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1 **EXPRESSION OF CANNABINOID SYSTEM IN MUSCLE: EFFECTS OF**
2 **HIGH FAT DIET AND CB1 RECEPTOR BLOCKADE**

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23

24 Abbreviations: ACOX, acyl-CoA oxidase; AdipoR1, adiponectin receptor 1; DAGLβ,
25 diacylglycerol lipase beta; CB₁/CB₂, cannabinoid receptor type 1 and 2; GHR, growth
26 hormone receptor; HFD, high-fat diet; MAGL, monoacylglycerol lipase;
27 PPARα/PPARγ, peroxisome proliferator-activated receptor alpha and gamma; SD,
28 standard/low-fat diet.

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1 **ABSTRACT**

2

3 The endocannabinoid system (ECS) plays an important role on the onset of obesity and
4 metabolic disorders implicating central and peripheral mechanisms predominantly via
5 CB₁ receptor. CB₁ receptor antagonist/inverse agonist treatment improves cardio-
6 metabolic risk factors and insulin resistance. However, the relative contribution of
7 peripheral organs to the net beneficial metabolic effects remains unclear. Here we have
8 identified the presence of the endocannabinoid-signaling machinery in the skeletal
9 muscle and also investigated the impact of a very high-fat diet (HFD) on lipid
10 metabolism-related genes and endocannabinoid-related proteins. Finally we tested
11 whether CB₁ inverse agonist AM251 administration restored the alterations induced by
12 HFD. Rats were fed either with a standard diet (STD) and a HFD for 10 weeks and then
13 treated with AM251 (3 mg/kg/day) for 14 days. The accumulated caloric intake was
14 progressively higher in HFD than STD, resulting in a divergence on body weight gain.
15 AM251 treatment reduced accumulated food/caloric intake and body weight gain, being
16 more marked in HFD. CB₂ and PPAR α gene expression was decreased in HFD rats,
17 while MAGL were upregulated. These data suggest an altered endocannabinoid-
18 signaling as results of HFD. AM251 treatment reduced CB₂, PPAR γ and adipoR1 gene
19 expression in STD, but only partially normalized CB₂ receptors in HFD. Protein levels
20 corroborated gene expression results, but also showed a decrease of DAGL β and
21 DAGL α after AM251 in STD and HFD respectively. Present data indicates a diet-
22 sensitive ECS in the skeletal muscle, suggesting that blockade of CB₁ receptors could
23 work towards restoration of the metabolic adaption imposed by diet.

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25 Key words: CB₁ inverse agonist AM251, endocannabinoid system, diet-induced
26 obesity, skeletal muscle oxidative metabolism.

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1 INTRODUCTION

2

3 The regulation that the endocannabinoid system (ECS) exerts over food intake and
4 energy metabolism involves not only the activation of CB₁ receptors present in central
5 circuits in the hypothalamus but also a complex interplay of peripheral organs such as
6 adipose tissue, liver, pancreas and the muscle [1,2,3]. A network of fatty acid-derived
7 signaling molecules including anandamide (AEA), oleylethanolamide (OEA) and 2-
8 arachidonoyl-glycerol (2-AG), exert their effect mainly binding to the cannabinoid
9 receptors type 1 and 2 (CB₁ and CB₂), or to the peroxisome proliferator-activated
10 receptor alpha (PPAR α) and gamma (PPAR γ), for which there is growing evidence of
11 their role in obesity and diabetes [4]. CB₁ receptors are among the most important
12 receptors expressed in the brain, but also in many peripheral organs involved in energy
13 metabolism [5] such as liver [6,7,8], adipose tissue [9,10], gut [11], pancreas [12,13,14]
14 and skeletal muscle [15,16]. CB₂ receptors are predominantly localized on peripheral
15 immune cells and hematopoietic system [17], but they have also recently been found in
16 the brain, liver, pancreas, adipose tissue and cardiac muscle [18,19]. It is well
17 demonstrated that the use of selective cannabinoid receptor inverse agonist/antagonists,
18 such as SR141716 (Rimonabant) and others with both a similar structure and
19 pharmacological properties like the CB₁ inverse agonist AM251 are able to regulate
20 energy balance and body weight gain. Studies in various animal models of obesity and
21 subsequent human clinical studies demonstrated that the treatment with CB₁ inverse
22 agonists result in weight loss attributable to a reduction of food intake but also to an
23 increase in whole body energy expenditure with the involvement of several peripheral
24 organs [11,20,21]. Blockade of central and peripheral CB₁ receptors by Rimonabant has
25 demonstrated to induce important weight loss and improvement in multiple metabolic
26 parameters correcting hyperinsulinemia, lowering free fatty acid levels and reversing
27 insulin resistance, all of which counteracting the adverse effects of an over-stimulated
28 ECS [21,22]. So, CB₁ receptor stimulation by an over-active ECS will cause an increase
29 in appetite and food intake together with a series of metabolic changes in peripheral
30 organs that can lead to weight gain and consequently to obesity and related disorders
31 [23,24].

32 However, the central action of CB₁ receptor antagonism has been associated with a
33 higher risk of anxiety and depression [25]. This fact leads to the suspension of the
34 marketing authorization for Rimonabant, the first commercialized selective CB₁

1 receptor antagonist, by the European Medicine Agency (EMA), highlighting the
2 importance of limiting CB₁ antagonism to peripheral organs in order to reduce the
3 potential central risks. So, further work is necessary to better understand the peripheral
4 mechanisms occurring in adipose tissue, liver and muscle as well as the possible
5 interactions between these organs, in order to establish whether peripheral cannabinoid
6 receptors are solely responsible for the metabolic changes observed in obese subjects
7 treated with cannabinoid CB₁ receptor inverse agonists, leaving apart the hypothalamic
8 control of the motivation to eat [26].

9 In this study we focused on the skeletal muscle, which is an important tissue for glucose
10 and fat oxidation, being an important site for insulin action [27]. However, despite the
11 fact that AEA can modify the pathways regulating fatty acid oxidation in the skeletal
12 muscle, probably via CB₁ receptor, suggesting that CB₁ antagonism would have an
13 important role on oxidative metabolism and energy regulation [28,29], there is still a
14 general lack of clarity regarding the physiological functions and molecular mechanism
15 implicated. In fact there are almost no studies demonstrating the presence of the
16 endocannabinoid signaling proteins and its sensitivity to high fat diets. Therefore, in the
17 present study, we have a) investigated the presence of the endocannabinoid signaling
18 machinery in skeletal muscle, b) analyzed the impact of a very high fat diet on lipid and
19 glucidic metabolism and endocannabinoid-related genes, and c) monitored the effects of
20 the CB₁ antagonists AM251 during standard chow and high fat diet on the
21 endocannabinoid machinery and the genes related to lipid oxidative metabolism on the
22 skeletal muscle of rats. Among the many molecules involved in lipid metabolism of the
23 skeletal muscle we evaluated changes on the gene and protein expression of relevant
24 components of the ECS such as the cannabinoid CB₁ and CB₂ receptors and some of
25 their synthesis (diacylglycerol lipase α and β , DAGL α/β), and degradation
26 (monoacylglycerol lipase, MAGL) enzymes, as well as for the PPAR α and PPAR γ
27 receptors, the adiponectin receptor 1 (AdipoR1), the Acetyl-CoA-oxidase (ACOX) and
28 the growth hormone receptor (GHR).

29

30 **EXPERIMENTAL**

31 **Animals**

1 All experimental procedures with animals were performed in compliance with the
2 European Communities directive 86/609/ECC and Spanish legislation (BOE 252/
3 34367-91, 2005) regulating animal research. Male Wistar rats (~250 g, 10-12 weeks
4 old) (Charles Rivers, Barcelona, Spain) were housed in groups of two in cages
5 maintained in standardized conditions of animal facilities (Servicio de Estabulario,
6 Facultad de Medicina, Universidad de Málaga) at 20±2°C room temperature, 40±5%
7 relative humidity and a 12h light/dark cycle with dawn/dusk effect.

