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Genome-wide expression profiling revealed peripheral effects of CB1 inverse agonists in improving insulin sensitivity and metabolic parameters

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Running Title: gene expression changes induced by CB1 inverse agonist in peripheral tissues.

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Abbreviations:

Regular chow: RC; High fat diet: HFD; Fatty acid β-oxidation: FAO; Wild-type: wt; Peroxisome proliferator-activated receptor: PPAR; Cannabinoid receptor 1: CB1; Epididymal white adipose tissue: eWAT; Knock-out: KO; Tumor necrosis factor: TNF; Diet induced Obesity: DIO; Intracerebroventricular: icv; Intraperitoneal: ip; Sterol regulatory element binding protein: SREBP; False discovery rate: FDR;

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Abstract

Inhibition of cannabinoid receptor 1 (CB1) has shown efficacy in reducing body weight and improving metabolic parameters, with the effects correlating with target engagement in the brain. Recently, the peripheral effects of inhibiting the CB1 receptor has been appreciated through studies in diet-induced obese and liver-specific CB1 KO mice. In this report, we systematically investigated gene expression changes in peripheral tissues of DIO mice treated with the CB1 inverse agonist AM251. CB1 receptor inhibition led to down-regulation of genes within the de novo fatty acid and cholesterol synthetic pathways, including SREBP-1 and -2, and their downstream targets in both liver and adipose tissue. In addition, genes involved in fatty acid β-oxidation were up-regulated with AM251 treatment, probably through the activation of PPARα. In adipose tissue, CB1 receptor inhibition led to the down-regulation of genes in the TNFα signal transduction pathway and possibly to the activation of PPARγ, both of which would result in improved insulin sensitivity.

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Introduction

Obesity has become a global epidemic health problem and it is estimated that in the United States, one third of the population are overweight or obese and fifteen percent are of morbid obesity (Bloom et al., 2008). However, current anti-obesity pharmacotherapy only demonstrates moderate efficacy and are often associated with adverse effects (Van der Ploeg, 2000). Thus there is a therapeutic need for safe and effective compounds to treat obesity.

Inhibition of the endocannabinoid receptor has shown promise in treating obesity and improving co-morbidities (Bloom et al., 2008). Two cannabinoid receptor 1 (CB1) antagonists/inverse agonists, Rimonabant and Taranabant, went through phase 3 clinical trials and have proved to be efficacious in body weight reduction and improvement of metabolic parameters (Addy et al., 2008; Despres et al., 2005; Pi-Sunyer et al., 2006; Scheen et al., 2006; Van Gaal et al., 2005). CB1 receptors are predominantly expressed in the central nervous system (CNS), which is believed to be the primary target tissue of Rimonabant and Taranabant. However, CNS engagement is also associated with psychiatric adverse effects, which limits the utility of CB1 antagonists/inverse agonists in treating obesity (Jones, 2008).

The CB1 receptor is also expressed at low levels in peripheral tissues such as liver and adipose (Bensaid et al., 2003; Kunos et al., 2009; Osei-Hyiaman et al., 2005). Several studies have investigated the hypothesis that the effects of CB1 receptor blockade on

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food intake and body weight may not be limited to a central mode of action. Antagonizing CB1 receptor suppresses the expression of the lipogenic transcription factor SREBP-1c and its target genes acetyl CoA carboxylase-1 (ACC1) and fatty acid synthase (FASN), as well as de novo fatty acid synthesis both in vivo and in primary hepatocytes (Osei-Hyiaman et al., 2005). Hepatic de novo lipogenesis was decreased in liver-specific CB1 KO mice (LCB1-/-), as in the global-KO mouse (Osei-Hyiaman et al., 2008), underscoring the role of liver CB1 receptor in mediating the effect. LCB1-/- mice also showed reduced liver steatosis, hyperglycemia, dislipidemia, and insulin and leptin resistance than wild-type controls, suggesting hepatic CB1 receptor signaling cascade contributes to the regulation of these metabolic pathways (Osei-Hyiaman et al., 2008).

To systematically investigate physiological pathways modulated by CB1 receptor inhibition in peripheral tissues, we performed gene expression profiling of liver and epididymal white adipose tissue (eWAT) isolated from lean and diet-induced obese (DIO) mice treated with a CB1 receptor inverse agonist. Our findings confirmed earlier reports of suppression of SREBP-1 responsive genes by CB1 inhibition, and revealed regulation of additional pathways that should lead to improved metabolic parameters.

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Materials and Methods:

In vivo treatment for transcriptional profiling: All animal experiments and euthanasia protocols were conducted in strict accordance with the National Research Council's Guide for the Care and Use of laboratory Animals. Animal experiment protocols were reviewed and approved by the Institutional Care and Use Committee of Merck Research Laboratories. The laboratory animal facilities of the Merck Research Laboratories are certified by the Association for the Assessment and Accreditation of Laboratory Animal Care International. Animals were housed in temperature-, humidity-, and light-controlled rooms (21-23^{0}C, 47-65%, 12-12 h light/dark cycle, respectively).

CB1-/- mice were obtained from A. Zimmer (University of Bonn) (Zimmer et al., 1999) and back-crossed onto C57BL/6J genetic background for ten generations by A. Zimmer before homozygous CB1-/- mice were re-derived at Taconic Farms (German Town, NY) onto the C57BL/6N genetic background. Male CB1-/- mice and control littermates (n=5-7 in each group) at 2 months of age were fed with regular chow (Teklad 7012, 13 % kcal from fat, 3.41 kcal/g, Harlan Laboratories, Indianapolis, IN, USA) or high fat diet (S3282, 59.4% kcal from fat; 24.5% kcal from carbohydrate; 16.2% kcal from protein; 5.29 kcal/g, Bio-Serv, Frenchtown, NJ, USA) for 14 weeks, and were individually caged 1 week before drug treatment. Vehicle (0.5% methylcellulose) or AM251 (10 mg/kg, Sigma, St Louis, MO, USA) were dosed by oral gavage at 5:00 PM daily for 2 days. Body weight was measured at 5:00 PM on day 1 and 2, and at 10:00 AM on day 3 before tissue collection. Food was measured at 5:00 PM on day 1 and at 10:00 AM on day 3 before

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tissue collection. Food intake is calculated as the difference in food weight at the start minus at the end of the study. Mice were euthanized by CO_2 asphyxiation at 10:00 AM following the second dose.

RNA extraction and hybridization: Total RNA was isolated from frozen tissues after homogenizing in TRIzol reagent (Invitrogen, Carlsbad, CA) and processed using RNeasy kits (QIAGEN, Valencia, CA) according to manufactures' instructions. Samples were amplified and labeled using a custom automated version of the RT/IVT protocol and reagents provided by Affymetrix (Santa Clara, CA). Hybridization, labeling and scanning were completed following the manufacturer's recommendations (Affymetrix). Sample amplification, labeling, and microarray processing were performed by the Rosetta Inpharmatics Gene Expression Laboratory in Seattle, Wash. The raw gene expression data has been deposited into the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/. Accession ID: GSE21069).

Statistical Analysis: One-way ANOVA and False Discovery Rate (FDR) analyses were performed with Matlab (The Mathworks, Natick, MA). Genes had to pass a pre-filter of Affymetrix MAS5 present call P-value < 0.05 in $> 50\%$ of the samples to qualify for further analysis. Ratios between a replicate and the virtual pool of control samples were calculated for each comparison. Differentially expressed genes (signature genes) were selected with ANOVA P value (P value < 0.01 or 0.05).

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FDR was estimated using Monte Carlo sample permutations. For each statistical test, a P value threshold (P value < 0.01) was used to select significant genes. Each sample was randomly permuted changing the treatment group to which the sample belongs. This process was repeated 100 times. The FDR was calculated as the ratio of the average number of significant genes identified in the randomized data to the number of significant sequences in the original data.

Pathway analysis: modulation of a certain pathway can be significant if most genes in the pathway are regulated in the same direction, even though the expression change of an individual gene is small. To capture those small changes, we applied a relaxed cut-off to select signature genes (P value < 0.05 and absolute fold change > 1.1) for pathway analysis. The Ingenuity Pathway Analysis software (Redwood city, CA, USA) was used to analyze the enrichment of canonical pathways in the signature genes. Public microarray data set was queried with the NextBio System (Cupertino, CA, USA).

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Results

AM251 induced most robust gene expression changes in DIO mice

Previous studies demonstrated that the appetite- and weight-reducing effects of CB1 receptor inverse agonists are more profound in obese, hyperphagic animals than in their lean controls (Hildebrandt et al., 2003; Vickers et al., 2003). Thus it is possible that gene expression changes induced by CB1 inverse agonists are dependant on diet. We designed experiments that will not only allow the evaluation of diet effect, but also possible off-target activity of CB1 receptor inverse agonists (Table 1). Two-month old male mice were fed with regular chow (RC) or high fat diet (HFD) for 14 weeks, and treated with either vehicle or AM251, a specific CB1 receptor inverse agonist (Gatley et al., 1996). CB1-/- mice were included to evaluate CB1 receptor mediated effects versus non-mechanism based activity of the compound. Two-day treatment with AM251 was sufficient to induce significant reduction in both food intake and body weight in wild-type (wt) but not CB1-/- mice, and the effect was much stronger in mice fed with HFD. In DIO mice, a decrease of 74% food intake (Figure 1A) and 8% body weight (Figure 1C) was observed in DIO mice, whereas in lean mice, a modest 17% reduction in food intake (Figure 1B) and 3% decrease of BW (Figure 1D) was detected. This was consistent with earlier observations that the body weight reduction effect of CB1 inverse agonist was more robust in mice fed with HFD compared to those on regular chow (Jbilo et al., 2005).

