



Cannabidiol promotes adipogenesis of human and mouse mesenchymal stem cells via PPAR γ by inducing lipogenesis but not lipolysis

Richard C. Chang^a, Chloe S. Thangavelu^a, Erika M. Joloya^a, Angela Kuo^a, Zhuorui Li^a, Bruce Blumberg^{a,b,c,*}

^a Department of Developmental and Cell Biology, USA

^b Department of Pharmaceutical Sciences, USA

^c Department of Biomedical Engineering, University of California, Irvine, CA 92697-2300, USA

ABSTRACT

Cannabidiol (CBD) is a non-psychoactive phytocannabinoid found in the *Cannabis sativa* plant. Human exposure to CBD can be through recreational marijuana use, commercially available CBD-containing products, and medical treatments. Previous studies found that cannabidiol may activate the master regulator of adipogenesis, peroxisome proliferator activated receptor gamma (PPAR γ). Here we investigated the effects of CBD on adipogenesis in human and mouse multipotent mesenchymal stromal stem cells (MSCs). We tested the effects of CBD on nuclear receptor activation and adipogenic potential to demonstrate the mechanism of CBD effects and employed the *in vitro* MSC differentiation models to assess adipogenic effects of CBD. Using transient transfection assays, we demonstrated that CBD activated mouse and human PPAR γ , but not its heterodimeric partner, the retinoid 'X' receptor, RXR. Our results showed that CBD increased lipid accumulation and the expression of adipogenic genes in mouse and human MSCs *in vitro*. Adipogenic differentiation induced by CBD was significantly decreased by the PPAR γ antagonist T0070907, supporting the hypothesis that CBD promoted differentiation via PPAR γ . Taken together, our results indicate that in humans and in mice, CBD induced adipogenic differentiation in MSCs through a PPAR γ -dependent mechanism.

1. Introduction

Cannabidiol (CBD) is a component of cannabis that is devoid of the typical psychotropic effects of the plant caused by its better known relative, Δ 9-tetrahydrocannabinol (THC) [1]. Whereas THC mediates its effects on the brain and peripheral tissues through activation of the classical G-protein-coupled cannabinoid receptors, cannabinoid CB1 and cannabinoid CB2 [2], CBD does not significantly activate either of these receptors [3]. CBD has also been shown to antagonize the psychoactive effects of THC by blocking its conversion to the more psychoactive 11-hydroxy-THC [4] and to have antipsychotic and anti-anxiety effects [5,6]. In addition to its anxiolytic properties, CBD has been shown to have analgesic [7], anti-inflammatory [8,9], anti-oxidant [10], and anti-cancer capabilities [10,11].

Due to the increased availability of CBD-containing products after the recent legalization of commercial hemp and the legalization and/or decriminalization of cannabis in many US states, a growing number of individuals are exposed to CBD. Exposure is through commercially available CBD-containing products and recreational marijuana use. 37 million Americans aged 12 years or older reported using cannabis in 2018 [12]. Consumption of a single 1 g marijuana cigarette resulted in a

blood serum CBD concentration of up to 56.1 ng/mL (0.178 μ M) [13]. Individuals can be exposed to higher and more continuous doses through prescribed medical treatments. For instance, CBD is now used in pharmaceutical products, such as the first cannabinoid-based drug developed, Sativex, to treat neuropathic pain in individuals with multiple sclerosis [14], rheumatoid arthritis [15], and cancer unresponsive to opiates [7]. In addition, the widespread availability of CBD-containing products across all 50 states has increased the overall exposure to CBD.

The molecular targets of CBD have been studied in an attempt to determine the mechanisms underlying its medicinal benefits. It is thought that CBD may mediate its effects by interacting with a variety of non-endocannabinoid receptors [16], such as the GPR55 Receptor [17], serotonin 5HT1A receptors [18,19], Adenosine A2A receptors [20], and the vanilloid receptors TRPV1, TRPV2, TRPA1 [21]. However a growing body of evidence indicates that many effects of CBD, notably its anti-inflammatory and anti-cancer actions, can be inhibited by PPAR γ antagonists suggesting that these actions may be PPAR γ -dependent [22–28].

Peroxisome proliferator activated receptor gamma (PPAR γ) is a member of a family of nuclear receptors containing three distinct

* Corresponding author at: University of California Irvine, 2011 BioSci 3, Irvine, CA 92697, USA.

E-mail address: blumberg@uci.edu (B. Blumberg).

<https://doi.org/10.1016/j.bcp.2022.114910>

Received 30 September 2021; Received in revised form 4 January 2022; Accepted 4 January 2022

Available online 10 January 2022

0006-2952/© 2022 The Authors.

Published by Elsevier Inc.

This is an open access article under the CC BY-NC-ND license

(<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

subtypes: PPAR α , PPAR δ and PPAR γ [29]. PPAR γ is primarily involved in the regulation of adipocyte differentiation and lipid storage [30]. PPARs bind to DNA sequences and regulate the expression of target genes after heterodimerizing with the retinoid X receptor (RXR) [31]. PPARs are endogenously activated by unsaturated fatty acids such as linolenic acid, linoleic acid, petroselinic acid and arachidonic acid [32]. PPAR γ is capable of binding and being activated by a wide variety of structurally diverse ligands including organotin compounds such as the biocide tributyltin (TBT) [33], phthalates which are commonly found in plastics [34], and thiazolidinediones [35]. Thiazolidinediones are a class of pharmaceutical PPAR γ -selective agonists that includes the drug rosiglitazone (ROSI), which was once used to treat insulin resistance and decrease blood glucose levels in type 2 diabetes [36]. Like cannabidiol, PPAR γ ligands exhibit anti-inflammatory actions, including the upregulation of anti-inflammatory cytokines and the downregulation of pro-inflammatory cytokines and nitric oxide synthase (iNOS) expression [37]. Other cannabinoids such as THC [38], ajulemic acid, a structural analog of a THC metabolite [39], and the endocannabinoids anandamide and 2-arachidonoylglycerol (2-AG) bind to PPAR γ [40] or have anti-inflammatory and other effects that can be inhibited with PPAR γ antagonists [41,42]. Taken together, these reports indicate that CBD, like other cannabinoid and pharmaceutical PPAR γ activators, may exert some of its physiological effects via a PPAR γ -dependent mechanism.

To assess whether CBD could regulate another important PPAR γ -dependent process we tested whether CBD promoted adipocyte differentiation in human and mouse multipotent mesenchymal stromal stem cells (a.k.a., MSCs). We found that CBD induced adipogenesis in MSCs by binding to and activating PPAR γ and that inhibition of PPAR γ with an antagonist also inhibited the adipogenic effects of CBD. These data confirm that CBD is a PPAR γ agonist and that PPAR γ activation may underlie the adipogenic effects of cannabidiol. These results are consistent with the possibility that consumption or use of CBD could lead to increased accumulation of white adipose tissue.