8

9 **Diet-induced obesity. Measurement of food/calorie intake and body weight gain**

10 Rats (n=16/group) were fed for 12 weeks with two different types of diets: a
11 standard/low-fat diet (STD, 10% fat diet-D1245B) and a very high-fat diet (HFD, 60%
12 fat diet-D12492, Brogaarden, Gentofke, Denmark) in order to induce obesity. The
13 standard/low-fat diet consisted of 3.85 kcal/g (with 20% protein, 70% carbohydrates
14 and 10% fat) and the very high-fat diet had 5.24 kcal/g (of which, 20% of the
15 metabolizable energy content was protein, 20% carbohydrates and 60% fat).

16 The accumulated food/caloric intake by each rat and their body weight gain were
17 measured every day for 12 weeks. Treatment was then administered when the weight
18 curves showed a clear divergence and stabilization between both diets was achieved.
19 This was accomplished after 10 weeks of diet (Supplementary figure 1).

20

21 **Treatment**

22 From week 10 on (stabilization of weight curves), each group of diet-fed rats
23 (n=8/group) received treatment which consisted of a daily intraperitoneal (i.p.) injection
24 of either vehicle (1 ml/kg of 10% Tocrisolve in saline) or the cannabinoid CB1 receptor
25 inverse agonist AM251 [*N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-
26 methyl-1*H*-pyrazole-3-carboxamide] (Tocris Bioscience, Bristol, UK), at 3 mg/kg in
27 10% Tocrisolve during 14 days, while the diets remained unchanged. The accumulated
28 food/caloric intake and the body weight gain were measured every day during the 14
29 days of treatment (Supplementary figure 1).

30

31 **Sample collection**

1 AM251- and vehicle-treated (control) animals were anesthetized (sodium pentobarbital,
2 50 mg/kg, i.p.) two hours after the last dose of treatment in a room separate from the
3 other experimental animals. Blood samples were briefly collected from the orbital
4 cavity and centrifuged (3000 r.p.m. for 8 min, 4°C), and all plasma samples were frozen
5 at -80°C for hormonal analysis. Skeletal muscle from the abdominal wall was dissected.
6 Part of each sample was fixated with 4% paraformaldehyde in 0.1 M phosphate buffered
7 saline (PBS) by immersion until immunohistochemical analysis. The remaining of each
8 sample was briefly frozen at -80 until RT-PCR analysis.

10 **RNA isolation and RT-PCR analysis**

11 RNA, RT and RT-PCR were performed as described in the Supplementary Material.
12 Primers for PCR reaction (table 1) were designed based on NCBI database sequences of
13 rat reference mRNA and checked for specificity with BLAST software from NCBI
14 website (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Quantification was carried out with a
15 standard curve run at the same time as the samples with each reaction run in duplicate.
16 Absolute values from each sample were normalized with regard to the housekeeping
17 gene actin beta.

19 **Western blot analysis**

20 In order to evaluate the presence of CB₁ and CB₂ receptors, DAGL α , DAGL β , MAGL,
21 and PPAR α receptor we displayed a Western blot analysis. For protein detection, each
22 blotted membrane lane was incubated separately with the specific primary antibody:
23 rabbit polyclonal antibody CB₁ (1:100; Abcam cat n° ab23703, Cambridge, UK), rabbit
24 polyclonal antibody CB₂ (1/200; ABR cat n° PA1-746A, Golden, USA), rabbit
25 polyclonal antibody MAGL (1/200; Cayman, cat. n° 100035), rabbit polyclonal
26 antibody PPAR α (1/200; ABR cat n° MA1-822), and rabbit polyclonal antibodies
27 DAGL α and DAGL β (both at 1/100; generated in our laboratory, Suárez et al., 2008),
28 all of them diluted in the blocking buffer (table 2). Western Blot procedures using these
29 antibodies were performed as previously described [30, see Supplementary Material].

31 **Immunohistochemistry**

1 Paraffined tissue microarray blocks (Manual Tissue Arrayer MTA-1, beecher
2 Instruments, Inc., Sun Prairie, WI, USA) of rat skeletal muscle (abdominal) were
3 analyzed for the presence and quantification of CB₁ and CB₂ receptors, DAGL α ,
4 DAGL β , MAGL and PPAR α receptor by immunohistochemistry and densitometry. The
5 primary antibodies were: rabbit polyclonal antibody CB₁ (diluted 1:100; Abcam cat n $^{\circ}$
6 ab23703, Cambridge, UK), rabbit polyclonal antibody CB₂ (diluted 1:100; ABR cat n $^{\circ}$
7 PA1-746A, Golden, USA), rabbit polyclonal antibody PPAR α (diluted 1:200; ABR cat
8 n $^{\circ}$ MA1-822), rabbit polyclonal antibody MAGL (diluted 1:100; Cayman, cat. n $^{\circ}$
9 100035, Chemical, Ann Harbor, MI, USA), and finally rabbit polyclonal antibodies
10 DAGL α and DAGL β (both diluted 1:50), generated in our laboratory [30].
11 Immunohistochemical procedures using these antibodies were performed as previously
12 described [30, see Supplementary Material].

13

14 **Statistical analysis**

15 All data are represented as mean \pm SEM (standard error of the mean). Differences
16 between the different diets and treatments were analyzed by Student's *t* test (two tailed,
17 paired groups). The level of statistical significance was set at *p* values of less than 0.05.

18

19 **RESULTS**

20 For this study, we investigated the effect of a very high-fat diet (60% fat) and CB₁
21 inverse agonist AM251 on the glucose and lipid metabolism of the skeletal muscle in a
22 rat model of diet-induced obesity. To get this aim, we evaluated changes on the gene
23 and protein expression of relevant molecules involved in skeletal muscle oxidative
24 metabolism. The selected enzymes and receptors represent a selection of molecules
25 implicated in lipid and glucidic metabolism within the skeletal muscle with a putative
26 role in energy metabolism as the adiponectin receptor 1 (AdipoR1), the Acetyl-CoA-
27 oxidase (ACOX), the growth hormone receptor (GHR) and the PPAR α and PPAR γ
28 receptors, but also the gene and protein expression of relevant components of the ECS
29 such as the cannabinoid CB₁ and CB₂ receptors and some of their synthesis (DAGL α
30 and DAGL β) and degradation (MAGL) enzymes. The endocannabinoid proteins were

1 selected on the basis of abundance of mRNA on PCR studies. In order to control the
2 effect of diet and treatment, we monitored the accumulated food/caloric intake and body
3 weight gain.

4

5 **Effect of diet on accumulated food/calorie intake and body weight gain**

6 The figure 1 represents either accumulated food or caloric intakes and body weight gain
7 on the rats fed with either a standard/low-fat diet (STD, 10% fat) or with a high-fat diet
8 (HFD, 60% fat) over the first 10 weeks of feeding period. We observed that rats fed
9 with STD accumulated more food intake than rats fed with a HFD (Figure 1A), in
10 contrast with the accumulated caloric intake (Figure 1B), and this difference was
11 statistically significant from the first day on. The lower food intake in HFD rats is due
12 to the higher caloric/energy content in the HFD than the STD, as was described in the
13 methodology. Accumulated caloric intake correlates with the body weight increase
14 (Figure 1C), but interestingly body weight gain also showed a significant divergence in
15 HFD rats in comparison with STD rats from the fourth week on (30 days). When body
16 weight gain began to be stabilized over time (after 10 weeks of either diet), AM251
17 treatment was scheduled for 14 additional days, while the diets remained unchanged.

18

19 **Effect of AM251 treatment on accumulated food/calorie intake and body weight** 20 **gain**

21 After a feeding period of 10 weeks, rats received a daily dose of either vehicle (1 ml/kg
22 of 10% Tocrisolve) or the CB1 inverse agonist AM251 (3 mg/kg in 10% Tocrisolve)
23 during 14 days (Figure 2). We observed that treatment with AM251 reduced
24 accumulated food and caloric intake in both diets (Figures 2A and 2B) from the 4-5
25 days of treatment on, and this correlated with a significant body weight decrease (Figure
26 2C). Interestingly, body weight decrease was more marked in HFD rats than in STD
27 rats, that is, we observed a significant decrease in HFD rats from the first day of
28 treatment on, whereas we did not detect a significant body weight decrease in STD
29 rats until the eighth day of treatment. This may be due to that, despite AM251
30 treatment, the accumulated food consumed continued to be slightly higher in STD rats
31 than in HFD rats.

