0.07</td>
<td>P=0.551</td>
<td></td>
</tr>
<tr>
<td>Adiponectin (µg/ml)</td>
<td>10.48 ±0.84</td>
<td>10.32 ± 0.70</td>
<td>-0.16±0.76</td>
<td>P=0.898</td>
<td></td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>0.96 ± 0.13</td>
<td>1.70± 0.24</td>
<td>0.73±0.16</td>
<td>P=0.0001</td>
<td></td>
</tr>
</tbody>
</table>

*signed rank test
Table 3: List of primers and UPL used in the RT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
<th>UPL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adiponectin</td>
<td>AGAGATGGCACCCTCGGT</td>
<td>CAGTGTCACCCCTTAGGACCAGAC 85</td>
<td></td>
</tr>
<tr>
<td>ADR1</td>
<td>TCCCCCTGGCTCTATTACTCTCT</td>
<td>CAGGACACAGACGATGGAGA      76</td>
<td></td>
</tr>
<tr>
<td>ADR2</td>
<td>CCAAAACACTCTCTTTTGGA</td>
<td>GCCCCAAGAAGAATAATCC       2</td>
<td></td>
</tr>
<tr>
<td>IDE</td>
<td>AAAGAAGCGCTGATGAGTT</td>
<td>ATGGAGAAGGGCTCTGAGTG      17</td>
<td></td>
</tr>
<tr>
<td>CEACAM1</td>
<td>TTCCAGAACATACCCCTGAA</td>
<td>AGTGCACTTTCAAATTTTTGGTT   47</td>
<td></td>
</tr>
<tr>
<td>CB1</td>
<td>CCTGGTTCTGATCTCTGGTG</td>
<td>ACCATACGCAAGCAGAGG        3</td>
<td></td>
</tr>
<tr>
<td>CPT1</td>
<td>ATGGGAATGACGGCAGAG</td>
<td>CAGGACGTACTCCACAGAGG      26</td>
<td></td>
</tr>
<tr>
<td>PPAR-α</td>
<td>GGAGCTAGATAGACACGGCA</td>
<td>GCGATCTCCACAGAACATG       5</td>
<td></td>
</tr>
<tr>
<td>UCP2</td>
<td>ACAGGTGCCTTGCGCTTG</td>
<td>CTTGGAACGCAGCTTTACTA      23</td>
<td></td>
</tr>
<tr>
<td>UCP3</td>
<td>TTGCTGATCCTCCTACCTTCTC</td>
<td>AAAATCCGCGTAGTGAAGCT      76</td>
<td></td>
</tr>
<tr>
<td>SCAD</td>
<td>ATCCCTATCTTTAGCTCTGTT</td>
<td>AAAAGAAGACAGAGGTGTAAG     38</td>
<td></td>
</tr>
<tr>
<td>MCAD</td>
<td>GAGCTTTGGATGAGCTTACCA</td>
<td>GCTCTACAGCACTCTCCCAA      5</td>
<td></td>
</tr>
<tr>
<td>LCAD</td>
<td>GAGAATGTATTTGGATTGGTCC</td>
<td>GCCATTTGTATTATCCTTGC      1</td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>CTCCACAAGCCCTTCTCTCT</td>
<td>CGGAGTGGGAAAAGGAGTA       9</td>
<td></td>
</tr>
<tr>
<td>TNFa</td>
<td>GCCGTCCTCTACAGACAA</td>
<td>GGGTGCTCCCTTTGGCAAG       53</td>
<td></td>
</tr>
<tr>
<td>SREBP1c</td>
<td>TGCTTCTGACAACCATGAAA</td>
<td>GCCCGGGAGGCTGATACC        8</td>
<td></td>
</tr>
<tr>
<td>FAS</td>
<td>ATTCGCTGGCATGAGGCC</td>
<td>CACCAGTCCCCATCACACG       59</td>
<td></td>
</tr>
</tbody>
</table>


34. **Najjar SM and Russo L.** CEACAM1 loss links inflammation to insulin resistance in obesity and non-alcoholic steatohepatitis (NASH). *Semin Immunopathol* 36: 55-71, 2014.


List of Figures and Figure Legends

Figure 1. MCR, CEACAM-1 gene and CEACAM1-P/CEACAM1 protein and IDE gene expressions. P values, versus pre-fat period: *$P < 0.05$ and versus HFD # $P < 0.05$. Data are means ± SEM.

Figure 2. Effect of RIM on the mRNA expression of adiponectin in the subcutaneous and visceral fat depots (a). Adiponectin receptor 1 and 2 gene expressions in the liver (b). CB1 gene and protein expressions in the liver (c). P values, versus pre-fat period: *$P < 0.05$ and versus HFD # $P < 0.05$. Data are means ± SEM.

Figure 3. a) Correlation between log CEACAM1 and ADR1 (left) and log CEACAM1 and ADR2 (right), b) Correlation between log IDE and ADR1 (left) and log IDE and ADR2 (right), at the end of the study. C) Correlation between MCR and plasma adiponectin

Figure 4. Effect of RIM on the liver AMPK-p/AMPK protein expression (a), liver mRNA of CPT1, PPARα, UCP2, UCP3 (b). Liver mRNA expression of SCAD, MCAD, LCAD (c). P values, versus pre-fat period: *$P < 0.05$ and versus HFD # $P < 0.05$. Data are means ± SEM.