2. Materials and methods

2.1. Chemicals

TBT, LG100268, dexamethasone, insulin, isobutylmethylxanthine, Nile Red, Hoechst 33,342 were purchased from Sigma-Aldrich (St. Louis, MO). ROSI was purchased from Cayman Chemical (Ann Arbor, MI). T0070907 was purchased from Enzo Life Sciences (Farmingdale, NY). Formaldehyde was purchased from Thermo Fisher Scientific (Waltham, MA). 99% pure CBD crystals were purchased from CBDistillery (Denver, CO) and diluted to 200 mM in DMSO for storage.

2.2. Cell culture

hTERT immortalized adipose derived mesenchymal stem cells (ATCC SCRC-4000) were purchased from ATCC (Manassas, VA) and stored at passage 4 in liquid N₂. Bone marrow-derived multipotent MSCs from the long bones of C57BL/6J mice (MSCs) were purchased at passage 6 (OriCell; Cyagen Biosciences, Santa Clara, CA) and stored at passage 8 or 9 in liquid N₂. Bone marrow-derived human MSCs were purchased from Promocell (Lot 429Z001) and stored in liquid N₂. Cells were maintained, as previously described [43], in Dulbecco's modified Eagle medium containing 10% calf bovine serum, 10 mM HEPES, 1 mM sodium pyruvate, 100 IU/mL penicillin, and 100 μ g/mL streptomycin. MSCs were plated at 15,000 cells/cm² in 12-well cell culture plates. For standard adipogenesis assays, cells were allowed to attach and acclimate for 24 h prior to 48 h of chemical treatment (day - 2 through day 0). Specific ligands [200 nM ROSI, 50 nM TBT, 200 nM LG268 or CBD (100 nM - 10 μ M)] were dissolved in DMSO and administered every 3 days throughout the duration of the assay. Antagonist assays were performed in parallel under the same conditions, but the antagonist T0070907 (100 nM-1 μ M) or DMSO vehicle control was added every 12 h. The amount of dimethyl

sulfoxide (DMSO) vehicle was kept at < 0.1% in all assays. No changes in cell viability were observed at the chemical doses used in this study.

2.3. Adipogenesis and staining

Once they reached 100% confluency, cells were induced to differentiate with an adipose induction cocktail (500 μ M isobutylmethylxanthine, 1 μ M dexamethasone, and 5 μ g/mL insulin) in minimal essential medium α containing 15% fetal bovine serum, 10 mM HEPES, 2 mM l-glutamine, 100 IU/mL penicillin, and 100 μ g/mL streptomycin as we described [44]. Cells were replenished with fresh media, differentiation factors, and chemical ligands every 3 days. Cells were differentiated over the course of 14 days for murine MSC cell lines and 21 days for human MSCs then fixed in buffered 3.7% formaldehyde.

Nile Red (1 μ g/mL) was used to stain neutral lipids and Hoechst 33,342 (1 μ g/mL) to stain DNA. Cells were fixed with 3.7% formaldehyde, washed once with PBS for 1 min, and maintained at 4 °C in PBS overnight to remove residual phenol red. For each biological replicate, Nile Red relative fluorescence units (RFU) was measured relative to Hoechst RFU using a SpectraMax Gemini XS spectrofluorometer (Molecular Devices, Sunnyvale, CA) using SoftMax Pro (Molecular Devices) [45].

2.4. RNA isolation and real-time RT-qPCR analysis of gene expression

Differentiated adipocytes were lysed with TriPure (Roche, Basel, Switzerland). Total RNA extracted with chloroform (Fisher Chemical, PA), precipitated with isopropanol (Fisher Chemical, PA). Complementary DNA was synthesized from 1 μ g total RNA using reverse transcriptase (Roche), according to the manufacturer's instructions. Gene expression was assessed with real-time quantitative polymerase chain reaction (qPCR) using SYBRTM Green PCR Master Mix (Thermo Fisher Scientific, MA) on a Roche LightCycler 480 II (Roche). Cycle threshold values were quantified as the second derivative maximum using LightCycler software (Roche). The 2^{- $\Delta\Delta$ Ct} method [46] was used to analyze RT-qPCR data and determine relative quantification. Standard propagation of error was used throughout for each treatment group [47]. The data were normalized to a housekeeping gene, the ribosomal protein 36B4, and compared to 0.1% DMSO vehicle for standard assays or a 0.1% DMSO vehicle plus T0070907 for antagonist assays. Error bars represent the SEM from three to four biological replicates, calculated using standard propagation of error [47]. Each assay was conducted at least twice.

2.5. Transient transfection assays

Nuclear receptor activation by CBD was tested using the luciferase reporter tk-(ApoA1) \times 4-luc, pCMX- β -galactosidase [48] and fusion constructs to nuclear receptor ligand-binding domains for human PPAR γ and RXR α [49]. Transient transfections were performed as described [50] in COS7 cells (ATCC[®] CRL-1,651TM). Briefly, COS7 cells were seeded in Dulbecco's Modified Eagle Medium (DMEM; HyClone) containing 10% CBS at a density of 15,000 cells per well in 96-well tissue culture plates. After becoming 90% confluent approximately 24 h after seeding, cells were transfected in Opti-MEM[®] using Lipofectamine[®] 2000 reagent (InvitrogenTM; Life Technologies), according to the manufacturer's recommended protocol. One microgram of effector plasmid, five micrograms of reporter, and five micrograms of pCMX- β -galactosidase [48] transfection control plasmids per 96-well plate and incubated overnight. Cells were then treated with ligands or vehicle control in DMEM containing 10% resin charcoal stripped FBS for an additional 24 h [51]. In transient transfection assays, the control compounds ROSI (PPAR γ agonist), LG268 (RXR agonist), and TBT (PPAR γ and RXR agonist) were tested from 10⁻¹⁰ M to 10⁻⁵ M, whereas CBD was tested up to 10⁻⁴ M to account for its weaker capacity as an agonist. To test the interaction between ROSI and CBD, ROSI or CBD was tested up to 10⁻⁴ M

in the presence of 100 nM CBD or 100 nM ROSI, respectively. Cells were lysed in 165 μ l of lysis buffer [25 mM Tris-phosphate (pH 7.8), 15% glycerol, 2% 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate (CHAPS), 1% lecithin, 1% bovine serum albumin (BSA), 4 mM ethylene glycol-bis(β -aminoethyl ether)-N, N, N', N'-tetraacetic acid (EGTA), 6 mM Magnesium Chloride ($MgCl_2$), 1 mM dithiothreitol (DTT), 1 mM and 4-(2- aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF)]. 24 h after adding the ligands to the media, media was removed and lysis buffer added, then cells were placed on a shaker at room temperature for 30 min. For luciferase assays, 100 μ l of luciferase solution [4.28 mM magnesium carbonate hydroxide pentahydrate ($(MgCO_3)_4Mg(OH)_2 \cdot 5H_2O$), 10.68 mM magnesium sulfate ($MgSO_4$), 80 mM Tricine (pH 7.8), 0.4 mM ethylenedinitrilo tetraacetic acid (EDTA) (pH 8.0), 5 mM DTT, 0.15 mg/mL Coenzyme A, 0.5 mM adenosine triphosphate (ATP), and 0.5 mM D-Luciferin] and 50 μ l of the lysate from each well was transferred to a well in a nontreated, white, flat-bottom, polystyrene, 96-well plate (Costar). The plate was shaken for 30 s at RT and placed in Dynatech ML3000 luminometer. Luminescence for the reporter was measured with luminometer ML3000 (version 3.07) software. For β -galactosidase assay, 100 μ l of β -galactosidase solution [60 mM sodium phosphate dibasic (Na_2HPO_4), 40 mM sodium phosphate monobasic (NaH_2PO_4), 10 mM potassium chloride (KCl), and 1 mM magnesium chloride ($MgCl_2$), 0.3% β -Mercaptoethanol, and 0.5–3 mg/mL o-nitrophenyl β -D-galactopyranoside (ONPG)] and 50 μ l of the cell lysate was transferred to a clear, 96-well plate (Thermo Fisher Scientific, MA). The plates were placed on a shaker for 30 s and allowed to incubate at room temperature for 15 min. A Versamax microplate reader (Molecular Devices) was used to measure absorbance at a wavelength of 405 nm using SOFTmaxPRO 4.0 software.