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One-way ANOVA analysis was performed between vehicle and AM251 treatment to define gene expression changes (signature genes) induced by AM251 in wt or CB1-/- mice fed with RC or HFD (Table 2). In both liver and eWAT, AM251 induced gene expression changes were more robust in wt mice fed with HFD as compared to regular chow (Table 2). In liver with P value < 0.01 and absolute fold change > 1.2, the AM251 signature size in wt DIO mice was over 757 genes with 10% false discovery rate (FDR), whereas in CB1-/- animals fed with the same diet, the signature size was only 101 with a much higher FDR (64%). There were only 6 commonly regulated genes between the wt and KO AM251 signatures under HFD (Figure 2A). Among those six genes, three belong to the cytochrome P450 family (Cyp2b10, Cyp2c29, Por), and are perhaps involved in compound metabolism. The other three genes were heatshock protein (Hspb1), aminoacylase (Aspa) and a gene of unknown function (4931406C07Rik). These observations suggest minimal off-target effect of AM251. In lean mice, the effect of AM251 was much more modest (110 genes with 64% FDR) than in DIO mice under the same threshold (ANOVA P value < 0.01 and absolute fold change > 1.2), underlining the diet effect to transcriptional regulation of AM251. There were only three commonly regulated genes between the AM251 signatures in lean and DIO animals (Figure 2B). The small overlap is not too surprising given the weak response and high false positive rate of AM251 signature in lean mice. Similar observations were made in eWAT, where no commonly regulated genes by AM251 between wt and CB1-/- mice were observed (both fed with HFD, Figure 2C), and only fourteen overlapping genes were found after AM251 treatment in either lean or DIO mice (Figure 2D).

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The experimental design also allowed for comparisons of gene expression differences between wt and CB1-/-, and between lean and DIO mice. Interestingly, many genes induced by AM251 in DIO mice (in liver and eWAT) were a reversal of the HFD induced changes when compared to wt mice fed with regular chow (Figure 3A and 3B). The regulation of these genes is probably accountable for the improvement in metabolic parameters by CB1 receptor inverse agonists in DIO mice. Mapping these changes to physiological pathways should aid our understanding of the mechanism of AM251 in peripheral tissues.

Liver fatty acid β-oxidation (FAO) pathway is up-regulated by AM251

We first compared the AM251 signature in DIO mice to over 10,000 microarray experiments deposited in public databases and curated by the NextBio System (Cupertino, CA, USA). An internal web-based search engine was used which generated a list of experiments with the most overlapping signature to our query gene set, ranked by hypergeometric P value. The top hits for liver DIO AM251 signature were from studies published by Rakhshandehroo et al, where they analyzed liver gene expression changes in PPARα KO versus wild-type mice, or induced by PPARα agonist treatment (Rakhshandehroo et al., 2007). PPARα is a nuclear receptor that has been shown to play a critical role in the regulation of cellular uptake, activation, and β-oxidation of fatty acids (Moller and Berger, 2003). An increase of 36% of PPARα gene expression in liver was detected in wt mice treated with AM251 (Figure 4, black bars), but not in CB1-/- animals (Figure 4, striped bars). In addition, genes encoding the key enzymes regulating

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β-oxidation including acyl-CoA dehydrogenase (ACAD), 3-oxoacyl CoA thiolase (ACAA2), alpha and beta subunit of the trifunctional enzyme composing of enoyl CoA hydratase, hydroxyacyl CoA dehydrognease and ketoacyl CoA thiolase activity (HADHA and HADHB), carnitine palmitoyltransferase (CPT2 and CPT1a), carnitine transporter (SLC22A5), carnitine/acylcarnitine translocase (SLC25A20), 2,3-enoyl CoA isomerase (DCI), 2,4-dienoyl CoA reductase (DECR1) were all up-regulated by AM251 in liver of wt mice (Figure 4, black bars), but not in CB1-/- mice (Figure 4, striped bars). None of these genes were significantly regulated in liver of wt lean mice treated with AM251 (data not shown). Malonyl CoA decarboxylase (MLYCD), a target of PPARα (Lee et al., 2004), was also up-regulated by AM251. An increase in MLYCD should decrease malonyl CoA levels, an inhibitor of CPT1a, and thus increase FAO (Ruderman et al., 1999). Osei-Hyiaman et al demonstrated that CB1 receptor inverse agonist treatment increased liver CPT1a mRNA, protein level and its enzymatic activity (Osei-Hyiaman et al., 2008), also supporting the up-regulation of fatty acid β-oxidation by inhibition of the CB1 receptor.

PPARα regulates fatty acid β-oxidation in both mitochondria and peroxisome (Lefebvre et al., 2006; Rakhshandehroo et al., 2007; Reddy and Rao, 2006). The peroxisomal β-oxidation is more versatile than the mitochondrial pathway and is capable of metabolizing a wide variety of fatty acid analogs as well as very-long-chain fatty acid. Examination of the key enzymes catalyzing peroxisomal β-oxidation also reveals a trend of increased expression by AM251 treatment. The bifunctional enzyme composed of both enoyl-CoA hydratase and 3-hydroxylacyl CoA dehydrogenase (EHHADH), carnitine O-

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octanoyltransferase (CROT), peroxisomal D3,D2-enoyl-CoA isomerase (PECI), peroxisomal 2,4-dienoyl CoA reductase 2 (DECR2), hydroxysteroid (17-beta) dehydrogenase 4 (HSD17B4) are also increased by AM251 treatment in liver of wt DIO mice (Figure 4, black bars), but not in CB1-/- mice fed with HFD (Figure 4, striped bars).

De novo fatty acid and cholesterol synthesis pathways are suppressed by AM251

Osei-Hyiaman et al demonstrated earlier that CB1 receptor antagonist decreased expression of the lipogenic transcription factor SREBP-1c (an isoform of SREBP-1) and its target genes acetyl CoA carboxylase-1 (ACC1) and fatty acid synthase (FASN). The suppression is accompanied by reduction in de novo fatty acid synthesis in liver, both in vivo and in vitro (Osei-Hyiaman et al., 2005). Our microarray data not only confirmed their observation, but also showed the down-regulation of additional SREBP-1 target genes crucial for lipogenesis. These include stearyol CoA desaturase 1 (SCD1), long-chain fatty-acid elongase 5 and 6 (ELOVL5 and ELOVL6), ATP citrate lyase (ACLY), fatty acid desaturase 1 and 2 (FADS1 and FADS2), and glycerol-3-phosphate acyltransferase (GPAM) (Figure 5A, black bars). In CB1-/- mice, expression of SREBP-1 and some of its target genes are also decreased compared to wt (Figure 5A, dotted bars), although the extent of regulation is not as profound as in AM251 treatment. This is probably attributed to difference between acute inhibition of CB1 receptor versus more chronic effect caused by embryonic inactivation of the gene. The latter could induce compensatory effect for loss of CB1 receptor activity. In mice fed with high fat diet, the SREBP-1 responsive genes are up-regulated compared to those on regular chow, which

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probably is reflective of steatosis observed in obese animals. SREBP-1 gene encodes two members, SREBP-1a and SREBP-1c, through the use of alternative transcription start site, and SREBP-1c is a weaker transcription activator than SREBP-1a (Brown and Goldstein, 1997; Shimano et al., 1997). The SREBP-1 probe on our Affymetrix chip is designed from the 3'UTR, which is shared by 1a and 1c, thus we are not able to discern the two isoforms.

The SREBP-1 isoforms are more selective in activating fatty acid biosynthetic genes, while SREBP-2 is more specific for controlling cholesterol biosynthesis (Shimano et al., 1999). In our experiments, SREBP-2 was also decreased by AM251 treatment, accompanied by down-regulation of cholesterol synthesis genes such as HMG CoA reductase (HMGCR), farnesyl diphosphate synthase (FDPS), and squalene synthase (SQLE) etc (Figure 5A, black bars). Again, this suppression was mediated by CB1 receptor and not observed in CB1-/- mice treated with AM251 (Figure 5, striped bars).

Interestingly, the down-regulation of SREBP-2 and its target genes was not limited to liver. In eWAT, SREBP-2 and genes involved in cholesterol synthesis were also decreased by AM251 (Figure 5B, black bars). Although SREBP-1 was not significantly regulated in eWAT, some of its target genes, e.g. fatty acid synthase (FASN), ATP citrate lyase (ACLY), long-chain fatty-acid elongase 6 (ELOVL6), cytosolic malic enzyme (ME1), and stearyol CoA desaturase 2 (SCD2) were all decreased by AM251. It is intriguing that the SREBP pathways were similarly regulated by the CB1 receptor inverse

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agonist in both liver and eWAT, though the mechanism leading to this down-regulation of SREBPs remains to be characterized.

TNFα signaling pathway is decreased by AM251 in epididymal white adipose tissue

Similar to the observation in liver, AM251 treatment reversed many gene expression changes caused by HFD treatment in eWAT (Figure 3B 1st and 3rd rows). In addition, the AM251 signature in eWAT has significant overlap with the gene expression changes between wt and CB1-/- mice (Figure 3B top two rows), suggesting these transcriptional regulations are mediated by CB1 receptor. Pathway Analysis of the signature genes was performed to investigate physiological pathways perturbed by these expression changes. One of the top hits was the TNFα signaling pathway. Activation of this pathway in adipose tissue is associated with insulin resistance, and TNFα has been shown to suppress PPARγ activity, impair insulin signaling, and increase lypolysis in adipocytes (Guilherme et al., 2008). In DIO mice, genes mediating the TNFα signaling pathway (Hayden and Ghosh, 2008) (Figure 6A) were up-regulated compared to lean (Figure 6B, grey bars): these include increases in gene expression of the TNF receptors (TNFR), receptor-interacting S/T kinases (RIPK), TNF receptor associated factors (TRAF), the NF-kB family of transcription factors (NF-kB2 and RELB), and the IkB family members (IkB-b and IkB-e). Treatment with AM251 reversed these regulations and decreased the TNFα signaling pathway (Figure 6B, black bars). In CB1-/- mice, the TNFα pathway was also decreased compared to wt (Figure 6B, dotted bars), further supporting the regulation was mediated through the CB1 receptor.

