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Lipid mediators as regulators of lipid and energy metabolism during energy balance derangement in animal models

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Abstract

Environmental factors play a key role in the development of obesity-induced metabolic syndrome and obesity, which are now true epidemics raising public health concerns. Both excess body fat in obesity, and fat and lean mass loss in undernutrition conditions or anorexia nervosa, are the result of energy imbalance. Therefore, maintaining energy balance is crucial for both the prevention of obesity and the treatment of anorexia nervosa and other forms of undernutrition. Lipid signals such as those mediated by endocannabinoidome mediators are deeply involved in the control of energy metabolism. In this thesis, within two different projects, we studied how different environmental factors including calorie restriction, physical activity, vitamin D supplementation and antipsychotic drugs, may lead to energy metabolism modification through modulation of the endocannabinoidome signaling. The results of the experimental work show how the different studied conditions cause changes in the tissue levels of endocannabinoidome lipid mediator as well as in the expression of their receptors and metabolic enzymes, which may contribute to the observed changes in body fat mass and energy metabolism within the models. We conclude that the investigated conditions may cause changes in energy balance through alteration of the endocannabinoidome.

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Abbreviations

Δ^9-THC	Δ9-tetrahydrocannabinol
1-AG	1-Arachidonoylglycerol
1,25(OH)2D3	1,25 dihydroxyvitamin D
2-AG	2-Arachidonoylglycerol
2-MG	2-monoglycerides
2-OG	2-Oleoylglycerol
6-keto PGF1-alpha	6-keto prostaglandin F1 alpha
ABCD1	ABC transporter
ABHD12	α,β -hydrolase-12
ABHD4	α,β -hydrolase-4
AC	adenylyl cyclase
ACOX	acyl-CoA oxidase
ACS	acyl-CoA synthetases
AEA	Anandamide
Agk	Acylglycerol kinase
Akr1B3	Aldo-keto reductase 1B3
Alox12	Arachidonate 12-lipoxygenase
Alox15	Arachidonate 15-lipoxygenase-1
AMPK	AMP-activated protein kinase
ATGL	Adipose triglyceride lipase
ATM	Adipose tissue macrophages
BAT	Brown adipose tissue
C/EBP	CCAAT/enhancer binding proteins
Cacna1b	Calcium channel/voltage dependent/N type, alpha 1 subunit
Cacna1h	Calcium channel, voltage-dependent, T type, alpha 1H subunit
CACT	Carnitine acylcarnitine translocase
CB1	Cannabinoid receptors of type 1
CB2	Cannabinoid receptors of type 2
Ces1d	Carboxylesterase 1d
CLO	Clozapine
Cnr1	Cannabinoid receptor 1 gene
Cnr2	Cannabinoid receptor 2 gene
CoA	Co-enzyme A
Comt	Catechol-O-methyltransferase
COX	Cyclooxygenase
cPGI2	Carbaprostacyclin
CPT1	Carnitine palmitoyl transferase 1
CPT2	Carnitine palmitoyl transferase 2
CREB	cAMP response element-binding protein
DAG	Diacylglycerol
DAGL	Diacylglycerol lipase
Dagla	Diacylglycerol Lipase alpha

Daglb	Diacylglycerol Lipase Beta
ddH2O	Double-distilled water
Dgke	Diacylglycerol Kinase Epsilon
DHA	Docosahexaenoic acid
DHA-1-G	DHA-1-glycerol
DHA-2-G	DHA-2-glycerol
DP2R	Dopamine receptor 2
EC	Endocannabinoid
eCBome	Endocannabinoidomes
EETs-EA	Epoxyeicosatrienoyl-ethanolamides
Elovl	Elongation of very long chain fatty acids
Enpp2	Ectonucleotide pyrophosphatase/phosphodiesterase 2
EPA	Eicosapentaenoic acid
FAAH	Fatty acid amide hydrolase
FABP4	Fatty acid binding protein 4
fam213b	Family with sequence similarity 213 member B
FAMEs	Fatty acid methyl esters
FAS	Fatty acid synthase
FATP	Fatty acid transport protein
FID	Flame ionization detector
Gapdh	Glyceraldehyde-3-Phosphate Dehydrogenase
GDE1	Glycerophosphodiester Phosphodiesterase 1)
Gde1	Glycerophosphodiesterase-1
Gdpd1	Glycerophosphodiester phosphodiesterase 1
GPCR	G protein-coupled receptor
GPR119	G protein-coupled receptor 119
GPR55	G protein-coupled receptor 55
HDL	High-density lipoproteins
HETEs-EA	Hydroxyeicosatetraenoyl-ethanolamides
HFHS	High fat high sucrose
Hprt	Hypoxanthine-guanine phosphoribosyltransferase
Hrasls5	HRAS-like suppressor
HSL	Hormone-sensitive lipase
Inpp5d	Inositol polyphosphate-5-phosphatase
LEA	Linoleoyl ethanolamide
LOX	Lysyl oxidase
LPS	Lipopolysaccharide
lyso-PI	Lysophosphatidylinositol
lysoPLD	Lysophospholipase D
MAG	Monoacylglycerol
MAPK	Mitogen-activated protein kinase
MGLL	Monoglyceride lipase
Mgll	Monoacylglycerol lipase
Mogat1	Monoacylglycerol acyltransferase 1
N-acyl-lysoPE	N-acyl-lysophosphatidylethanolamine
NAAA	N-acylethanolamide-hydrolyzing acid amidase

NAE	N-acylethanolamines
NAGly	N-arachidonyl glycine
NAPE	N-arachidonyl-phosphatidylethanolamine
NAPE-PLD	N-arachidonyl-phosphatidylethanolamine phospholipase
NF-κB	Kappa-light-chain-enhancer of activated B cells
NVD	Normal vitamin D
OAT	Ovarian adipose tissue
OEA	Oleoylethanolamide
Pam	Positive allosteric modulator
PCR	Polymerase chain reaction
PEA	Palmitoylethanolamide
PGD2	Prostaglandin D2
PGE2	Prostaglandin E2
PGF2-alpha	Prostaglandin 2-alpha
PGI2	Prostaglandin I2
PGs-EA	Prostaglandin-ethanolamides
PI3K	Phosphoinositide 3-kinases
PKB/Akt	The serine/threonine protein kinase B/
PLA1	Phospholipase A1
Pla1a	Phospholipase A1 member A
PLA2	Phospholipase A2
PLC	Phospholipase C
Plcb1	Phospholipase C beta
PPAR-α	Peroxisome proliferator–activated receptor α
PPAR-γ	Peroxisome proliferator-activated receptor γ
Ppt1	Protein thioesterase 1
Ptges	Prostaglandin E synthase
Ptgfr	Prostaglandin F receptor
Ptpn22	Protein tyrosine phosphatase non-receptor type 22
RIS	Risperidone
Rps13	Ribosomal Protein S13
SCFA	Short chain fatty acid
SEA	Stearoylethanolamide
SFA	Saturated fatty acid
sPLA2	Secretory phospholipase A2
sub AT	Subcutaneous adipose tissue
TAG	Triacylglycerols
Tbp	TATA-Box Binding Protein
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline, 0.1% Tween 20
TCA	Tricarboxylic acid cycle
TG	Triglyceride
TRPA1	Transient Receptor Potential Cation Channel Subfamily A Member 1
TRPM8	Transient Receptor Potential Cation Channel Subfamily M Member 8

TRPV1	Transient receptor potential cation channel subfamily V
UCP-1	Uncoupling protein 1
UFAs	Unsaturated fatty acid
VAT	Visceral adipose tissue
VDD	Vitamin D deficiency
VDR	Vitamin D receptor
WAT	White adipose tissue

1. Introduction

The survival of any organism requires efficient energy substrate utilization; therefore, it becomes crucial to be able to both compartmentalize and store energy resources as a function of supply and demand. Humans utilize mainly dietary-derived glucose and long-chain fatty acids as sources of energy (Ahmed et al., 2007). Feeding has a crucial role in survival: besides providing the macronutrients (carbohydrates, lipids, and proteins) and micronutrients (minerals and vitamins), it is considered as an essential aspect of energy homeostasis as it allows storage of energy in the adipose tissue. This latter process occurs when the amount of energy intake is higher than energy expenditure (Margetts et al., 2003). For this reason, calorie restriction and exercise are common factors for losing weight in overweight or obese people (Curioni & Lourenço, 2005).

Once the energy intake decreases, an adaptive reduction of energy expenditure occurs, therefore there is a decrease in body weight in relation to a loss of free fat and fat mass (Yoo, 2018).

Malnutrition is the imbalance between food intake and nutrient requirements, which results in different metabolism (Norman et al., 2008). It refers to overnutrition, undernutrition, specific nutrient deficiencies, or imbalances (Margetts et al., 2003)

Undernutrition is caused by insufficient intake of dietary energy, regardless of any specific nutrient. Starvation results from war or poverty, psychological disorders like anorexia nervosa, intestinal disorders and infections, which may cause malabsorption of nutrients, etc. and hence undernutrition (Shetty, 2003).

Insufficient dietary intake is characterised by increasing demand and decreasing intake of special requirements, namely vitamins. This malnutrition might be caused by lack of adequate food intake containing the essential nutrients or by some illnesses, which cause decreasing the ingestion, absorption, or metabolization of specified nutrients (Chen et al., 2001). In particular, insufficient intake or intestinal absorption of vitamin D, calcium and magnesium reduces bone mass as well as body weight (Saunders et al., 2019).

Another form of malnutrition named as overnutrition, results from excessive dietary intake, and leads to accumulation of body fat in the form of triglycerides produced by the overconsumption of fats and carbohydrates. in different tissues, overnutrition has negative effects on body health and increases the risk of becoming overweight or obese (Svacina, 2008; Mathur & Pillai, 2019)

It has been suggested that regulation of body composition through food Intake and body metabolism is influenced by a balance between the endocannabinoid (EC) and the PPAR-α systems (Naughton et al., 2013).

The Endocannabinoidome (eCBome) refers to the endocannabinoid system, related mediators and EC-like molecules (Iannotti & Piscitelli, 2018). Endocannabinoids including Anandamide (AEA) and 2-Arachidonoylglycerol (2-AG) and EC-like molecules like other N-acylethanolamines (NAEs) and 2-monoglycerides (2-MGs) are derivatives of fatty acids and they may have effects on lipid metabolism (Piscitelli, 2015). Except for the two main cannabinoid receptors of type 1 and 2 named as CB1 and CB2, which act as preferential receptors for AEA and 2-AG, NAEs like oleoylethanolamide (OEA) and palmitoylethanolamide (PEA) have different molecular targets including PPAR-α, TRPV1, GPR119 and GPR55, which are concerned with the control of appetite and energy metabolism (Godlewski et al., 2009; Lambert & Muccioli, 2007). Endocannabinoids including AEA, as well as Δ^9-THC, effect on appetite and lead to overeating (Williams & Kirkham, 1999). CB1 receptor and its agonists control energy intake in the brain and regulates energy hemostasis in the metabolic tissues of liver and adipose tissue (Després, 2007; Matias & Di Marzo, 2007). Although, the weight gain in diet induced obesity rats was accompanied with lower density of CB1 receptor in different brain regions including hippocampus, the entopeduncular nucleus, and the nucleus accumbent (Harrold et al., 2002). Another study has shown increasing levels of 2-AG levels in the visceral adipose tissue of diet-induced obesity mice and obese patients but there were no changes in AEA levels (Matias et al., 2006)

Eating disorders also are related psychiatric pathologies characterized by abnormal eating patterns and behavior and based on its role in the regulation of energy metabolism and feeding, the eCBome might have role on these disorders, as in the case of Anorexia nervosa (Monteleone et al., 2005).

Based on the above background, in this thesis I have investigated the role of the eCBome in two animal different models of malnutrition and dysmetabolism:

1. CRR rats which have acute calorie restriction combined with a sustained physical activity related to malnutrition.

2. Vitamin D/Olanzapine administrated mice which are highly correspondent with metabolic disorders.

1.1. The Endocannabinoidome

Cannabis sativa has been cultivated and used for, among others, medicinal purposes throughout the centuries. The plant cannabinoid (phytocannabinoid) Δ9-tetrahydrocannabinol (Δ9-THC) is the main psychoactive component in cannabis. In addition to phytocannabinoids, two other classes of THC-related compounds have been widely investigated: synthetic cannabinoids and the endocannabinoids (Wu, 2019).

The eCBome includes the classical EC system - consisting of the two ECs, AEA and 2-AG, their main molecular targets, the cannabinoid CB1 and CB2 receptors, and their anabolic and catabolic enzymes - and the EC-like molecules, with their own receptors and enzymes, which partly overlap with those of the ECs (figure 1-1) Use a more recent representation of the eCBome. The eCBome is involved in various physiological and pathological aspects of the body and can be the target of many drugs (Iannotti & Piscitelli, 2018; Aizpurua-Olaizola et al., 2017). It is a signalling system found in several animal species, indicating an essential role in vital functions.

1.1.1. Endocannabinoids and Endocannabinoid-like molecules

The EC system includes the arachidonic acid-containing, arachidonic acid-derived mediators capable of binding to two cannabinoid receptors of CB1 and CB2, and the enzymes responsible for EC biosynthesis and degradation (Batetta et al., 2009).

N-arachidonoylethanolamine, better known as anandamide (AEA), is a fatty acid mediator derived from the metabolism of arachidonic acid essential omega-6 fatty acid and was the first EC to be discovered (Devane et al., 1992). Then, the other endocannabinoid, 2-Arachidonoylglycerol (2-AG) is present in most tissues in much greater concentrations than AEA (Stella et al., 1997; Matias et al., 2008).

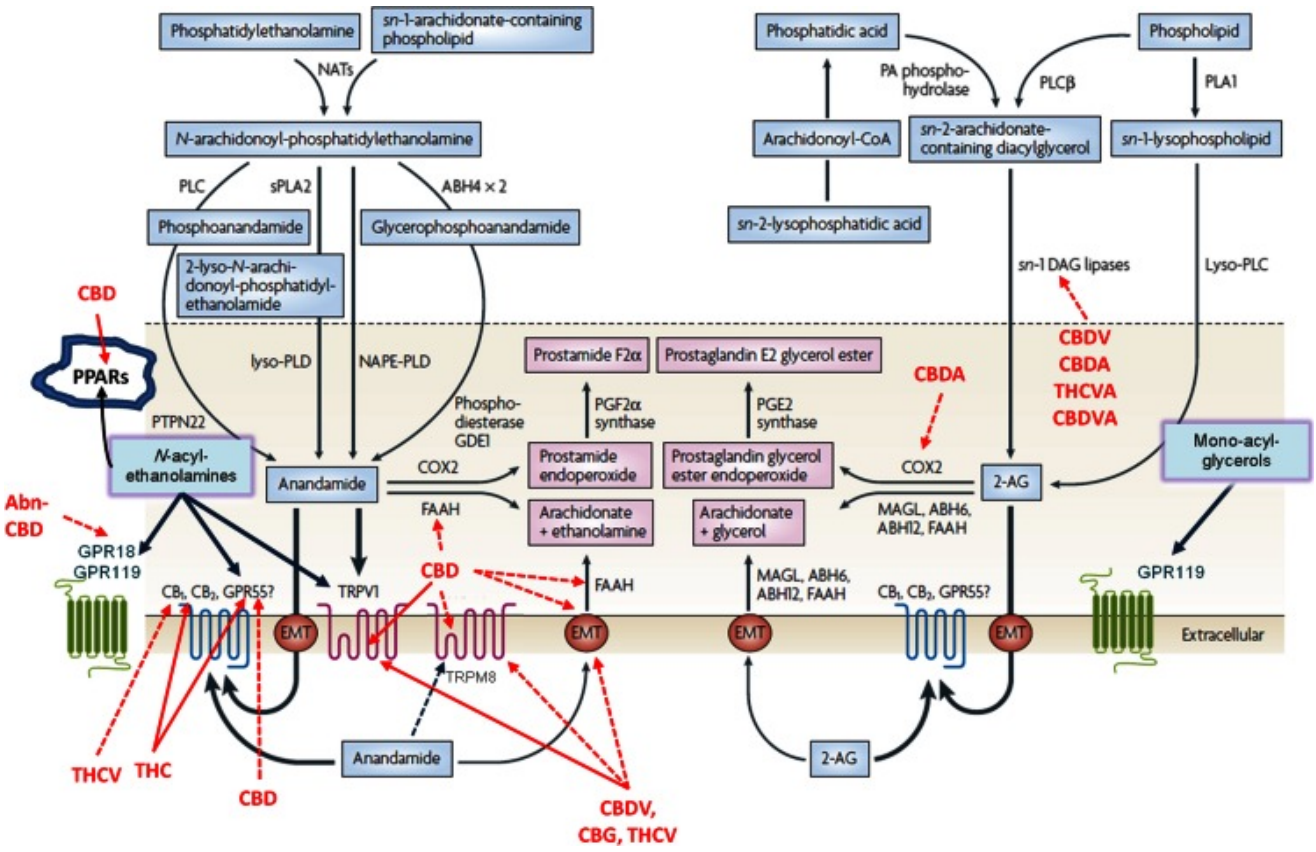

Figure 1-1. Endocannabinoidome system (Di Marzo & Piscitelli, 2015)

Endocannabinoids are an arachidonate-containing NAE or 2-MG, but there are similar molecules containing other fatty acids including oleic acid, palmitic acid, linoleic acid, stearic acid which are known as non-EC NAEs and 2-MGs. These molecules usually cannot activate the cannabinoid receptors, but target other receptors including GPR119, TRPV1 and PPAR-α. The synthesis and degradation pathways and enzymes of these molecules are common to those of the ECs (Figure 1) (Hansen et al., 2015; Overton et al., 2006).

The main synthetic pathway of AEA includes two main steps: 1) synthesis of N-arachidonyl-phosphatidylethanolamine (NAPE) from arachidonic acid and phosphatidylethanolamine catalyzed by specific N-acyltransferases, and 2) synthesis of AEA and phosphatidic acid from NAPE via the N-arachidonyl-phosphatidylethanolamine phospholipase (NAPE-PLD) (Maccarrone, 2017). Through another pathway, NAPE can be converted to AEA via the sequential action of α,β -hydrolase-4 (ABHD4) and phosphodiesterase GDE1 (Di Marzo & Piscitelli, 2015)

AEA and non-endocannabinoid NAEs may also be formed by the sequential action of a sPLA2 and lysoPLD (Sun et al., 2004). Finally, NAPE has also been suggested to be transformed to AEA by a another two-step process including the production of phosphoanandamide by a PLC and its dephosphorylation to AEA via Ptpn22 (J. Liu et al., 2006).

There are also different pathways suggested for synthesis of 2-AG from arachidonic acid-containing membrane phospholipids. The first and most important pathway is the hydrolysis of inositol phospholipids containing arachidonic acid at the sn-2 position by PLC, followed by the hydrolysis of the resulting arachidonic acid-containing DAG by DAGL to produce 2-AG. In the second pathway, phosphatidylinositol may be converted to lyso-PI via PLA1, then lyso PI-specific PLC catalyze the production of 2-AG from lyso-PI (Ueda et al., 2011).

Degradation of AEA into AA and ethanolamine is linked to various enzymes. The first and main pathway is catalysed by fatty acid amide hydrolase (FAAH) (Maccarrone, 2017). N-acylethanolamide-hydrolyzing acid amidase (NAAA) is a NAE-hydrolyzing enzyme with a preference for PEA, a NAE that exerts anti-inflammatory and analgesic effects via stimulation of nuclear receptor of PPAR-α (Y. Li et al., 2012). Furthermore, AEA oxidation through cyclooxygenase-2 (COX-2) and prostaglandin synthases results in prostaglandin-ethanolamides (PGs-EA) or prostamides. In another pathway, lipoxygenases convert AEA into hydroxy-

anandamides or hydroxyeicosatetraenoyl-ethanolamides (HETEs-EA). Cytochrome P450 oxygenases instead convert AEA to epoxyeicosatrienoyl-ethanolamides (EETs-EA) from AEA (Maccarrone, 2017).

Degradation of 2-AG and other MAGs is controlled by the main hydrolysing pathway via MGLL, a serine hydrolase, to produce fatty acids and glycerol (Dinh et al., 2002). MGLL also functions as a key lipolytic enzyme and catalyzes the last step in the hydrolysis of lipid storage (Karlsson et al., 1997). ABHD6 and ABHD12 enzymes, belonging to the α/β-hydrolase family, are two other serine hydrolases that can hydrolase 2-AG to a smaller extent than MGLL (Savinainen et al., 2012; Wei et al., 2016)

1.1.2. Receptors linked to the endocannabinoidome

1.1.2.1. CB1 and CB2

Cannabinoid receptors, including CB1 and CB2, are G-proteins coupled receptors (GPCRs). CB1 receptors are the most abundant metabotropic receptors in the central nervous system and in neurons; however, they have been identified also in peripheral tissues. CB_2 receptors are very abundant in immune tissues and cells (Galiègue et al., 1995; Pertwee, 1997; Demuth & Molleman, 2006).

Following CB1 and CB2 receptor activation by their agonists, multiple intracellular signal transduction pathways are triggered. The principal mechanisms triggered by activation of cannabinoid receptors are inhibition of the adenylate cyclase(AC)/cAMP cascade, voltage-gated calcium channels, stimulation of potassium channels and promotion of mitogen-activated protein kinase (MAPK) (Aizpurua-Olaizola et al., 2017).

The AC/cAMP cascade is an important intracellular mechanism controlling cell functions including cell survival, differentiation and proliferation. Furthermore, cAMP regulates the activity of various ion channels, including voltage-gated K^+ and Ca^{2+} (Iannotti et al., 2016). Through coupling of the receptor to $G_{i/0}$ proteins, the α_i subunits of the latter inhibit AC and the synthesis of cAMP, which results in less stimulation of PKA and boosted activation of potassium channels type A. Furthermore, α_0 subunits of G proteins can decrease activation of voltage dependent

Ca^{2+} channels. β_γ subunits interact with other intracellular pathways which are linked to PLC, PI3K or PKB/Akt (Fernández-López et al., 2013)

1.1.2.2. Other GPCRs

Other three additional orphan G-protein-coupled receptors including GPR18, GPR55 and GPR119, are also known to be members of the endocannabinoidome (O'Sullivan et al., 2011).

GPR55 seems to be activated by Δ^9-THC, some synthetic cannabinoids, and low micromolar concentrations of AEA, 2-AG and PEA. GPR55 is linked to different molecular signaling cascade including Ca^{2+} release, nuclear factor of activated T cells, cAMP response element-binding protein (CREB)- and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)-induced transcription (Iannotti et al., 2016).

GPR18 is localized in spleen, bone marrow, thymus, lung, cerebellar tissue and endometrial cells. It may be activated Δ^9-THC and N-arachidonoyl glycine (NAGly). Activation of GPR18 by its agonists enhances MAPK activity, increases intracellular calcium and ERK1/2 phosphorylation (Console-Bram et al., 2014).

The GPR119 receptor has a key role in the secretion of metabolic hormones known as incretins in response to food consumption. Endocannabinoidome agonists of GPR119 include OEA, LEA and 2-OG (Syed et al., 2012). Activation of GPR119 through effects on Gs leads to increases in AC activity and hence activates PKA. Moreover, ATP-sensitive K^+ and voltage-dependent Ca^{2+} channels may be involved in GPR119-mediated responses (Godlewski et al., 2009).

1.1.2.3. Transient receptor potential (TRP) channels including TRPV1

Transient receptor potential (TRP) channels, a group of membrane proteins, including TRPV1, TRPV2, TRPV3, TRPV4, TRPA1, and TRPM8, been reported to mediate cannabinoid and EC activity, and are known as ionotropic cannabinoid receptors (Muller et al., 2019).

The transient receptor potential vanilloid type-1 (TRPV1) channel, also known as the capsaicin receptor or vanilloid receptor 1, is a nonselective ion channel with six transmembrane domains and a short, pore-forming hydrophobic stretch between the fifth and sixth transmembrane domains. TRPV1 is activated by the vanilloid capsaicin, noxious heat (>43°C) and low pH, voltage,

various lipids (Rosenbaum & Simon, 2007), and eCBome mediators (Meletis, 2019). TRPV1 activation allows Ca^{2+} to pass from the extracellular side of the membrane to the intracellular side, thus increasing intracellular calcium, which can activate a series of signaling pathways in the cell (Du et al., 2019). TRPV1 is involved in different processes, including thermosensation and energy homeostasis. It may affect energy homeostasis, either in the control of food intake or energy expenditure, through controlling appetite and producing heat, respectively (Christie et al., 2018).