2.6. Statistical analysis

GraphPad Prism 7.0 (GraphPad Software, Inc.) was used to perform statistical analysis for all datasets. A one-way analysis of variance

(ANOVA) followed by Dunnett's post-hoc test was performed to compare the different concentrations of CBD to the vehicle and an unpaired *t*-test was performed to compare the positive controls ROSI, TBT, LG268 to the vehicle in adipogenesis assays. In antagonist assays, treatment groups without T0070907 were compared to a corresponding treatment group treated with T0070907 using a student's *t*-test. $P \leq 0.05$ was considered statistically significant.

3. Results

3.1. CBD activates PPAR γ but not RXR α to induce adipogenesis in human and mouse model in vitro

Accumulated studies showed the effects of CBD may be mediated through a variety of non-endocannabinoid receptors; however, nuclear receptors such as PPAR γ and RXR α were not well-studied. We tested the effects of CBD on PPAR γ and its heterodimeric partner, RXR in transient transfection assays using luciferase reporter genes. Statistically significant activation of human and mouse PPAR γ started from 100 nM CBD, which was similar in potency to the positive control, 100 nM ROSI, albeit with much lower efficacy (Fig. 1A, 1C). However, CBD showed no activity toward RXR α (Fig. 1B, 1D). Since CBD was less efficacious than ROSI in activating PPAR γ , we tested whether CBD could antagonize PPAR γ activation by ROSI or if ROSI could antagonize PPAR γ activation by CBD. We found that CBD and ROSI showed an additive effect on human and mouse PPAR γ activation in a dose-dependent manner (Fig. 1E, 1F). PPAR γ is known to be a key regulator of adipogenesis. Therefore, we tested whether CBD promoted adipogenesis by activating PPAR γ by testing the ability of CBD to induce adipogenesis in MSCs and if any activity observed could be inhibited by co-treatment with the selective PPAR γ antagonist, T0070907. In human cells, CBD showed a dose-dependent effect on adipogenesis which was suppressed by 100 nM T0070907 (Fig. 1G). In mouse cells, both 5 and 10 μ M CBD increased adipogenesis; 100 nM T0070907 significantly suppressed this induction (Fig. 1H).

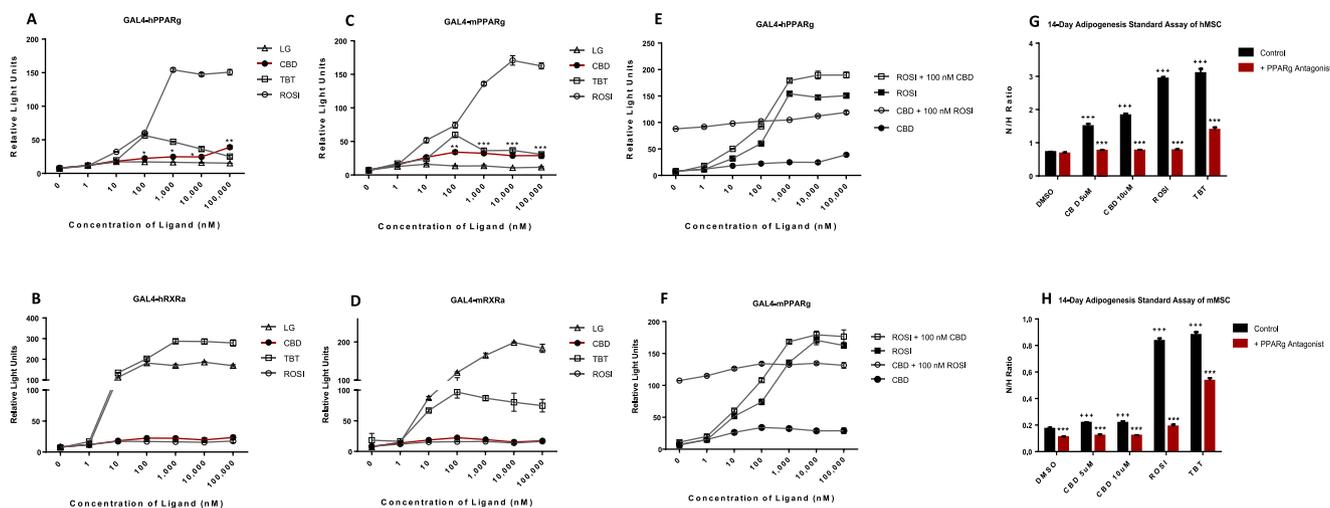


Fig. 1. CBD bound to PPAR γ and induce adipogenesis in human and mice MSC. (A) The ability of CBD to activate PPAR γ and RXR α was assessed in COS7 cells transiently transfected with luciferase reporter plasmid and either human PPAR γ and (B) RXR α . (C) The ability of CBD to activate PPAR γ and RXR α was assessed in COS7 cells transiently transfected with luciferase reporter plasmid and either mouse PPAR γ and (D) RXR α . The ability of CBD to antagonize PPAR γ by ROSI, or ROSI to antagonize PPAR γ activation by CBD was assessed in COS7 cells transiently transfected with luciferase reporter plasmid and either (E) human PPAR γ or (F) mouse PPAR γ . The PPAR γ activators ROSI and TBT were used as positive controls for activation of PPAR γ , while the RXR activators LG268 and TBT were used as positive controls for activation of RXR α . Luciferase values were normalized to β -galactosidase transfection values to account for transfection efficiency. Triplicates for each chemical and concentration were averaged and plotted \pm SEM. (G) A standard adipogenesis assay was performed in human and (H) mouse MSCs in the presence of adipogenesis cocktail (MDI). Cells were treated with CBD (5 μ M or 10 μ M), a 100 nM ROSI, or 50 nM TBT, positive control with or without PPAR γ antagonist, 100 nM T0070907. All treatment groups were compared to 0.1% DMSO (vehicle). Lipid accumulation is shown as the ratio between fluorescence units (RFU) of Nile Red and Hoechst, which were used to quantify lipid content and the number of cells per well, respectively. Each bar represents the average of 3 replicates \pm SEM * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared to control; † $P < 0.05$, †† $P < 0.01$ and ††† $P < 0.001$ compared to control). One-way analysis of variance (ANOVA) statistical test and a Dunnett's post-hoc test were performed to compare the different concentrations of CBD to DMSO.