1

2 **Presence of the endocannabinoid system in the skeletal muscle**

3 Before evaluating any changes on the gene and protein expression of relevant molecules
4 involved in skeletal muscle oxidative metabolism by quantitative RT-PCR and
5 immunohistochemistry, we analyzed the presence of CB₁, CB₂, DAGL α , DAGL β ,
6 MAGL, and PPAR α protein and gene expression by Western blotting and RT-PCR.
7 Figure 3A shows a comparative study of the gene expression distribution for both
8 skeletal muscle and adipose tissue, represented as number of copies of PCR product,
9 from STD rats. We observed lower levels of gene expression for CB₁ (15-fold, $P<0.01$),
10 CB₂ (6-fold, $P<0.05$), DAGL α (5-fold) and DAGL β (3.5-fold) in the skeletal muscle
11 when compared with the levels obtained in the adipose tissue. In contrast, MAGL and
12 PPAR α gene expression was higher in the skeletal muscle than in adipose tissue (7.5-
13 fold, $P<0.01$; and 2-fold, respectively). In order to demonstrate that antibodies
14 recognized the corresponding antigens in the rat skeletal muscle we perform a Western
15 blot analysis. Membrane protein extracts from Wistar male rat skeletal muscle revealed
16 prominent immunoreactive bands of expected molecular masses of 60 kD for CB₁, 55
17 kD for CB₂, 120 kD for DAGL α , 74 kD for DAGL β , 37 kD for MAGL, and 52 kD for
18 PPAR α (Figure 3B).

19

20 **Effect of diet and AM251 treatment on gene expression of the molecules implicated** 21 **in skeletal muscle glucidic and lipidic metabolism**

22 Gene expression of receptors and enzymes involved in metabolism on the skeletal
23 muscle as well as the components of the ECS showed significant changes according to
24 the use of both diets (STD vs HFD) on the levels of CB₂, PPAR α and MAGL (Figure
25 4). The expression of both CB₂ receptor and PPAR α was reduced significantly when
26 using a very high-fat diet as shown in figures 4B and 4E. Meanwhile, MAGL
27 expression, the 2-AG-degradation enzyme, was increased significantly in HFD rats
28 (Figure 4F). So the tendency for the synthesis enzyme DAGL α and the receptors
29 analyzed was to the decrease as opposite to the gene expression of the degradation
30 enzyme MAGL, suggesting the existence of lower productions of 2-AG in the skeletal
31 muscle, but desensitization of the receptors.

9

1 Regarding the use of a standard diet and AM251 treatment (Figure 5), most genes
2 studied showed a decrease in their levels of expression after treatment with the
3 antagonist, being statistically significant for CB₂, PPAR γ and adipoR1 gene expression
4 only (Figures 5B, 5G and 5I). However, as in the case of HFD, the tendency for MAGL
5 gene expression was to increase as opposite to the rest of genes analyzed.

6 When rats were fed with a very high-fat diet and treated with AM251 (Figure 6), we
7 observed a differential response of the gene expression of the molecules studied.
8 Changes observed in STD were not observed and CB₁ and CB₂ gene expression tend to
9 be normalized (upregulated) to that of STD levels, again in contrast with the tendency
10 of MAGL gene expression. PPAR γ , GHR, AdipoR1 and ACOX tended to be down-
11 regulated.

12

13 **Effect of diet and AM251 treatment on protein expression of the molecules** 14 **implicated in skeletal muscle glucidic and lipidic metabolism**

15 Figure 7 shows a panel of representative high-magnification images from the skeletal
16 muscle immunostained for CB₁, CB₂, DAGL α , DAGL β , MAGL and PPAR α in the four
17 rat groups (STD-Veh, STD-AM251, HFD-Veh, HFD-AM251). Results in the
18 quantification of immunoreactivity on tissue microarray blocks showed similar changes
19 to that of gene expressions (Figure 8). CB₂ and PPAR α showed a significant decrease
20 on their protein expression levels in HFD rats (Figures 8B and 8F). On the other hand,
21 after AM251 treatment, DAGL α and DAGL β protein levels showed a significant
22 decrease in HFD and STD rats respectively (Figure 8C and 8D).

23

24 **DISCUSSION**

25 Over recent years, animal models of obesity and clinical trials with the cannabinoid CB₁
26 blocker Rimonabant have demonstrated that CB₁ antagonism helps improving several
27 cardio-metabolic risk factors, insulin sensitivity and glucose uptake which are found to
28 be deregulated in obesity states and related disorders [24]. Independently of the
29 lowering effects on food intake by central CB₁ antagonism of CB₁ receptors, the long
30 term effects on body weight, visceral fat reduction and metabolic regulation appeared to

1 be mediated by stimulation of energy expenditure and by effects on glucidic and lipid
2 metabolism in peripheral organs such as liver, adipose tissue and skeletal muscle [29,
3 31]. The skeletal muscle is known to be the primary site for nutrient oxidation,
4 including fatty acid oxidation and glucose uptake and it has been suggested that insulin
5 resistance and impaired glucose uptake seem to be associated with a loss of oxidative
6 capacity in this tissue in response to an increase in dietary fat intake [28]. Insulin
7 resistance in the skeletal muscle might ultimately play an important role in the
8 pathogenesis of the metabolic syndrome and type 2 diabetes [32,33]. Consequently,
9 blocking of CB₁ receptors in the skeletal muscle will have a direct effect on energy
10 expenditure and oxidative metabolism as it has been demonstrated in various studies
11 with Rimonabant [16, 34]. However, due to a lack of clarity regarding the molecular
12 mechanisms implicated, this area would benefit from further investigation. This lack of
13 knowledge starts from the nearly absence of studies addressing the overall expression in
14 skeletal muscle and changes of endocannabinoid signaling machinery, which has been
15 addressed on the present experimental design.

16 In this study we aimed to identify molecular changes occurring within the metabolic
17 pathways of the skeletal muscle by a very high-fat diet (60% fat) and the subsequent
18 response to a subchronic treatment with the CB₁ receptors inverse agonist AM251. This
19 issue is important because of several data suggesting that endocannabinoids might affect
20 insulin resistance in skeletal muscle, as well as participating in the metabolic
21 consequences (i.e. insulin resistance) of high-fat-induced obesity. In accordance with
22 previous results [5,15,35], we have confirmed that in addition to cannabinoid receptors,
23 CB₁ and CB₂, skeletal muscle cells express the enzymes to locally produce (DAGL α
24 and DAGL β) and degrade (MAGL) endogenous cannabinoids by
25 immunohistochemistry, western blotting and quantitative RT-PCR (Figures 3 and 7).
26 These observations give biological support to an endocannabinoid regulation of genes
27 controlling skeletal muscle metabolism. Indeed, in our study we could observe that the
28 most important changes induced by a very high-fat diet on several molecules implicated
29 in lipid metabolism of the skeletal muscle of male Wistar rats were at the transcriptional
30 level. As it could be expected, a very high-fat diet caused an important weight gain in
31 these animals. At such level, chronic treatment with a very high-fat diet reduced the
32 gene expression of CB₂ receptors and the nuclear receptor PPAR α significantly. We
33 detected the same effect at the protein expression analysis by immunohistochemistry in

1 both CB₂ and PPAR α receptors. To the contrary, the gene expression of MAGL, the 2-
2 AG-degradation enzyme with lipolytic action, showed a significant increase as a result
3 of feeding the animals with this type of diet. Although we have centered the study on
4 the endocannabinoid system we have detected also that the protein expression of
5 NAPE-PLD, the enzyme that synthesizes the non-cannabinoid PPAR α receptor agonist
6 oleylethanolamide and FAAH, the enzyme that degrades it, showed a significant
7 reduction in HFD rats (data not shown).