1.1.2.4. Peroxisome proliferator-activated receptors (PPARs)

The peroxisome proliferator-activated receptors (PPARs) are a group of nuclear receptors. Three forms are known: PPAR-γ, PPAR-α, and PPAR- β/δ which are encoded by different genes. These receptors act as ligand-regulated transcription factors to control the gene expressions. The PPARs have an important physiological role in energy homeostasis and metabolic function (Tyagi et al., 2011).

A variety of saturated and unsaturated fatty acids fatty acids, such as linoleic acid, linolenic acid, palmitic acid, oleic acid, arachidonic acid, eicosapentaenoic acid and fatty acid derivatives, such several eCBome molecules, are recognised as natural ligands for the PPARs (Berger & Moller, 2002; Ramer et al., 2019).

The PPAR- γ isotype is principally expressed in the WAT, where it is an essential component of the adipocyte differentiation program, and in macrophages, to modulates differentiation and cytokine production. The expression of PPAR-β is observed in many tissues, but its function is not still clear. PPAR-α, is mainly expressed in the liver and brown adipose tissue (BAT), kidneys, muscle, and heart (Kersten et al., 1999).

Project 1

Changes in the eCBome following food restriction and excessive exercise

2.1. Literature review

2.1.1. Imbalance in energy intake and expenditure

Body energy is controlled by both the central and peripheral nervous system. The central nervous system controls metabolism through hypothalamic satiety and hunger centers in order to normalize energy balance (Koch & Horvath, 2014)

Since a long time, there has been an interest in food intake and energy expenditure to understand more about the mechanisms that relate intake to expenditure (Edholm et al., 1955). Energy expenditure is to a great extent the result of physical activity which is defined as any bodily movement produced by skeletal muscle. Physical activity in daily life can be categorized into occupational, exercise, conditioning, household, etc. (Caspersen, Powell, & Christenson, 1985).

The body mass regulation complies with the first law of thermodynamics, i.e. that energy can be converted but not produced or destroyed (Jéquier & Tappy, 1999). Thus, to understand the regulation of body mass, the concept of energy balance is important and is often calculated by the difference between energy intake including food and drink consumption and energy expenditure (Spiegelman & Flier, 2001). If there is an imbalance between energy intake and expenditure, body mass will change. A positive energy balance in which energy intake exceeds expenditure, leads to an increase in body mass, while a negative energy balance leads to a decrease in body mass (Swinburn & Ravussin, 1993).

There are highly integrated systems involved in the coordination of energy storage and release processes that act at several physiological levels. As for many complex physiologic systems, the hypothalamus has an essential adjusting role in energy homeostasis through regulation of food intake by hunger and satiety, energy expenditure by thermogenesis, and by acting as a target of peripheral hormones such as insulin, leptin, ghrelin etc. (Flier, 1995; Gale, Castracane, & Mantzoros, 2004).

Thus, exercise is an important factor in the maintenance of an optimal body composition when it is coupled with dietary intake of macro and micronutrients, meeting the daily energy requirement in order to preserve the fat-free mass (Hill et al., 1987). An important source of fuel for endurance exercise are triacylglycerols (TAG). During exercise there is an increase in the

oxidation of TAG-derived fatty acids to meet the energy requirements of muscles. Exercise increases the oxidation of non-plasma-derived fatty acids, which, in the absence of sufficient fat stores in the adipose tissue, might be taken from intramuscular triacylglycerol stores (Horowitz & Klein, 2000). Lipid mediators of the eCBome are and are important modulators of energy homeostasis, are involved in controlling food intake and satiety, and are regulated by physical activity (Di Marzo & Matias, 2005; Jaromin et al., 2019)

2.1.2. Fat metabolism

The adipose tissue plays various roles in vertebrate physiology, such as thermal insulation, mechanical role, fuel storage, anti-inflammatory actions and glucose homeostasis. There are two forms of adipose tissue: white adipose tissue (WAT) and brown adipose tissue (BAT). WAT accounts for the majority of the fat mass in the body and is critical for energy homeostasis and insulin signaling; however, BAT is principally responsible for thermogenesis through a mitochondrial pathway. WAT provides a long-term fuel storage linked to the size of the adipocytes, which is enlarged with positive energy balance and reduced with excess of energy expenditure (Trayhurn & Beattie, 2001). Most energy reserves in the body are stored in adipocytes as TAGs. Mechanisms that control the deposition or mobilization of TAGs in adipocytes are principal regulators of fat accumulation (Lafontan & Langin, 2009). Fat accumulation is linked to the balance of differentiation of pre-adipocytes into mature fat cells, known as adipogenesis, fat synthesis via lipogenesis and fat breakdown through lipolysis and subsequent fatty acid oxidation (S Kersten, 2001; Hristov et al., 2019).

2.1.2.1. Adipogenesis

Adipogenesis occurs in 2 steps: 1) increasing the numbers of preadipocytes, and 2) differentiation of preadipocytes into mature adipocytes (de Ferranti & Mozaffarian, 2008).

Mesenchymal stem cells undergo mitosis and represent a stable pool of preadipocytes to be differentiated into adipocytes (MacDougald & Mandrup, 2002). The peroxisome proliferator-activated receptor γ (PPAR-γ), and the CCAAT/enhancer binding proteins (C/EBPs) including C/EBPα, β and δ are the main early regulators of adipogenesis, whereas fatty acid binding protein

4 (FABP4), adiponectin, and fatty acid synthase (FAS) are considered to produce mature adipocytes (Moseti et al., 2016).

2.1.2.2. Lipogenesis

Lipogenesis is a pathway in liver and adipose tissue that converts additional non-lipid nutrients such as carbohydrates, into fatty acids and then esterifies them to TAGs for energy storage (Moseti et al., 2016).

In conditions of positive energy balance, extra glucose is transformed to pyruvate through glycolysis, then pyruvate is transported into the mitochondria and to become the substrate for the tricarboxylic acid cycle (TCA) and produce Citrate, which is imported into the cytosol and converted to acetyl-CoA by ATP citrate lyase (Browning & Horton, 2004). Acetyl-CoA, by the catalysis of acetyl-CoA carboxylase through ATP-dependent carboxylation is converted to malonyl-CoA (Kim, 1983), which donates two-carbon to the acetyl-CoA primer by FAS and eventually produces palmitic acid (16:0), the principal fatty acid formed through de novo lipogenesis. Palmitic acid, through the elongation process by fatty acyl-CoA elongase (Elovl) family members, can be converted to long-chain fatty acids with over 16 carbon chains (X. Liu et al., 2017). These fatty acids are then esterified into TGs and, via very low-density lipoproteins, transported to and stored in the adipose tissues as an energy reservoir (van der Spek et al., 2012; Ameer et al., 2014).

2.1.2.3. Lipolysis

During energy deprivation, lipolysis is a catabolic process leading to the breakdown of TAGs stored in the adipose tissue into free fatty acids and glycerol via the consecutive actions of adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), and monoglyceride lipase (MGLL) (Duncan et al., 2007), with the liberation of fatty acids in two sequential steps via the formation of diacylglycerol (DAG) and monoacylglycerol (MAG) intermediates by the enzymes ATGL and HSL, respectively. In the last step, MAG is hydrolyzed via MGLL to release fatty acids and glycerol (Bolsoni-Lopes & Alonso-Vale, 2015)

2.1.2.4. Beta-oxidation

After their release into the bloodstream, fatty acids are transported and taken up by other tissues to be utilized for beta-oxidation and subsequent ATP generation (Adeva-Andany et al., 2019). In different cells, peroxisomes and mitochondria develop beta-oxidative pathways that have many similarities, particularly in terms of enzymatic reactions; however they are different in substrates, metabolic implication and final products (Demarquoy & Le Borgne, 2015). Mitochondrial β-oxidation of fatty acids is an anaerobic catabolic pathway that includes activation of various enzymes and generates acetyl-coA, $NADH^+$ and $FADH_2$ (Adeva-Andany et al., 2019)

Long chain fatty acids need to be chemically modified to enter the mitochondria. Therefore, they are initially activated to fatty acyl-CoAs by acyl-CoA synthetases (ACSs). Since, ACSL1 knockout mice during exercise have shown less fatty acid oxidation than WT once, it seems that this isoform is crucial for fatty acid beta-oxidation in the skeletal muscle (L. O. Li et al., 2015).

Next, at the external mitochondrial membrane, the fatty acyl-CoAs are converted to their fatty acyl carnitines, through a reaction that requires carnitine and the enzyme carnitine palmitoyl transferase 1 (CPT1). Later, the fatty acyl carnitines translocate across the inner mitochondrial membrane and enter into the mitochondrial matrix via carnitine acylcarnitine translocase (CACT) and then are converted back to acyl-CoAs via carnitine palmitoyl transferase 2 (CPT2) (Bonnefont et al., 2004; Dasgupta & Wahed, 2014).

In the mitochondrial matrix, beta-oxidation occurs by cleaving two carbons in each cycle to form final product of acetyl-CoA. The acyl-CoAs are dehydrogenated to create a trans double bond between the second and third carbons via acyl CoA dehydrogenase, to produce trans-delta 2-enoyl CoA, which is hydrated at the double bond to produce L-3-hydroxyacyl CoA by enoyl-CoA hydratase. Later, L-3-hydroxyacyl CoA is dehydrogenated to create 3-ketoacyl CoA by 3-hydroxyacyl CoA dehydrogenase enzyme. Finally, β-ketothiolases by using a free CoA produce a new acyl-CoA molecule which has two carbons less, and an acetyl-CoA (Goetzman, 2011).

In peroxisomal beta-oxidation, through the first step, fatty acids are converted to acyl-CoA, which influx to the peroxisomal matrix via the ABC transporter (ABCD1). Then, acyl-CoA oxidase (ACOX) catalyzes the first reaction, which is same as the first mitochondrial beta-oxidation reaction with a difference in hydrogen transfer. The multifunctional enzyme is a catalyzing enzyme in the

second and third peroxisomal beta-oxidation reactions that result in the formation of 3-ketoacyl-CoA. The final step of this pathway is catalyzed by thiolase to produce acetyl-CoA and Cn-2-acyl-CoA (Demarquoy & Le Borgne, 2015). Peroxisomal beta-oxidation leads to synthesis of DHA and its retroconversion to EPA (Kanamori et al., 2018). It has been also shown in an in-vitro study that non-neural cells have a greater rate of retroconversion of DHA to EPA compared to neurons (Park et al., 2016). Furthermore, an in vivo study in rats demonstrated that DHA can increase the peroxisomal beta-oxidation rate and that EPA with hypotriglyceridemic action may cause a stimulation of both mitochondrial and peroxisomal oxidation (Willumsen et al., 1993).

2.1.3. PPAR-α

PPAR-α is mainly expressed in tissues with elevated mitochondrial and peroxisomal fatty acid oxidation rates. In the metabolic tissues, especially liver, PPAR-α regulates the expression of proteins involved in the transport and beta-oxidation of free fatty acids (FFAs). PPAR-α upregulates proteins that facilitate the uptake of long chain fatty acids by the liver, named as fatty acid transport protein (FATP), after which fatty acids are then submitted to hepatic beta-oxidation (van Raalte et al., 2004).

PEA, is a NAE which selectively activates PPAR-α in vitro and promotes the mRNA expression of PPAR-α and reduces inflammation (Lo Verme et al., 2005). OEA is a more potent NAE agonist of PPAR-α, through which it decreases of appetite and weight gain mostly through peripheral mechanisms (Fu et al., 2003). Administration of OEA (5 mg kg–1) also stimulates lipolysis in both rats and wild-type mice, but not in PPAR-α knockout mice, which shows that this mediator may cause fat loss, for example in the adipose tissue and skeletal muscle, via activation of PPAR-α (Guzman et al., 2004).

2.2. Hypothesis

We hypothesized that calorie restriction and physical activity together, through dramatic changes in tissue lipid deposition, influence the tissue concentrations of eCBome mediators that may mediate the physiological adaptation of the body to acute and extreme adipose tissue depletion. Therefore, we employed an animal model of anorexia nervosa, in which both calorie restriction and excessive exercise are administered to rats, to investigate changes in eCBome mediator levels in metabolic tissues in response to this treatment.

This part of project was performed in university of Cagliari, Italy.

2.3. Experimental approach and methodology

2.3.1. Animals and experimental design

In this experiment 32 female Sprague Dawley rats for 6 days were divided to two groups: 1- Acute calorie restriction combined with a sustained physical activity (CRR) in rats fed for 1.5 h/d and 22.5h/d activity wheel access, and 2- Control (Crtl) i.e. rats that had food ad libitum and no access to the activity wheel. After 6 days each group was divided to two groups of eight rats: two subgroups of CRR and Ctrl rats were sacrificed and the other two subgroups continued the second phase for another 10 days under the following situation: 3- refeeding phase (REF), in which rats were allowed food access ad libitum and 24 h access to the activity wheel and 4- control refeeding phase, in which rats had food ad libitum and no access to the activity wheel. Also, these two subgroups were then sacrificed by decapitation on the day 16 (Figure 2-1).

Aliquots of liver and muscle and visceral adipose tissue (VAT) were collected and stored in -80$^{\circ C}$.

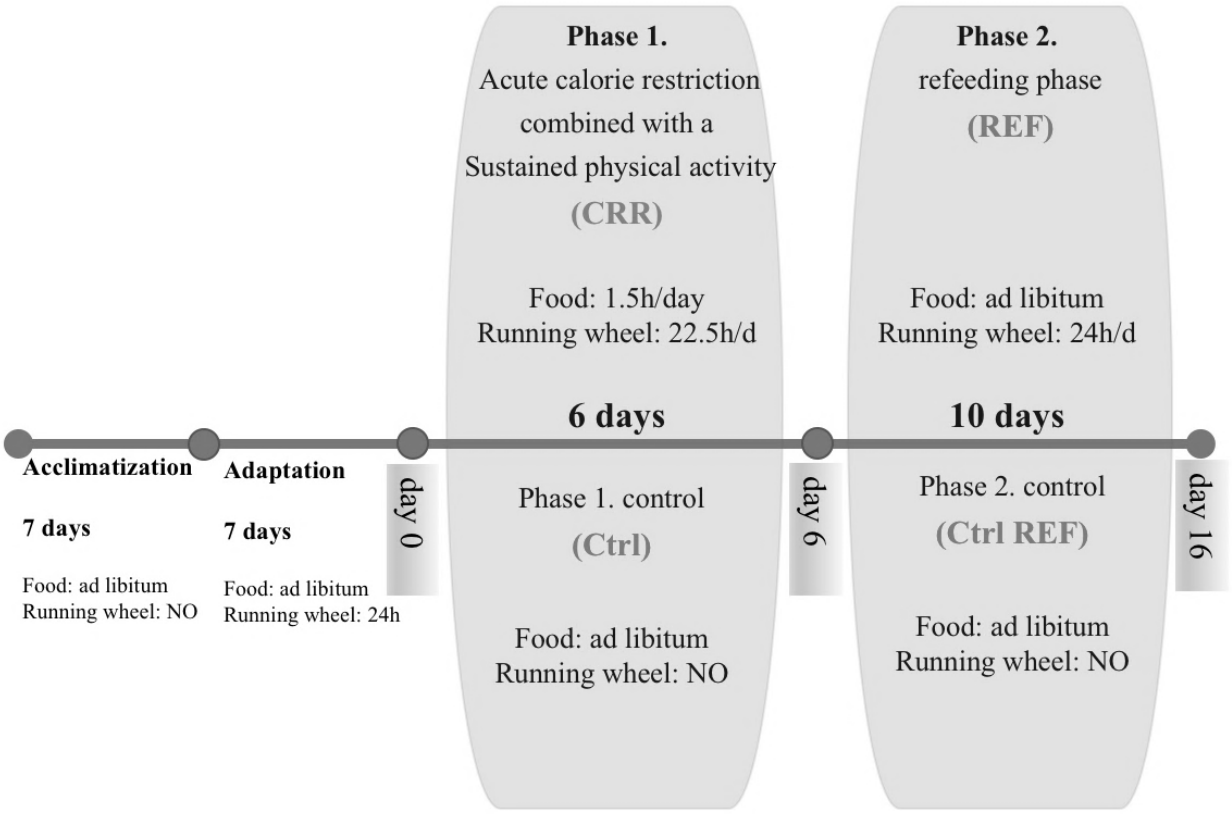

Figure 2-1. Experiment design

2.3.2. Tissue analysis of lipids

Acetonitrile (CH3CN), methanol (CH3OH), chloroform (CHCl3), *n*-hexane (C6H14), ethanol (C2H5OH), acetic acid (CH3COOH) were HPLC grade and purchased from Sigma Chemicals Co., St. Louis, MO, USA. All standards of SFAs, UFAs were purchased from the same company.

Ascorbic acid, potassium hydroxide (KOH), hydrochloric acid (HCl) were purchased from Carlo Erba, Milano, Italy. Deferoxamine mesylate (desferal) was purchased from CIBA-Geigy, Basel, Switzerland. Internal deuterated standards for AEA, 2-AG, PEA and OEA quantification by isotope dilution ([2H]8AEA, [2H]52AG, [2H]4 PEA, [2H]4 OEA) were purchased from Cayman Chemicals, MI, USA.

2.3.3. Extraction of total lipids

Total lipids were extracted by the method of Folch (Folch et al., 1957). Samples of liver (~0.4 g), and adipose tissue (~0.06 g), were homogenized each into a 2:1 chloroform-methanol solution containing 2 µg of vitamin E and deuterated AEA (200 ng), 2-AG (300 ng), OEA (200 ng), and PEA (100 ng).

Tubes containing lipids under extraction were kept one hour in the dark, added an equal volume of double-distilled water (ddH2O) to that of methanol present, then left another hour in the dark. Samples were centrifuged for one hour at 900 x *g* to facilitate the separation of the chloroform phase from the aqueous methanol.

The lower chloroform phase containing lipids was collected, divided in three different aliquots for subsequent analyses and evaporated under vacuum by a rotator evaporator at room temperature.

2.3.4. Quantitative determination of total lipids

Total lipid quantification was performed by the method of Chiang (Chiang et al., 1955) on evaporated aliquots of chloroform phase containing lipids, collected after initial lipid extraction. 1.5 ml of the Chiang reagent (2 g of K2Cr2O7 into 4 ml ddH2O, make up to a final volume of 100 ml adding H2SO4) was added, and the samples incubated for 30 min. at 100 $^{\circ}$C. Finally, 1.5 ml of ddH2O was added, and the absorbance measured at the wavelength of 600 nm by a

spectrophotometer. To determine the concentration of total lipids a standard curve of corn oil was used, and the range of reliability of the method was between 100 and 800 mg of lipids.

2.3.5. **Fatty acid analysis of tissue lipid fraction**

An aliquot of the lipid fraction for each sample was mildly saponified using a procedure in order to obtain UFAs for HPLC analysis. Lipid extracts were dissolved in 5 ml of ethanol, 100 µl of desferal (25 mg/ml ddH2O), 1 ml of a 25% solution of ascorbic acid in water, 0.5 ml of 10N KOH, and left 14 hours in the dark at room temperature. Later, 10 ml of *n*-hexane and 7 ml of ddH2O were added, then the samples were acidified with 0.35 ml of 37% HCl, to a pH rang of 3 to 4. Samples were centrifuged for 1h at 900 43 xg. The hexane phase containing free fatty acids was collected, the solvent evaporated, and the residue was dissolved in 0.5 ml of CH3CN/0.14% of CH3COOH (v/v).

Separation of fatty acids was carried out with an Agilent 1100 HPLC system (Agilent, Palo Alto, Calif., USA) equipped with a diode array detector. A C-18 Inertsil 5 ODS-2 Chrompack column (Chrompack International BV, Middleburg, The Netherlands), 5 µm particle size, 150 x 4.6 mm, was used with a mobile phase of CH3CN/H2O/CH3COOH (70/30/0.12, v/v/v) at a flow rate of 1.5 ml/min (Melis et al., 2001). Conjugated diene unsaturated fatty acids were detected at 234 nm. Spectra (195–315 nm) of the eluate were obtained every 1.28 s and were electronically stored. Second-derivate UV spectra of the conjugated diene fatty acids were generated using Phoenix 3D HP Chemstation software (Agilent, Palo Alto, CA). These spectra were acquired to confirm identification of the HPLC peaks (Angioni et al., 2002).

Since SFAs are transparent to UV, after derivatization, they were measured as fatty acid methyl esters (FAMEs), by a gas chromatograph (Agilent, Model 6890, Palo Alto, CA) equipped with split ratio of 20:1 injection port, a flame ionization detector (FID), an autosampler (Agilent, Model 7673), a 100 m HP-88 fused capillary column (Agilent). Finally, data were analyzed by the Agilent ChemStation software system. The injector and detector temperatures were set up at 250°C and 280°C, respectively. H2 served as carrier gas (1 ml/min), and the FID gases were H2 (30 ml/min), N2 (30 ml/min), and purified air (300 ml/min). The temperature program was as follows: initial

temperature was 120°C, programmed at 10°C/min to 210°C and 5°C/min to 230°C, then programmed at 25°C/min to 250°C and held for 2 min (Batetta et al. 2009).

2.3.6. **Analysis of the NAE mediators**

NAEs were quantified in aliquots of organic phase (chloroform) containing extracted lipids were evaporated to dryness under vacuum and reconstituted with 0.4 ml of 100% methanol tissue samples by 6460C Triple Quadrupole LC/MS(Agilent).

2.3.7. Western blot

Western blot analysis was done to evaluate of total PPAR-α protein levels using the Lowry protein assay method (Lowry et al., 1951; Carta et al., 2018). Briefly, bovine serum albumin was used as the standard. Tissue homogenates were prepared by a solution of sodium dodecyl sulfate (2%-SDS) which contained a complete protease inhibitor cocktail (Roche, Basel, Switzerland).

40 µg of each tissue homogenates were diluted 3:1 in 4× NuPAGE LDS Sample Buffer (Novex by Life Technologies, Carlsbad, CA, USA) as a loading buffer and kept in 95°C for 7 min. Then, proteins were separated through SDS-polyacrylamide gel electrophoresis (SDS-PAGE), using NuPAGE 4–12% Bis-Tris Gel Midi gel (Novex by Life Technologies) in the XCell4 Sure LockTM Midi-Cell chamber (Life Technologies). In the meantime, Internal mw standards of Precision Plus ProteinTM WesternCTM Standards (Bio-Rad, Hercules, CA, USA) were run. In order to immunoblotting, proteins were placed on a polyvinylidene fluoride membrane (Amersham HybondTM-P, GE Healthcare, Little Chalfont, U.K.) via CriterionTM Blotter (Bio-Rad).

After the gel immersion in a solution containing three different bases of 20 mM Tris base and 137 mM sodium chloride (TBS) (containing 5% milk powder) and 0.1% Tween 20 (TBS-T), for 60 min at room temperature, blots were blocked. Later, blocked blots incubated overnight at 4 ◦C with PPAR-α (Thermo Scientific, Waltham, MA, USA), diluted 1:1000 in TBS containing 5% milk powder and 0.02% sodium azide. The day after, TBS-T rinse was done, then blots were incubated for 60 min, at room temperature, with peroxidase-conjugated goat anti-rabbit serum (Sigma Aldrich, Milan, Italy), diluted 1:10,000 in TBS-T. Stripping and immunostaining the membranes with a

mouse monoclonal antibody against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping protein (EMD Millipore, Darmstadt, Germany), diluted 1:1000, as primary antiserum, and a peroxidase-conjugated goat anti-mouse serum (EMD Millipore-Merck, Darmstad, Germany), diluted 1:5000, as secondary antiserum, resulted to obtaining loading controls. Then, blots were stripped and incubated with the relevant secondary antiserum to control non-specific staining.

protein bands were visualized by means of the ECL chemiluminescent system according to the manufactures protocol (GE Healthcare), under ImageQuant LAS 4000. Approximate molecular weight (mw) and relative optical density (O.D.) of immunolabeled protein bands were measured by a "blind" examiner and were assessed by comparing the position of relevant bands on the digital images with those of the GAPDH bands, respectively. The O.D. was measured through Image Studio Lite Software (Version 5.2, Li-Cor).

The intensity ratio of PPAR-α-positive bands to the intensity of GAPDH-positive ones was used to compare relative expression levels of these proteins following BCCAO/R procedure.