3.2. CBD enhanced adipogenesis by increasing expression of lipogenic genes

During adipogenesis, increased expression of adipogenic markers such as PPAR γ 2, fatty acid binding protein-4 (FABP4), and fat-specific protein-27 (FSP27) promote lipid accumulation. We performed adipogenesis assays on human MSCs using CBD (2.5, 5, or 10 μ M), compared with 100 nM ROSI, 50 nM TBT, or 100 nM LG268 as positive controls. In hMSCs, Nile Red staining revealed a dose-dependent increase in lipid accumulation by CBD (Fig. 2A). Expression levels of mRNAs encoding three adipogenic genes, PPARG2, FABP4, and FSP27 were increased by both CBD and the PPAR γ agonist, ROSI, whereas TBT and LG268 failed to increase expression of PPAR γ 2 mRNA (Fig. 2B-D). In mMSCs, 2.5 μ M CBD did not increase lipid accumulation after a 14-day adipogenesis assay but 5 and 10 μ M CBD did (Fig. 2E). All three doses of CBD increased PPAR γ 2 mRNA levels but FABP4 and FSP27 genes responded to CBD only at 10 μ M (Fig. 2F-H). TBT and LG268 did not induce PPAR γ 2 expression in hMSCs or mMSCs (Fig. 2B & 2F).

3.3. CBD showed no effects on lipolysis or expression of lipolysis genes during adipogenesis

During adipogenesis, cells utilize both lipogenic and lipolytic pathways to acquire fatty acids and reach a net balance to accumulate fat content. Although ROSI induced the expression of the lipolysis genes, adipose triglyceride lipase (PNPLA2) and hormone-sensitive lipase (LPL), CBD treatment did not alter expression of PNPLA2 or LPL mRNAs in human MSCs (Fig. 3C-D). We measured the free fatty acid content in the culture medium by the end of 14-day adipogenesis assay and found that ROSI and TBT groups showed increased free fatty acid level in the medium whereas the 10 μ M CBD treated group accumulated less compared to controls (Fig. 3A). To examine lipolytic function, isoproterenol was added to induce lipolysis in adipocytes differentiated with DMSO, 100 nM ROSI, 50 nM TBT, or 100 nM LG268. Isoproterenol increased free fatty acid levels in the culture medium whereas CBD

showed no difference in response to isoproterenol (Fig. 3B). Similarly, CBD did not affect lipolysis gene expression or free fatty acid accumulation in the culture medium of mMSCs (Fig. 3E-H).

3.4. The PPAR γ antagonist T0070907 suppressed CBD-induced lipogenic gene expression in both human and mouse MSCs

After excluding the association between CBD-induced PPAR γ activation and lipolysis, we next tested whether the expression of lipogenesis genes, FABP4, FSP27 and PPARG2 in response to CBD treatment was dependent on PPAR γ by treating with the PPAR γ antagonist, T0070907. ROSI and TBT induced the expression of lipogenesis genes in hMSCs and served as positive controls. 5 and 10 μ M CBD induced expression of the lipogenesis genes which were significantly suppressed by co-treatment with the PPAR γ antagonist (Fig. 4A-C). Lipogenesis genes were also induced by ROSI and CBD in mMSCs. T0070907 was able to suppress the effects of ROSI and CBD close to the baseline suggesting PPAR γ plays a dominant role in CBD induced lipogenesis gene expression. Collectively, these observations indicate that CBD can promote adipogenesis by regulating lipogenesis but not lipolysis in both human and mouse MSCs.

4. Discussion

The purpose of this study was to determine the effects of CBD on adipogenesis in mouse and human MSCs and whether this can be attributed to PPAR γ activation. We found that CBD activated PPAR γ in transient transfection assays and promoted the differentiation of human MSC's into adipocytes. The action of CBD could be inhibited by co-treatment with the PPAR γ antagonist T0070907. These results are consistent with previous findings which showed that CBD promoted differentiation of mouse 3 T3-L1 preadipocytes into adipocytes [52]. CBD had a stronger adipogenic effect in the human adipose-derived MSCs than in mouse MSCs. This suggests that although mouse body weight did not change to a significant extent following 30 mg/kg CBD exposure [53], this might not be the case for humans exposed to CBD in