8 This MAGL regulation can be explained by an ECS over-activity caused with the use of
9 fatty diets as it has been observed in previous studies and/or in conditions of unbalanced
10 energy homeostasis (obesity and type 2 diabetes) [23,31,36,37]. A relevant previous
11 work [38] found elevated levels of 2-AG in the soleus muscle of mice after HFD,
12 particularly when a HFD with high amount of saturated fat is used. Such work came
13 after results in the brain showed that DAGL α was found to be a major biosynthetic
14 enzyme for 2-AG in the nervous system revealing a regulatory role of this enzyme in
15 retrograde synaptic plasticity and adult neurogenesis [39,40]. However, the dysregulated
16 ECs levels found in obesity and/or hyperglycemic states could be affected differently
17 depending on the type of diet and tissue involved. Whatever the circumstances protein
18 expression followed the same tendency as the gene expression results, but differences in
19 the regulatory pathways involved in mRNA and protein stability in the muscle cell
20 could be responsible for the observed variations within the experiment.

21 Fatty acids and eicosanoids are natural ligands for PPARs, and considering both that
22 endocannabinoids chemical structure is derived from arachidonic acid and that the
23 anorectic lipid oleylethanolamide acts on PPAR α , can lead to hypothesize that
24 endocannabinoids might also act through binding to PPARs as well as the
25 endocannabinoid receptors. Recent evidence [41] suggests that endocannabinoids are
26 natural activators of PPAR α , which could mediate many of the biological effects of
27 cannabinoids including feeding and lipid metabolism. According to our results, a
28 condition of energy overload caused by a very high-fat diet could be the reason for
29 PPAR α to be negatively regulated in the skeletal muscle. Conversely, the degrading
30 enzyme MAGL would be up-regulated in order to compensate for over-activation of
31 endocannabinoids as a result of chronic feeding with a high-fat diet.

1 In our experiment, diet exerted more important effects on the skeletal muscle
2 metabolism than treatment with the inverse agonist AM251. However, AM251 caused
3 an important weight loss in these animals as it could be expected from a CB₁ receptor
4 blocker in accordance with previous historical work [21, 24]. Such weight loss would be
5 reflected on muscle cell dynamics which will require further investigation. High
6 endocannabinoid levels are associated with insulin resistance and with a decrease in
7 glucose uptake by skeletal muscle cells. Rimonabant has been found to reverse this
8 situation and that may account for the better use of energy on these HFD-fed animals
9 treated with AM251. The blockade of cannabinoid receptors and the reversion of
10 DAGL/MAGL overexpression may account for this. It is important to mention two
11 important studies based on the possible role played by CB₁ receptors in modulating
12 insulin sensitivity within the skeletal muscle cells: in the first one [42] the authors found
13 that treatment with CB₁ inverse agonist SR141716 alone can improve insulin sensitivity
14 in skeletal muscle as determined by its ability to enhance both insulin-stimulated PKB-
15 and ERK1/2-directed signaling. In the other study [43], authors found that treatment of
16 skeletal muscle cells with AEA increases IRS-1 (Ser307) phosphorylation, thus,
17 disturbing insulin signaling at an early point in the cascade. The study of these
18 important signaling pathways would have added important information regarding
19 insulin resistance and glucose uptake with a HFD.

20 In relation to any effect of treatment with AM251 on gene expression we observed
21 changes on the levels of the CB₂ receptor, the nuclear PPAR γ receptor and the
22 adiponectin receptor (AdipoR1), in the case of animals fed with a standard/low-fat diet
23 (10% fat) and treated with AM251 at 3 mg/Kg dose. However, such doses of AM251
24 did not have any effect when animals were fed with a high-fat diet. It is possible that
25 despite the blockade of the endocannabinoid signaling system in the muscle may not be
26 sufficient to normalize the changes observed after a short AM251 treatment and that
27 extended periods of administration or additional diet modifications might be necessary
28 to observe changes on the genes selected. The immunohistochemical results showed
29 that there is a contrasting tendency for the synthesis enzyme DAGL α and DAGL β to
30 the decrease and increase after treatment with AM251, respectively. This could suggest
31 the existence of mechanisms implicated in the maintenance of the balance in the levels
32 of 2-AG in the skeletal muscle.

1 In addition to the endocannabinoid system, we have studied the effects of diet on two
2 hormonal modulators of muscle function, adiponectin and GH. Adiponectin, produced
3 in adipose tissue, helps improving hyperglycemia, hyperinsulinemia, and insulin
4 resistance via activation of AdipoR1, which is abundantly expressed in skeletal muscle
5 [44]. It is important to note that skeletal muscle is one of the most important sites for the
6 actions of adiponectin and insulin and it has also been observed that PPAR γ agonists
7 have a marked effect on plasma adiponectin [45]. However, although acute doses of
8 AM251 results on insulin increase in plasma (Crespillo *et al.* unpublished observations)
9 plasma adiponectin levels remain unchanged after subchronic treatment with AM251.
10 Some reports have suggested that CB $_1$ antagonism with Rimonabant might exert a direct
11 effect on adiponectin stimulating its mRNA expression [46]. In contrast, other studies
12 did not find any difference in plasma adiponectin levels between animals treated with
13 CB $_1$ antagonist and control animals, or in animals fed with a high-fat or a control diet
14 [47, 48]. Whether the chronic treatment with AM251 is capable of induce alterations on
15 plasma adiponectin production on the adipose tissue remains conclusively determined.

16 The observed effects of AM251 on the muscle might be indirect and a mere
17 consequence of an AM251-induced reversal of an HFD-induced increase in body
18 weight; however, the opposite, AM251 having an intrinsic effect on skeletal muscle,
19 cannot be discarded. It is important to note that we did not find significant changes in
20 CB $_1$ and DAGL α gene expression in either liver or adipose tissue (supplementary table
21 S1). This could be the reason why no significant changes were seen in other genes
22 included in our study (ACOX, GHR, etc). As an example, it has been proposed that
23 increased lipid synthesis in the liver may produce insulin resistance in other tissues such
24 as muscle. Overactive endocannabinoids produce liver lipogenesis accounting then for
25 this hypothesis. In addition, endocannabinoids may also influence insulin secretion
26 directly in islets β -cells via CB $_1$ [14] and CB $_2$ receptors [49].

27 As a final note, the regulatory mechanisms may be different at rest and during exercise
28 and may change as the exercise intensity increases, this could be influential in
29 endocannabinoid production [31,50]. It would be interesting to repeat this type of
30 experiment combining exercise and diet in its original design. Regulation of skeletal
31 muscle fat and glucose metabolism is clearly multifactorial, and different mechanisms
32 may dominate in different conditions; besides, potential variations may exist between
33 individuals in response to stimulating or blocking CB $_1$ receptors. This could cause

1 differences in response to treatment with CB₁ antagonists between different obese
2 states. In conclusion, we have helped identifying important relevant players involved in
3 the signaling pathways of CB₁ receptors antagonism in the skeletal muscle and
4 determined the extent of changes in this system associated with either high fat diet or
5 CB₁ receptors blockade.

6

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9

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1 LEGENDS TO FIGURES

2 **Figure 1.** Effect of a standard/low fat diet (STD) and a very high-fat diet (HFD) on
3 accumulated food/caloric intake and body weight gain during 10 weeks. We observed
4 that rats fed with STD accumulated more food intake than rats fed with a HFD (**A**), in
5 contrast with the accumulated caloric intake (**B**), and this difference was statistically
6 significant from the first day on. Body weight gain showed a significant divergence in
7 HFD rats in comparison with STD rats from the fourth week on (**C**). When body weight
8 gain stabilized over time (at least, for 10 weeks), AM251 treatment was administered,
9 while the diets remained unchanged. Histograms represent the mean \pm SEM (16 animals
10 per diet group). Student's *t* test: **P* < 0.05, ***P* < 0.01 versus SD rats.