2.3.8. **Statistical analysis**

The data are expressed as the mean±SD or s.e.m as specified in the legends. Differences between two groups were assessed using an unpaired, two-tailed Student's t-test. Data sets involving more than two groups were assessed by ANOVA followed by a Tuckey's post hoc test. Data with different superscript letters were significantly different ($P<0.05$) according to the post hoc ANOVA statistical analysis. Data were analysed using graphpadprism (Broadway – Nedlands, Western Australia). The results were considered statistically significant for $P<0.05$.

2.4. Results

2.4.1. Body weight and Food intake

In the CRR group there was a significant decrease of body weight from day one, which increased further through the six days. This reduction in body weight might be related to the significant less food intake and more physical activity through the activity wheel that we recorded daily during the protocol.

Then, after starting the second phase, the REF group started to have more food intake than the Ctrl REF and both groups showed an in increase in body weight ($p < 0.05$) (Figure 2-2).

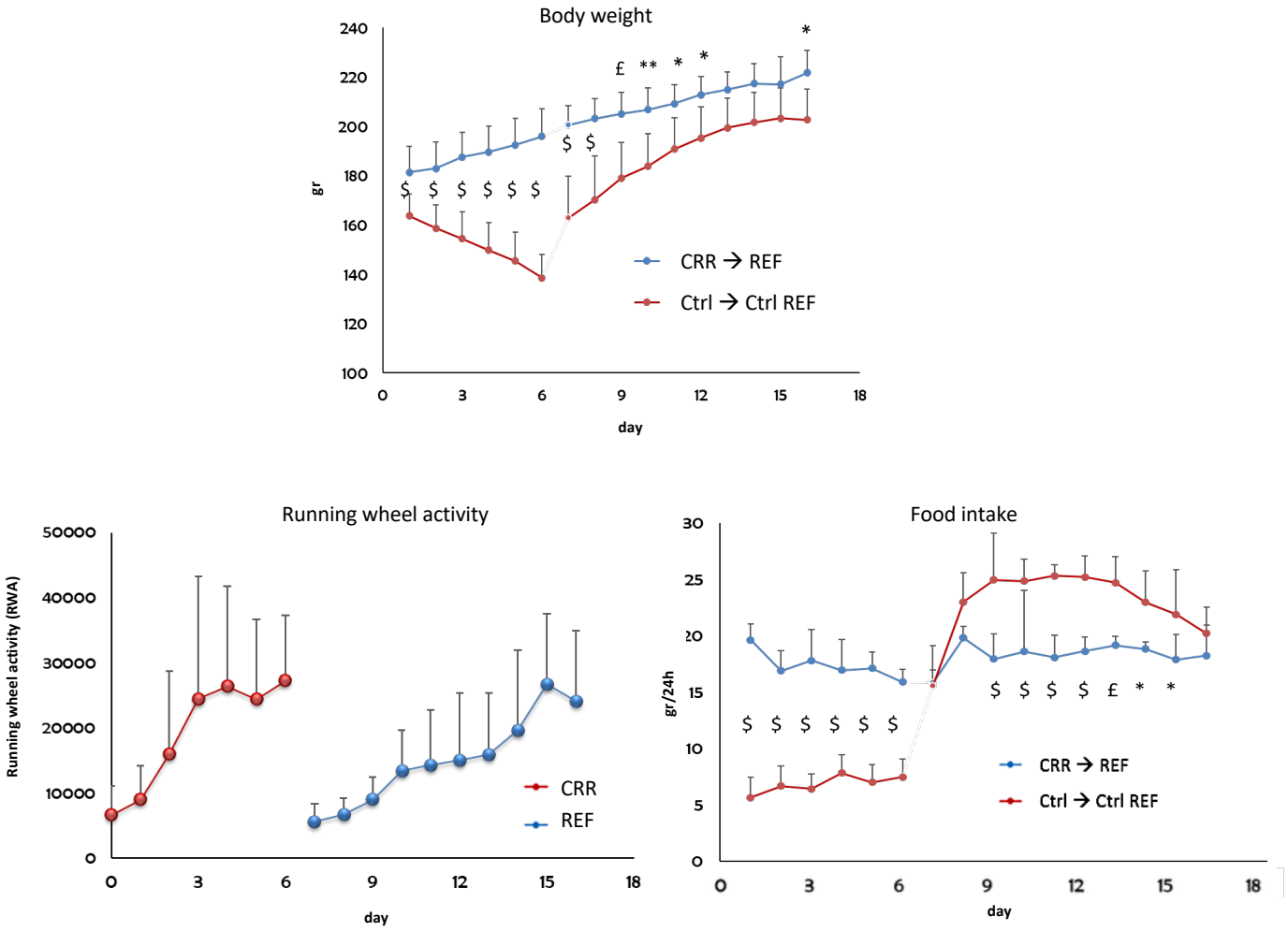

Figure 2-2. food consumption, physical activity and body weight gain.

2.4.2. Metabolic tissue analysis

Food restriction and excessive exercise resulted in a significant decrease in the weight of liver and visceral adipose tissue. In addition, there was a seven-fold fat depletion per gram of adipose tissue, but there were no changes in the total fat amount of liver; however, after the refeeding phase (REF) weight and the mg lipid of both tissues increased ($p < 0.05$) (Figure 2-3)

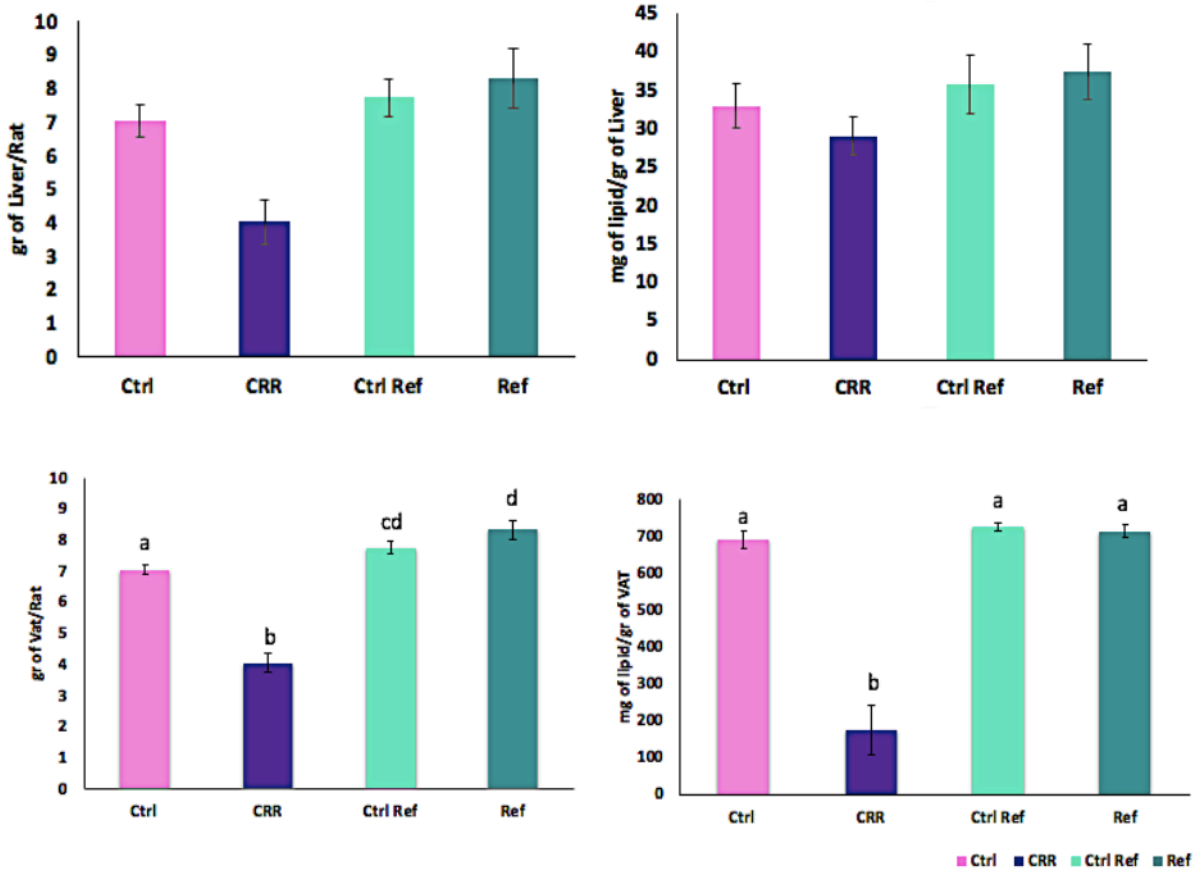

Figure 2-3. total weight and total fat amount in liver and visceral adipose tissue.

2.4.3. Endocannabinoidome analysis in Liver and Visceral adipose tissue

In both liver and visceral adipose tissue, analysis of NAEs showed a strong increase in the CRR group compared to the other groups ($p<0.001$ and $p<0001$, respectively) (Figure 2-4).

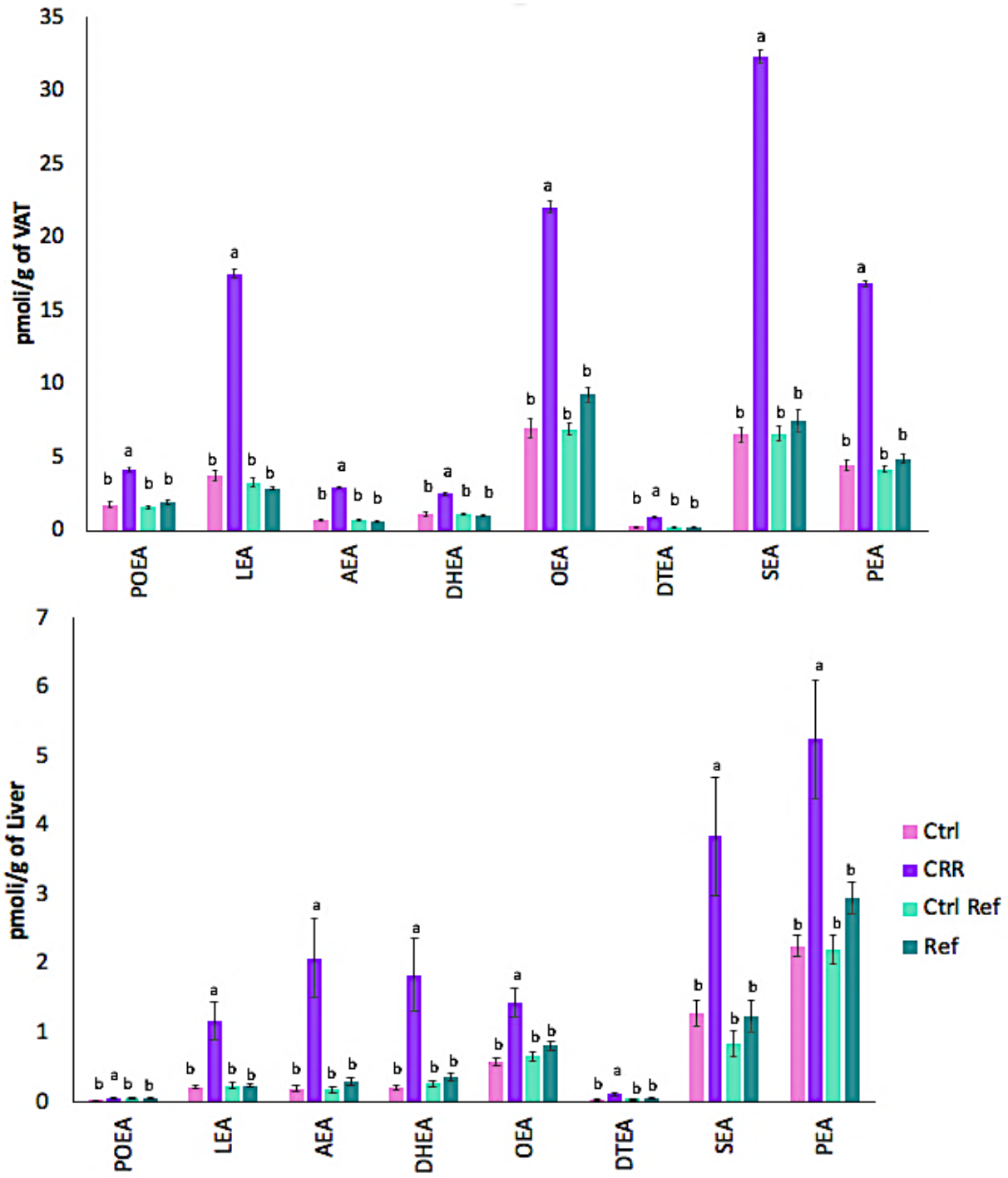

Figure 2-4. NAE levels in liver and muscle

2.4.4. Peroxisomal beta-oxidation index: DHA/EPA

The ratio of DHA/EPA, which shows the rate of peroxisomal beta-oxidation, was significantly increased in the CRR group, and later, in the REF groups, returned to its normal level (figure2-5).

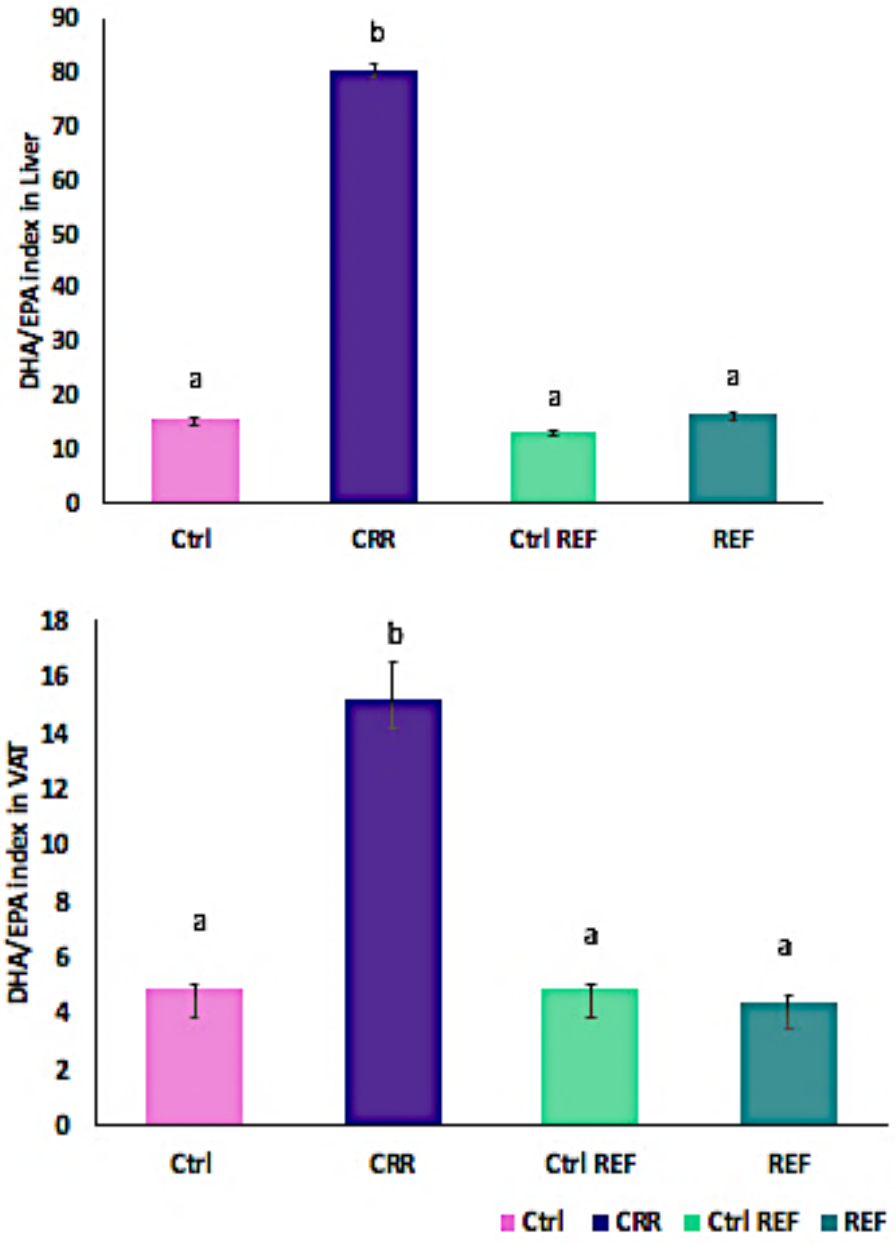

Figure 2-5. Proxisomal beta-oxidation index in both liver and muscle

2.4.5. Western blot analysis of PPAR-α

Densitometric Western blot analysis of PPAR-α in the liver showed an increase in the CRR group and then a subsequent decrease in the REF groups, which returned to their previous levels ($p<0.05$) (figure 2-6).

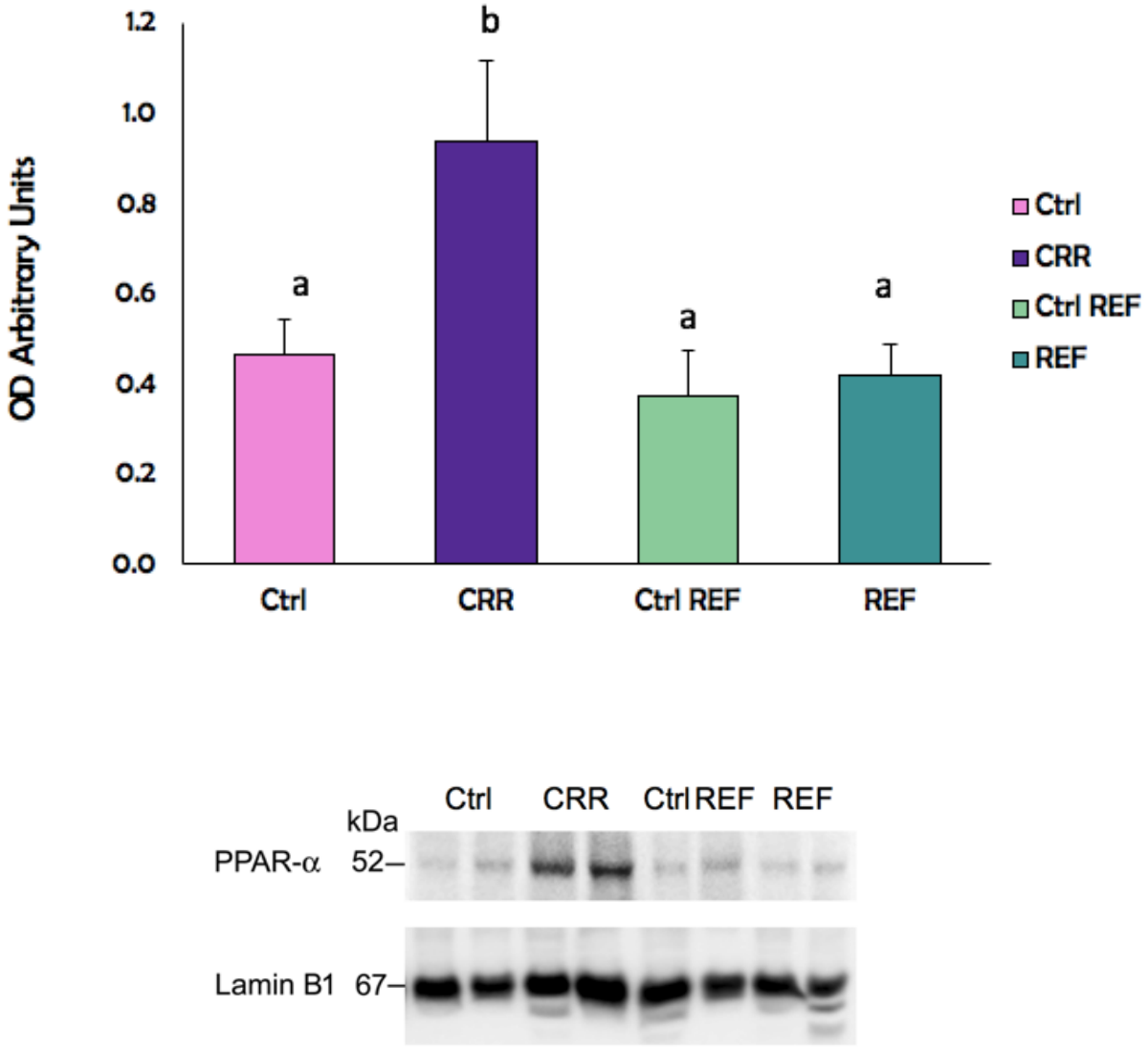

Figure 2-6. Western blot analysis of PPAR- α

2.5. **Discussion**

Our data clearly show that a strong negative energy unbalance results in a steep increase of NAEs in the two key tissues for lipid metabolism, i.e. the liver and adipose tissue. PPARs, including PPAR-α, are molecular targets that regulate genes involved in lipid and carbohydrate metabolism. Some eCBome molecules, such as PEA and, particularly, OEA, by acting through the activation of PPAR-α, can affect lipid metabolism (Bazwinsky-Wutschke et al., 2019). Therefore, this steep increase in NAE levels may represent an adaptive PPARα-mediated response necessary to mobilise energy as a result of the release of fatty acids from TAGs and subsequent fatty acid beta oxidation in tissues such as skeletal muscle and liver (Longo et al., 2016). Given the previously described positive correlation between the plasma levels of NAEs and their corresponding free fatty acids (Sihag & Jones, 2018), it is possible that part of the long chain fatty acids derived from enhanced PPARα-induced lipolysis may feed the increased levels of the corresponding NAEs, thus generating a positive feed-back loop.

In fact, since some NAEs, including OEA and PEA, have strong stimulatory effects on PPAR-α activity (Guzman et al., 2004; Lo Verme et al., 2005), the strong increase, particularly of these two eCBome mediators, may enhance PPAR-α alpha activity, which facilitates the use of fatty acids as a fuel to meet the energy needs during excessive exercise (Rubino, Zamberletti, & Parolaro, 2015). Additionally, PEA, SEA and, particularly, OEA may exert anorexigenic activity via peripheral mechanisms (Terrazzino et al., 2004), which may explain some of the effects shown in anorexia nervosa, where the excess of physical activity is accompanied by anorexia.

Furthermore, the results of our study confirmed the significant increase of the DHA:EPA ratio, linked to the increase of peroxisomal beta-oxidation levels. Both DHA and EPA are agonists of PPAR-α and increase the rate of beta-oxidation (Berge et al., 1999). Peroxisomal fatty acid β-oxidation can be regulated by PPARs, especially PPARα (Latruffe et al., 2000). Thus, increasing activity/levels of PPAR-α and the DHA/EPA ratio in the CRR group, may both underlie and be the consequence of increased NAE levels in the liver. Interestingly, in agreement with the observed increase in the DHA:EPA ratio, and with the aforementioned correlation between NAEs and

corresponding fatty acids, we detected in the CRR group relatively high levels of DHAE but not of EPAE.

Remarkably, in the recovery (refeeding) phase, a prompt recovery of fat deposition was associated to normal levels of NAEs, as well as of the other parameters studied here, such as lipid levels in the adipose tissue, DHA:EPA ratio and PPARα expression in the liver.

In summary, our results are consistent with the underlying hypothesis of this study that food restriction coupled to excessive exercise may impact on fatty acid metabolism via alterations in NAE levels, and that the rate of fatty acid metabolism and the concentrations of eCBome mediators in metabolic tissues are closely regulated by undernutrition and excessive exercise.

Project 2

Investigation into the effects of vitamin D and antipsychotic drug metabolic side effects: focus on the endocannabinoidome - gut microbiome axis.

3.1. Literature review and hypothesis

3.1.1. Vitamin D

Vitamin D deficiency represents a global health issue. Exposure to natural sunlight is the general source of vitamin D, but also some foods including oily fish naturally contain it. (Chen & Holick, 2008; Holick et al., 2011) Technically, vitamin D is a secosteroid which has an endocrine mechanism of action and it is synthesized in body (Pérez-López, 2007). There are two major types of Vitamin D: vitamin D2 (Ergocalciferol) and vitamin D3 (Calciferol) (Holick, 2006).