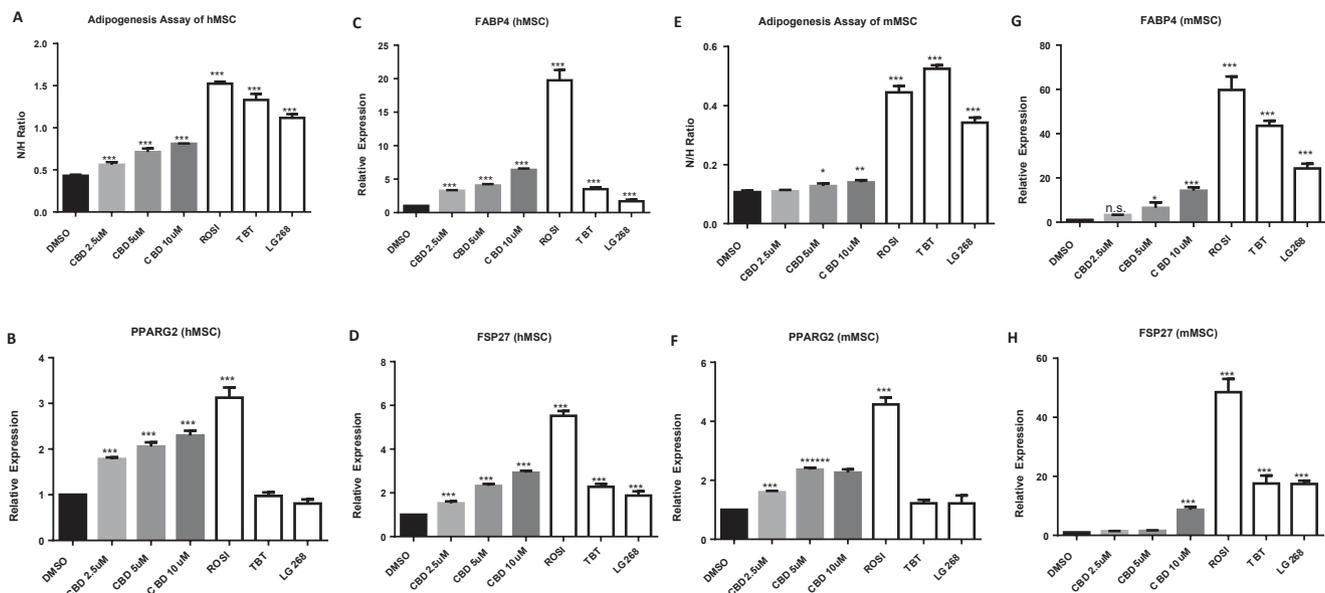


Fig. 2. CBD enhanced adipogenesis and induced lipogenesis gene in human and mouse MSC. (A) A standard adipogenesis assay was performed in human MSC in the presence of adipogenesis cocktail (MDI). Cells were treated with CBD (2.5 μ M, 5, or 10 μ M), a 100 nM ROSI, 50 nM TBT, or 100 nM LG268 positive control. All treatment groups were compared to 0.1% DMSO (vehicle). Lipid accumulation is shown as the ratio between fluorescence units (RFU) of Nile Red and Hoechst, which were used to quantify lipid content and the number of cells per well, respectively. Each bar represents the average of 3 replicates \pm SEM. (B) qRT-PCR validation of lipogenesis genes, PPARG2, (C) FABP4, and (D) FSP27, of human MSC differentiated adipocytes. (E) A standard adipogenesis assay was performed in the mouse MSC. (F) qRT-PCR validation of lipogenesis genes, PPARG2, (G) FABP4, and (H) FSP27, of mouse MSC differentiated adipocytes. For qRT-PCR analyses, measured Ct values were normalized to 36B4 and graphed relative to the control treatment. Error bars represent SEM *P < 0.05, **P < 0.01 and ***P < 0.001). Data analyzed using either an unpaired *t*-test or a one-way ANOVA followed by Dunnett's post hoc analysis.

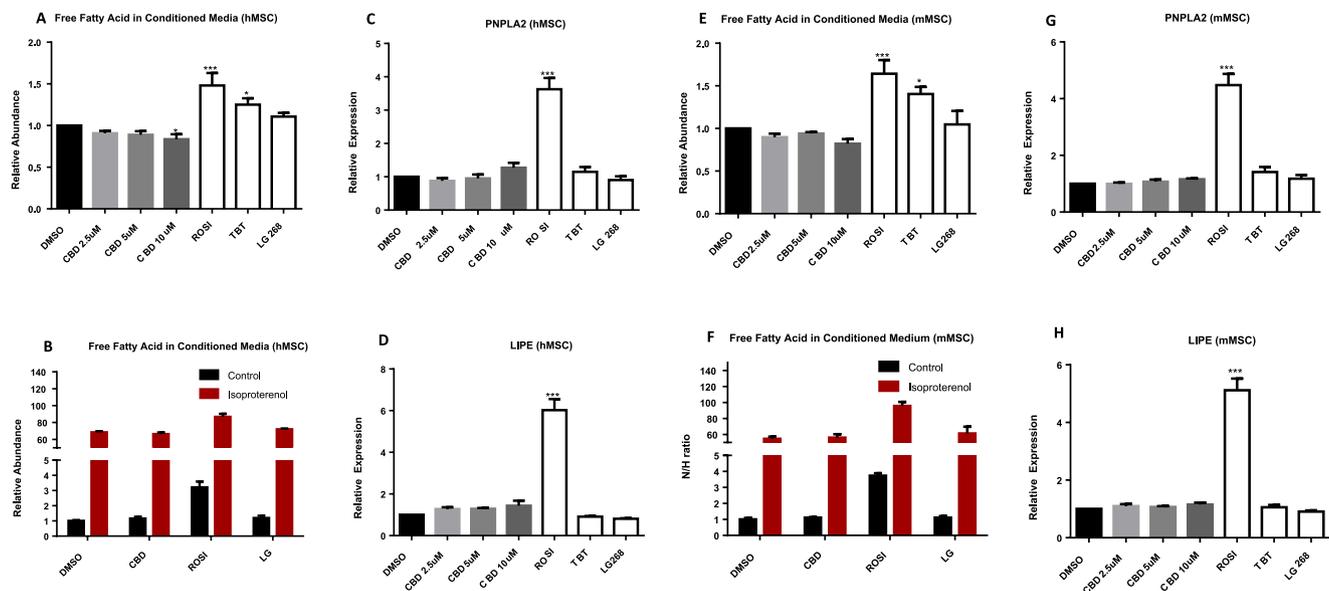


Fig. 3. CBD did not affect lipolysis in human or mouse MSC. (A) The cultured medium of human MSC at the end of adipogenesis or (B) 24 h post-isoproterenol treatment after 14-day adipogenesis were collected to measure free fatty acids levels. (C) qRT-PCR validation of lipolysis genes, PNPLA2 and (D) LIPE, of human MSC differentiated adipocytes. (E) The cultured medium of mouse MSC at the end of adipogenesis or (F) 24 h post-isoproterenol treatment after 14-day adipogenesis were collected to measure free fatty acids levels. (G) qRT-PCR validation of lipolysis genes, PNPLA2 and (H) LIPE, of mouse MSC differentiated adipocytes. For qRT-PCR analyses, measured Ct values were normalized to 36B4 and graphed relative to the control treatment. Error bars represent SEM * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$). Data analyzed using either an unpaired t -test or a one-way ANOVA followed by Dunnett's post hoc analysis.