11 **Figure 2.** Effect of a standard/low fat diet (STD) and a very high-fat diet (HFD) 10 days
12 before AM251 administration and during 14 days of AM251 treatment (3 mg/kg) on
13 accumulated food/caloric intake and body weight gain, while the diets remained
14 unchanged. Treatment with AM251 reduced accumulated food (**A**) and caloric (**B**)
15 intake in both diets from the 4-5 days of treatment on, and this correlated with a
16 significant body weight decrease (**C**). Body weight decrease was more marked in HFD
17 rats than in STD rats, that is, a significant decrease in HFD rats was detected from the
18 first day of treatment on, whereas a significant body weight decrease was not detected
19 in STD rats until the eighth day of treatment. Arrows point to the beginning of AM251
20 administration. Histograms represent the mean \pm SEM (8 animals per treated group).
21 Student's *t* test: **P* < 0.05, ****P* < 0.001 versus HFD rats treated with AM251; #*P* <
22 0.05, ###*P* < 0.01 versus SD rats treated with AM251.

23 **Figure 3.** Presence of relevant components of the ECS and PPAR α receptor in the
24 skeletal muscle by RT-PCR and Western blot analysis. **A**) Comparative study of the
25 gene expression distribution for both skeletal muscle and adipose tissue, represented as
26 number of copies of PCR product, from STD rats. Lower levels of gene expression for
27 CB₁, CB₂, DAGL α and DAGL β in the skeletal muscle were observed when compared
28 with the levels obtained in the adipose tissue. MAGL and PPAR α gene expression was
29 higher in the skeletal muscle than in adipose tissue. **B**) Membrane protein extracts from
30 Wistar male rat skeletal muscle revealed prominent immunoreactive bands of expected
31 molecular masses of 60 kD for CB₁, 55 kD for CB₂, 120 kD for DAGL α , 74 kD for
32 DAGL β , 37 kD for MAGL, and 52 kD for PPAR α (Fig. 3B). Positions of molecular

1 markers (MW) are indicated at the left. For abbreviation see list. Histograms represent
2 the mean \pm SEM (8 animals per treated group). Student's *t* test: **P* < 0.05, ***P* < 0.01
3 of skeletal muscle versus adipose tissue.

4 **Figure 4.** Effect of diet on gene expression of the molecules implicated in skeletal
5 muscle glucidic and lipidic metabolism. The expression of both CB₂ receptor (**B**) and
6 PPAR α (**E**) was reduced significantly when using a very high-fat diet. MAGL
7 expression was increased significantly in HFD rats (**D**). Absolute values from each
8 sample were normalized with regard to the housekeeping gene actin beta. Histograms
9 represent the mean \pm SEM (8 animals per diet group). Results were compared using
10 Student's *t* test: **P* < 0.05 versus SD. For abbreviation see list.

11 **Figure 5.** Effect of CB₁ inverse agonist AM251 using a standard/low-fat diet (STD) on
12 gene expression of the molecules implicated in skeletal muscle glucidic and lipidic
13 metabolism. Most genes studied showed a decrease in their levels of expression after
14 treatment, being statistically significant for CB₂ (**B**), PPAR γ (**G**) and adipoR1 (**I**) gene
15 expression only. MAGL gene expression showed a tendency to increase. Absolute
16 values from each sample were normalized with regard to the housekeeping gene actin
17 beta. Histograms represent the mean \pm SEM (8 animals per treated group). Results were
18 compared using Student's *t* test: **P* < 0.05 versus vehicle. For abbreviation see list.

19 **Figure 6.** Effect of CB₁ inverse agonist AM251 using a very high fat diet (HFD) on
20 gene expression of the molecules implicated in skeletal muscle glucidic and lipidic
21 metabolism. We observed no significant changes on the gene expression, but CB₁ (**A**)
22 and CB₂ (**B**) gene expression tend to be normalized (upregulated) to that of STD levels.
23 Absolute values from each sample were normalized with regard to the housekeeping
24 gene actin beta. Histograms represent the mean \pm SEM (8 animals per treated group).
25 Results were compared using Student's *t* test: **P* < 0.05 versus vehicle. For
26 abbreviation see list.

27 **Figure 7.** Representative high-magnification microphotographs showing
28 immunohistochemistry for CB₁, CB₂, DAGL α , DAGL β , MAGL and PPAR α in Wistar
29 rat skeletal muscle in the four rat groups (SD-Veh, SD-AM251, HFD-Veh, HFD-
30 AM251).

1 **Figure 8.** Quantification of CB₁, CB₂, DAGL α , DAGL β , MAGL and PPAR α
2 immunoreactivity in the skeletal muscle in the four rat groups (SD-Veh, SD-AM251,
3 HFD-Veh, HFD-AM251). CB₂ (**B**) and PPAR α (**F**) showed a significant decrease on
4 their protein expression levels in HFD rats. On the other hand, after AM251 treatment,
5 DAGL α and DAGL β protein levels showed significant increases in HFD and STD rats
6 respectively (**C**, **D**). Histograms represent the mean \pm SEM (8 animals per group).
7 Results were compared using Student's *t* test: **P* < 0.05 versus SD; # *P* < 0.05 versus
8 vehicle. For abbreviation see list.

Table1. Primers sequences used for real time polymerase chain reaction (RT-PCR)¹.

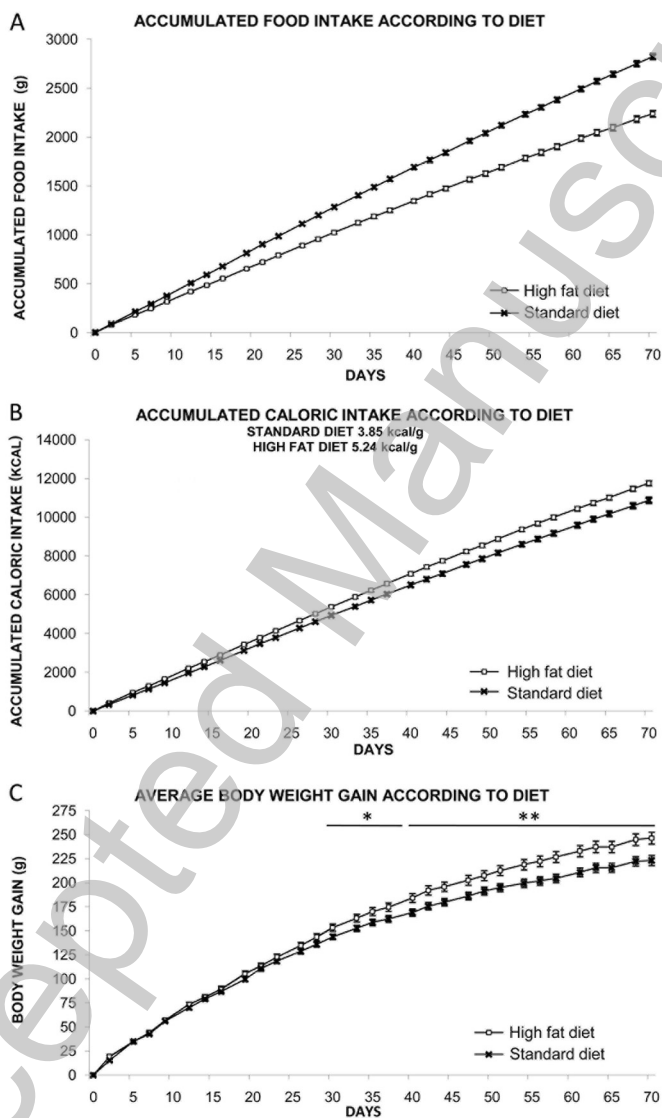
Gene	Oligosense 5'→3'	Oligoantisense 5'→3'	N° access Genbank	Product size (bp)	Annealing T ^a (°C)
ACTB	CAGGCTGTGTTGTCCCTGTA	GCTGTGGTGGTGAAGCTGTA	NM_03144.2	203	51,4
CB1	AGACCTCCTCTACGTGGGCTCG	GTACAGCGATGGCAGCTGCTG	NM_012784.2	314	58
CB2	GCAGCCTGCTGCTGACTGCTG	TGCTTTCAGAGGACATAACC	NM_020543.3	284	58,9
DAGL α	GGGTACCTAATGGCTGCTCA	AGGACTGACCATCCAACCTG	NM_001005886	220	60,5
DAGL β	TGATAGGCCCAAAGATGCTG	AAGTCCATTGTGCTCGTCAG	NM_001107120.1	161	58,9
MAGL	CATGGAGCTGGGGAACACTG	GGAGATGGCACCGCCATGGAG	NM_13852.1	240	58,1
PPAR α	TGCTGTCTCCTTGATGAAC	GCTTGAGCACGTGCACAATC	NM_013196.1	270	58
PPAR γ	GACCACTCCCATTCCTTGA	CGCACTTTGGTATTCTTGGAG	NM_013124.1	153	61,4
ACOX	GGACCATGGTTTTTGTGAGG	AGTGAAGGCATAGGCAGTG	NM_017340.2	201	58,9
AdipoR1	TGATTATGGGGAGCTTCGTC	ACAACACCACTCAAGCCAAG	NM_207587.1	196	60,4
GHR	ACAAATGGTGATTTGTTGGA	TCACTTGGATATCTCCACGGA	NM_017094.1	130	57