Generally, 7-dehydrocholesterol, the inactive form of vitamin D, produces pre-vitamin D3 in the skin, then, the formed pre-vitamin D3 or digested vitamin D2, bind to the vitamin D binding protein and are transported to the liver. Chemical reactions in the liver result in the conversion of pre-vitamin D3 or Vitamin D2 to the 25(OH)D by D-25-hydroxylase, which is still biologically inactivated. In the kidney 25(OH)D is converted to 1,25 dihydroxyvitamin D (1,25(OH)2D3), the active form of vitamin D, by 1-alpha-hydroxylase (Cutolo et al., 2007; Sirajudeen et al., 2019). The biological reactions of vitamin D are mediated by the vitamin D receptor (VDR), which is a DNA-binding transcription factor (Haussler et al., 2011).

Vitamin D status is defined by four different levels of: deficiency at <20ng/ml, insufficiency at 20-30 ng/ml sufficiency at 30-50 ng/ml and toxicity at >150ng/ml (Ashique et al., 2018)

Several aspects of the metabolic syndrome like as obesity, insulin resistance and hypertension which are clustering of conditions in response to overnutrition and sedentary lifestyles, are linked with vitamin D deficiency, even in areas with high levels of sun exposure (Steig et al., 2008; Strange et al., 2015; Al-Dabhani et al., 2017).

There have been an growing number of statistical and experimental studies on the association between vitamin D deficiency/insufficiency and obesity which confirm that obesity is highly prevalent in individuals with vitamin D deficiency (Chang & Kim, 2017; Pereira-Santos et al., 2015; Turer et al., 2013). Obese subjects are well-known to have lower levels of vitamin D compared to non-obese ones (Liel et al., 1988). There is also a link between increasing BMI and lower serum vitamin D concentrations (Vanlint, 2013). Furthermore, adiposity and body fat content have an inverse correlation to serum vitamin D concentration, which are stronger than those between vitamin D and BMI and body weight (Arunabh et al., 2003).

Whereas, the mechanism which shows the effects of vitamin D on metabolic syndrome is not still clear, it supposed that vitamin D, by activating vitamin D receptors or via the regulation of intra- and extra-cellular calcium pathway, may alter gene expression related to changes in adiposity, glucose tolerance, lipid profile, etc. (Parker et al., 2010). Studies on vitamin D supplementation on lean and obese male mice have shown slower rates of decline in serum vitamin D concentration and lower serum calcitriol in obese mice compare to lean mice, but there was no significant weight gain in lean or obese mice of with vitamin D insufficiency compared with sufficiency, although lean mice with high supplementation of vitamin D had a greater calories consumption with no apparent BMI increase (Seldeen et al., 2017).

Vitamin D may have effects on food digestion and energy metabolism through modulating the variety and abundancy of microorganisms presented in gastrointestinal tract known as the gut microbiota (microbiota) (LeBlanc et al., 2013; Bashir et al., 2016). Vitamin D supplementation on vitamin D insufficient/deficient adult subjects modified bacterial family levels; increasing the relative abundance of *Akkermansia* and decreasing the relative abundance of *Porphyromonas* and at the phylum levels showed dose-dependent increases in the relative abundance of *Bacteroides*, which suggests that the beneficial effects of increased levels of vitamin D may be correlated to the increase of beneficial bacteria and decrease of pathogenic bacteria (Charoenngam et al., 2020).

Vitamin D deficiency is associated with some mental illnesses as depression and schizophrenia (Gracious et al., 2012). Activation of VDRs on neurons stimulates the release of neurotrophin and protects brain through anti-inflammatory defenses, which explain the linkage of vitamin D and improvement of mental illnesses, including depression, bipolar disorder and schizophrenia (Dursun, 2010). However, activation of vitamin D receptor is not the only link between vitamin D and the pathology of cognition and mental illness: it has been shown that extreme vitamin D deficiency is prevalent in the acute state of schizophrenia, which may be a result of low sunlight exposure, intake of antipsychotic medications, poor mobility, extreme alcohol intake, and/or smoking in psychotic patients (Yüksel et al., 2014; Cuomo et al., 2019).

Vitamin D deficiency may also effect eCBome system by changing the expression of the eCBome receptors including CB1, CB2 and PPAR-alpha, and increasing the levels of some mediators such as AEA and DHEA (Guida et al., 2020).

3.1.2. Gut microbiota

In the last decade, many studies investigated the microbiota of a variety of bodily niches, including the skin, the oral, vaginal and nasal cavities, but nowadays a lot of researches are focused on the gut microbiota, which is by far the largest and most diverse group of microorganisms inhabiting the human body (Seldeen et al., 2017). Due to its numerous functions, it is considered a metabolic organ (Méndez-Salazar et al., 2018).

Gut microbiota is a term that refers to the group of microorganisms, which includes bacteria, Archea, fungi, protists and viruses that are inhabitants of the gut. Bacteria make up the largest proportion of these and estimates on their number suggest that there are about as many as 1 to 10 times the number of bacteria in the gut as there are cells in the human body (PLoS Biol. 2016 Aug; 14(8): e1002533.). This complex ecosystem of nearly 300 to 500 bacterial species, includes nearly 2 million genes, making it a much richer genetic source than the human genome (Quigley, 2013).

The intestinal tract is a totally sterile before birth, at which point it is colonized by maternal and environmental bacteria, most significantly through feeding. The microbiota community architecture remains relatively stable in adulthood but is different between individuals due to lifestyle and cultural and dietary habits. (Rinninella et al., 2019; Sekirov et al., 2010). Each individual's unique gut microbiota profile can have different effects on the metabolism of the host.

Gut microbiota comprise different bacteria species that taxonomically classified by phyla, classes, orders, families, genera, and species (figure 3-1). The main gut microbial phylum, representing 90% of gut microbiota, is Firmicutes. The Firmicutes phylum consists of more than 200 different genera such as Lactobacillus, *Bacillus*, *Clostridium*, *Enterococcus,* and *Ruminicoccus*, but the *Clostridium* genus is by far the most abundant genera in Firmicutes phylum. Bacteroidetes is the

second most common phylum and is composed of *Bacteroides* and *Prevotella* as its main genera (Rinninella et al., 2019).

Dysbiosis is an imbalance between protective and harmful species of the intestinal bacteria and may reduce the benefits of gut microbiota or even harm the host (Méndez-Salazar et al., 2018).

The two phyla of *Bacteroidetes* and the *Firmicutes* are dominant beneficial bacteria in human gut, however, it is believed that ratio of *Firmicutes* to *Bacteroidetes* can be significantly relevant to human gut microbiota composition and reduction in this ratio is associated with changes in physiologic actions including superior glucose levels, less fat accumulation and less body gain weight (Ley et al., 2006; Lin et al., 2019) , during obesity there are increased levels of *Bacteroidetes and* a higher ratio of Firmicutes to Bacteroidetes as compared to non-obese subjects (Mitsuoka & Hayakawa, 1973; Kasai et al., 2015). The results of studies in mouse models of diet-induced obesity have shown mucosal breakdown within the ileum, endotoxemia and dysbiosis of the gut microbiome often characterized by an increase in the Firmicutes:Bacteriodetes ratio and increased levels of pathogenic *Helicobacter hepaticus* and decreased levels of metabolically beneficial *Akkermansia muciniphila* among others (Tamboli et al., 2004)

Gut microbiota may modify host metabolic balance and be an important factor for the development of some conditions associated with the metabolic syndrome (Festi et al., 2014).

Dysbiosis as well as vitamin D deficiency provoke factors of the metabolic syndrome, including insulin resistance and fatty liver (Su et al., 2016). Variation in the human *VDR* gene effects gut microbiome. Intestinal epithelial VDR conditional knockout mice showed dysbiosis, and their gut bacterial abundance is significantly changed by decreases in the genus *Lactobacillus* and butyrate-producing bacteria (Sun, 2018). Another study on VDR knock out mice showed dysbiosis, characterized by elevating the ratio of Bacteroidetes and Firmicutes phyla with increases in *Lactobacillaceae* and *Lachnospiraceae* families (Ooi et al, 2013). Vitamin D deficient diets changes the gut microbiota taxa through increasing the relative abundance of the *Enterobacteriaceae* *Prevotella* and *Actinomyces,* and decreasing *Odoribacteraceae*,

however, it does not cause generalized dysbiosis (Robles-Vera et al., 2019). Supplementation of vitamin D in vitamin D deficient, obese individuals didn't show any changes in α-diversity but there were some changes in genus levels of bacteria related in response to the vitamin D supplementation level which suggests that vitamin D supplementation has effects on human fecal microbiota (Naderpoor et al., 2019). In addition, germ free mice have lower levels of vitamin D (Bora et al., 2018) which shows an interplay with the gut microbiota.

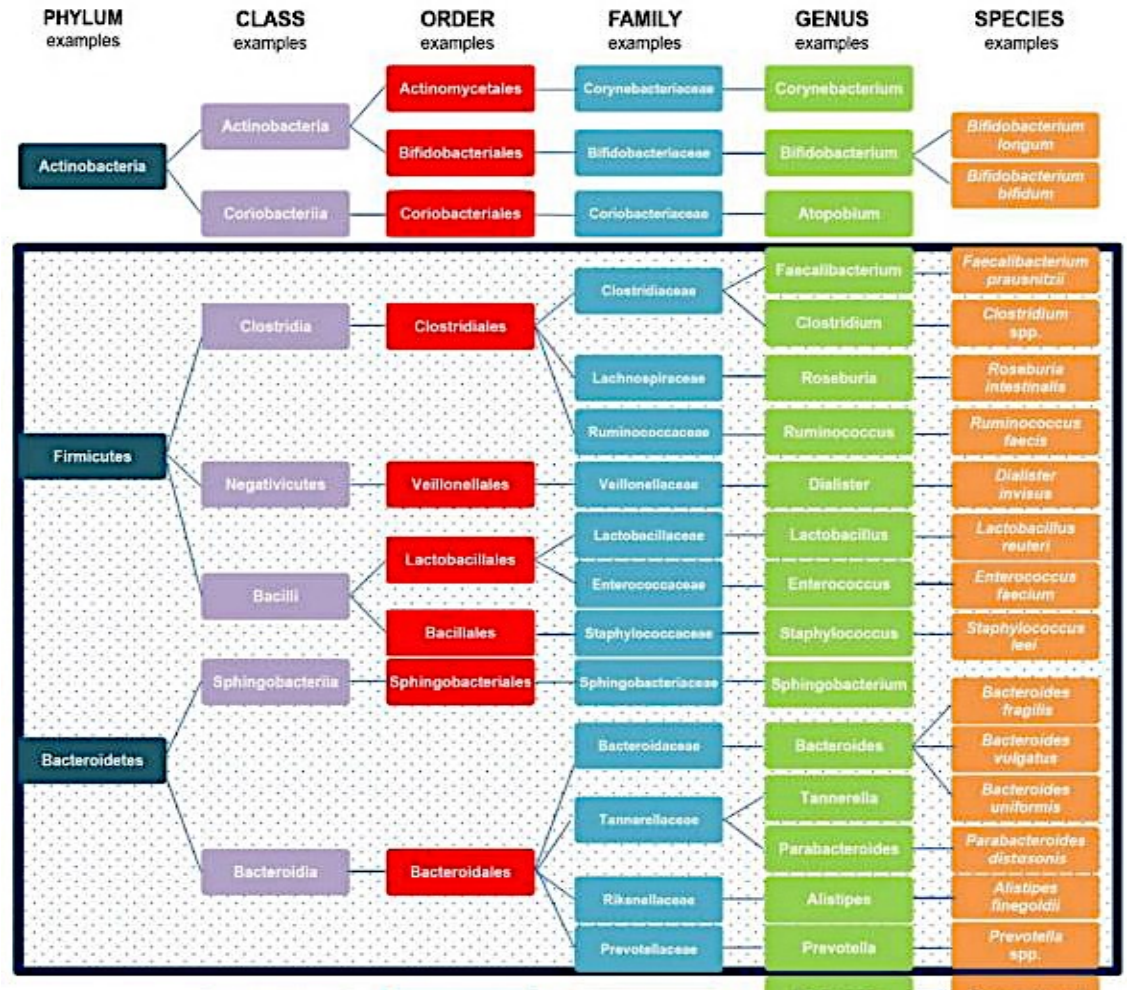

Figure 3-1. mamalians gut microiota classification (Rinninella et al., 2019)

Furthermore, a study on interactions between the brain and gut microbiota has shown the relationship between these systems named as brain–gut–microbiome axis, in which gut microbiota effects on psychological behaviors, including anxiety and depression but its mechanism of action is not still clear well (Allen et al., 2017); although it may originate from specific signaling alternation of hormones, neurotransmitters and immunological factors which are released from the gut and send signals to the brain (Clapp et al., 2017).

3.1.2.1. Gut microbiome/endocannabinoidome axis

The eCBome appears to have a bi-directional interaction with gut microbiota which has important consequences on the regulation of metabolism (Cani et al., 2016; Cristiano et al., 2018; Guida et al., 2018).

Fecal microbiome transfer from conventional donor mice to germ free mice has shown that alterations in the ileum and jejunum eCBome can be reverted by reintroduction of gut microbiota (Manca et al., 2019). While altering gut microbial composition through the use of prebiotics modified CB1 receptor levels in obese mice (Muccioli et al., 2010). Further, mice treatedt with *A. muciniphila,* a species belong to *Akkermansia* genus, increase 2-MG levels including 2-AG, 2-OG and 2-PG, in the intestinal track, which confirms the linkage between gut microbiota and eCBome (Everard et al., 2013).

Blocking CB1 receptor activity pharmacologically in diet induced obese mice, decreased intestinal permeability and LPS circulating levels along with inhibiting obesity development (Mehrpouya-Bahrami et al., 2017). Furthermore, chronic infusion of CB1 agonist in wild type mice increase gut permeability while treating obese mice by CB1 antagonist results in reduction in plasma LPS levels and decreasing gut permeability (Muccioli et al., 2010). These data suggest that the interaction between the gut microbiome and eCBome is indeed bidirectional.

3.1.3. Antipsychotic drugs

Antipsychotic drugs are used to treat psychotic disorders and are classified broadly as typical or first-generation and atypical or second-generation (Abou-Setta et al., 2012). Antipsychotic drugs are mostly antagonist of dopamine-2 receptors and they reduce psychotic symptoms as hallucinations and delusions (Wisner & Schaefer, 2015).

3.1.3.1. Typical or first-generation antipsychotics

Antipsychotics were first discovered in the 1950s, when chlorpromazine was shown to have sedative properties and was used as a calmative agent prior to surgery, which led it being studied in patients with chronic mental illness (Shen, 1999). Typical antipsychotics were initially used for psychosis such as schizophrenia, but were they were shown to be effective in the treatment of agitation, acute mania, and bipolar disorder (Abou-Setta et al., 2012).

The calming effects of antipsychotics begins quickly but their action on psychotic symptoms is slower. Generally, symptoms are initially treated with low doses and then it is increased considering their side effects and the patient's clinical response (Stevens & Rodin, 2011).

Typical antipsychotics include chlorpromazine, thioridazine, haloperidol, etc. (Wisner & Schaefer, 2015) and all of them increase extrapyramidal symptoms as a side-effect (Holloway & Peirce, 1998). They also can cause dystonia, neuroleptic malignant syndrome and akathisia, which may seriously affect the patient’s daily functioning and general heath (Advokat et al., 2000)

3.1.3.2. Atypical or second-generation antipsychotic

The atypical antipsychotic drugs including quetiapine, clozapine, olanzapine and ziprasidone in use since the 1990s are used to treat psychotic disorders, especially the acute phase of schizophrenia. These atypical drugs versus typical ones, have wider receptor affinity profiles as antagonists and have more affinity at the serotonin-2 receptors in comparison with D2 dopamine receptors (Seeman, 2004; Tamminga, 2000; Mackin & Thomas, 2011). Apart from the several notable benefits of atypical antipsychotics over typical ones, such as improved cognitive function, prevention of deterioration and the quality of life; they have variety of side effects including obesity, insulin resistance and diabetes as well as dyslipidemia which are associated with

potential long-term cardiovascular health risks (Nasrallah, 2007). Indeed, there are 3-times more incidence rate of metabolic syndrome in Schizophrenia patients who use atypical antipsychotic drugs (De Hert et al., 2008). Olanzapine administration in severe mental patients causes different metabolic perterbations such as obesity and diabetes (Citrome et al., 2011).

The gut microbiome plays a role in the dysfunction of metabolic cycle with olanzapine. A study on rats suggested that changing in fecal microbiota profile in rats which treated with olanzapine might be related to the aspects of olanzapine's metabolic effects (Davey et al., 2013)

In addition, studies on rats confirmed that vitamin D deficiency affects metabolic disorders which cause by antipsychotic drugs, and vitamin D supplementation can reduce these dysfunctional metabolic effects (Figure 3-2; Dang et al., 2015).

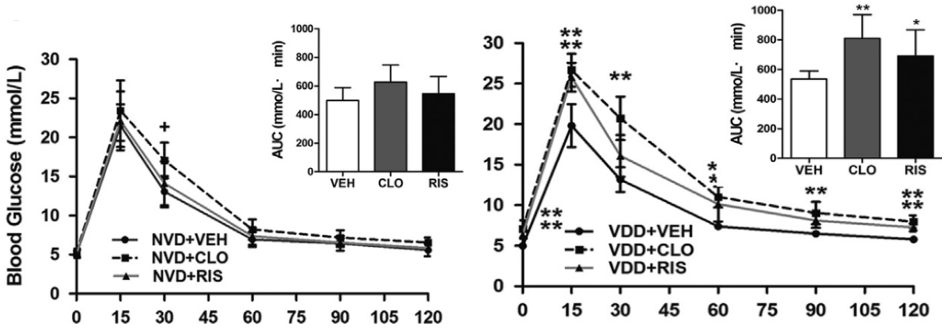

Figure 3-2. Glucose tolerance in rats treated with antipsychotic drugs and fed diets with two different levels of vitamin D. Normal vitamin D (NVD), vitamin D deficiency (VDD), clozapine (CLO), risperidone (RIS). (Dang et al., 2015).

Both endocannabinoidome mediators and their receptors demonstrate dysregulation in animal models of psychosis and in schizophrenic patients (Zamberletti et al., 2012). Schizophrenic patients show higher levels of AEA and PEA compare to healthy controls (Leweke et al., 1999), and an increase of AEA in patients administrated atypical antipsychotic drugs is higher than individuals administrated typical antipsychotics and antipsychotic-free schizophrenic patients(Giuffrida et al., 2004). It also has been shown, that the increased level of AEA after the clinical remission decrease in blood as along with changes in the expression of CB2 and FAAH (De Marchi et al., 2003).

Schizophrenic rat models, which are treated with phencyclidine (PCP), have shown more functionality of the CB1 receptor, increases in the levels of 2-AG and decrease of AEA in the brain particularly in the prefrontal cortex (Vigano et al., 2009). Olanzapine treated rats which have demonstrated weight gain and increased blood glucose didn't have any difference in CB1 expression compare to the control rats, but activation of CB1 receptor through its invers agonist of Rimonabant decreased CB1 mRNA expression (Lazzari et al., 2017).

3.2. Hypothesis

It is not yet clear if Vitamin D status affects severity of antipsychotic drug metabolic side effects. We intend to examine if vitamin D status affects the severity of metabolic abnormalities associated with atypical antipsychotic drug use. As both gut microbiota and the eCBome play critical roles in metabolism, we hypothesize that the gut microbiota-eCBom axis may play a role in mediating the metabolic effects of antipsychotics and potential modulation by Vitamin D.

This part of project was performed in University Laval, Canada

3.3. Experimental approach and methodology

3.3.1. Animals and treatment

Sixty three-week-old female C57BL/6J mice which have weight gain by consuming the atypical antipsychotics in linkage with the gut microbiota (Morgan et al, 2014) divided into 3 groups and fed by low fat diet for 3 weeks (n=20): 1. Control, 2. With vitamin D deficiency (0.06 IU D3 / g), 3. supplemented with vitamin D (10 IU D3 / g).

After three weeks, each group divided into two groups (n=10): 1. Vehicle, 2. Olanzapine for a further 9 weeks and the diet changed to high fat high sucrose.

The olanzapine treatment administered by daily gavage in the morning and dosage of olanzapine for the first 5 weeks was 2mg/kg/day, then it increased to 4mg/kg/day for other 4 weeks (figure 3-3).

For the preparation of the Olanzapine slurry, each tablet of 10mg was easily dissolved in a 1 ml of 0.1 N citric acid, and then adjusted to pH 5.5 with sterile PBS, while the vehicle was prepared using the same proportion of citric acid and PBS (Coccurello et al., 2006)

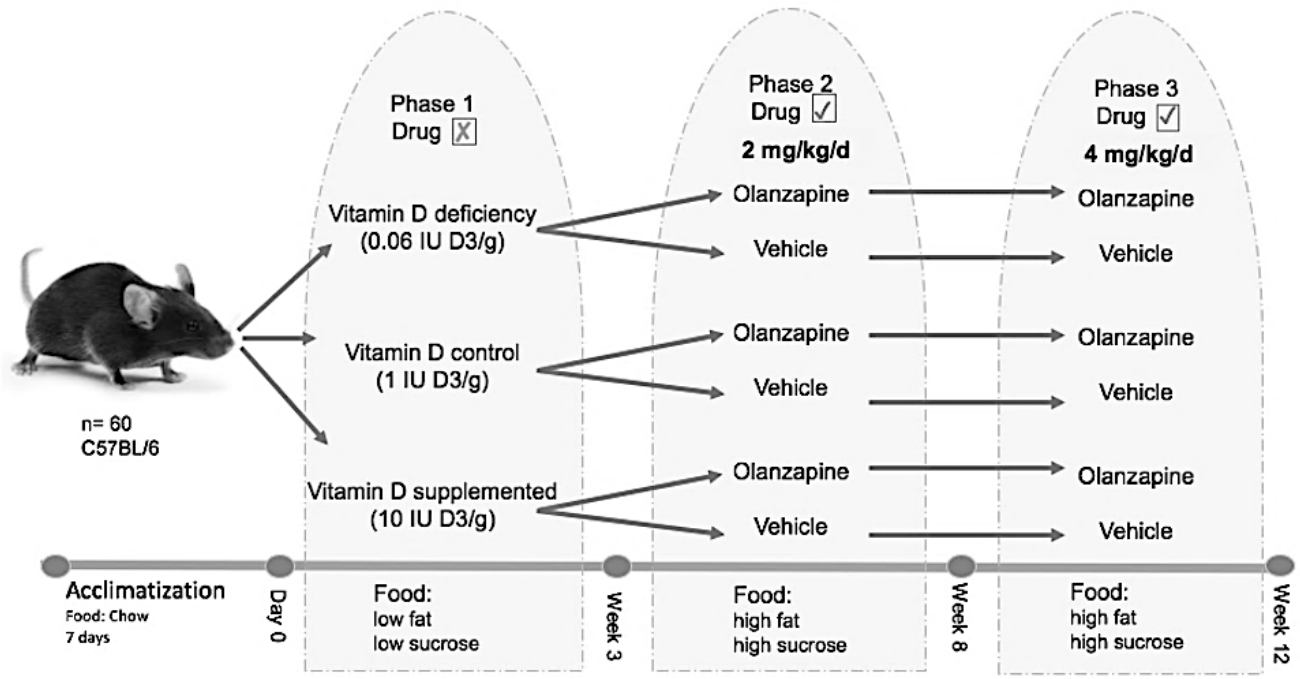

Figure 3-3. Experimental design

3.3.2. Determination of food intake, body weight, body composition

Weight monitoring and food consumption were done twice a week. Body composition of live animals determined using the NMR Analyser Minispec (Bruker Optik) at days 0 and 77.

3.3.3. Samples collection and analysis

Mice feces were collected at d 21 and 84 in order to characterize the intestinal microbiota and immediately stored in -80°C.

Blood was collected by the day of dissection through intra-cardiac blood samples were taken from animals during deep isoflurane anesthesia followed by cervical dislocation in order to measurement different plasma parameters and eCBome mediators. Tubes contain EDTA (K3) were used for blood collection.

On dissection day, the necessary tissues and organs including different adipose tissues namely brown, ovarian and inguinal adipose tissues were collected. Named tissues immediately divided to two part: one for fatty acids and endocannabinoids measurement by LCMS which put immediately in liquid nitrogen and then kept in -80°C, the other part placed in the tubes containing RNA later for RNA extraction and stored in -20°C.

3.3.4. Oral glucose tolerance test (OGTT)

On day 77 of protocol all the animals were fasted in new cages with water supply for 6 hours from 7 a.m. to 1 p.m. to perform the OGTT test and controlling glucose metabolism.