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PPARγ mediates part of the AM251 effect in adipose tissue

PPARγ is a key nuclear receptor regulating adipocyte differentiation and lipid storage, and its activation leads to an improvement in insulin sensitivity in diabetic patients and animal models (Berger et al., 2005). TNFα decreases PPARγ activity at multiple levels including at the transcription level (Ruan et al., 2002a; Ruan et al., 2002b; Zhang et al., 1996). Although we did not detect a significant change of PPARγ expression level, a handful of PPARγ target genes, such as PEPCK, CD36, FATP-1, UCP3, PDK4 (Berger and Moller, 2002; Kelly et al., 1998; Martin et al., 1997; Sfeir et al., 1997; Tontonoz et al., 1995) were all up-regulated in eWAT by AM251 treatment of DIO mice. Muise et al reported systematic identification of PPARγ agonist-responsive genes in eWAT of *dbdb* mice via transcriptional profiling of two structurally distinct PPARγ agonists (Muise et al., 2008). We compared the AM251 eWAT signature in DIO mice (a total of 1553 genes with ANOVA P value < 0.05 and absolute fold change > 1.1) to the PPARγ agonist signature in eWAT of *dbdb* (339 genes) as reported previously (Muise et al., 2008) and found a significant overlap (a total of 80 genes with a hypergeometric P value < 4E-15). A few of these genes are listed in Table 3. Consistent with speculation that AM251 suppressed TNFα signaling and thus an increase of PPARγ target genes, many genes activated by PPARγ agonists were also increased by AM251 treatment in eWAT of DIO mice. These include genes regulating lipid uptake (CD36 and FATP-1), glycerol synthesis/lipogenesis (PEPCK1 and PDK4), lipoprotein hydrolysis (MGLL), fatty acid β-oxidation and energy expenditure (MLYCD, ACAA1B and UCP3). However, genes

catalyzing de novo fatty acid synthesis were differentially regulated between AM251 and PPARγ agonists. Fatty acid synthase (FASN) and cytosolic malic enzyme (ME1) were increased by PPARγ agonist treatment, but decreased by AM251 treatment. The down-regulation of de novo fatty acid synthesis genes observed in AM251 treatment was probably due to SREBP suppression, and the different animal models used (DIO versus *dbdb*).

PPARγ also modulates the secretion of several adipokines that function as hormones and regulate insulin sensitivity and energy homeostasis. Leptin was reported to be negatively regulated by PPARγ, and we also detected a decrease of leptin gene expression in AM251 treatment. Adiponectin is another hormone secreted by adipocytes and stimulates fatty acid oxidation in liver through AMPK (Yamauchi et al., 2002), and it is also a downstream target of PPARγ (Muise et al., 2008; Nawrocki et al., 2006). It was reported that AM251 treatment leads to an increase in adiponectin mRNA level in adipose tissue and adipocytes (Bensaid et al., 2003; Jbilo et al., 2005). We were unable to detect gene expression changes of adiponectin, probably because the relative short treatment of AM251 in our studies (two-day). In the paper published by Bensaid et al, significant increase of adiponectin mRNA was only detectable after at least four days of treatment with CB1 receptor inverse agonist.

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Discussion

Increasing experimental and clinical data are demonstrating peripheral effects of CB1 inverse agonists. To understand the molecular mechanisms underlying this we performed genome-wide gene expression analysis of lean and DIO mice treated with the CB1 inverse agonist AM251. We demonstrate that AM251 induced robust signatures in DIO mice but had much more modest effects in lean mice. This is consistent with earlier reports that the CB1 receptor inverse agonist is more potent in food intake and body weight reduction in obese mice (Hildebrandt et al., 2003; Vickers et al., 2003). In addition, there was almost no overlap in the AM251 signatures in either wt or CB1-/- mice, ruling out the potential off-target effect in gene expression studies.

Our microarray data showed that AM251 decreased the expression of both SREBP-1 and -2, leading to the down-regulation of their target genes in de novo fatty acid and cholesterol synthesis. The down-regulation of SREBP-1 target genes is consistent with an earlier report that SR141716, a CB1 inverse agonist, suppresses de novo fatty acid synthesis in liver and indeed the decrease of SREBP-1c, FASN and ACC1 mRNA were confirmed by Northern blot assays (Osei-Hyiaman et al., 2005). The simultaneous suppression of both de novo fatty acid and cholesterol synthesis pathways probably contributes to the known beneficial effects of CB1 inverse agonists in improving insulin sensitivity and cardio-metabolic risk factors in both human and rodent models (Addy et al., 2008; Despres et al., 2005; Gary-Bobo et al., 2007; Osei-Hyiaman et al., 2008; Pi-Sunyer et al., 2006; Scheen et al., 2006; Van Gaal et al., 2005).

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Chronic treatment of obese rodent models with CB1 inverse agonists induces sustained weight loss even though it causes less reduction in food intake after the second week of treatment (Doyon et al., 2006; Gary-Bobo et al., 2007; Ravinet Trillou et al., 2003; Serrano et al., 2008), suggesting a contribution of energy expenditure towards weight reduction. Indeed, several groups showed CB1 inverse agonists increase oxygen consumption and/or reduce respiratory quotient in both rodent models (Flamment et al., 2009; Herling et al., 2007; Liu et al., 2005) and humans (Addy et al., 2008). We detected a significant increase in the expression of genes involved in fatty acid β-oxidation in liver of DIO mice treated with AM251, possibly mediated through PPARα activation. Osei-Hyiaman et al demonstrated that CB1 inverse agonism increases CPT1a mRNA, protein and enzymatic activity (Osei-Hyiaman et al., 2008), suggesting transcriptional regulation of this pathway translates into physiological effects.

The effect of adipose CB1 receptor signaling is less validated than liver, though there is evidence suggesting CB1 receptor antagonists/inverse agonists induce adiponectin expression (Bensaid et al., 2003), suppress lipogenesis (Cota et al., 2003) and promote mitochondrial biogenesis (Tedesco et al., 2008) in adipose tissue or cultured adipocytes. Recent studies of respiratory quotient and total energy expenditure in wt, global and liver-specific CB1 receptor knock-out mice clearly demonstrated an extrahepatic contribution towards energy expenditure (Osei-Hyiaman et al., 2008). Similar to the observation in liver, we see down-regulation of expression of genes involved in de novo fatty acid and cholesterol synthesis in eWAT, which is probably mediated through

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SREBPs. This observation aligns with earlier reports that treatment with CB1 inverse agonists lead to smaller adipocytes (Jbilo et al., 2005). We also noticed a modest up-regulation of genes involved in fatty acid β-oxidation and energy expenditure in eWAT (Figure 7). The extent of the increase in eWAT was not as robust as in liver, probably due to the relative low content of mitochondria in white adipose tissue. Yet the observation indicates the roles of extra-hepatic tissues such as adipose in mediating energy expenditure by CB1 inverse agonists. Liu et al reported that SR141716 increases oxygen consumption and glucose uptake in soleus muscle of *ob/ob* mice (Liu et al., 2005). Although it is not clear whether this is mediated through direct action on muscle CB1 receptors or a secondary effect, the data do support contribution of skeletal muscle towards increased energy expenditure induced by CB1 inverse agonist. We did not collect muscle samples in this experiment. The regulation of genes and pathways in muscle should be interrogated in future studies.

Our observation that AM251 caused a down-regulation in the TNFα signaling pathway in eWAT highlights a mechanism underlying improved insulin sensitivity by CB1 inverse agonists, given the well-established association between inflammation and insulin resistance. Suppression of the TNFα pathway could lead to activation of PPARγ and downstream target genes. Although we were not able to detect a significant increase of PPARγ mRNA after a two-day treatment with AM251, many of its target genes involved in lipid uptake and lipogenesis were up-regulated. While activation of PPARγ clearly improves insulin sensitivity, it could also lead to increased adipocyte differentiation in adipose tissue. This may sound paradoxical given the fact that CB1 inverse agonists

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decrease adiposity. We speculate that the potential adipocyte differentiation effect of PPARγ activation can be counteracted by suppression of SREBPs and increase of energy expenditure in both liver and adipose tissue. Our hypothesis of activation of PPARγ target genes is also supported by the report that adiponectin is increased by CB1 inverse agonist treatment (Bensaid et al., 2003; Jbilo et al., 2005). We were unable to detect significant regulation of adiponectin mRNA, probably because the relative short treatment of AM251 in our experiments.

Restriction of food intake was reported to suppress SREBP target genes and increase fatty acid β-oxidation pathways in liver or adipose tissues (Desert et al., 2008; Nishikawa et al., 2008; Nogueiras et al., 2008; Wang et al., 2009). In our experiment, the animals were not pair-fed and there is a significant food intake difference between AM251 and vehicle treatment, thus one can not exclude the contribution of hypophagia towards the regulation of de novo fatty acid synthesis and fatty acid β-oxidation pathways. However, we believe that reduction of food intake only partially account for the observed effect. In the CB1-/- versus wt comparison, many of the gene regulations observed by AM251 treatment were also detected in CB1-/- compared to wt, and this is unlikely caused entirely by hypophagia since the food intake effect in CB1-/- is more subtle than the food intake effect of AM251 treatment.