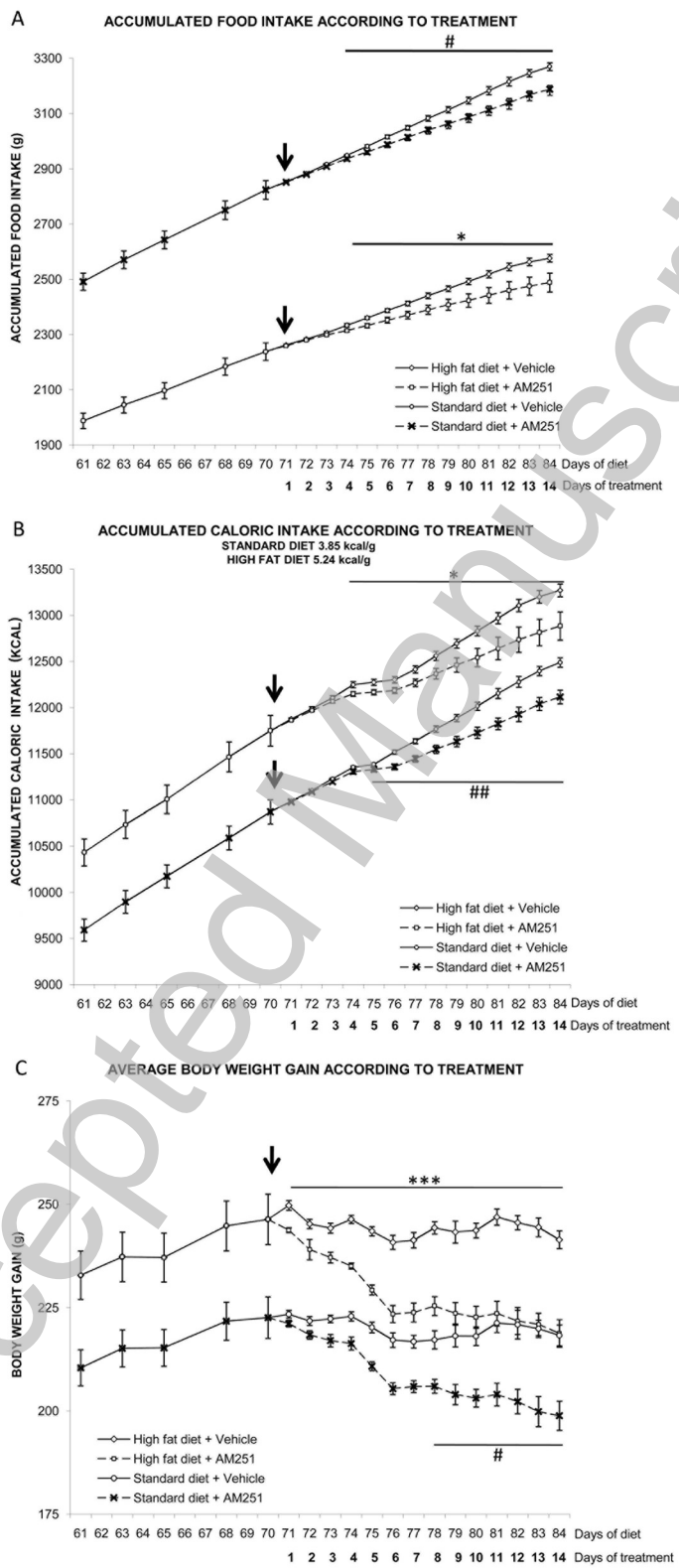
¹ACTB, β -actin; CB1, cannabinoid receptor type 1; CB2, cannabinoid receptor type 2; DAGL α/β , diacylglycerol lipase α/β ; MAGL, monoacylglycerol lipase; PPAR α , peroxisome proliferator-activated receptor α ; PPAR γ , peroxisome proliferator-activated receptor γ ; ACOX, acetyl-CoA-oxidase; AdipoR1, adiponectin receptor 1; and GHR, growth hormone receptor.

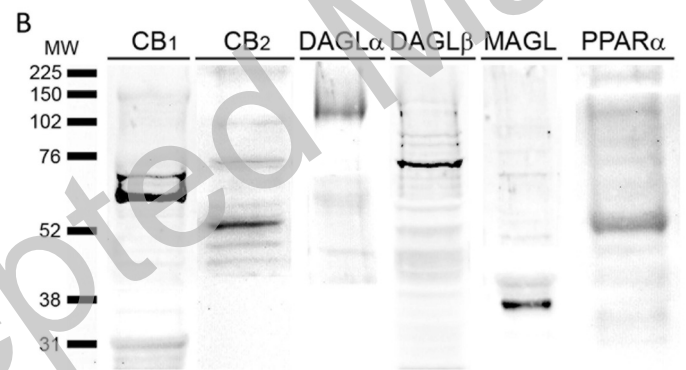
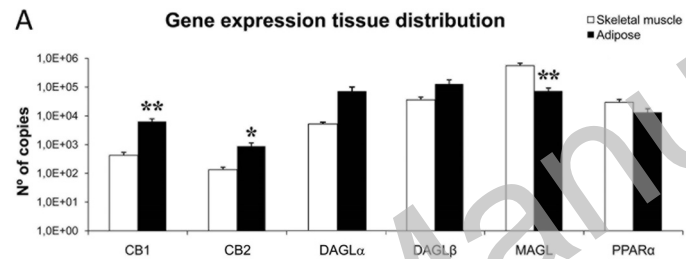
Table 2. Antibodies used in this study with the dilution used and molecular weight for each one of them.

Name	Commercial company	Dilution	Size (kDa)
CB1	Abcam. Anti-rabbit polyclonal antibody	1/100	60
CB2	ABR. Anti-rabbit polyclonal antibody	1/200	55
DAGLα	Produced in our laboratory. Anti-rabbit polyclonal antibody	1/100	120
DAGLβ	Produced in our laboratory. Anti-rabbit polyclonal antibody	1/100	73.6
MAGL	Cayman. Anti-rabbit polyclonal antibody	1/200	37
PPARα	ABR. Anti-rabbit polyclonal antibody	1/200	52