Before starting the experiment, monitoring of body weight for each mouse was done and considering the weight of each mouse, the injection solution prepared by 2mg of 50% dextrose per gram of mouse calculated.

The base line blood glucose level was measured by the blood of tail vein before gavage the mice by the prepared solution (Time 0) and then after gavage blood glucose levels measured at 15, 30, 60, 90 and 120 min from the same punching.

In times 0, 15, 30, 60 and 120 in addition to measuring the glucose levels, the blood was collected to measure Insulin by Elisa.

Then mice had a week of rest to de-stress before sacrifice.

3.3.5. Insulin quantification via ELISA

Mouse Ultrasensitive Insulin ELISA kit (Alpo, Salem, NH, USA) was used to quantify blood insulin concentration of OGTT test. After equilibrating all reagents to room temperature, 5 µL of each standard, control, and samples were pipet to each well of ELISA plate. 75 µL of Working Strength Conjugate was added into each well and then incubated for 2 hours at room temperature on a shaker. After 2 hours, plate contents were discarded, and wells washed with 350 µL of provided wash buffer using a microplate washer for total of 6 time. Then, 100 µL of TMB substrate was added to each well and incubated for other 30 min. At the last step 100 µL of Stop Solution was added to each well, and the plate was read at absorbance of 450 nm in a microplate reader.

3.3.6. Diabetes-related biomarkers quantification

Different diabetes-related biomarkers were assessed in the plasma using the Bio-Plex Pro™ mouse diabetes assays kit (Bio-Rad Laboratories Canada Ltd., Mississauga, ON, Canada). Samples (diluted 1:4) and standards (serial dilution) were prepared following the manufacturer's instruction. 50 µL of beads were added to each well. Then the plate washed twice by 100 µL of wash buffer. Later, 50 µl of standards, blank, samples added to each well and incubated for 1 hr on a shaker at room temperature. Three times of wash was done after 1 hr incubation time, 25 µl of 1x detection antibody added to each well and incubated for other 30 min on a shaker. By finishing second incubation, 50 µl of 1x streptavidin-PE was added to each well and after other 10 min of incubation on shaker, plate was washed for three times. For the last step, 125 µl of assay buffer was added to each well, waited for 30 sec on shaker and the prepared plate was read via a Bio-Plex® 200 system with Bio-Plex Manager™ software version 6.0 (Bio-Rad Laboratories Canada Ltd. Mississauga, ON, Canada).

3.3.7. RNA isolation, Reverse Transcription and qPCR based TaqMan Open Array

RNA was extracted from ovarian and subcutaneous adipose tissues samples with the RNeasy Lipid Tissue Mini Kitc(Qiagen, Hilden, Germany). Before stating extraction, RPE buffer prepared following the manufacturer's instruction.

Initialy, 1 ml of QIAzol Lysis Reagent (Qiagen, Hilden, Germany) added to each tube which were containing a 5mm single-bead dispenser and sample (Qiagen, Hilden, Germany). Then, tissues were disrupted and homogenized through a TissueLyser for 10 min. After 5 min incubation of the homogenates in room temperature, 200 µl of chloroform was added and shaked for 15 s. Following 3 min of incubation, tubes were centrifuge at 12,000 x g for 15 min at 4°C. The upper phase was transferred to new tubes and 1 volume of 70% ethanol was added and vortexed. 700 µl of the sample were transferred to RNeasy Mini spin column in 2 ml collection tube and centrifuged at room temperature for 15 s at ≥8000 x g and flow-through were discarded. Other 700 µl of the sample were added to the same RNeasy Mini spin column, centrifuged and flow-through discarded. Afterward, 350 µl of Buffer RW1 was added to the spin column centrifuged for 15 s at 8000 x g (10,000 rpm) to wash the membrane and the flow-through was discarded. 80 µl of DNase I incubation mix (prepared following the manufacturer's instruction) (Qiagen, Hilden, Germany) directly added to the spin column membrane and incubated in the room temperature for 15 min. After finishing the incubation time 350 µl of Buffer RW1 was added to the spin column and centrifuged for 15 s at 8000 x g. Then, 500 µl of Buffer RPE was added to column and centrifuged as previous step and the flow-through discarded. Again 500 µl of Buffer RPE was added to column and centrifuged for 2 min at ≥8000 x g. Finally, RNeasy columns were placed in new 1.5 ml tubes, 30 µl UltraPure Distilled Water (Invitrogen, CA, USA) was added and centrifuged for 1 min at ≥8000 x g to collect the total RNA. The concentration and purity of RNA was determined by measuring the absorbance of the RNA in a Biodrop at 260nm and 280nm.

1µg of total RNA was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad, CA, USA) in a reaction volume of 20µl. 60ng of starting RNA was used to evaluate the expression of the 52 eCBome-related genes and 4 housekeeping genes of *Gapdh, Hprt, Tbp* and *Rps13* using a custom-designed qPCR-based TaqMan Open Array on a QuantStudio 12K Flex Real-Time PCR System

(Thermo Fisher Scientific, CA, USA) following the manufacturer's instruction. Gene expression levels were evaluated by the $2^{-\Delta\Delta Ct}$ method and represented as fold increase with respect to baseline within each tissue section for each age.

3.3.8. Lipid extraction and HPLC-MS/MS for the analysis of eCBome mediators

Lipids were extracted from tissue samples according to the Bligh and Dyer method (Bligh & Dyer, 1959). Briefly, about 10mg of each tissue were homogenized in 1ml of Tris-HCl 50mM pH 7 and 1 ml of methanol using a tissue homogenizer. 5 µl of deuterated standards and 5.75 µl of 0.1M acetic acid were added to each tube and then 1ml of chloroform was added to each sample, vortexed for 30 seconds and centrifuged at 3000×g for 5 minutes. This was repeated twice for a total addition of 3ml of chloroform. The organic phases were collected and evaporated under a stream of nitrogen and then suspended in 50µl of mobile phase containing 50% of solvent A (water + 1mM ammonium acetate + 0,05% acetic acid) and 50% of solvent B (acetonitrile/water 95/5 + 1mM ammonium acetate + 0.05% acetic acid). 40µl of each sample was finally injected onto an HPLC column (Kinetex C8, 150 × 2.1mm, 2.6µm, Phenomenex) and eluted at a flow rate of 400µl/min using a discontinuous gradient of solvent A and solvent B (Everard et al., 2019). Finally, Quantification of eCBome-related mediators was carried out by HPLC system interfaced with the electrospray source of a Shimadzu 8050 triple quadrupole mass spectrometer and using multiple reaction monitoring in positive ion mode for the compounds and their deuterated homologs.

3.3.9. DNA extraction and 16S rRNA gene sequencing

DNA was extracted from feces using the Qiagen DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany) according to the manufacturers' instructions. Briefly, one piece of feces
and 800 µl of Solution CD1 was added to Each PowerBead Pro Tube and then the tubes placed into the TissueLyser in maximum speed for 10 min. Afterward, tubes were Centrifuged at 15,000 x *g* for 1 min at room temperature and supernatant transferred to a clean 2 ml tubes. 200 µl of Solution CD2 was added and Centrifuged at 15,000 x *g* for 1 min. New supernatant were transferred to another clean 2 ml tube and 600 µl of Solution CD3 and vortex for 5 s. From

prepared lysate, 650 µl was loaded onto MB Spin Column, centrifuged at 15,000 x *g* for 1 min and flow-through were discarded for two time.

Then, Spin Columns were placed into clean 2 ml tubes, 500 µl of Solution EA added and Centrifuged at 15,000 x *g* for 1 min and he flow-throughs were discarded. 500 µl of Solution C5 was added to the MB Spin Column, Centrifuged at 15,000 x *g* for 1 min and the flow-through discarded. For the last step, 50 µl of UltraPure Distilled Water (Invitrogen, CA, USA) was added and centrifuged at 15,000 x *g* for 1 min to collect DNA content.

The DNA concentrations of the extracts were measured fluorometrically with the Quant-iT PicoGreen dsDNA Kit (Thermo Fisher Scientific, MA, USA) and the DNAs were stored at −20°C until 16S rDNA library preparation.

Briefly, 1ng of DNA was used as template and the V3-V4 region of the 16S rRNA gene was amplified by polymerase chain reaction (PCR) using the QIAseq 16S Region Panel protocol in conjunction with the QIAseq 16S/ITS 384-Index I (Sets A, B, C, D) kit (Qiagen, Hilden, Germany). The 16S metagenomic libraries were eluted in 30µl of nuclease-free water and 1µl was qualified with a Bioanalyser DNA 1000 Chip (Agilent, CA, USA) to verify the amplicon size (expected size ~600 bp) and quantified with a Qubit (Thermo Fisher Scientific, MA, USA). Libraries were then normalized and pooled to 2nM, denatured and diluted to a final concentration of 6pM and supplemented with 5% PhiX control (Illumina, CA, USA). Sequencing (2 × 300 bp paired-end) was performed using the MiSeq Reagent Kit V3 (600 cycles) on an Illumina MiSeq System. Sequencing reads were generated in less than 65 h. Image analysis and base calling were carried out directly on the MiSeq.

3.3.10. Statistical analysis

Mixed linear regressions (nlme R package) followed by Tukey HSD post hoc test or Kruskal-Wallis test followed by Dunn's multiple comparison tests, for parametric and non-parametric data respectively, were used to identify significant vitamin D status and/or Olanzapine treatment effects on gut microbiota composition and eCBome levels. Spearman correlations will be used to investigate for associations between microbiota composition and eCBome mediators. Adjustments for multiple testing will be obtained using False Discovery Rate (FDR). All results will

be considered statistically significant at $p<0.05$ or FDR-adjusted $p<0.1$. Analyses will be performed with R.

3.4. Results

3.4.1. Body weight and Food intake

Although the food intake didn't change between our different animal groups, the body weight gain started to present significant decreases in all groups with olanzapine exposure compare to their vehicle in the same vitamin D level from week 10 of animal protocol ($p < 0.05$) (Figure 10) Looking at area under the curve analysis for the body gain weight, shows less difference in decreasing level of weight in olanzapine group which we under supplementation of vitamin D, shows the beneficial effect of vitamin D (Figure 3-4).

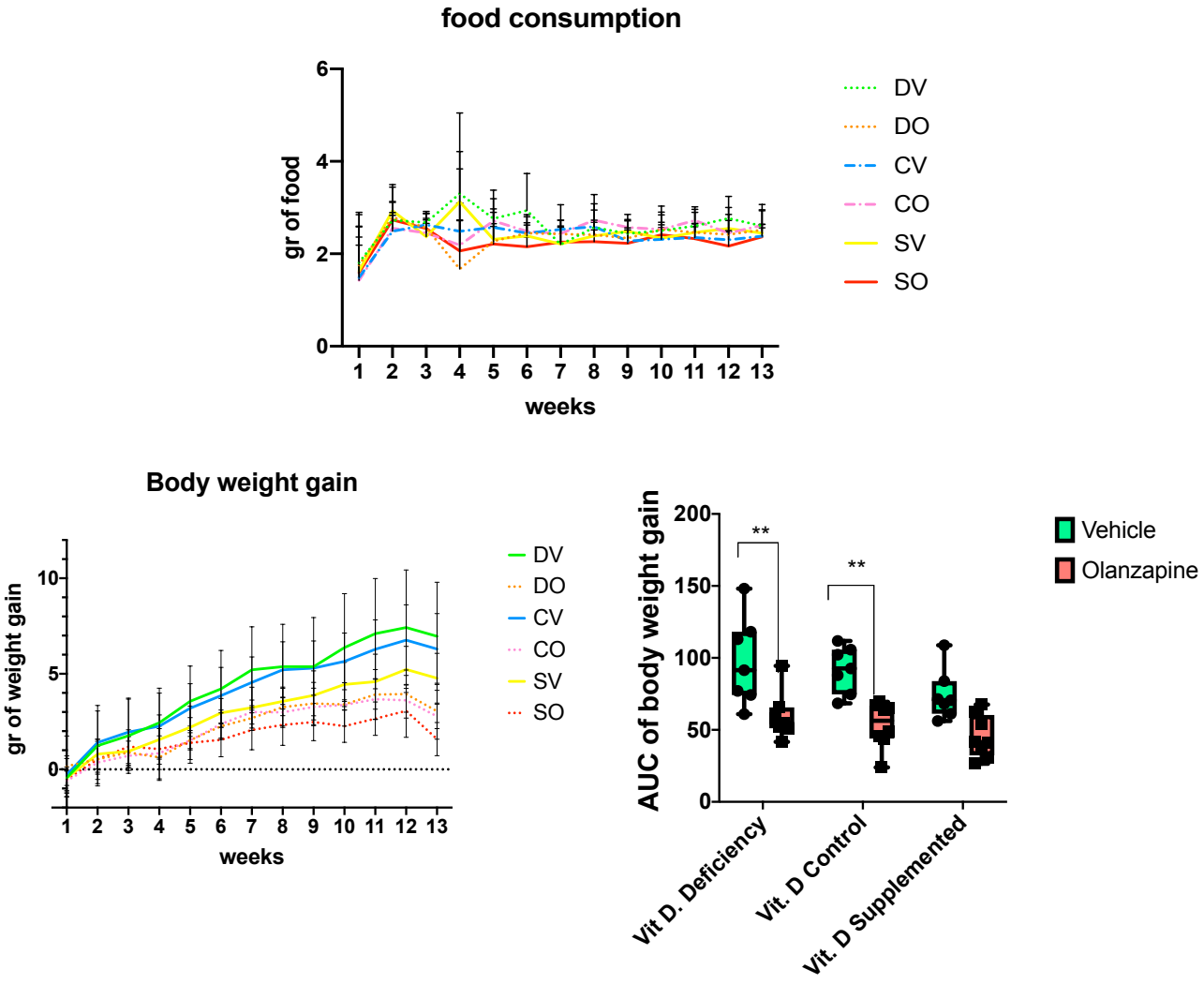

Figure 3-4. Food consumption and body gain weight during 13 weeks of animal protocol.

3.4.2. Body composition

According to what we saw in weight gain of our different groups, studying the body composition through NMR machine to see changes in lean or fat mass showed significantly greater reduction in fat mass of olanzapine treated groups after 75 days ($p < 0.01$). There was a trend toward a larger loss of fat mass in the olanzapine/vitamin D supplemented group ($p < 0.01$). Instead, there was not any difference in the lean mass of our experimental groups (figure 3-5).

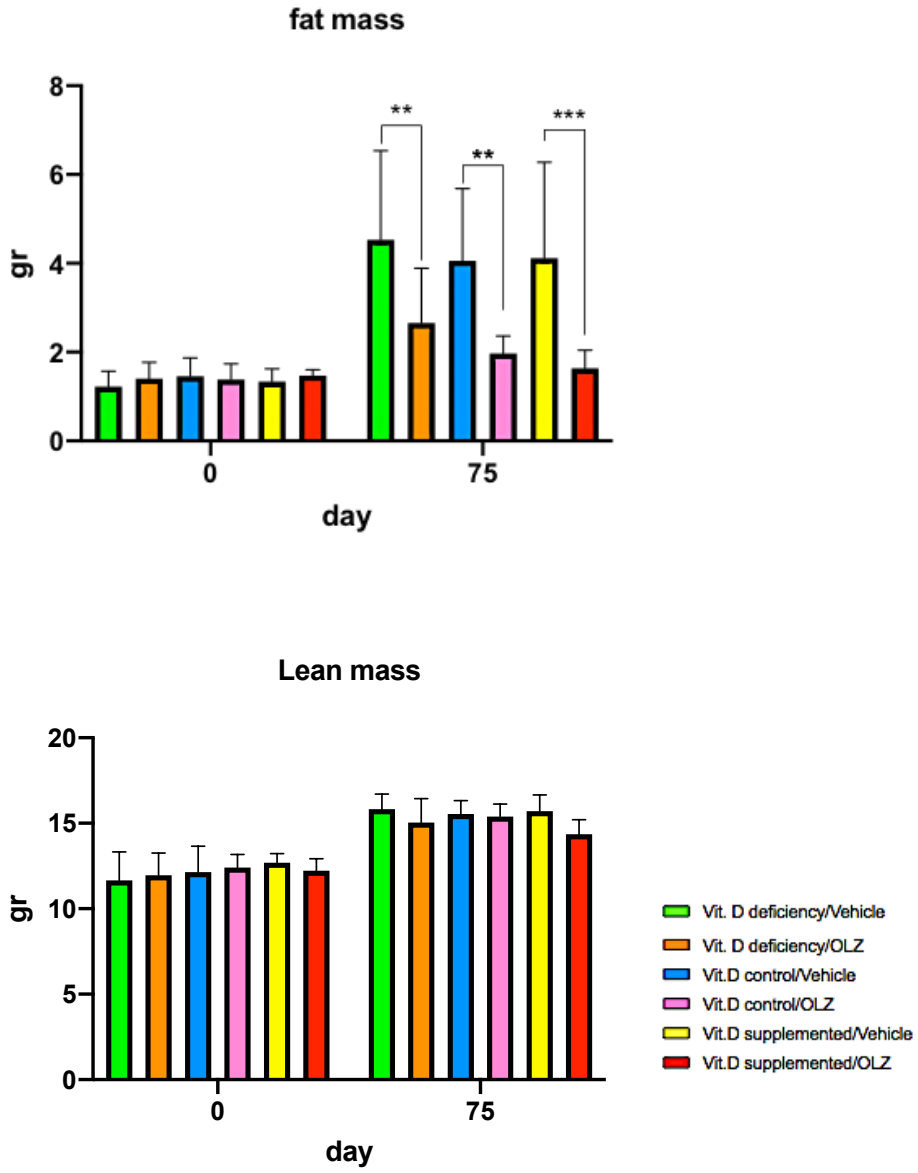

Figure 3-5. NMR body composition of mice on days 0 and 75 of animal protocol.

3.4.3. Biochemical markers

Since different biochemical markers related to obesity may be affected by olanzapine or different vitamin D levels, we measured different blood biochemical markers including glucose, three glycerides, cholesterol and HDL; but there was not any difference in the named markers between six different groups (figure 3-6).

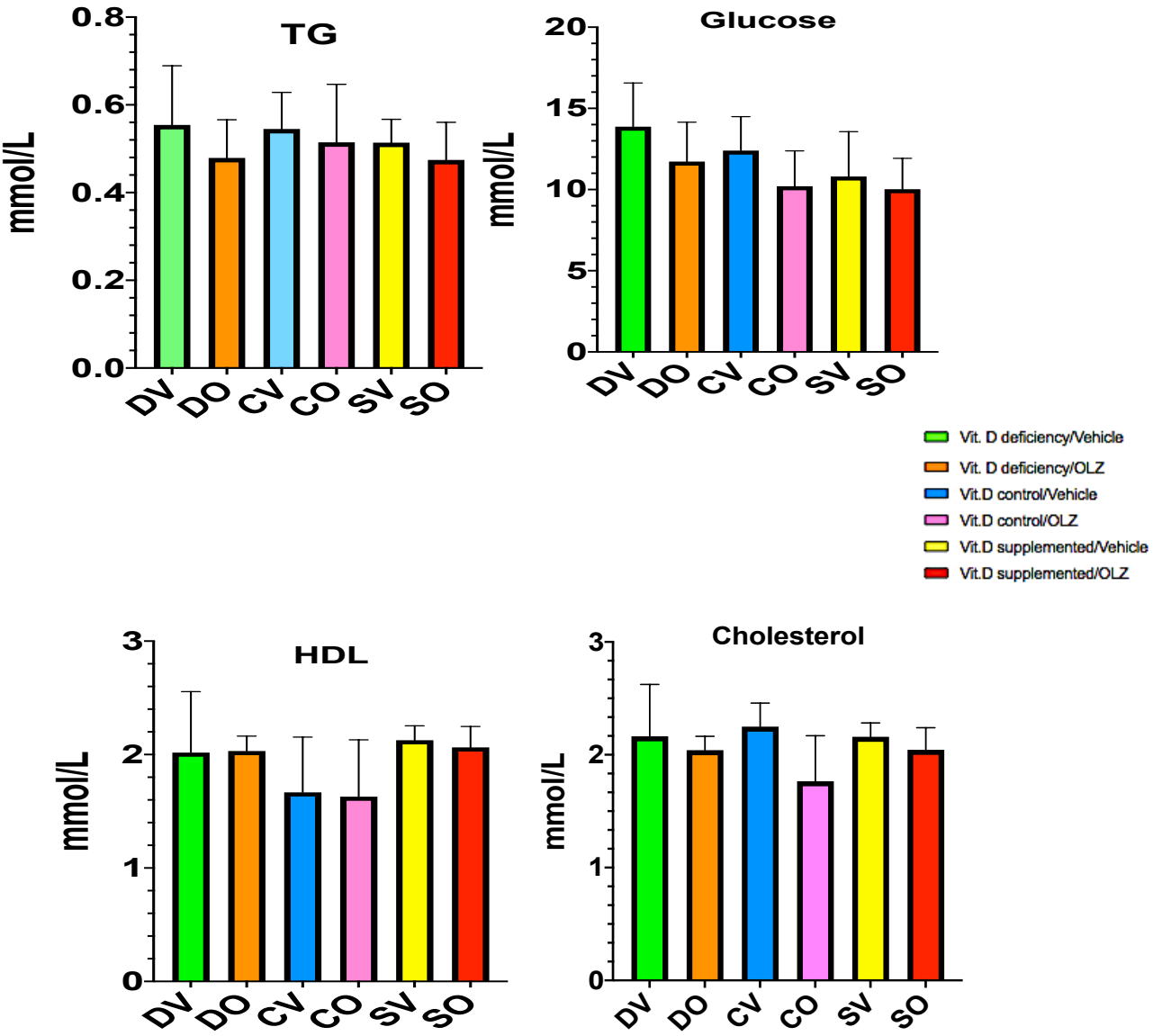

Figure 3-6. Analysis of different blood biochemical markers related to obesity.

3.4.4. OGTT test

Results from OGTT test always showed a decreasing trend in olanzapine treated group in both glucose and insulin levels, and also the insulin resistance index which was determined by multiplying the area under the curve of both blood glucose (0 to 120 min) and plasma insulin (0and 120 min) obtained following the oral glucose tolerance test. The only significant changes were presented in control level of vitamin D for glucose level and insulin resistance index by a great reduction in olanzapine group ($p < 0.01$) (figure 3-7).

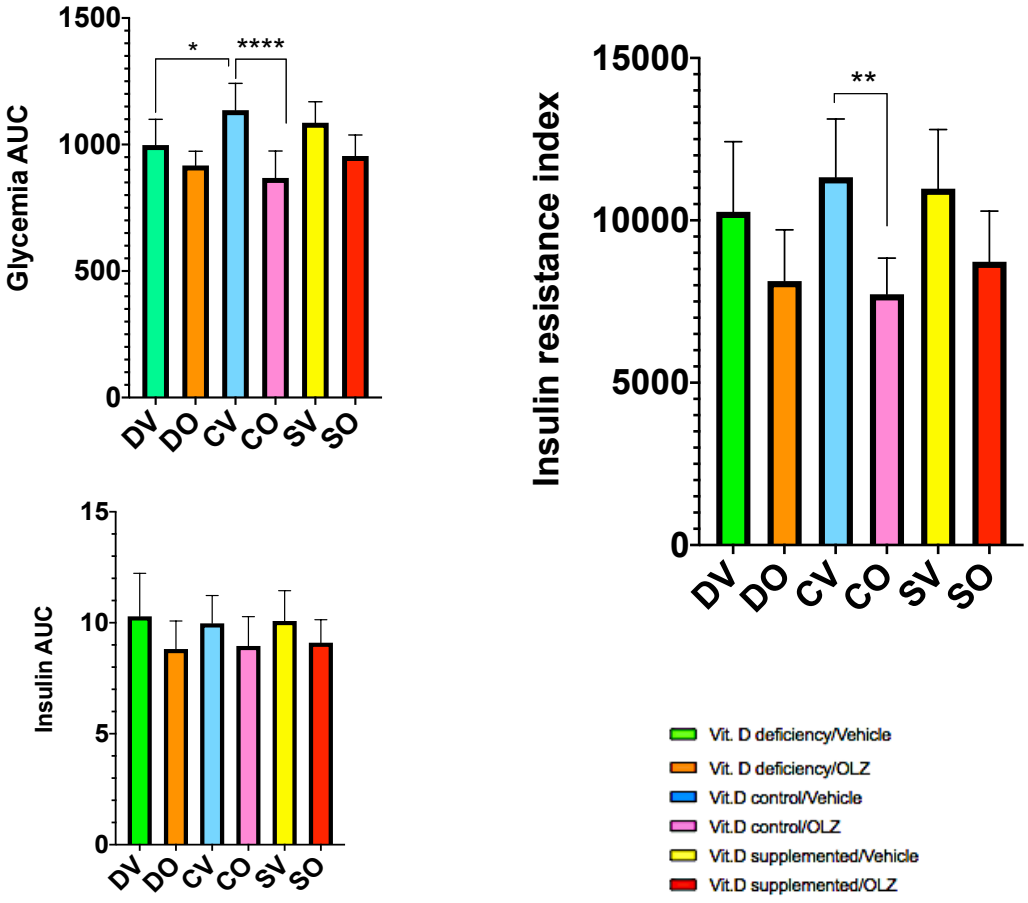

Figure 3-7. Oral glucose tolerance test showed some changes in insulin resistance level.