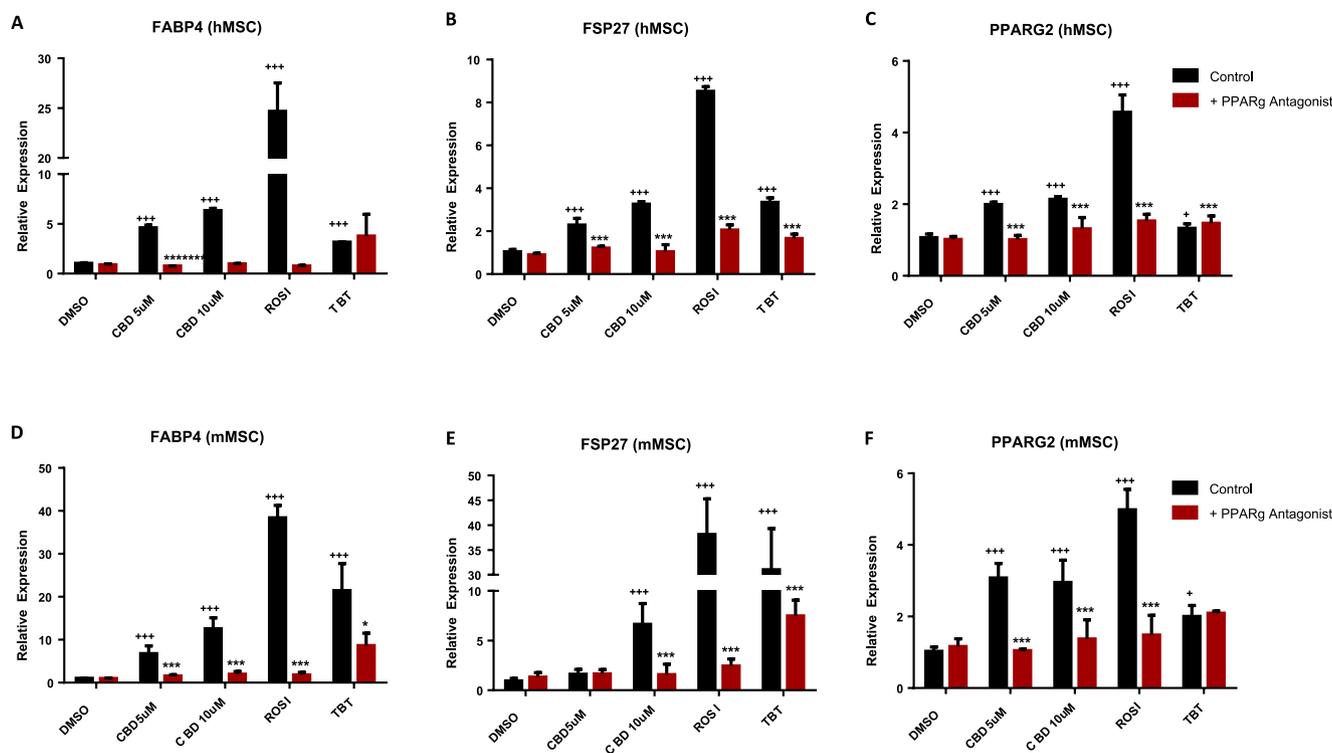


Fig. 4. CBD-induced adipogenesis was inhibited by PPAR γ antagonist T007097 in human and mouse MSCs. (A) qRT-PCR validation of lipogenesis genes, FABP4, (B) FSP27, and (C) PPARG2 of human MSC differentiated adipocytes. (D) qRT-PCR validation of lipogenesis genes, FABP4, (E) FSP27, and (F) PPARG2 of mouse MSC differentiated adipocytes. For qRT-PCR analyses, measured Ct values were normalized to 36B4 and graphed relative to the control treatment. Error bars represent 3 replicates \pm SEM * $P < 0.05$ (** $P < 0.01$ and *** $P < 0.001$ compared to control; † $P < 0.05$, †† $P < 0.01$ and ††† $P < 0.001$ compared to control). Data analyzed using either an unpaired t -test or a one-way ANOVA followed by Dunnett's post hoc analysis.

commercial products with concentrations above 5 μ M. In addition, the mouse study did not examine body composition (% fat vs % lean); and we previously demonstrated that the obesogen tributyltin could elicit

increased white adipose tissue weight without increasing total body weight [44]. Other PPAR γ activators, such as rosiglitazone and pioglitazone, were associated with human weight gain [36,54] and our gene

expression analyses showed that CBD-induced adipogenic genes including PPARG2, FABP4, and FSP27. Taken together, these studies indicated that CBD acted as a weak PPAR γ agonist in MSCs and up-regulated target genes, resulting in an increase in adipogenesis.

Two major processes in adipogenesis are lipogenesis and lipolysis which contribute to a net balance of triglyceride accumulation in fat cells. Lipogenesis is the processes of fatty acid synthesis and subsequent triglyceride synthesis, which take place in both liver and adipose tissues [55]. FABP4 encodes the fatty acid binding protein found in adipocytes. Fatty acid binding proteins binds long-chain fatty acids and other hydrophobic ligands to perform fatty acid uptake, transport, and metabolism [56]. Fat-specific protein 27 (FSP27) was reported to promote lipid droplet expansion by lipid trafficking at lipid droplet contact sites [57]. These two lipogenesis markers were positively regulated by PPAR γ to enhance adipogenesis [58]. ROSI induced FABP4 and FSP27 expression which was abolished by PPAR γ agonist as many reported. Our data showed that 10 μ M CBD induced FABP4 and FSP27 expression (~3 fold and ~6 fold, respectively) in human MSCs. We also observed similar induction of FABP4 and FSP27 expression (~10 fold and ~7 fold, respectively) in mouse MSCs. Those effects of CBD were all suppressed by the PPAR γ antagonist, T0070907, suggesting that PPAR γ responds to CBD and further promotes lipogenesis. Surprisingly, we found that CBD showed no effects on lipolysis genes, PNPLA2 and LIPE, which were induced by ROSI in our study and in other reports [59]. We confirmed this observation by validating the lipolysis capacity of adipocytes matured by treating MSCs with CBD, ROSI, TBT, and LG. However, the strong lipolysis inducer, isoproterenol, induced lipolysis well in all groups, including CBD groups, which showed no changes in lipolysis gene expression. We therefore inferred that CBD regulated lipogenesis but not lipolysis during adipogenesis in both human and mouse MSCs.

Several studies have suggested CBD can act as an agonist for PPAR γ [60–62]. For example, Fellous et al., used mouse bone marrow derived mesenchymal stem cells to examine the effects of CBD on adipocyte regulatory genes and insulin induced AKT phosphorylation [62]. Our findings revealed a similar effect of CBD and we further tested the effects in both murine and human models. In addition, our data excluded the effects of CBD in lipolysis and reinforced the role of lipogenesis in CBD-induced adipogenesis.

Taken together, our findings indicate that CBD has the ability to promote adipogenesis in mouse and human MSCs and that this may be the result of it inducing lipogenesis that is not balanced by lipolysis. Our findings will help in understanding possible unexpected effects resulting from the widespread use of CBD in both recreational cannabis and in personal care products.

CRedit authorship contribution statement

Richard C. Chang: Methodology, Software, Validation, Writing – original draft. **Chloe S. Thangavelu:** Conceptualization, Validation. **Erika M. Joloya:** Writing – review & editing, Validation. **Angela Kuo:** Writing – review & editing. **Zhuorui Li:** Writing – review & editing. **Bruce Blumberg:** Supervision, Project administration, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