Our studies demonstrated a profound effect of AM251 in peripheral tissues, with the liver gene expression changes also confirmed by Agilent ink-jet oligonucleotide arrays (data not shown), a microarray platform ((Hughes et al., 2001) different from the Affymetrix

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chips used in this report. AM251 is a brain-penetrating compound, thus we cannot exclude the impact of CNS to peripheral tissues. Nogueiras et al (Nogueiras et al., 2008) attempted to address the central versus peripheral effect of CB1 inverse agonist via comparing intracerebroventricular (icv) and intraperitoneal (ip) administration of Rimonabant (SR141716) in DIO rats. They observed an increase of glucose uptake and expression of CPT1a, as well as a decrease of SCD1 in adipose tissues that can not be explained by reduction of food intake in the ip treatment only. While their results are consistent with the contribution of peripheral effect towards increased energy expenditure and glucose homeostatsis, the possibility that the ip administered inverse agonist reaches populations of brain CB1 receptors that are not reached by icv administration cannot be ruled out. Ideally central versus peripheral mode of action of CB1 inverse agonists should be addressed by profiling of a non-brain penetrating compound, and validating in tissue-specific CB1 KO models. Although gene expression profiling alone is not sufficient to reveal the mechanism of CB1 inhibition leading to improved insulin sensitivity and metabolic parameters, our analysis sets the foundation for future hypothesis-driven validation.

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FOOTNOTES

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Legends for Figures

Figure 1: Food intake and body weight changes of mice treated with AM251 for 41-hr. Student t-test was applied to evaluate significance. Treatment with AM251 reduced food intake (A) and body weight (C) in wt DIO mice, but not in CB1-/- mice fed with HFD. Decrease of food intake (B) and body weight (D) was also detected in lean wt mice, although the extent of reduction is less than those observed in DIO mice.

Figure 2: Venn diagrams of AM251 signatures (ANOVA P value < 0.01 and absolute fold change > 1.2) between wt and CB1-/- mice, DIO and lean mice. (A) In liver and under HFD, there are only six overlapping AM251 signature genes between wt and CB1-/- mice, three of which belong to the cytochrome P450 family and are perhaps involved in AM251 metabolism. (B) Small overlap between AM251 signatures in wt DIO and lean mice in liver. (C) In eWAT under HFD, no commonly regulated genes were found between wt and CB1-/- mice. (D) There are fourteen overlapping AM251 signature genes between wt DIO and lean mice in eWAT.

Figure 3: Many of the gene expression changes induced by two-day treatment of AM251 in DIO mice are shared in CB1-/- mice (compared to wt), and are regulated in opposite direction by high-fat diet (HFD) feeding (compared to regular chow). Ratio experiments were generated with vehicle (1st row), wt (2nd row) or mice fed with regular chow (RC, 3rd row) as the baselines. Shown in the clustergram are Log(Ratio) values at a range of +/- 0.6 (or +/- 4 fold change), with magenta and cyan signifying up- and down-regulation,

respectively. (A) Liver. A total of 757 AM251 signature genes are shown (ANOVA P value < 0.01 and absolute fold change > 1.2). (B) eWAT. A total of 526 AM251 signature genes are shown (ANOVA P value < 0.01 and absolute fold change > 1.2).

Figure 4: In liver, PPARα and its target genes regulating mitochondrial and peroxisomal fatty acid β-oxidation are increased by AM251 treatment in wt DIO mice (black bars), but not in CB1-/- mice fed with HFD (striped bars). Y-axis represents percentage of gene expression changes as measured by microarray. Only genes with significant expression changes (ANOVA P value < 0.05) are shown.

Figure 5: SREBP-1 and SREBP-2 target genes are suppressed by AM251 treatment in wt (black bars), but not in CB1-/- mice (striped bars). (A) Liver. (B) eWAT. Only genes with significant expression changes (P value < 0.05) are shown. In liver, SREBP-1 and target genes are also decreased in CB1-/- compared to wt controls (dotted bars), whereas they are up-regulated by high fat diet compared to regular chow (gray bars).

Figure 6: In eWAT, expression of genes in the TNFα signaling pathway (A) is increased by high fat diet (HFD) relative to regular chow (RC) feeding (B, gray bar). Two-day treatment of DIO mice with AM251 decreased gene expression of this pathway (B, black bar). TNFα signaling pathway is also down-regulated in CB1-/- mice fed with HFD compared to wt DIO mice (B, dotted bars). Only significant gene expression changes (ANOVA P value < 0.05) are shown.

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Figure 7: Genes catalyzing fatty acid β-oxidation and energy expenditure are increased by AM251 treatment in eWAT of wt DIO mice (black bars), but not in CB1-/- mice fed with HFD (striped bars). Most of these genes are regulated in same direction in CB1-/- mice, compared to wt controls (dotted bars). Only genes with significant expression changes (ANOVA P value < 0.05) are shown.

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Table 1: Design of gene expression profiling experiments. Male CB1-/- or wild-type mice were fed with regular chow (RC) or high-fat diet (HFD) for 14 weeks, and treated with vehicle or AM251 for two days. Each treatment group has 5-7 animals.

Diet	Genotype	Treatment	Number of animals
HFD	WT	vehicle	7
HFD	WT	AM251	7
HFD	CB1-/-	vehicle	5
HFD	CB1-/-	AM251	6
RC	WT	vehicle	7
RC	WT	AM251	7
RC	CB1-/-	vehicle	5
RC	CB1-/-	AM251	6

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Table 2: Gene expression changes induced by AM251 in each treatment group. One-way ANOVA was performed between vehicle and AM251 treated animals in each comparison. Differentially expressed genes were selected with ANOVA P value (<0.01) and absolute fold change (>1.2). False discovery rate (FDR) was estimated with Monte Carlo permutation.

Tissue	Comparison	P value	|Fold change|	Signature size	FDR
Liver	WT HFD: vehicle vs. AM251	< 0.01	> 1.2	757	10%
	CB1-/- HFD: vehicle vs. AM251	< 0.01	> 1.2	101	64%
	WT RC: vehicle vs. AM251	< 0.01	> 1.2	110	64%
	CB1-/- RC vehicle vs. AM251	< 0.01	> 1.2	133	66%
eWAT	WT HFD: vehicle vs. AM251	< 0.01	> 1.2	526	20%
	CB1-/- HFD: vehicle vs. AM251	< 0.01	> 1.2	69	100%
	WT RC: vehicle vs. AM251	< 0.01	> 1.2	265	42%
	CB1-/- RC: vehicle vs. AM251	< 0.01	> 1.2	141	60%

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Table 3: Many PPARγ target genes are regulated similarly by AM251 and PPARγ agonists in eWAT. AM251 signature genes (ANOVA P value < 0.05 and absolute fold change > 1.1) were compared to PPARγ target genes reported in literature (Berger and Moller, 2002; Kelly et al., 1998; Martin et al., 1997; Muise et al., 2008; Sfeir et al., 1997; Tontonoz et al., 1995). Genes regulated in opposite directions are underlined. Regulation of these genes in CB1-/- animals compared to wt controls were also included, many of the genes are regulated in same trend between AM251 treatment and CB1 knock-out. Genes that did not show significant expression changes are indicated by "nc" (no change).

Pathway	Gene	AM251	PPARg agonists	CB1-/-
fatty acid transport	CD36	up	up	nc
	FATP-1	up	up	up
lipoprotein hydrolysis	MGLL	up	up	up
lipid synthesis	FASN	down	up	nc
	ME1	down	up	nc
	PEPCK	up	up	up
	PDK4	up	up	nc
adipokine	LEP	down	down	down
fatty acid b-oxidation	MLYCD	up	up	up
	ACAA1B	up	up	nc
energy expenditure	UCP3	up	up	up