3.4.5. Endocannabinoidome genes expression

Real time quantitative PCR analysis to determine the expression of genes encoding eCBome anabolic and catabolic enzymes and receptors in different tissues of the mice was performed. The most significant changes were observed in ovarian and subcutaneous white adipose tissues (table 3-1).

Table 3-1. Different gene expression levels in ovarian and subcutaneous adipose tissues. All levels are calculated compare to control vitamin D/vehicle group. Red color is showing increasing trend and blue represents decreasing trend.

	Sub AT						OAT					
	DV	DO	CV	CO	SV	SO	DV	DO	CV	CO	SV	SO
Abhd12	1.08	0.90	1.00	0.72	1.12	0.84	0.89	0.83	1.00	0.82	0.97	0.89
Abhd16a	1.10	1.07	1.00	0.95	1.13	0.89	0.74	0.95	1.00	0.75	0.92	0.95
Abhd4	1.32	1.16	1.00	1.01	1.17	1.11	1.03	0.90	1.00	0.89	0.88	1.01
Abhd6	1.06	1.05	1.00	0.86	1.10	1.01	0.87	1.20	1.00	1.06	0.81	0.95
Agk	0.97	0.99	1.00	1.04	1.06	1.00	1.00	0.80	1.00	0.76	0.84	0.73
Akr1b3	0.87	0.70	1.00	0.59	0.93	0.65	0.61	0.76	1.00	0.50	0.72	0.60
Alox12	1.09	0.43	1.00	0.45	0.93	0.51	0.74	0.47	1.00	0.61	0.74	0.76
Alox15	1.86	1.77	1.00	1.27	0.80	1.42						
Cacna1b	0.71	1.28	1.00	1.16	0.69	0.00						
Cacna1h	0.71	1.31	1.00	0.79	1.39	0.76	1.82	1.69	1.00	1.00	1.27	0.76
Ces1d	1.13	1.24	1.00	0.88	1.04	1.00	1.02	1.49	1.00	1.42	1.28	1.69
Cnr1	1.03	1.25	1.00	0.89	0.79	0.81	0.92	1.04	1.00	0.87	0.62	0.66
Cnr2	0.35	0.22	1.00	0.35	0.37	0.26	0.60	2.37	1.00	0.99	0.89	0.70
Comt	0.75	0.66	1.00	0.54	1.07	0.62	0.93	0.67	1.00	0.68	0.95	0.52
Dagla	1.26	0.94	1.00	1.01	1.29	0.95	1.37	1.05	1.00	1.02	1.20	0.98
Daglb	1.30	1.22	1.00	0.79	1.27	0.71	1.16	0.96	1.00	0.86	1.03	0.81
Dgke	0.91	0.84	1.00	0.76	1.07	0.87	1.01	0.88	1.00	1.17	1.31	1.28
Enpp2	1.14	0.87	1.00	0.68	0.95	0.76	0.87	1.19	1.00	0.99	0.80	1.12
Faah	0.80	0.53	1.00	0.59	0.62	0.79	1.82	1.41	1.00	0.76	0.52	0.68
Fam213b	0.55	0.63	1.00	0.38	0.74	0.31	0.79	0.60	1.00	0.39	0.87	0.35
Gde1	1.17	1.13	1.00	1.25	1.10	1.17	0.95	1.34	1.00	2.02	0.94	2.68
Gdpd1	1.63	2.99	1.00	3.18	1.21	3.63	1.02	1.05	1.00	1.66	1.03	1.37
Hrasls5	1.02	1.00	1.00	0.76	0.94	1.00	1.09	1.64	1.00	1.07	0.90	1.18
Inpp5d	0.69	0.66	1.00	0.65	0.80	0.71	1.27	0.77	1.00	1.04	1.06	1.09
Mgll	0.89	0.65	1.00	0.50	1.08	0.42	0.77	0.65	1.00	0.64	1.03	0.52
Mogat1	1.06	1.09	1.00	1.02	0.98	0.94	0.84	1.25	1.00	1.66	1.10	1.56
Naaa	0.88	0.73	1.00	0.51	0.84	0.53	1.00	0.98	1.00	0.62	0.90	0.71
Napepld	0.83	0.79	1.00	0.62	0.98	0.67	0.72	0.84	1.00	0.73	0.90	0.78
Pam	0.85	0.66	1.00	0.55	1.01	0.53	1.15	0.87	1.00	0.67	0.95	0.63
Pla1a	1.52	1.79	1.00	1.81	1.17	1.49	0.75	0.86	1.00	0.72	0.66	0.64
Plcb1	1.02	1.05	1.00	0.78	1.00	0.98	1.00	1.05	1.00	0.78	1.00	1.21
Ppara	1.19	1.73	1.00	1.21	1.45	1.36	0.87	0.96	1.00	1.54	1.13	1.45
Pparg	1.07	0.92	1.00	0.67	0.82	0.73	0.72	0.78	1.00	0.91	0.68	1.18
Ppt1	0.90	0.88	1.00	0.79	0.95	0.83	1.18	0.87	1.00	0.85	1.00	0.90
Ptges	1.10	1.30	1.00	1.17	1.06	1.06	0.61	0.61	1.00	0.73	0.81	0.53
Ptgfr	1.66	0.85	1.00	0.61	0.95	0.80	1.50	1.04	1.00	1.06	0.99	1.50
Ptpn22	0.48	0.55	1.00	0.56	0.48	0.65	1.81	0.83	1.00	0.78	0.71	0.81
Trpv1	0.75	0.51	1.00	0.32	0.99	0.28	0.57	0.51	1.00	0.40	0.77	0.37
Trpv2	0.71	0.76	1.00	0.63	1.10	0.56	1.97	1.01	1.00	0.78	1.28	0.85
Trpv4	0.92	0.99	1.00	1.00	1.46	0.97	0.96	0.73	1.00	0.91	1.02	1.07

3.4.6. Genes related to anabolic pathway

Gdpd1 and *Gde1* which are enzymes responsible for the NAE production showed an increasing trend in olanzapine groups of different vitamin D levels. But the levels of this increment were different between these two white adipose tissues. In subcutaneous adipose tissue, *Gdpd1* significantly increased in olanzapine treated groups under all diets with different vitamin D levels. On the other hand, *Gde1* had a same trend in ovarian adipose tissue and this enhancement were significant under the control and supplemented vitamin D levels ($p < 0.01$) (figure 3-8).

Ark1b3, is responsible enzyme to produce prostamide F2α from AEA and PGF2α from arachidonic acid, showed a decreasing trend in both ovarian and subcutaneous adipose tissues but this decrease was significant only in mice obtaining the control level of vitamin D ($p < 0.05$) (figure 2-8).

Also, *Fam213b*, another anabolic enzyme for prostamides F2α, which works in the same pathway of Akr1b3, was decrease under control level of vitamin D in response to olanzapine ($p < 0.05$) (figure 3-8).

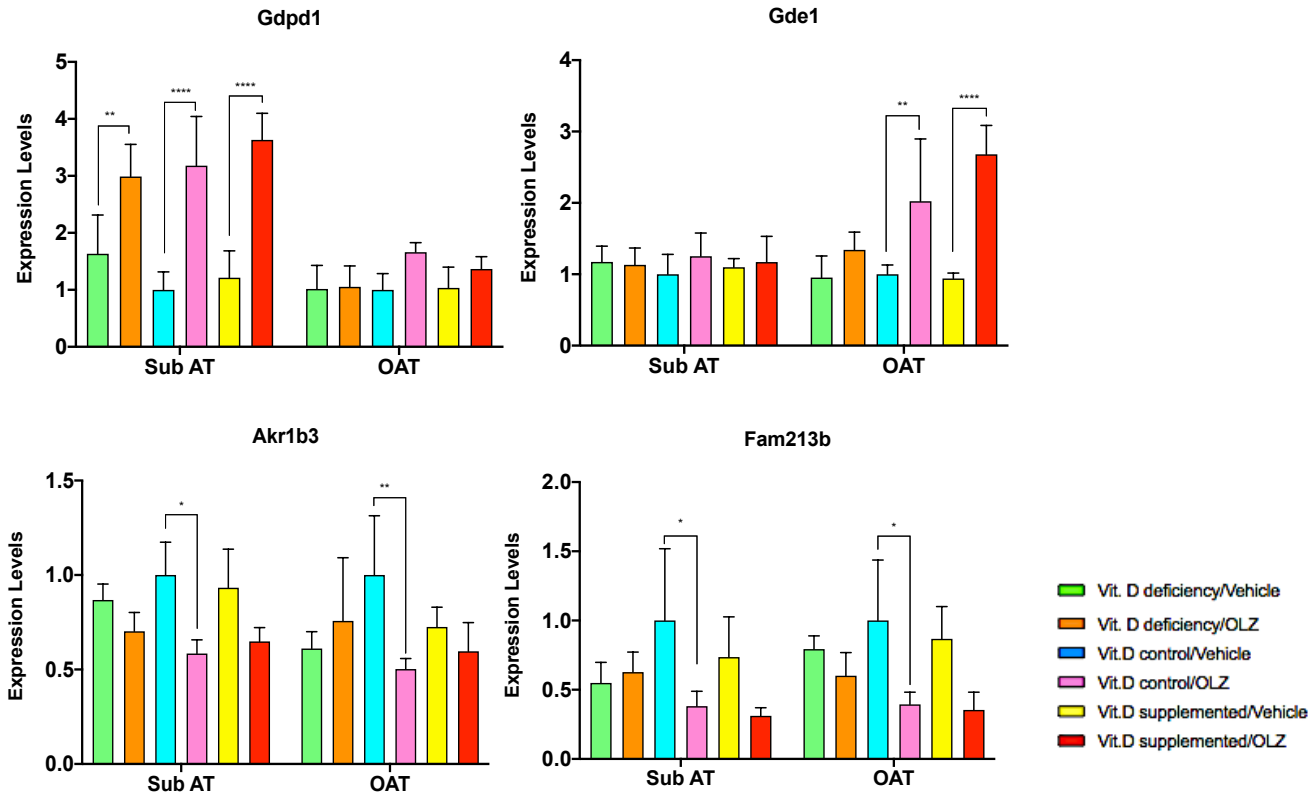

Figure 3-8. gene expression levels of eCBome anabolic enzymes. * ($p<0.05$); ** ($p<0.01$); *** ($p<0.001$); **** ($p<0.00001$)

3.4.7. Genes related to catabolic pathway

Results from real time quantitative PCR for genes encoding eCBome catabolic enzymes showed a decreasing trend for of *Naaa* and *Mgll* in olanzapine-treated groups.

Naaa, a catabolic enzyme degrading PEA to palmitic acid and ethanolamide, decreased significantly in response to olanzapine under control vitamin D levels in subcutaneous adipose tissue ($p<0.01$) (figure 3-9). *Mgll*, the enzyme responsible to degradation of 2-AG and other monoacylglycerols, thus playing a key role in lipolysis, decreased significantly in the olanzapine group, particularly with supplementation of vitamin D in subcutaneous adipose (figure 3-9).

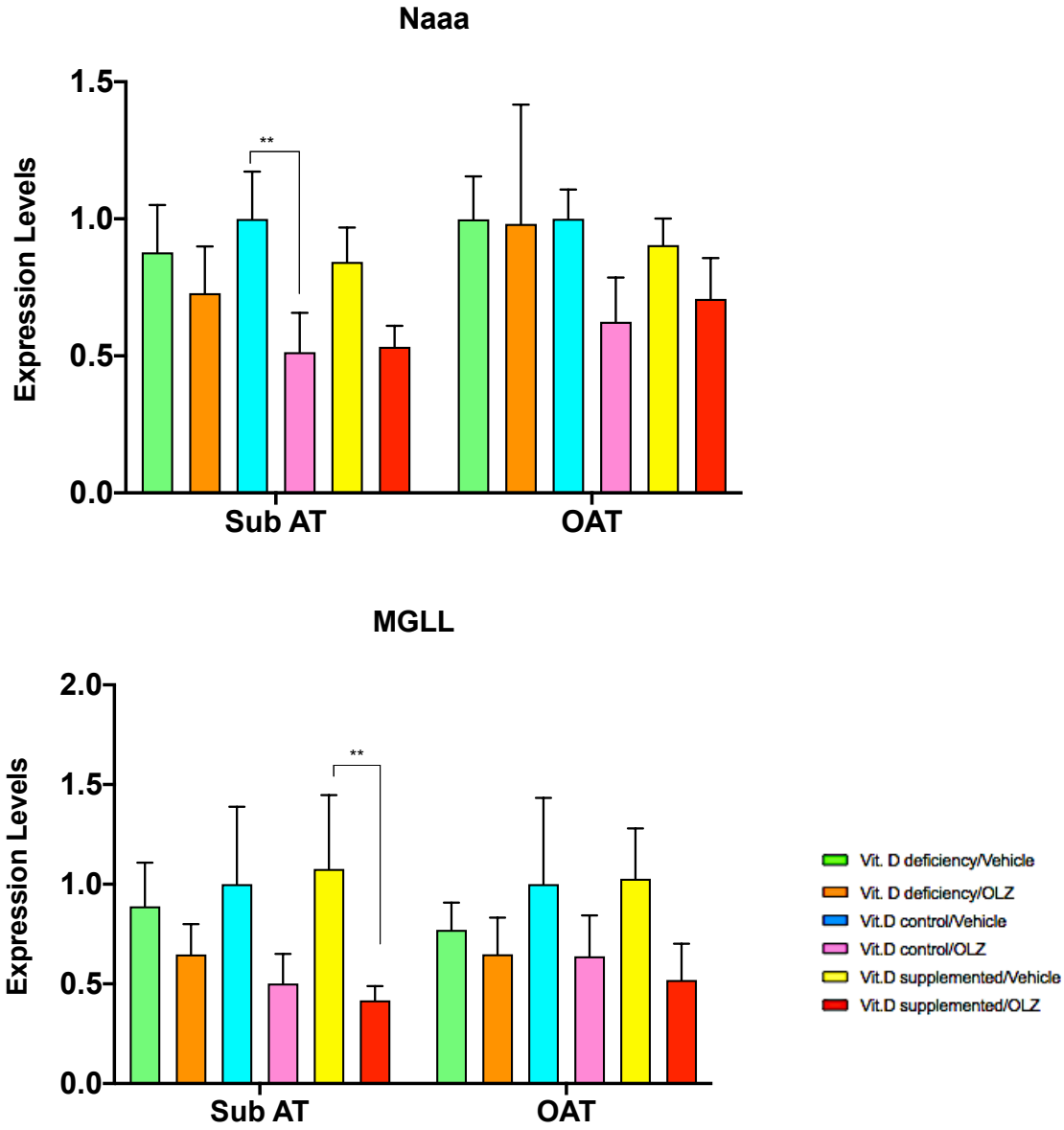

Figure 3-9. Increasing trend in catabolic enzymes of eCBome system. ** ($p<0.01$)

3.4.8. TRPV1 receptor gene expression

The expression level of *Trpv1* had a decreasing trend in all olanzapine groups compare to their vehicle controls under the same vitamin D levels, however it significantly decreased under control and supplemented vitamin D levels in subcutaneous adipose ($p<0.01$) but only under the control level of vitamin D in ovarian adipose tissue ($p<0.05$) (figure 3-10)

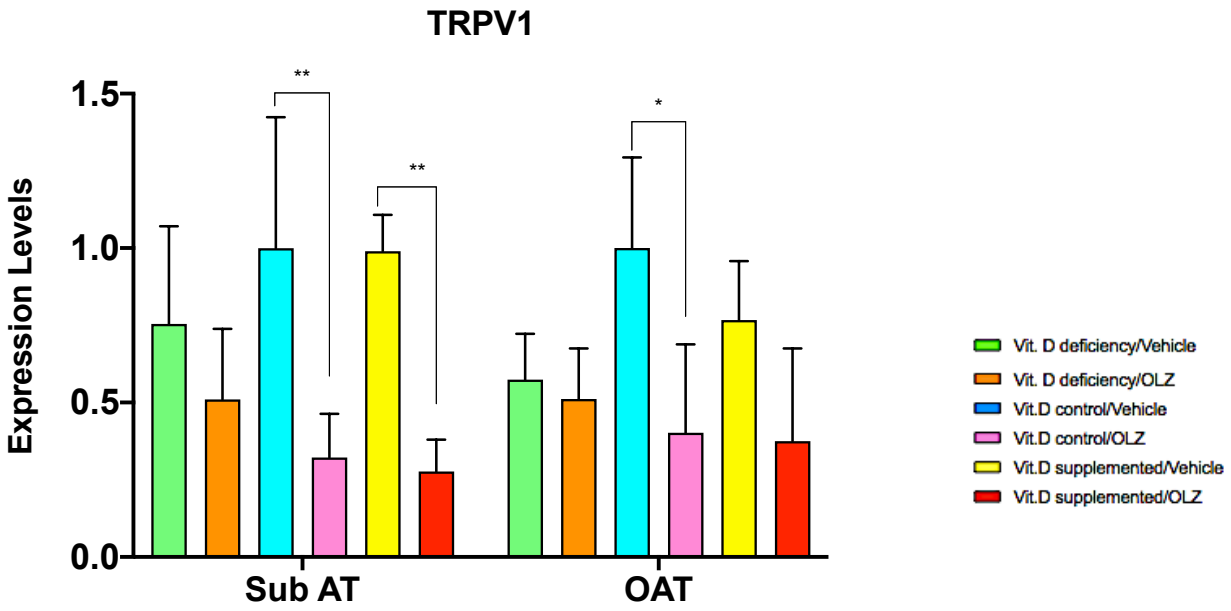

Figure 3-10. Gene expression level of Trpv1, the eCBome receptor which has role in fat metabolism. * ($p<0.05$); ** ($p<0.01$)

3.4.9. Endocannabinoidome lipid mediators

3.4.9.1. Ovarian adipose tissue

We observed several trends related to olanzapine treatment groups for increased levels of eCBome NAEs and 2-MGs lipid mediators in ovarian adipose tissue. Among the NAEs, both LEA and OEA showed and increasing trend in all levels of vitamin D in the olanzapine group compared to their vehicle, but this enhancement was significant only under the vitamin D supplemented diet ($p < 0.05$) (figure 3-11).

2-MG levels showed the same increasing trend in olanzapine administrated groups, but the trend of increase in different mediators were different (figure 3-12). Olanzapine under the vitamin D supplemented diet showed significantly increased DHA-2-G, DHA-1-G and 2-Oleoyl-G + 1-Oleoyl-G ($p< 0.05$), supplementation of vitamin D reduced the increasing trend of 2-AG + 1-AG in the olanzapine group.

Prostaglandin levels in ovarian adipose tissue didn't show much significancy, however olanzapine groups always showed increasing trend (figure 3-13). Only PGF2-alpha in control level of vitamin D increased significantly in response to olanzapine ($p < 0.001$).