- S.E. O'Sullivan, Y. Sun, A.J. Bennett, M.D. Randall, D.A. Kendall, Time-dependent vascular actions of cannabidiol in the rat aorta, *Eur. J. Pharmacol.* 612 (1–3) (2009) 61–68.
- R.G. Pertwee, Pharmacology of cannabinoid CB1 and CB2 receptors, *Pharmacol. Ther.* 74 (2) (1997) 129–180.
- Howlett, A.C., et al., *International Union of Pharmacology. XXVII. Classification of Cannabinoid Receptors*. *Pharmacological Reviews*, 2002. 54(2): p. 161–202.
- Russo, E. and G.W. Guy, A tale of two cannabinoids: the therapeutic rationale for combining tetrahydrocannabinol and cannabidiol. *Med Hypotheses*, 2006. 66(2): p. 234–46.
- Moreira, F.A., D.C. Aguiar, and F.S. Guimaraes, Anxiolytic-like effect of cannabidiol in the rat Vogel conflict test. *Prog Neuropsychopharmacol Biol Psychiatry*, 2006. 30 (8): p. 1466–71.
- Zuardi, A.W., et al., Cannabidiol, a Cannabis sativa constituent, as an antipsychotic drug. *Brazilian Journal of Medical and Biological Research*, 2006. 39: p. 421–429.
- J.R. Johnson, et al., Multicenter, double-blind, randomized, placebo-controlled, parallel-group study of the efficacy, safety, and tolerability of THC:CBD extract and THC extract in patients with intractable cancer-related pain. *J. Pain Symptom Manage.* 39 (2) (2010) 167–179.
- B. Costa, A.E. Trovato, F. Comelli, G. Giagnoni, M. Colleoni, The non-psychoactive cannabis constituent cannabidiol is an orally effective therapeutic agent in rat chronic inflammatory and neuropathic pain. *Eur. J. Pharmacol.* 556 (1–3) (2007) 75–83.
- G. Esposito, et al., Cannabidiol in vivo blunts beta-amyloid induced neuroinflammation by suppressing IL-1beta and iNOS expression. *Br. J. Pharmacol.* 151 (8) (2007) 1272–1279.
- M. García-Arencibia, S. González, E. de Lago, J.A. Ramos, R. Mechoulam, J. Fernández-Ruiz, Evaluation of the neuroprotective effect of cannabinoids in a rat model of Parkinson's disease: importance of antioxidant and cannabinoid receptor-independent properties. *Brain Res.* 1134 (2007) 162–170.
- A. Ligresti, A.S. Moriello, K. Starowicz, I. Matias, S. Pisanti, L. De Petrocellis, C. Lazea, G. Portella, M. Bifulco, V. Di Marzo, Antitumor activity of plant cannabinoids with emphasis on the effect of cannabidiol on human breast carcinoma. *J. Pharmacol. Exp. Ther.* 318 (3) (2006) 1375–1387.
- S. Steigerwald, P.O. Wong, B.E. Cohen, J.H. Ishida, M. Vali, E. Madden, S. Keyhani, Smoking, Vaping, and Use of Edibles and Other Forms of Marijuana Among US Adults. *Annals Internal Med.* 169 (12) (2018) 890, <https://doi.org/10.7326/M18-1681>.
- R. Pacifici, S. Pichini, M. Pellegrini, M.C. Rotolo, R. Giorgetti, A. Tagliabracchi, F. P. Busardó, M.A. Huestis, THC and CBD concentrations in blood, oral fluid and urine following a single and repeated administration of "light cannabis". *Clin. Chem. Lab. Med.* 58 (5) (2020) 682–689.
- D.J. Rog, T.J. Nurmikko, T. Friede, C.A. Young, Randomized, controlled trial of cannabis-based medicine in central pain in multiple sclerosis. *Neurology* 65 (6) (2005) 812–819.
- Blake, D.R., et al., Preliminary assessment of the efficacy, tolerability and safety of a cannabis-based medicine (Sativex) in the treatment of pain caused by rheumatoid arthritis. *Rheumatology (Oxford)*, 2006. 45(1): p. 50–2.
- Ibeas Bih, C., et al., *Molecular Targets of Cannabidiol in Neurological Disorders*. Neurotherapeutics, 2015. 12(4): p. 699–730.
- Ryberg, E., et al., The orphan receptor GPR55 is a novel cannabinoid receptor. *Br J Pharmacol*, 2007. 152(7): p. 1092–101.
- Russo, E.B., et al., Agonistic properties of cannabidiol at 5-HT1a receptors. *Neurochem Res*, 2005. 30(8): p. 1037–43.
- Pazos, M.R., et al., Mechanisms of cannabidiol neuroprotection in hypoxic-ischemic newborn pigs: role of 5HT(1A) and CB2 receptors. *Neuropharmacology*, 2013. 71: p. 282–91.
- E.J. Carrier, J.A. Auchampach, C.J. Hillard, Inhibition of an equilibrative nucleoside transporter by cannabidiol: a mechanism of cannabinoid immunosuppression. *Proc. Natl. Acad. Sci. USA* 103 (20) (2006) 7895–7900.
- B. Costa, G. Giagnoni, C. Franke, A.E. Trovato, M. Colleoni, Vanilloid TRPV1 receptor mediates the antihyperalgesic effect of the nonpsychoactive cannabinoid, cannabidiol, in a rat model of acute inflammation. *Br. J. Pharmacol.* 143 (2) (2004) 247–250.
- S.E. O'Sullivan, D.A. Kendall, M.D. Randall, Time-dependent vascular effects of Endocannabinoids mediated by peroxisome proliferator-activated receptor gamma (PPARgamma). *PPAR Res* 2009 (2009), 425289.
- G. Esposito, et al., Cannabidiol reduces Abeta-induced neuroinflammation and promotes hippocampal neurogenesis through PPARgamma involvement. *PLoS ONE* 6 (12) (2011), e28668.
- D. De Filippis, G. Esposito, C. Cirillo, M. Cipriano, B.Y. De Winter, C. Scuderi, G. Sarnelli, R. Cuomo, L. Steardo, J.G. De Man, T. Iuvone, S. Gaetani, Cannabidiol reduces intestinal inflammation through the control of neuroimmune axis. *PLoS ONE* 6 (12) (2011) e28159, <https://doi.org/10.1371/journal.pone.0028159>.
- R. Ramer, et al., COX-2 and PPAR-gamma confer cannabidiol-induced apoptosis of human lung cancer cells. *Mol. Cancer Ther.* 12 (1) (2013) 69–82.
- C. Scuderi, L. Steardo, G. Esposito, Cannabidiol promotes amyloid precursor protein ubiquitination and reduction of beta amyloid expression in SHSY5YAPP+ cells through PPARgamma involvement. *Phytother. Res.* 28 (7) (2014) 1007–1013.
- V.L. Hegde, et al., Critical Role of Mast Cells and Peroxisome Proliferator-Activated Receptor gamma in the Induction of Myeloid-Derived Suppressor Cells by Marijuana Cannabidiol In Vivo. *J. Immunol.* 194 (11) (2015) 5211–5222.
- W.H. Hind, T.J. England, S.E. O'Sullivan, Cannabidiol protects an in vitro model of the blood-brain barrier from oxygen-glucose deprivation via PPARgamma and 5-HT1A receptors. *Br. J. Pharmacol.* 173 (5) (2016) 815–825.
- P. Ferre, The biology of peroxisome proliferator-activated receptors: relationship with lipid metabolism and insulin sensitivity. *Diabetes* 53 (Suppl 1) (2004) S43–S50.
- P. Tontonoz, B.M. Spiegelman, Fat and beyond: the diverse biology of PPARgamma. *Annu. Rev. Biochem.* 77 (2008) 289–312.