Figure 1A

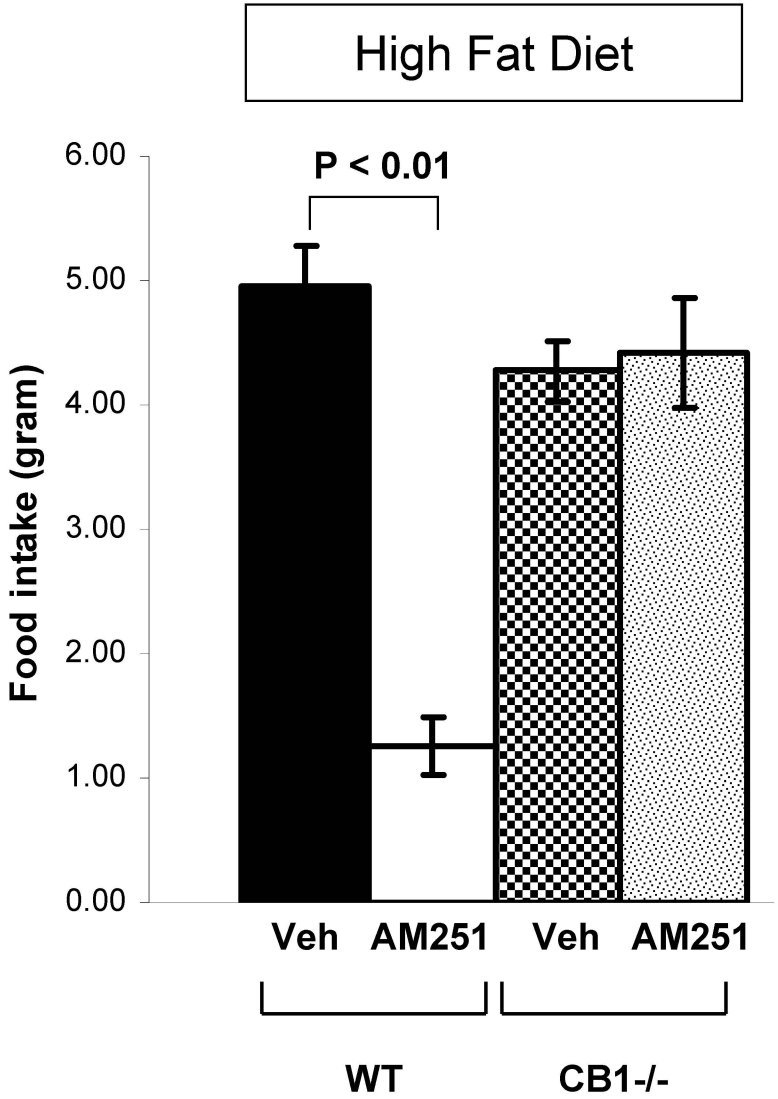

Figure 1B

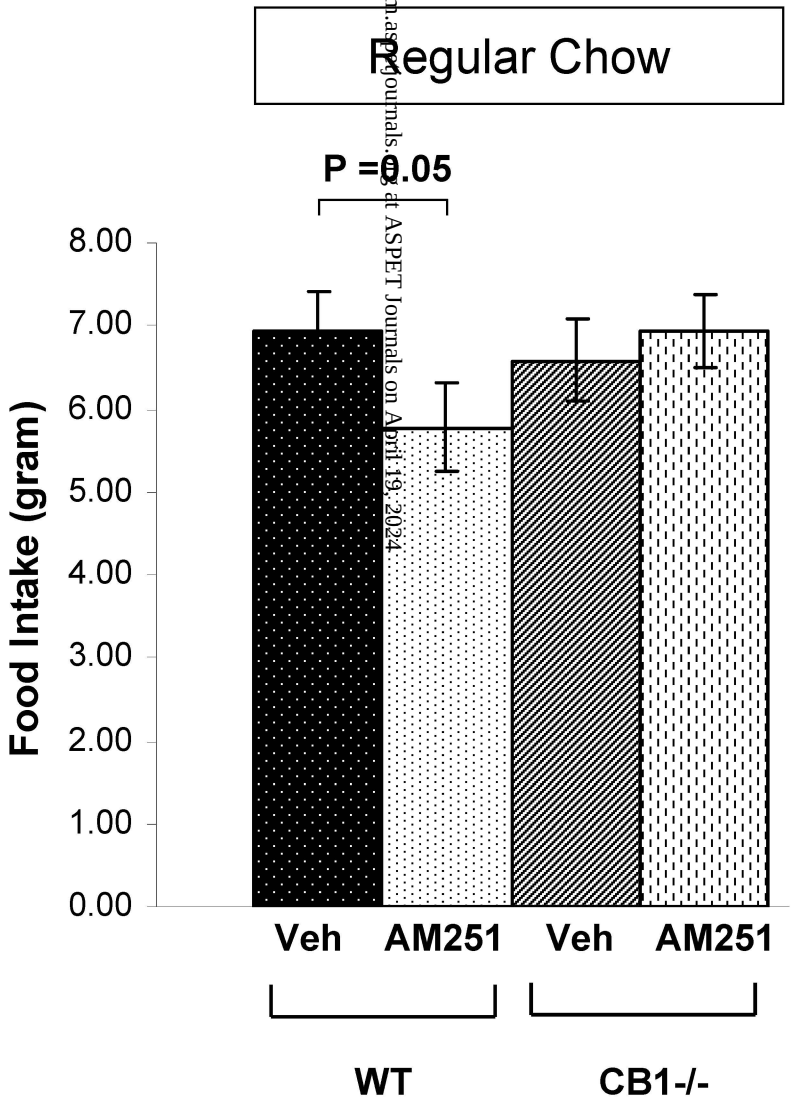

Figure 1C

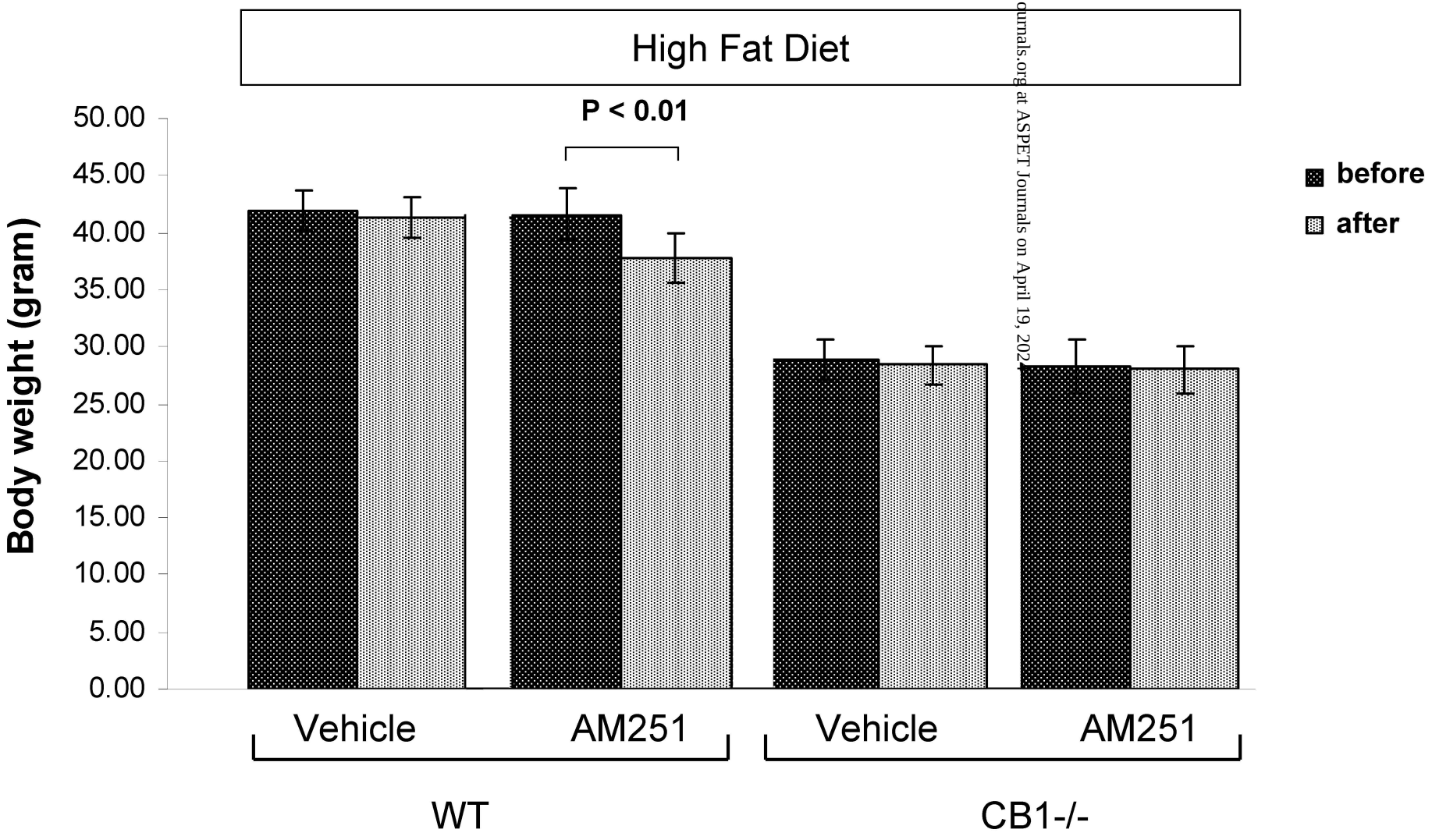

Figure 1D

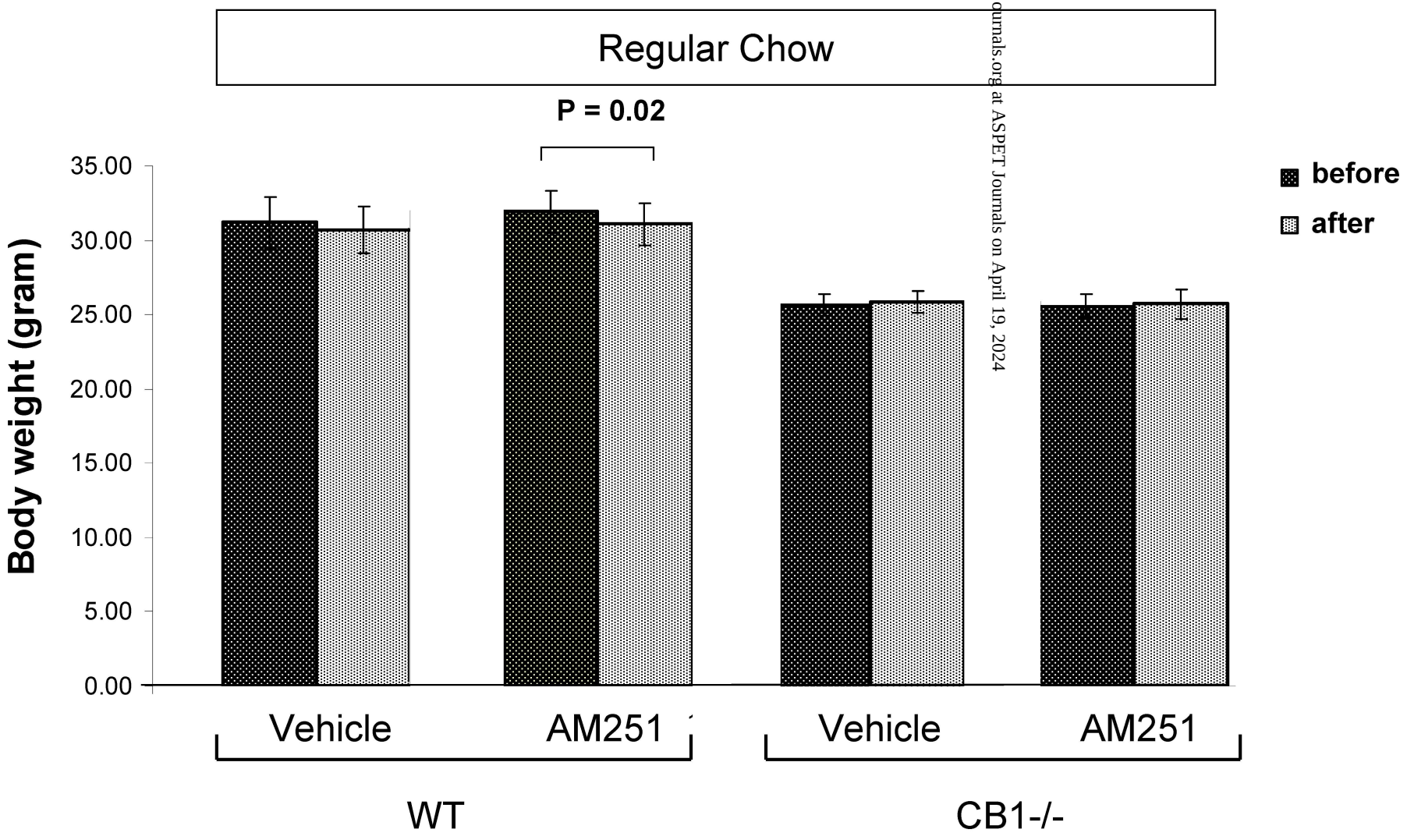