Figure 5. Effects of RIM on the liver mRNA and plasma concentrations of inflammatory cytokines IL-6 and TNFα. P values, versus pre-fat period: *$P < 0.05$ and versus HFD # $P < 0.05$. Data are means ± SEM.
Figure 6. Staining with hematoxylin and eosin (H&E) (a) and Red Oil O (b) of liver sections. TG (c) measurements in the liver tissue.

Figure 7. Effect of RIM on the liver lipogenic enzymes gene expression. FAS tended to decrease after RIM treatment ($P < 0.08$). SREBP1c decreased significantly by RIM treatment ($P < 0.01$). $P$ values, versus pre-fat period: *$P < 0.05$ and versus HFD # $P < 0.05$. Data are means ± SEM.
**Figure 1.**

a) **Metabolic clearance rate**

```
<table>
<thead>
<tr>
<th>Condition</th>
<th>Metabolic Clearance Rate (ml·kg⁻¹·min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-Fat</td>
<td>0</td>
</tr>
<tr>
<td>HFD</td>
<td>*</td>
</tr>
<tr>
<td>HFD+ PL</td>
<td>#</td>
</tr>
<tr>
<td>HFD+ RIM</td>
<td></td>
</tr>
</tbody>
</table>
```

b) **CEACAM-1 gene expression**

```
<table>
<thead>
<tr>
<th>Condition</th>
<th>mRNA Relative to 18S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-Fat</td>
<td></td>
</tr>
<tr>
<td>HFD</td>
<td>1</td>
</tr>
<tr>
<td>HFD+ PL</td>
<td>#</td>
</tr>
<tr>
<td>HFD+ RIM</td>
<td></td>
</tr>
</tbody>
</table>
```

c) **IDE gene expression**

```
<table>
<thead>
<tr>
<th>Condition</th>
<th>mRNA Relative to 18S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-Fat</td>
<td></td>
</tr>
<tr>
<td>HFD</td>
<td>*</td>
</tr>
<tr>
<td>HFD+ PL</td>
<td>#</td>
</tr>
<tr>
<td>HFD+ RIM</td>
<td></td>
</tr>
</tbody>
</table>
```

**CEACAM1**

```
<table>
<thead>
<tr>
<th>Condition</th>
<th>CEACAM1 Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-Fat</td>
<td></td>
</tr>
<tr>
<td>HFD+ PL</td>
<td>*</td>
</tr>
<tr>
<td>HFD+ RIM</td>
<td></td>
</tr>
</tbody>
</table>
```

**CEACAM1-P/CEACAM1 protein**

```
<table>
<thead>
<tr>
<th>Condition</th>
<th>CEACAM1-P/CEACAM1 Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-Fat</td>
<td></td>
</tr>
<tr>
<td>HFD+ PL</td>
<td>*</td>
</tr>
<tr>
<td>HFD+ RIM</td>
<td></td>
</tr>
</tbody>
</table>
```
Figure 2.

(a) Adiponectin levels in subcutaneous and visceral fat. HFD+, PL, and RIM groups show differences in adiponectin expression.

(b) mRNA expression of ADR1 and ADR2. HFD+ PL and RIM groups have significantly higher expression compared to HFD and Pre-Fat.

(c) CB1R gene and protein expression. CB1R gene expression is significantly higher in the HFD group, while protein expression shows a trend in the same direction.
Figure 3.

a) $r = 0.69, P < 0.001$

b) $r = 0.71, P < 0.005$

c) $r = 0.43, P = 0.052$
Figure 4.

a) AMPK-p/AMPK

b) CPT1, PPARα, UCP2, UCP3

c) SCAD, MCAD, LCAD
Figure 5.

a) **IL-6 gene expression**

b) **IL-6 plasma**

![Bar graph showing IL-6 gene expression](chart1)

![Bar graph showing IL-6 plasma levels](chart2)

a) **TNF-α gene expression**

b) **TNF-α plasma**

![Bar graph showing TNF-α gene expression](chart3)

![Bar graph showing TNF-α plasma levels](chart4)
Liver Triglyceride Content

\[ \text{mM/g wet weight} \]

\( 0 \quad 1 \quad 2 \quad 3 \quad 4 \quad 5 \quad 6 \quad 7 \)

H&E staining, 20x

Oil Red O, 40x

Figure 6.

(a) Pre-Fat

(b) Pre-Fat

HFD+ PL

HFD+ RIM

(c) Liver Triglyceride Content

\[ \mu M/g \text{ wet weight} \]

\( 0 \quad 1 \quad 2 \quad 3 \quad 4 \quad 5 \quad 6 \quad 7 \)

Pre-Fat

HFD+ PL

HFD+ RIM
Figure 7.

**FAS**

- mRNA relative to 18S

- Pre-Fat: 2.2
- HFD: 2.0
- HFD+ PL: 1.8
- HFD+ RIM: 1.6

**SREBP1-c**

- mRNA relative to 18S

- Pre-Fat: 3.9
- HFD: 3.4
- HFD+ PL: 2.7
- HFD+ RIM: 1.9

# indicates a significant difference.