- [31] S.A. Kliewer, K. Umehono, D.J. Noonan, R.A. Heyman, R.M. Evans, Convergence of 9-cis retinoic acid and peroxisome proliferator signalling pathways through heterodimer formation of their receptors, *Nature* 358 (6389) (1992) 771–774.
- [32] S.A. Kliewer, et al., Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors alpha and gamma, *Proc. Natl. Acad. Sci. USA* 94 (9) (1997) 4318–4323.
- [33] A. le Maire, M. Grimaldi, D. Roeklin, S. Dagnino, V. Vivat-Hannah, P. Balaguer, W. Bourguet, Activation of RXR-PPAR heterodimers by organotin environmental endocrine disruptors, *EMBO Rep.* 10 (4) (2009) 367–373.
- [34] C.H. Hurst, D.J. Waxman, Activation of PPARalpha and PPARgamma by environmental phthalate monoesters, *Toxicol. Sci.* 74 (2) (2003) 297–308.
- [35] J.M. Lehmann, et al., An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor gamma (PPAR gamma), *J. Biol. Chem.* 270 (22) (1995) 12953–12956.
- [36] S.M. Rangwala, M.A. Lazar, Peroxisome proliferator-activated receptor gamma in diabetes and metabolism, *Trends Pharmacol. Sci.* 25 (6) (2004) 331–336.
- [37] L. Szeles, D. Torocsik, L. Nagy, PPARgamma in immunity and inflammation: cell types and diseases, *BBA* 1771 (8) (2007) 1014–1030.
- [38] S.E. O'Sullivan, et al., Novel time-dependent vascular actions of Delta9-tetrahydrocannabinol mediated by peroxisome proliferator-activated receptor gamma, *Biochem. Biophys. Res. Commun.* 337 (3) (2005) 824–831.
- [39] J. Liu, et al., Activation and binding of peroxisome proliferator-activated receptor gamma by synthetic cannabinoid ajulemic acid, *Mol. Pharmacol.* 63 (5) (2003) 983–992.
- [40] V. Gasperi, F. Fezza, N. Pasquariello, M. Bari, S. Oddi, A.F. Agrò, M. Maccarrone, Endocannabinoids in adipocytes during differentiation and their role in glucose uptake, *Cell. Mol. Life Sci.* 64 (2) (2007) 219–229.
- [41] C.E. Rockwell, N.E. Kaminski, A cyclooxygenase metabolite of anandamide causes inhibition of interleukin-2 secretion in murine splenocytes, *J. Pharmacol. Exp. Ther.* 311 (2) (2004) 683–690.
- [42] C.E. Rockwell, et al., Interleukin-2 suppression by 2-arachidonyl glycerol is mediated through peroxisome proliferator-activated receptor gamma independently of cannabinoid receptors 1 and 2, *Mol. Pharmacol.* 70 (1) (2006) 101–111.
- [43] Shoucri, B.M., et al., Retinoid X Receptor Activation Alters the Chromatin Landscape To Commit Mesenchymal Stem Cells to the Adipose Lineage. *Endocrinology*, 2017. **158**(10): p. 3109-3125.
- [44] Chamorro-Garcia, R., et al., *Ancestral perinatal obesogen exposure results in a transgenerational thrifty phenotype in mice.* *Nat Commun*, 2017. **8**(1): p. 2012.
- [45] Shoucri, B.M., et al., Retinoid X Receptor Activation During Adipogenesis of Female Mesenchymal Stem Cells Programs a Dysfunctional Adipocyte. *Endocrinology*, 2018. **159**(8): p. 2863-2883.
- [46] Livak, K.J. and T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*, 2001. **25**(4): p. 402-8.
- [47] Bevington, P.R., D.K. Robinson, and G. Bunce, *Data Reduction and Error Analysis for the Physical Sciences, 2nd ed.* American Journal of Physics, 1993. **61**(8): p. 766-767.
- [48] Forman, B.M., et al., Unique response pathways are established by allosteric interactions among nuclear hormone receptors. *Cell*, 1995. **81**(4): p. 541-50.
- [49] Chamorro-García, R., et al., Effects of Perinatal Exposure to Dibutyltin Chloride on Fat and Glucose Metabolism in Mice, and Molecular Mechanisms, in *Vitro. Environ Health Perspect*, 2018. **126**(5): p. 057006.
- [50] A.S. Janesick, G. Dimastrogiovanni, L. Vanek, C. Boulos, R. Chamorro-García, W. Tang, B. Blumberg, On the Utility of ToxCast and ToxPi as Methods for Identifying New Obesogens, *Environ. Health Perspect.* 124 (8) (2016) 1214–1226.
- [51] R. Chamorro-García, S. Kirchner, X. Li, A. Janesick, S.C. Casey, C. Chow, B. Blumberg, Bisphenol A diglycidyl ether induces adipogenic differentiation of multipotent stromal stem cells through a peroxisome proliferator-activated receptor gamma-independent mechanism, *Environ. Health Perspect.* 120 (7) (2012) 984–989.
- [52] A. Vallee, et al., Effects of cannabidiol interactions with Wnt/beta-catenin pathway and PPARgamma on oxidative stress and neuroinflammation in Alzheimer's disease, *Acta Biochim. Biophys. Sin. (Shanghai)* 49 (10) (2017) 853–866.
- [53] R.K. Carvalho, M.R. Souza, M.L. Santos, F.S. Guimarães, R.L.H. Pobbé, M. L. Andersen, R. Mazaro-Costa, Chronic cannabidiol exposure promotes functional impairment in sexual behavior and fertility of male mice, *Reprod. Toxicol.* 81 (2018) 34–40.
- [54] S.M. Strowig, P. Raskin, The effect of rosiglitazone on overweight subjects with type 1 diabetes, *Diabetes Care* 28 (7) (2005) 1562–1567.
- [55] J.M. Schwarz, R.A. Neese, S. Turner, D. Dare, M.K. Hellerstein, Short-term alterations in carbohydrate energy intake in humans. Striking effects on hepatic glucose production, de novo lipogenesis, lipolysis, and whole-body fuel selection, *J Clin Invest* 96 (6) (1995) 2735–2743.
- [56] M. Furuhashi, S. Saitoh, K. Shimamoto, T. Miura, Fatty Acid-Binding Protein 4 (FABP4): Pathophysiological Insights and Potent Clinical Biomarker of Metabolic and Cardiovascular Diseases, *Clin Med Insights Cardiol* 8s3 (2014) CMC.S17067, <https://doi.org/10.4137/CMC.S17067>.
- [57] P. Keller, J.T. Petrie, P. De Rose, I. Gerin, W.S. Wright, S.-H. Chiang, A.R. Nielsen, C.P. Fischer, B.K. Pedersen, O.A. MacDougald, Fat-specific protein 27 regulates storage of triacylglycerol, *J. Biol. Chem.* 283 (21) (2008) 14355–14365.
- [58] T. Garin-Shkolnik, et al., FABP4 attenuates PPARgamma and adipogenesis and is inversely correlated with PPARgamma in adipose tissues, *Diabetes* 63 (3) (2014) 900–911.
- [59] D. Jin, et al., Lipocalin 2 is a selective modulator of peroxisome proliferator-activated receptor-gamma activation and function in lipid homeostasis and energy expenditure, *FASEB J.* 25 (2) (2011) 754–764.
- [60] Jung, T., et al., Functional interactions between cannabinoids, omega-3 fatty acids, and peroxisome proliferator-activated receptors: Implications for mental health pharmacotherapies. *Eur J Neurosci*, 2020.
- [61] Sonogo, A.B., et al., Cannabidiol prevents haloperidol-induced vacuolar chewing movements and inflammatory changes in mice via PPARgamma receptors. *Brain Behav Immun*, 2018. **74**: p. 241-251.
- [62] Fellous, T., et al., Phytocannabinoids promote viability and functional adipogenesis of bone marrow-derived mesenchymal stem cells through different molecular targets. *Biochem Pharmacol*, 2020. **175**: p. 113859.