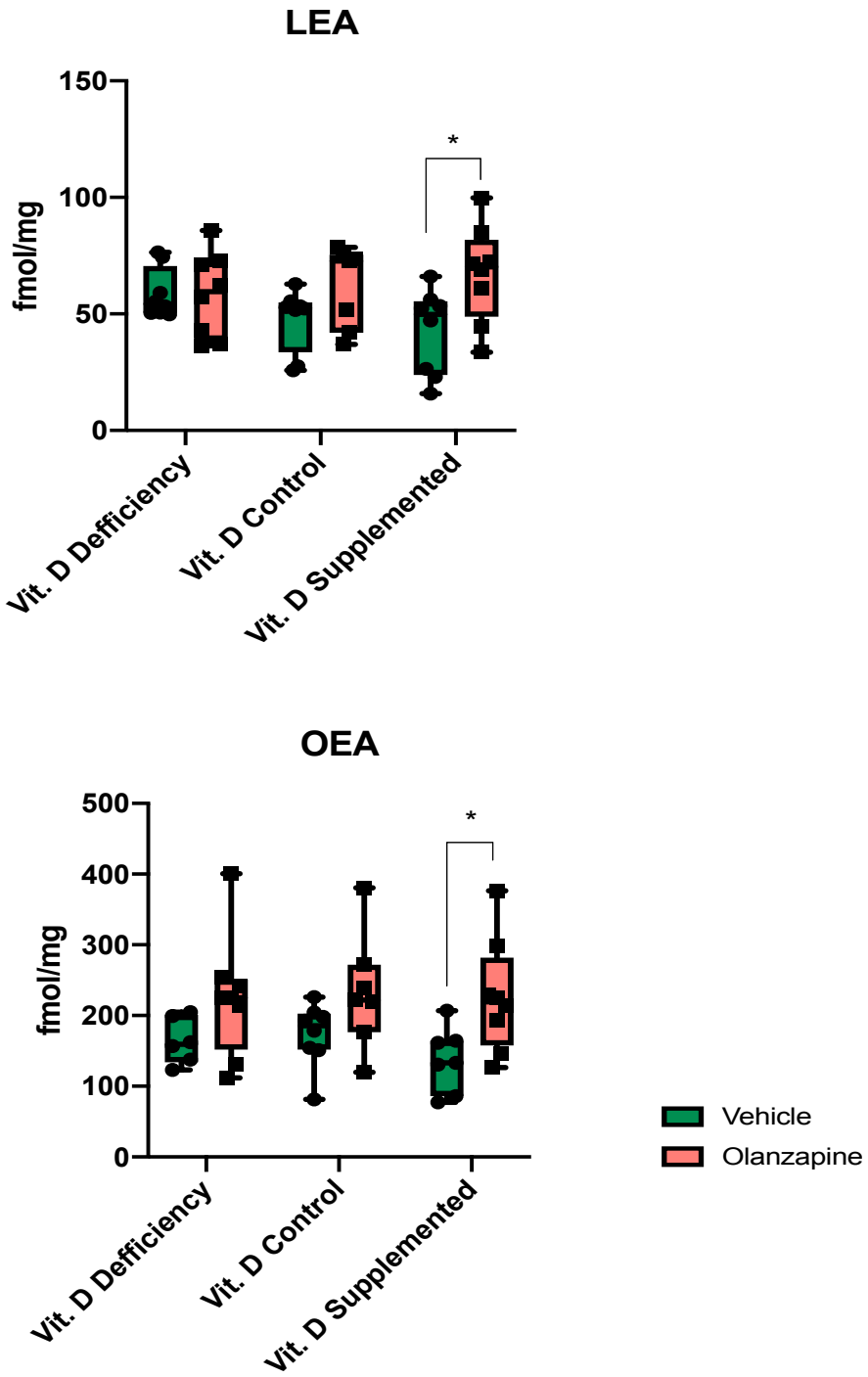

Figure 3-11. NAEs lipid mediators in ovarian adipose tissue

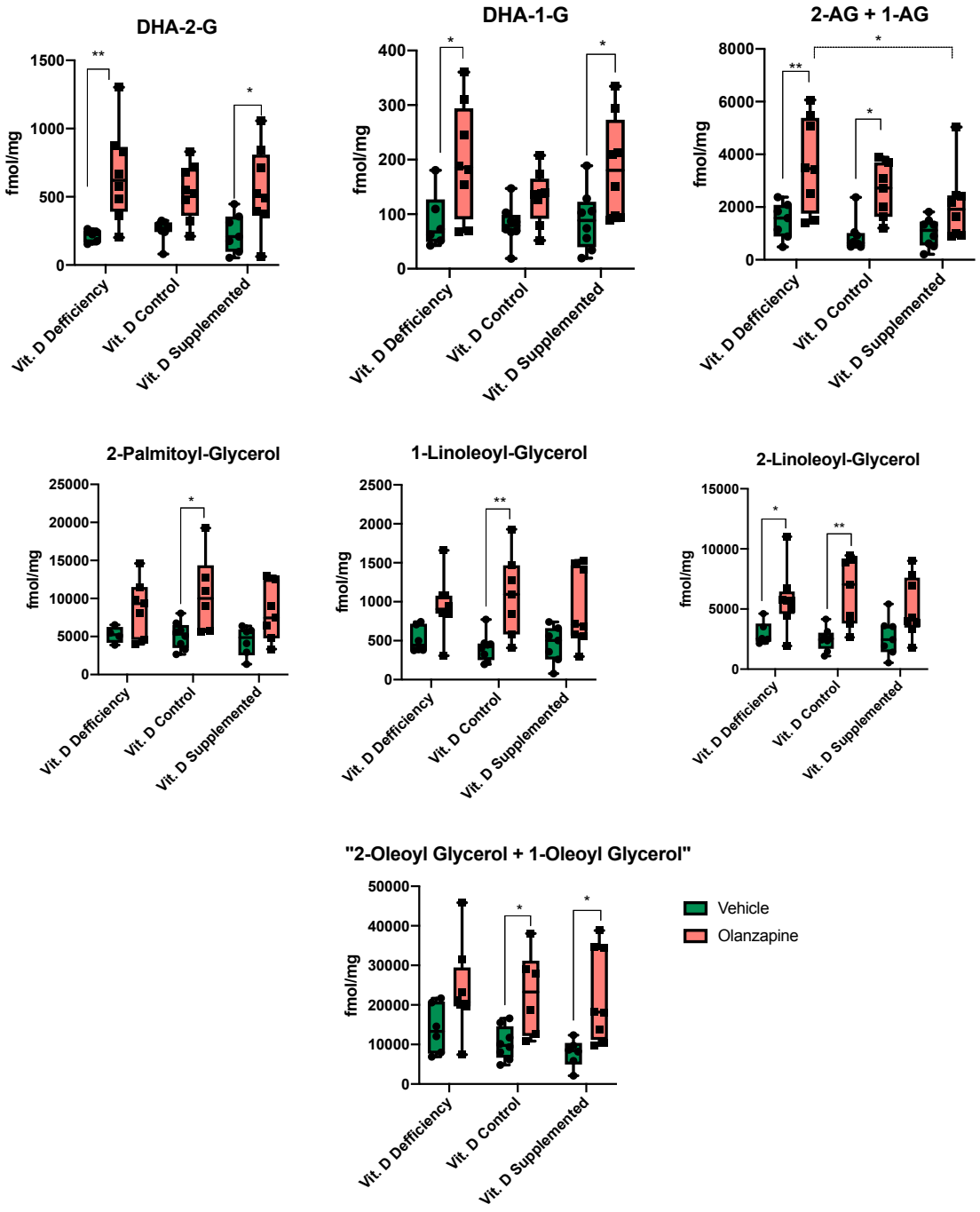

Figure 3-12. 2-MGs lipid mediators in ovarian adipose tissue

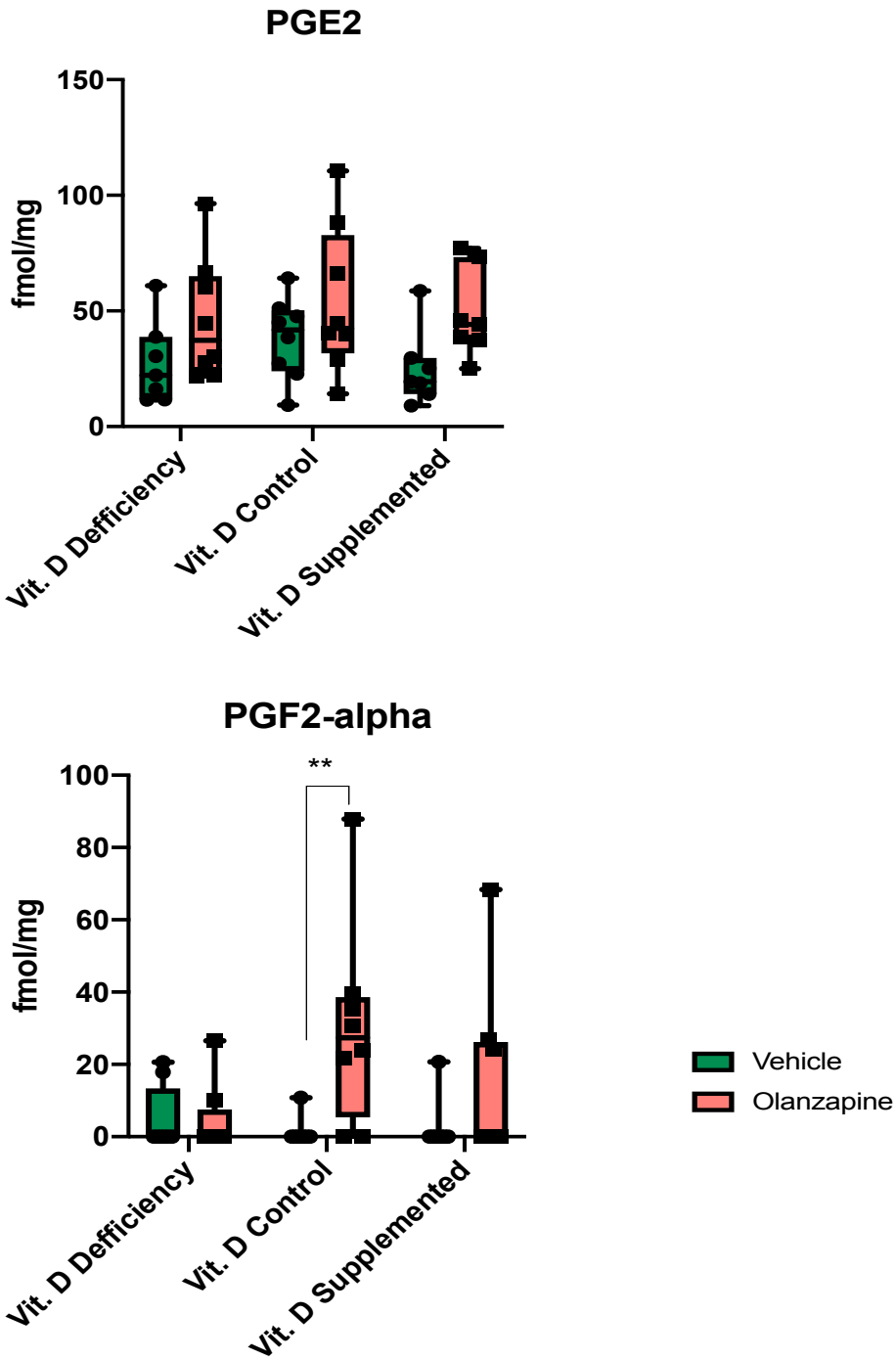

Figure 3-13. prostaglandin levels in ovarian adipose tissue

3.4.9.2. Subcutaneous adipose tissue

As for the ovarian adipose tissue, there was increment of NAE levels in olanzapine groups among different vitamin D levels (figure 3-14). This increase was significant in conjunction with the supplementation of vitamin D for OEA ($p < 0.05$).

2 -MGs had same increasing trend, but it was just significant in supplemented vitamin D level of DHA-2-G and 2-Palmitoyl-G ($p < 0.001$) (figure 3-15).

Olanzapine increased prostaglandin levels in sub AT (figure 3-16). Although, this increase in PGE2 and PGD2 were significant under deficiency and control levels of vitamin D. There was almost no PGF2-alpha detected in any of the vehicle groups, but olanzapine increased the level of this prostaglandin, particularly in the control vitamin D diet ($p< 0.05$). 6-keto PGF1-alpha levels increased greatly with supplementation of vitamin D in response to olanzapine ($p < 0.001$).

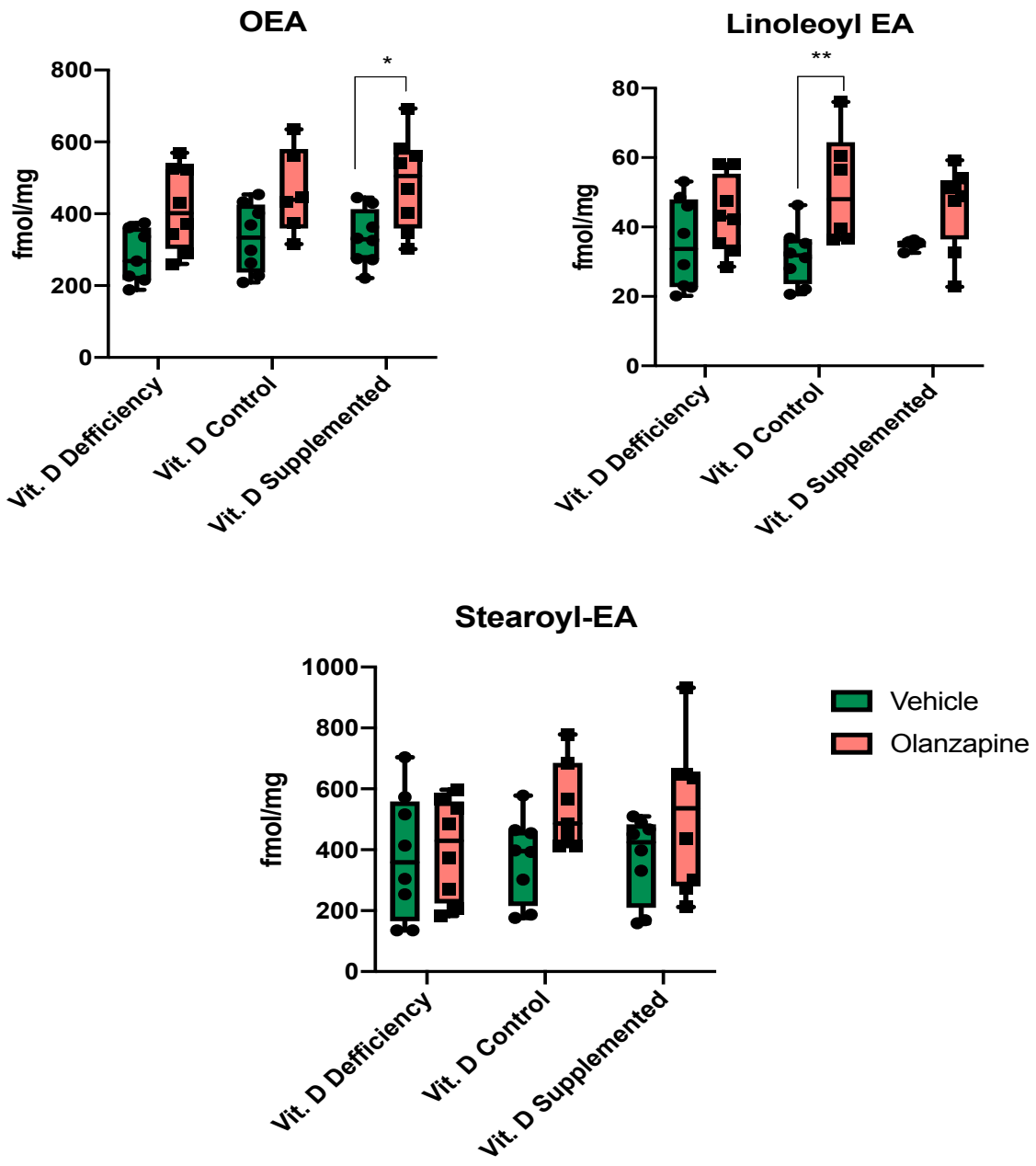

Figure 3-14. NAEs lipid mediators in subcutaneous adipose tissue

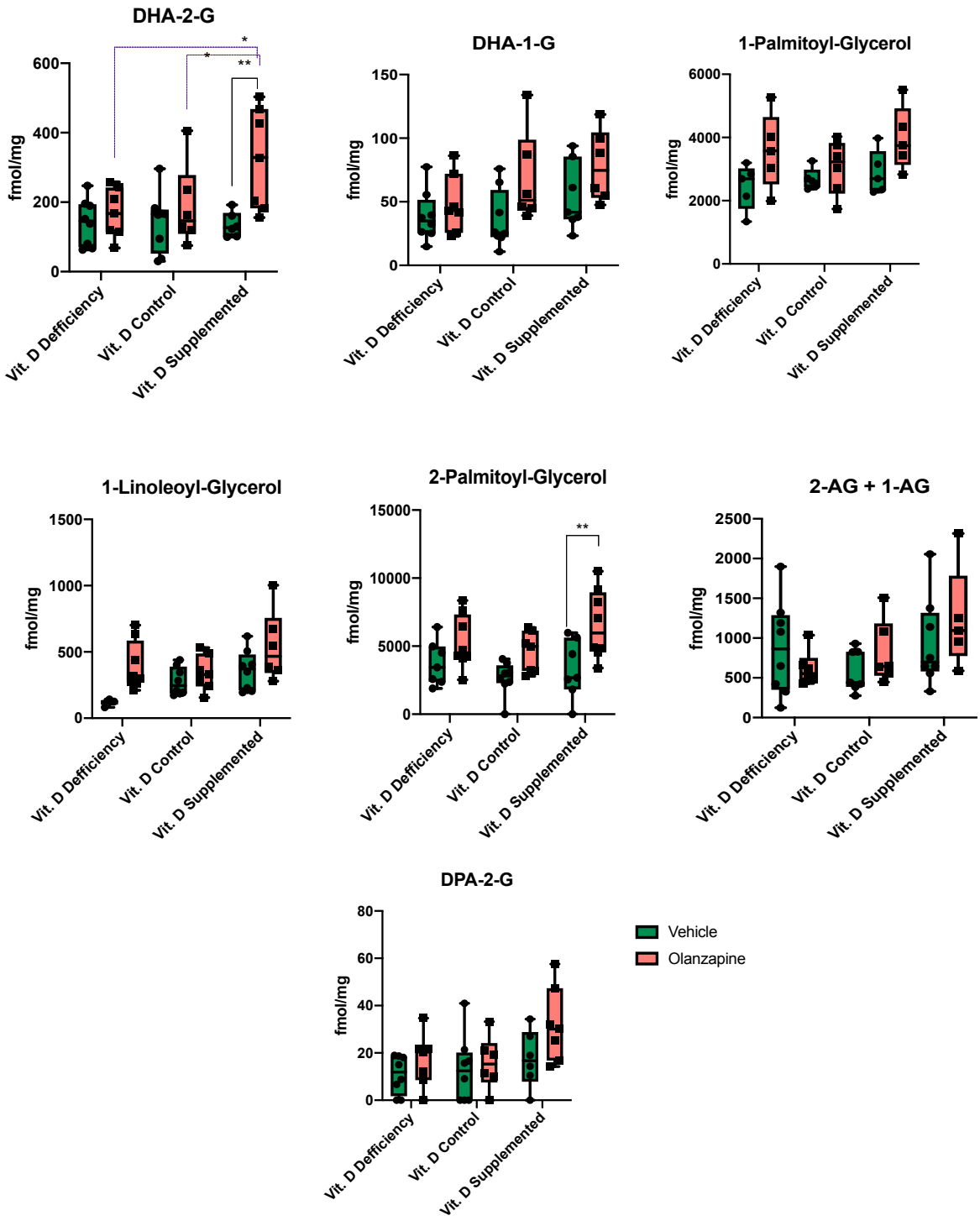

Figure 3-15. 2-MG lipid mediators in subcutaneous adipose tissue

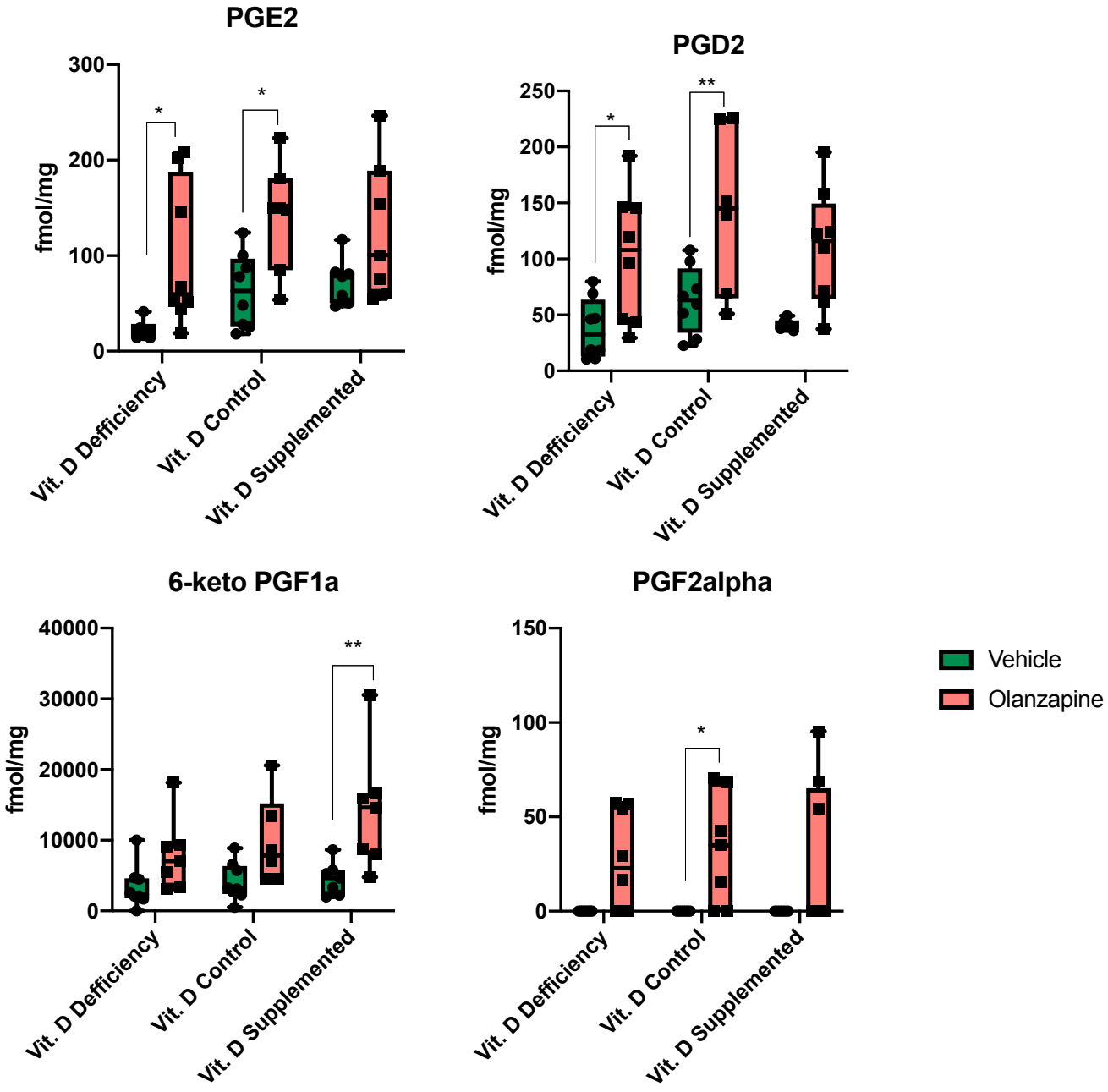

Figure 3-16. Prostaglandin levels in subcutaneous adipose tissue

3.4.10. Gut microbiota

Principal Coordinate Analysis (PCoA) are useful to display between-sample differences in gut microbiota composition (B-diversity). Here, Bray-Curtis dissimilarity indexes were used to estimate B-diversity.

According to the PERMANOVA p-values, there was a significant difference between day 21 and day 84, linked to diet changes from low fat-low sucrose to the high fat-high sucrose ($p<0.001$), but there were no differences between our experimental groups (figure 3-17).

The relative abundance of the most prevalent bacterial taxa (at least 1%) shows some changes in the family level (figure 3-18, 3-19).

Different levels of vitamin D after 21 days under LFLS diet, made changes in the family levels of Peptococcaceae, Enterobacteriaceae, Clostridiales_vadinBB60_group, Clostridiaceae_1 and Lachnospiraceae (table 3-2).

Following day 21, by changed the diet to HFHS and administration of olanzapine the family levels of bacteria changed again, and we had changes in different families of Atopobiaceae ($p< 0.01$), Muribaculaceae ($p< 0.01$) and Bifidobacteriaceae ($p< 0.05$) (figure 3-19)

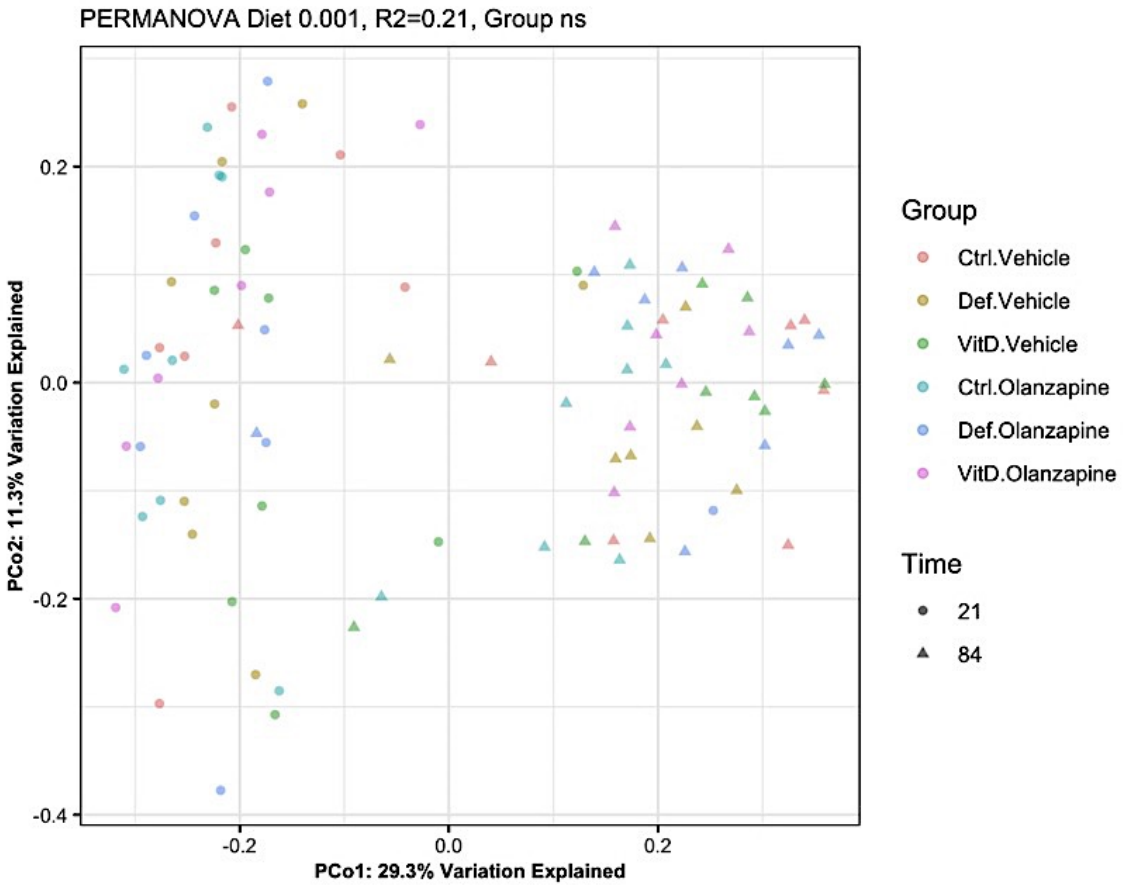

Figure 3-17. Dissimilarity or beta-diversity of gut microbiota between groups in days 21 and 84.