Figure 2A

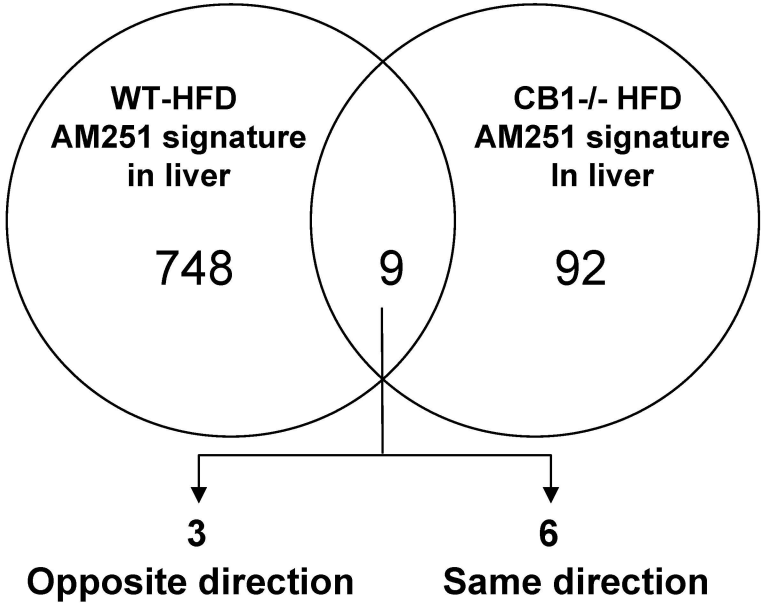

Figure 2B

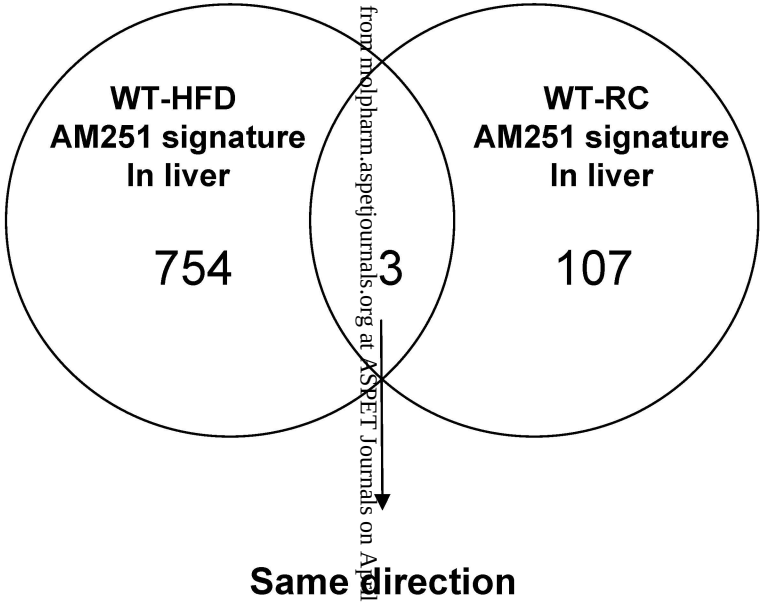

Figure 2C

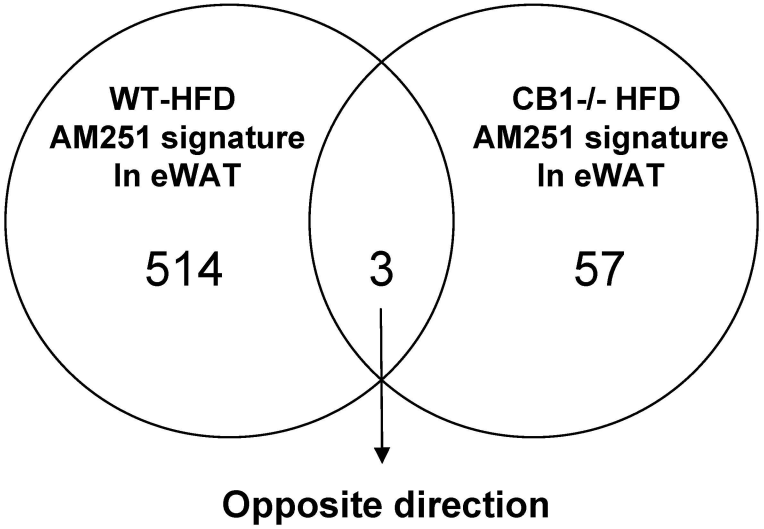

Figure 2D