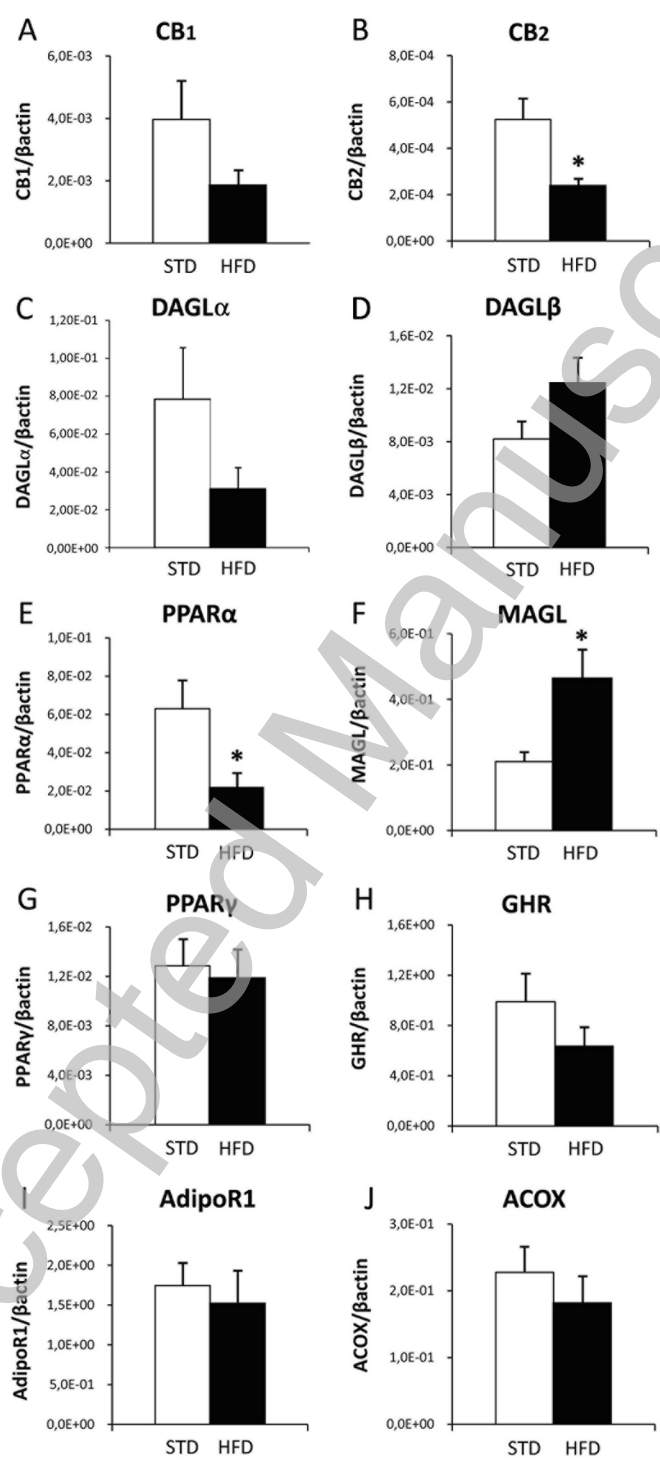
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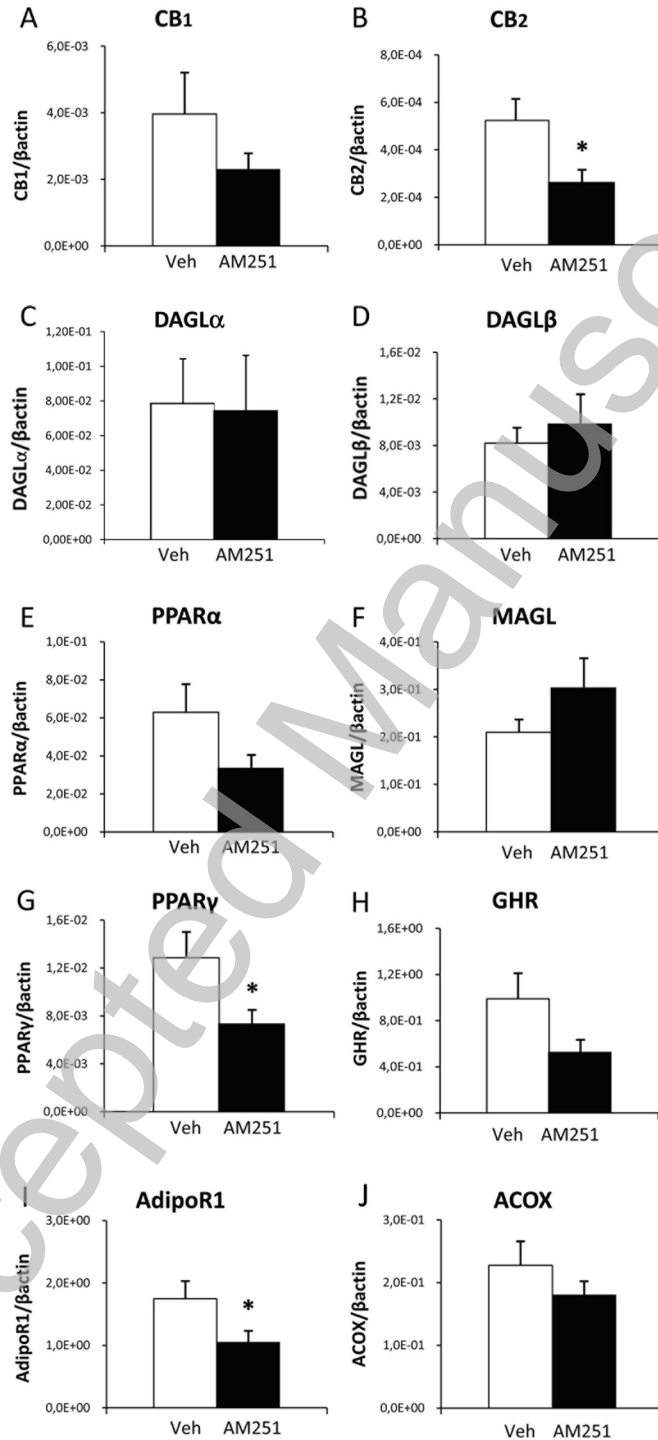