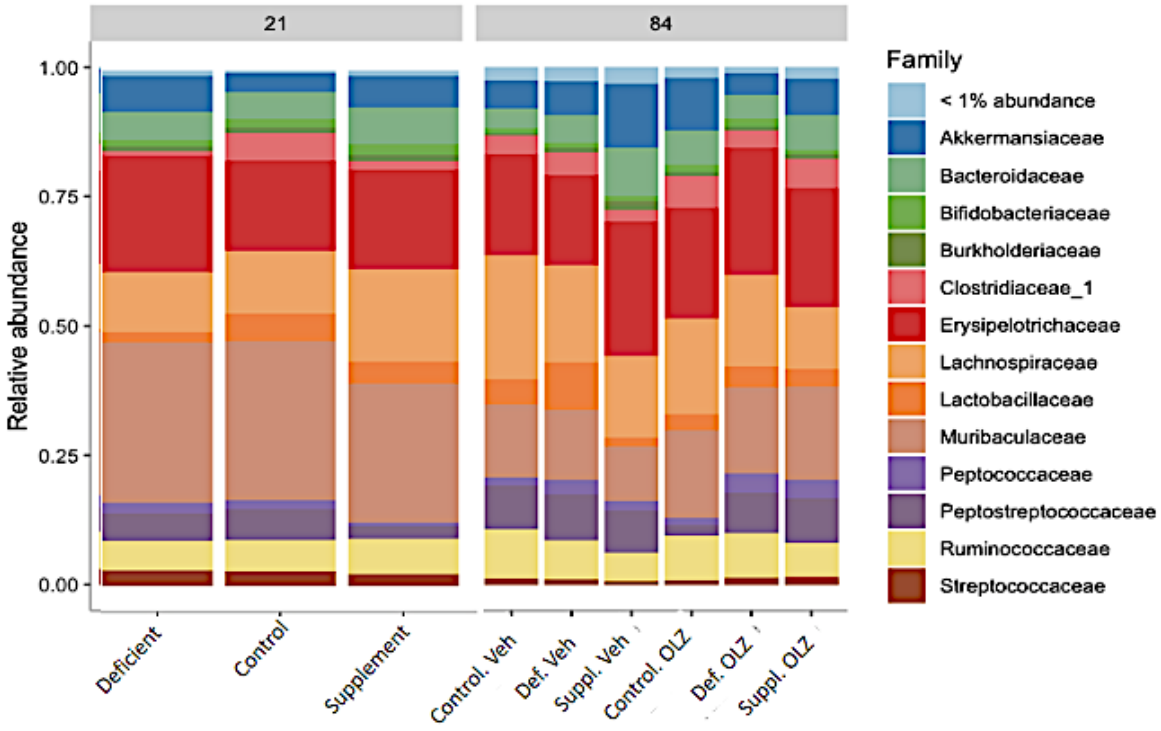

Figure 3-18. relative abundances of bacterial taxa in d 21 and 84

Table 3-2. differences in family level of bacteria according to the vitamin D levels on day 21

	Phylum	Family	Control vs Deficiency	Supplementet vs Deficiency	Supplemented vs Control
day 21	Furmicates	Clostridiales_vadinBB60_group	ns	0.09 ↑	0.0011 ↑
	Furmicates	Peptococcaceae	ns	ns ↓	0.037 ↓
	Furmicates	Clostridiaceae_1	0.091 ↑	ns	ns ↓
	Furmicates	Lachnospiraceae	ns	0.1 ↑	ns ↑
	Proteobacteria	Enterobacteriaceae	0.05 ↓	ns	0.06 ↓

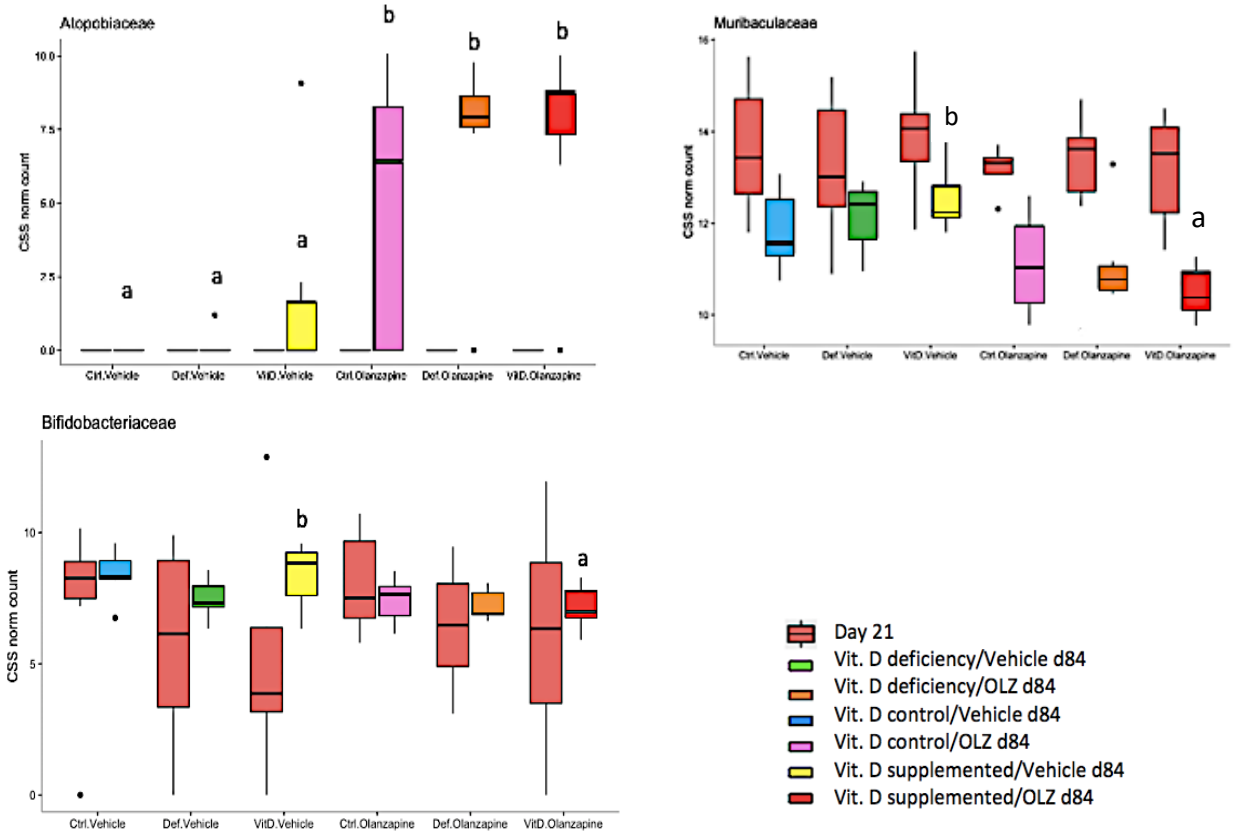

Figure 3-19. differences in family level of bacteria after olanzapine administration on day 84.

3.5. Discussion

It is currently accepted that obesity is a global health issue as it is causally related to numerous chronic disorders, including cardiovascular disease, diabetes, cancer, osteoarthritis, and chronic kidney disease (Ng et al., 2014).

Obesity is linked to adipose tissue dysfunction, including adipocyte hypertrophy, adipose tissue inflammation, impaired lipid metabolism, and insulin resistance. Evidence supports the association of obesity with different parameters such as vitamin D deficiency, psychotic disorders, dysbiosis of gut microbiota and endocannabinoids (Annamalai et al., 2017; Argueta & DiPatrizio, 2017; Pramono et al., 2019; Aron-Wisnewsky et al., 2019).

Vitamin D deficiency is a health problem that has been implicated in a wide variety of diseases including psychotic disorders and metabolic syndrome (Botella-Carretero et al., 2007; Benetti et al., 2018; Patrick & Ames, 2015). Vitamin D is involved in neurotransmitter synthesis, neuroprotection in response to injury and inflammation, and neurodevelopment. Epidemiological evidence suggests that low vitamin D levels are associated with of schizophrenia (Cieslak et al., 2014).

Vitamin D might play an anti-obesity role by inhibiting adipogenesis during early adipocyte differentiation; in addition, hyperparathyroidism due to a low vitamin D may cause the overload of calcium into adipocytes, increasing lipogenesis, and reducing lipolysis, and so vitamin D deficiency represents a risk factor of and potential treatment for, obesity (Migliaccio et al., 2019). Indeed, vitamin D supplementation in healthy overweight and obese women, increased vitamin D blood concentrations and led to body fat mass reduction (Salehpour et al., 2012).

Olanzapine is an effective second generation/atypical antipsychotic and produces less adverse effects for movement including akathisia, dystonia and hypertonia, however tends to cause more weight gain than typical antipsychotics (Fulton & Goa, 1997). Atypical antipsychotic drugs are correlated with a major risk of metabolic side effects, leading to dyslipidemia and obesity. Studies on the effect of risperidone, an atypical antipsychotic drug, on female mice showed an augmentation on food intake and an induction of body weight gain accompanied with an increase in visceral adipose tissue (Auger et al., 2014); another study reported administration of

olanzapine (8mg/kg/d) on female mice under the high fat- high sugar diet after three weeks causes appetite stimulation and weight gain (Coccurello et al., 2006).

A study of risperidone, another second-generation antipsychotic that causes weight gain, and its linkage to the gut microbiota in female mice, has shown that risperidone-mediated weight gain due to decreased energy expenditure that correlated with dysbiosis, and fecal transplant from risperidone-treated mice to naïve recipients was concomitant with less energy expenditure and weight gain in the recipient mice (Bahr et al., 2015a).

Human study of chronic treatment with risperidone reported an increase in BMI and decrease in the Bacteroidetes:Firmicutes ratio compared to the antipsychotic-naïve control psychotic patients (Bahr et al., 2015b)

A study on female schizophrenic patients being treated with olanzapine found that administration of vitamin D was generally effective in reducing obesity (Kaviyani et al., 2018). Whereas, another study has shown that schizophrenic patients with vitamin D deficiency who were under treatment of clozapine, after vitamin D supplementation for 8 weeks, exhibited higher levels of vitamin D on their serum, but this vitamin D increment did not affect patient metabolic profile nor psychotic status (Krivoy et al., 2017).

Based on the above, we supposed that vitamin D status could modify the severity of olanzapine-induced side effects; with increased food intake and weight gain in response to olanzapine in vitamin D deficiency and less weight gain in in response to olanzapine with vitamin D supplementation as compared to control levels of vitamin D. Surprisingly, results of our study showed a significant decrease in weight gain and body fat mass and decreased insulin resistance in olanzapine groups compare to their controls. Increasing vitamin D levels showed trends for a reduction in body weight gain and fat mass in both vehicle and olanzapine treated groups, confirming the effect of vitamin D on obesity. However, no such trend was observed for insulin resistance, and in fact, vitamin D supplementation

In obesity, excess of adipose tissue modulates metabolism by releasing non-esterified fatty acids and hormones including adiponectin and leptin with changing the production of many of these products. Instead, adiponectin acts as an insulin activator, through stimulation of AMP-activated

protein kinase (AMPK) and PPAR-α dependent manner, all these factors are involved in the development of insulin resistance (Kahn et al., 2006).

Insulin has many physiological activities including reduction of blood glucose, stimulation of fatty acids synthesis, promoting mitochondrial function and inducing cell proliferation (Straus, 1981; Wong & Sul, 2010; Cheng et al., 2010; Röder et al., 2016). In obesity, over production and decreased clearance of insulin may results in hyperinsulinemia (Templeman et al., 2017). In obesity, the number and function of β cells in pancreatic islets are increased through the stimulation of β cells by fatty acids/glucose (Ye, 2013). Considering the interaction between body fat mass and insulin resistance, our results suggest that under our high fat diet, the increased insulin sensitivity in response to olanzapine is may in part be a result of olanzapine-induced reductions in fat mass and weight gain.

The endocannabinoidome, a complex lipid signaling system including eCBs, other fatty acid-derived mediators, their receptors and anabolic and catabolic enzymes, which are involved in the control of energy metabolism and body weight (Di Marzo & Silvestri, 2019). The role of eCBs in body weight control is shaped early in life, 2-AG and other eCB-like compounds were found in many mammalian species' milks. Oral administration of AEA during the nursing period to the mouse pups has showed increase in body weight, with increased adiposity, insulin resistance and higher levels of *CB1* expression in adipose tissue later in their adult life (C A Aguirre et al., 2015; Carolina A Aguirre et al., 2012; Horn et al., 2018). The importance of the eCB system's role in the body weight control has been further demonstrated in $CB1^{-/-}$ mice with significant decrease of caloric intake and body weight compare to the control mice (Pi-Sunyer et al., 2006; Horn et al., 2018).

TRPV1 channels are modulated by several eCBome mediators including long-chain-saturated NAEs, MAGs, N-acyldopamines, and N-acyltaurines (Di Marzo & Silvestri, 2019).Multiple members of the N-acyl ethanolamides including AEA, OEA and PEA have been shown to activate TRPV1 (Di Marzo et al., 2001; Ho et al., 2008). Additionally, different MAGs including 1-monoacylglycerols (1-MGs) having C18 and C20 unsaturated and C8–C12 saturated fatty acid (FA) and 2-MGs having C18 and C20 unsaturated fatty acids activateTRPV1 receptors (Iwasaki et al., 2008; Zygmunt et al., 2013).

The role of TRPV1 in obesity and dysregulation of glucose homeostasis is complex, dietary capsaicin, an exogenous agonist of TRPV1, has been shown to reduce obesity-induced inflammation and insulin resistance in obese mice fed with a high-fat diet. It has been shown that administration of dietary capsaicin for a 10-week period resulted in lower fasting glucose levels, lower insulin and leptin levels, and improved glucose tolerance (Kang et al., 2010). Activation of TRPV1 through capsaicin promotes energy metabolism and suppresses visceral fat accumulation. MGs, having an unsaturated long-chain fatty acid, another TRPV1 agonist in foods, increases the expression of UCP-1 and causes a decrease in the weight of epididymal white adipose tissue, serum glucose, total cholesterol and free fatty acid levels in male mice under a high fat-high sucrose diet (Iwasaki et al., 2011). Activation of TRPV1 facilitates Ca^{2+} entry into cells and promotes the binding of PPAR-γ and PRDM16 to the promoter of UCP1, consequently stimulating browning of WAT and thermogenesis in BAT (Gao et al., 2019). In contrast, it has been shown that *Trpv1*$^{-/-}$ mice on high-fat diet have lower body weight compare to the wild type mice, which demonstrates a role for TRPV1 in stimulating fat accumulation and weight gain (Motter & Ahern, 2008). It has been shown that the two nonpsychotropic cannabinoids cannabidivarin and cannabidiol dose-dependently activate and then desensitize TRPV1 channels *in vitro* (Iannotti et al., 2014). Increasing the levels of NAEs including PEA and OEA is accompanied with TRP channel desensitization (Lowin et al., 2015). Another in *vitro* study on TRPV1, has demonstrated that administration of either capsaicin or PEA increase the desensitization of TRPV1, but this desensitization is more extent in PEA-induced TRPV1 (Ambrosino et al., 2013).

It has been shown, *Napepld* knockout mice reduce PEA and OEA levels, downregulate *Ucp1* expression in the WAT, which may suggests the role of these mediators on WAT browning (Geurts et al., 2015).

Results from our study show that olanzapine decreases the expression of *Trpv1* and increases its agonists including NAEs and 2-MGs which may cause desensitization of TRPV1, then weight loss and decrease in fat mass through stimulation of browning the WAT.

eCBome gene expression analysis in our study has demonstrated an increase in the eCBome gene encoding the catabolic enzyme of MGLL in olanzapine groups. This enzyme not only hydrolyses bioactive 2-MG eCBome mediators, but is also the last and rate limiting step of lipolysis,

hydrolysing MAGs to fatty acids and glycerol (Clapper et al., 2018; Hofer et al., 2020). In our study, an increase in the levels of 2-MGs may be as a result of lower expression level of *MGLL* and since in our study, olanzapine treated mice had less fat mass there may be lower rates of lipolysis, which may be link to the decrease of MGLL in this groups.

Further, we have seen an increase in the expression of the NAE anabolic enzymes *Gde1* And *Gdpd1* in olanzapine treated groups which might be the cause of enhancement in eCBome mediators and prostaglandins.

Vitamin D supplementation can decrease PGE2 production human lung fibroblasts through stimulation of 15-hydroxy prostaglandin dehydrogenase which is a PGE2 degradation enzyme, *in vitro* (Xiangde Liu et al., 2013) . Another study of the effect of Vitamin D on chondrocytes, *in vitro*, has demonstrated the regulation of PGE2 through Vitamin D administration, which may be linked to changes in the activity of phospholipase A2 , and thus regulation of free arachidonic acid levels, andconsequentlyPGE2 production (Schwartz et al., 1992). However, in our study, we didn't see any changes between groups under different vitamin D levels.

Previous studies have demonstrated administration of olanzapine reduces PGE2 levels in different parts of brain *in vivo* and *in vitro* (Cheon et al., 2011; Faour-Nmarne & Azab, 2015), but our results has demonstrated enhancement in different prostaglandins including PGE2, PGF2-alpha, PGD2 and 6-keto PGF1-apha particularly in subcutaneous adipose tissue.

Prostaglandins including PGD2, PGE2 and PGF2-alpha are synthesized from arachidonic acids and are involved in the differentiation, maturation and function of white adipocytes (Pisani et al., 2014)

PGF2-alpha is a potent inhibitor of adipocyte differentiation, it reduces adipogenesis via a Gαq-Ca2+-calcineurin-dependent signaling pathway that blocks expression of PPAR-γ and C/EBPα by a mechanism that appears to involve an HDAC-sensitive step (L. Liu & Clipstone, 2007).

PGE2 has a key role on browning the WAT, a study on PPAR-γ knockout mice have shown increase of UCP-1 and PGE2 in WAT, in turn, increasing the levels of PGE2 suppressed expression of PPAR-γ and blocked rosiglitazone-induced pre-adipocyte differentiation toward white adipocytes however increased UCP-1 expression and pre-adipocyte differentiation toward brown adipocytes (García-Alonso et al., 2013). Conversely, PGE2, by inhibiting the activity of Hormone-sensitive

lipase (HSL), the most important lipolytic enzyme in WAT, has an anti-lipolytic action on obese WAT (García-Alonso et al., 2016). Lipolysis stimulation increased release of PGE2 and PGD2, and enhanced COX-2 expression on obese WAT cells *in vitro*. PGE2 is suggested to mediated macrophage migration into adipose tissue but without inducing inflammation during weight loss (Hu ref).. Reconstituted medium with PGE2 effectively stimulated macrophage migration, while GD2 had less effects. Conversely, in fasted WAT cells, macrophage accumulation in adipose tissue coincided with upregulation of PGE2 levels and COX1 expression, suggesting that , PGE2 originating from adipocytes plays an important role in mediating adipose tissue macrophage accumulation but suppresses inflammation during lipolysis and modulates insulin sensitivity (Hu et al., 2016). PGD2, another prostaglandin which has role on adipose tissue metabolism, enhances lipid accumulation in adipocytes. PGD2 prevents lipolysis by repression of the cAMP-PKA-HSL axis through DP2R in adipocytes and enhances adipogenesis via activation of PPAR-γ (Geurts et al., 2015; Mazid et al., 2006). However, its molecular mechanism and complex interactions requires further investigation. PGI2 or prostacyclin is another prostaglandin that rapidly hydrolyzed into 6-keto-PGF1-alpha in biological fluids. PGI2 has a potent stimulation effect on differentiation of preadipocyte cells via up-regulation of C/EBPβ and C/EBPδ in preadipocyte cells for initial differentiation and activation of PPAR-γ to initiate the terminal differentiation of adipocytes (Rahman, 2019). Although, carbaprostacyclin (cPGI2), another stable analog of PGI2, stimulates the differentiation of white to beige adipocyte (Ghandour et al., 2016; Rahman, 2019).

Decreased fat mass accumulation in our experimental mice in the olanzapine treatment groups, aside from the effect of PGF2-alpha on adipogenesis inhibition, may be linked to increased levels of other prostaglandins within various adipose tissues (PGE2, PGD2 and 6-keto-PGF1-alpha), which may result in the stimulation of pathways to induce differentiation of white-to-brown adipocytes, modify lipid metabolism and affect adipogenesis.

Gut microbiota has a crucial role in energy regulation and obesity. The major part of human gut microbiota belong to three phyla; *Firmicutes*, *Bacteroidetes* and *Actinobacteria* (Mariat et al., 2009). Increasing *Firmicutes* and decreasing the proportion of *Bacteroidetes* and increasing *Actinobacteria* develop risk of obesity (Turnbaugh et al., 2006, 2009).It is possible that some

treatments, by altering gut microbiota composition could ameliorate olanzapine-induced weight gain (Davey et al., 2013). Vitamin D regulates the gut microbiome and its deficiency can results in dysbiosis in gut (Luthold et al., 2017; Ooi et al., 2013). Our results indicate that while chronic olanzapine under a HFHS diet does not appear to induce global changes in gut bacterial community structure, it does significantly alter the levels of *Atopobiaceae*, *Muribaculaceae* and *Bifidobacteriaceae*, the latter two of which were only modulated under conditions of vitamin D supplementation.

A study on high fat diet feeding demonstrated a decreased level of *Muribaculaceae* family, belong to *bacteriodates* phylum, in common with significant weight gain in mice (Gong et al., 2020). However, here, we show that olanzapine decreased this family, though only significantly so when administered in conjunction with vitamin D supplementation, suggesting that this little described family may not necessarily be obesogenic.

The level of *Actinobacteria* is higher in obese people (ref). *Although, Bifidobacteriaceae* , belong to *Actinobacteria,* this family is known as a generally protective in that it is associated with reduced intestinal inflammation, and amelioration of insulin resistance and glucose tolerance (Gonai et al., 2017; LE et al., 2014; McCabe et al., 2019), even though the relative abundances of *Bifidobacteriaceae* increase in diet induce obesity (Kulecka et al., 2016). , In a correlation analysis between gut microbiota family and metabolic biomarkers the *Atopobiaceae* family (also belonging to the *Actinobacteria* phylum) was found to have a negative relationship with TG and fasting blood glucose, and positive correlation with SCFAs of acetic acid, propionic acid, butyric acid and iso-butyric acid (Chakraborti, 2015; Wang et al., 2020).

SCFAs, are nutrients produced by gut microbiota that have role in the regulation of energy metabolism and adipose tissue function (Delzenne & Cani, 2011) Acetate and propionate are the main products of *Bacteroidetes* and the most abundantly produced SCFAs in the gut, butyrate is mostly produced by *Firmicutes* which is considered as a favourable marker of metabolic health (Chakraborti, 2015). *Bifidobacteriaceae* produce great amounts of acetate, but do not produce propionate which are mainly produced by *Bacteriodetes* phylum (Binda et al., 2018). Furthermore, the *Muribaculaceae* family is intensely correlated with propionate production (Smith et al., 2018). Acetate and propionate signal via activation of GPR43, the short-chain fatty

acid receptor, resulting in the inhibition of lipolysis and adipocyte differentiation, thereby promotes fat mass in high-fat-diet-fed (Bjursell et al., 2010; Ge et al., 2008). The SCFA acetate has regulatory effects in several pathways involved in energy expenditure and fat utilization, and increased acetate levels is proposed to be an approach for the prevention and management of obesity and glucose metabolism (Canfora & Blaak, 2017).

We observed a decrease in the level of *Muribaculaceae* and *Bifidobacteriaceae* families and an increase of *Atopobiaceae* through16S analysis of our experiments, which may result in changes of propionate and acetate levels in response to olanzapine, which may contribute to the decreased accumulation of fat mass through increasing energy expenditure and lipolysis in the olanzapine treated mice.

Future studies will be devoted to measuring short chain fatty acids (SCFAs) levels in mice feces to evaluating the possible effects of olanzapine and vitamin D status on SCFA levels and their impact on energy expenditure and adipose tissue metabolism.

In conclusion, results of this study suggest that under HFHS diet consumption, olanzapine may have a protective effect by ameliorating body metabolism with concomitant changes in eCBome system and gut microbiota, results in decreased body fat mass. These observations are constant with our findings that olanzapine decrease Trpv*1* expression and increase the levels of its agonists, including NAEs and 2-MGs. These changes were identified in correlation with alterations of various microbial family levels. Additionally, vitamin D supplementation consistently showed trends for improved the metabolic status of the mice, however the changes were generally not statically significant. Taken together, changes of eCBome and gut microbiota families in our experiment could contribute to the olanzapine-mediated inhibition of weight gain in mice on an HFHS diet.

4. Conclusion

These studies suggest that different environmental parameters like high fat diet, olanzapine, vitamin D, calorie restriction and exercise individually and in pair may have different effects on body metabolism and obesity through changes in various factors, we propose that alteration of the endocannabinoidome may affects fat metabolism at the molecular level in response to various environmental parameters and is a viable target for the treatment of obesity potentially by increasing lipolysis and beta-oxidation, browning of adipose tissue and decreasing adipogenesis.

5. **Bibliography**

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