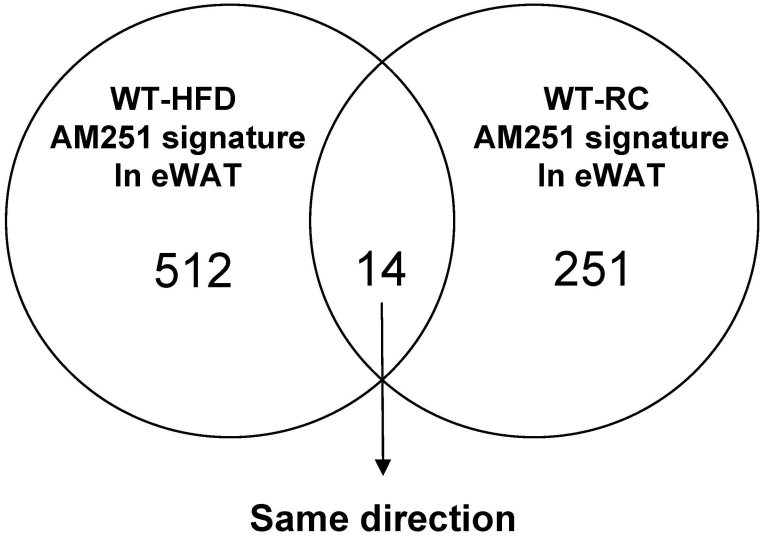

Figure 3A

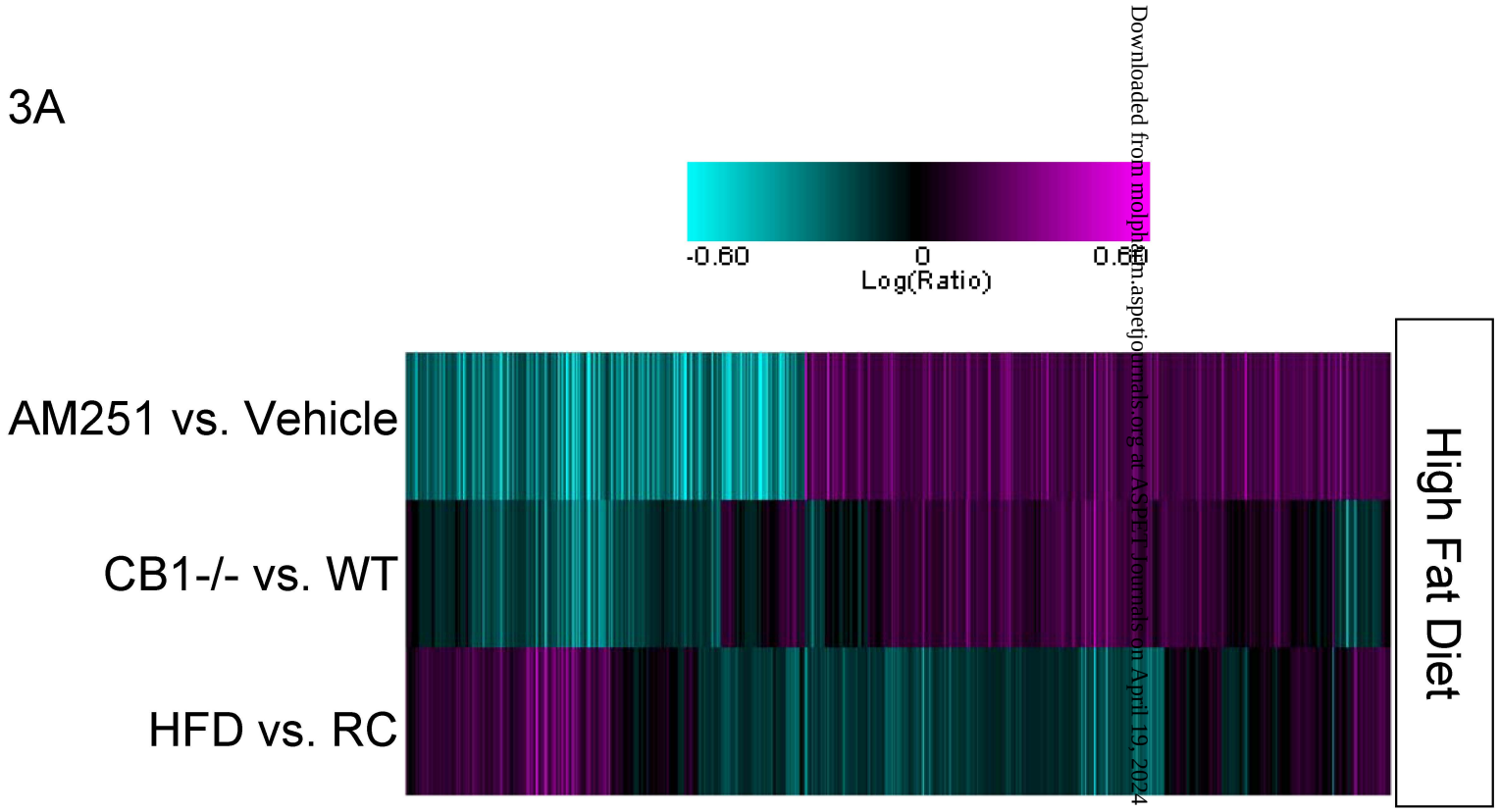

Figure 3B

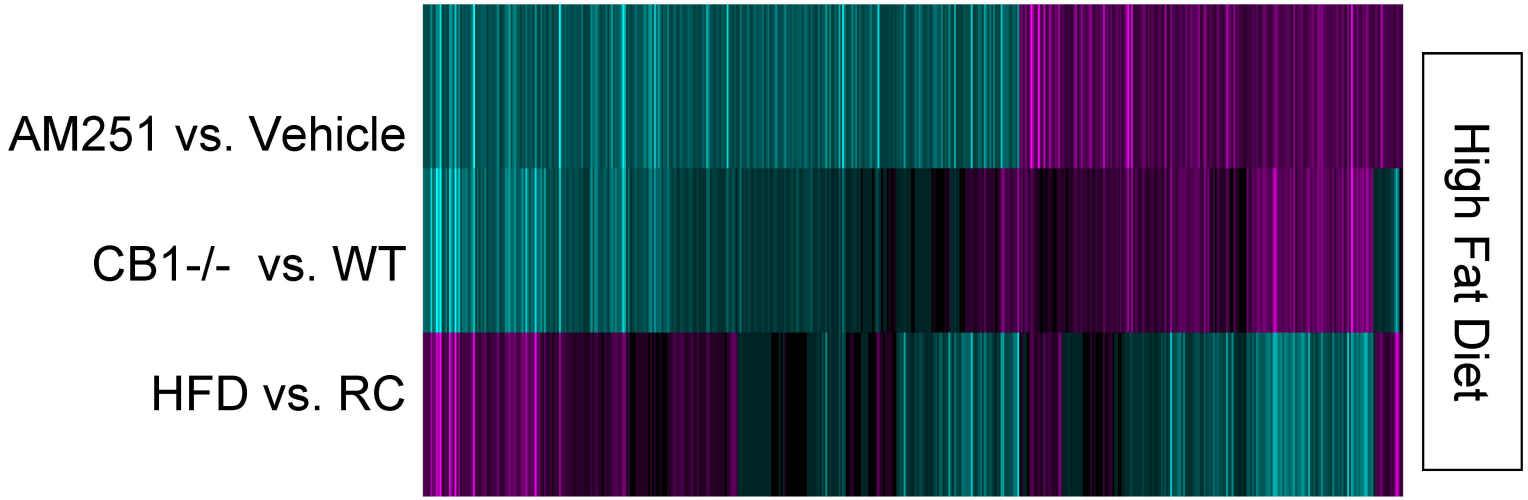

Figure 4

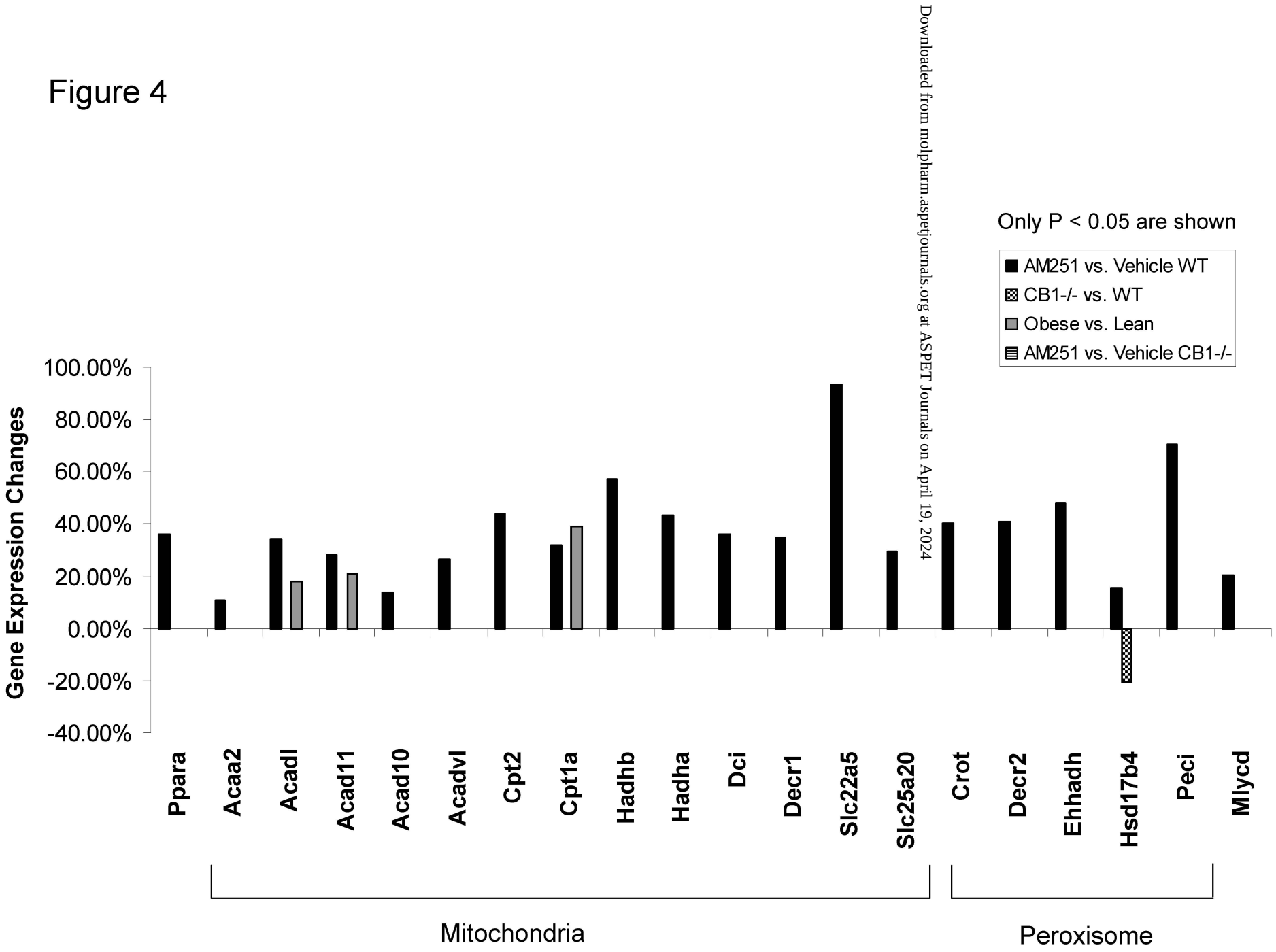

Figure 5A