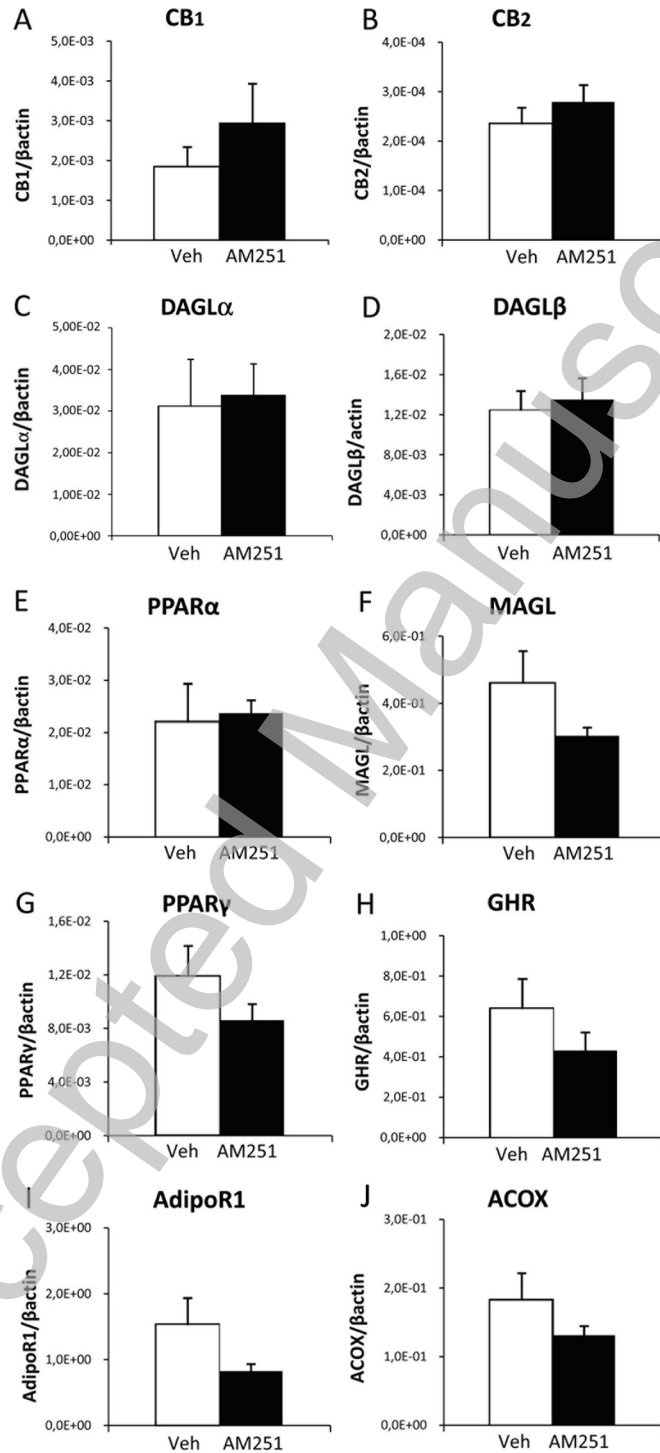




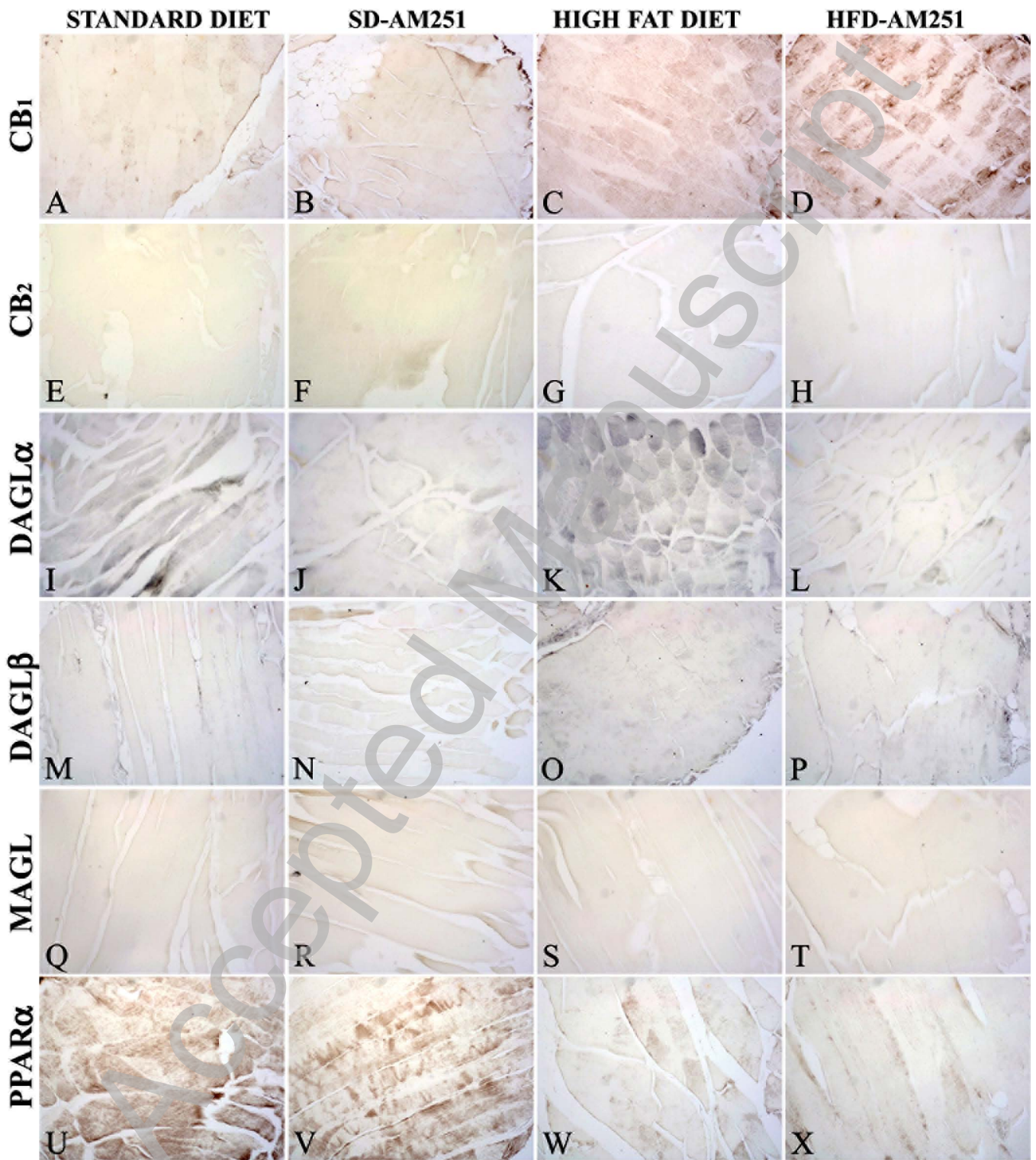
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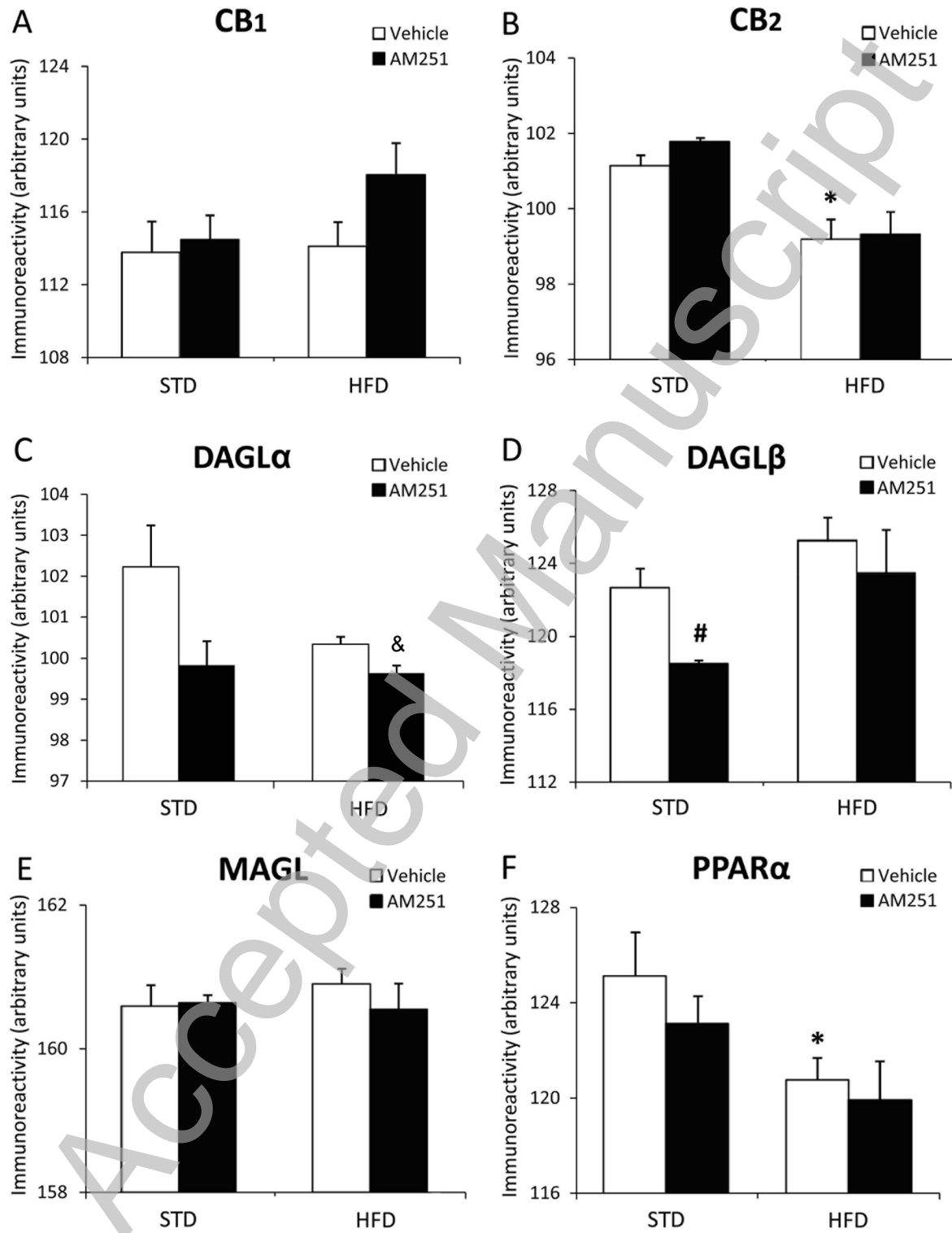






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