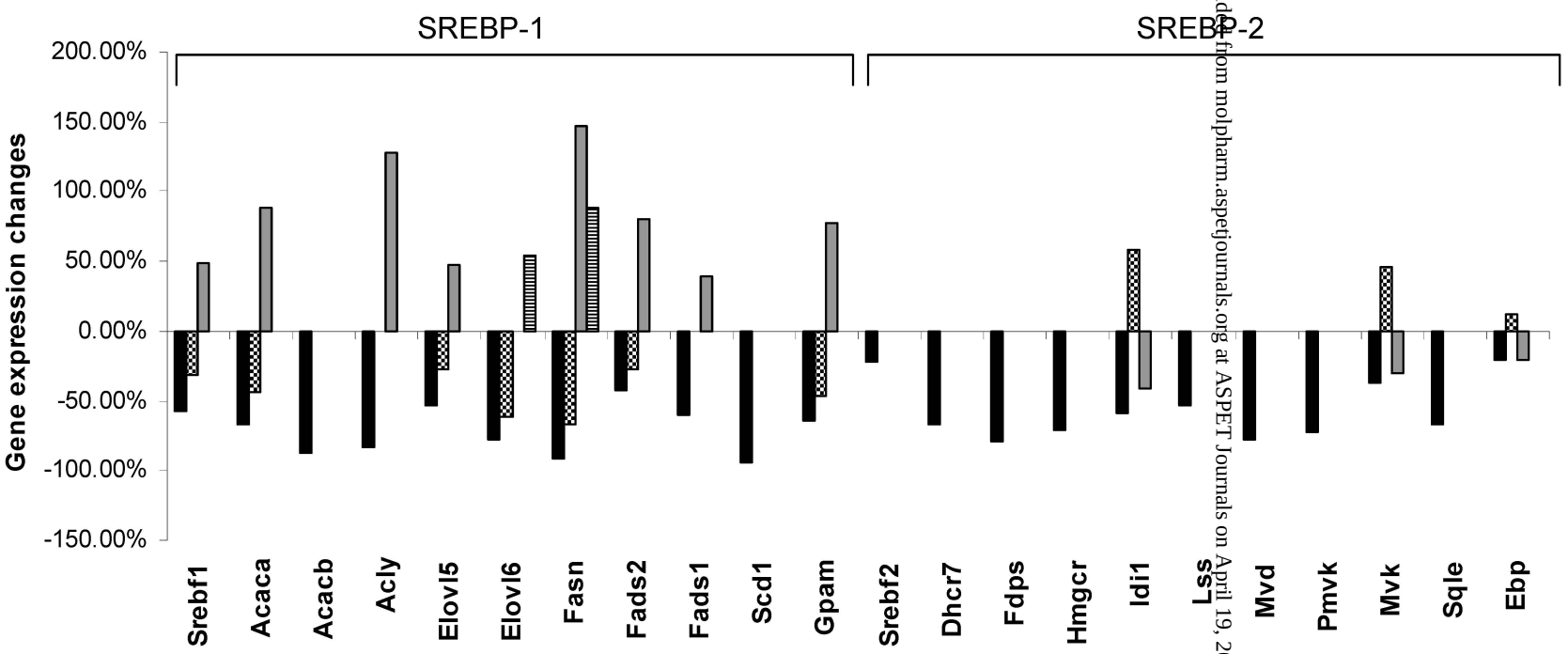

Figure 5B

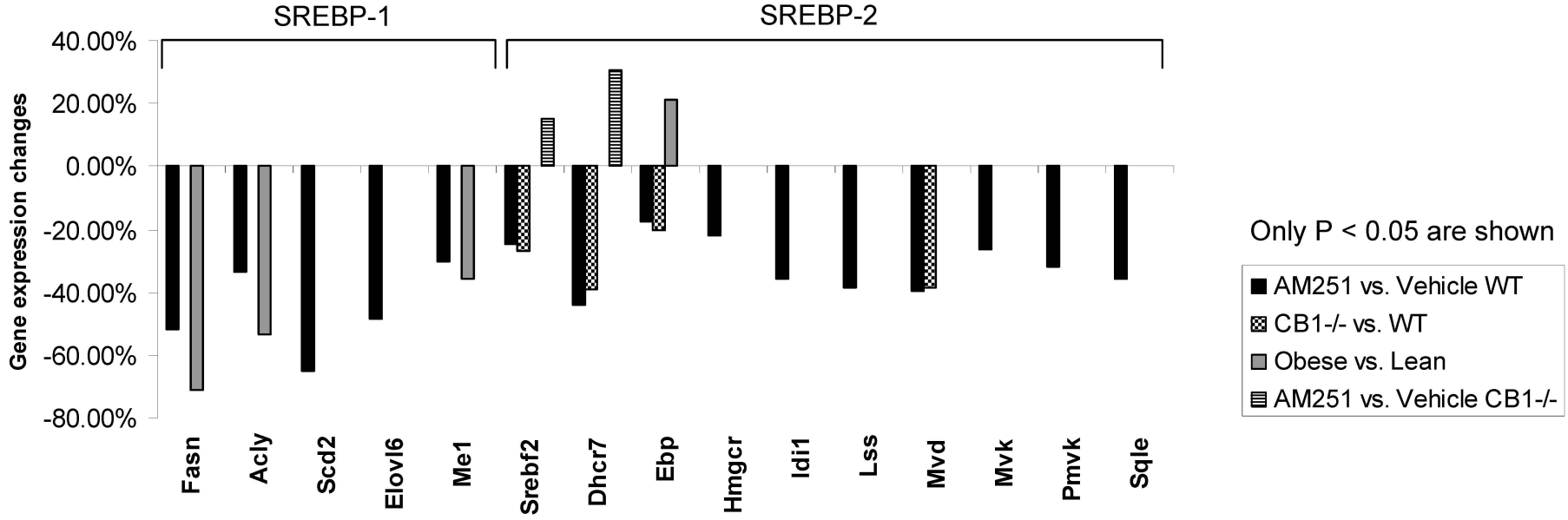

Figure 6A

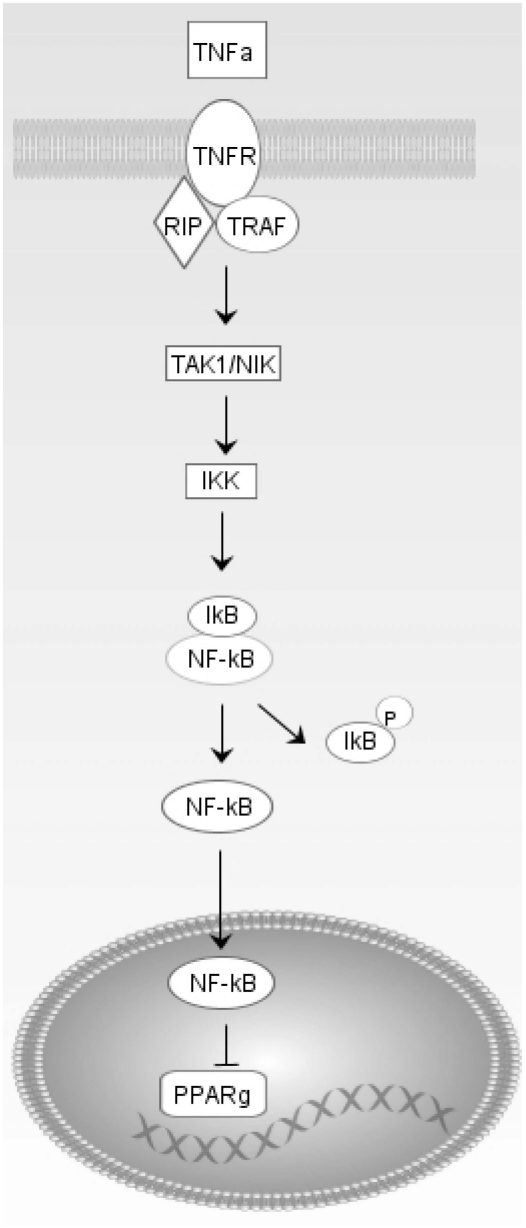

Figure 6B

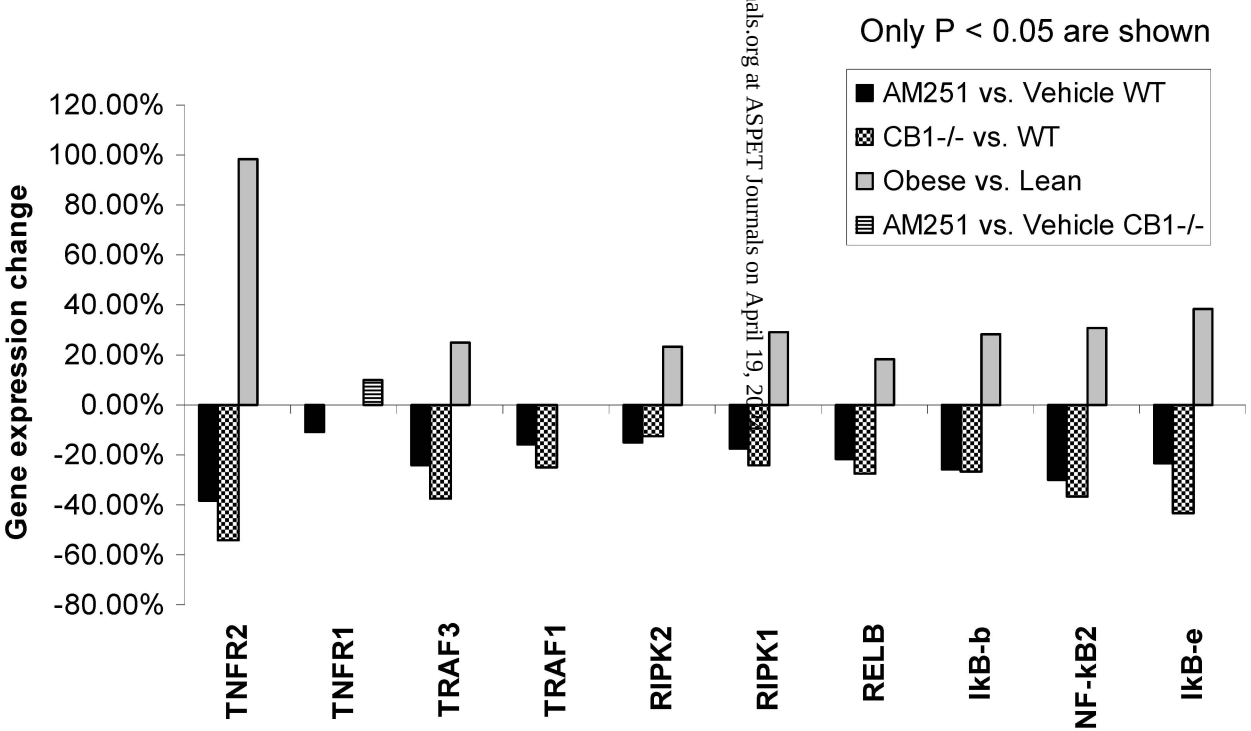

Figure 7