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Impact of dietary conjugated linoleic acid (CLA) on fatty acid metabolism and endocannabinoid biosynthesis

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Abstract

Background: Conjugated linoleic acid (CLA) refers to a group of positional and geometric isomers of linoleic acid (LA) mainly found in the meat and dairy products of ruminants. CLA has been shown to possess different biological activities such as anti-carcinogenic and anti-atherogenic properties, and also to influence body weight, energy and lipid metabolism, immune response, and inflammation. The endocannabinoid system (ECS) is involved in a variety of physiological processes, including the regulation of feeding behavior and energy homeostasis. ECS includes neuromodulatory archidonate-based lipids, the best characterized are 2-arachidonoyl-glycerol (2-AG) and N-arachidonoylethanolamine (AEA or anandamide), which activate specific cannabinoid receptors (CB1 and CB2). *N*-oleoylethanolamide (OEA) and *N*-palmitoylethanolamide (PEA) are generally termed as endocannabinoid-related compounds. OEA has been shown to decrease food intake and body weight gain, while PEA is known to possess anti-inflammatory activity. Conversely to endocannabinoids, OEA and PEA don't exert their actions by activating cannabinoid receptors, but mostly through binding the peroxisome proliferator activated receptors (PPARs).

Methodology: The aim of the present thesis was to evaluate the effects of dietary intake of CLA on fatty acid metabolism, tissue fatty acid incorporation and endocannabinoid biosynthesis in liver of obese Zucker rats and in plasma of healthy volunteers. 32 male rats were fed with an equimolar mix of *c*9,*t*11 and *t*10,*c*12 CLA isomers (about 1%) supplemented or not with two different background diets, one based on fat of vegetal origin, PO, and the other made with a fat content of animal origin, OF. Treatment lasted 3 months. In a randomized cross-over study we also evaluated the effects of very high doses of CLA on lipid profile and endocannabinoid levels in plasma of 24 healthy men and women (aged 18 to 65) who consumed each three distinct diets for three weeks. Diets were identical except for 7% of total energy (approximately 20 g/day) which was provided either by: CLA isomers (80% *c*9,*t*11 and 20% *t*10,*c*12), industrial trans fatty acids, or oleic acid (OA).

Principal Findings: In Zucker rats, irrespective of the background diet CLA affected body and liver weight, with a reduced hepatic lipid deposition. CLA intake increased concentration of arachidonic acid (AA) with both PO or OF diets, and docosahexaenoic acid (DHA) only when CLA was included in the OF diet. CLA feeding in combination with PO diet only, increased retinol level. CLA intake resulted in a decrease of Δ9 desaturase index, which was inversely correlated to n-3 highly unsaturated fatty acid (HUFA) score in OF diet. In human plasma, CLA compared to OA diet decreased the metabolism and the incorporation of the LA metabolites, and influenced alpha linolenic acid (ALA) metabolism, which resulted in an increased n-3 HUFA score. Also, we confirmed as high doses of CLA reduced elongation of long chain fatty acids (LCFAs). CLA intake did not cause significant changes in the profile of endocannabinoids in liver of Zucker rats and in human plasma, but we found increased levels of PEA in OFCLA fed rats, and OEA in both OF and PO background diets supplemented with CLA.

Conclusions: Based on our results, we conclude that the effects of CLA on fatty acid metabolism and endocannabinoid biosynthesis are strongly influenced by the background diet, which may also explain the differences found between experimental animals and humans. By improving n-6/n-3 HPUFA balance and sustaining PPARα activity, directly and indirectly through OEA and PEA, CLA may exert its beneficial actions on human health and protection against some diseases, especially those characterized by chronic inflammation due to an impaired body fat deposition.

Keywords: Conjugated linoleic acid, Endocannabinoids, Fatty acid composition, Fatty acid metabolism

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Paulilatino, March 2014

Antonio Piras

1. Introduction

For many years, the research in the fields of nutrition and food science has been specifically focused on the safety and toxicity of foods in relation to their conservation, as well as for obtaining the proper nutritional requirements to maintain an optimal state of health. Today, however, the quality and quantity of food intake is seen as a possible mean to preserve health in humans at different times in life, when physiologically, some nutritional needs are different (growth, pregnancy, lactation, special conditions of stress, etc.) and to slow or prevent the onset of chronic and degenerative diseases that are on the rise, also due to the elongation of life expectancy. This implies the recognition and characterization of substances in foods with specific biological activities and the study of their action in different physiological and pathological conditions. Once the target population suitable to these biologically-active compounds has been determined, the natural application of biotechnology research is the formulation of products enriched in these substances, the so-called functional foods. They are not necessarily "built" foods, more often are foods already present in our diet, but for technology and marketing needs have never been considered. Nutrition research is increasingly interested in the identification and characterization of molecules naturally present in foods with beneficial properties for human health. Of remarkable interest has been the discovery of the biological properties of a particular group of fatty acids, the conjugated linoleic acids (CLAs). These unusual fatty acids have shown multiple positive activities in different experimental models. Dairy products are particularly rich in CLA. The understanding of the mechanisms by which CLA exerts its protective effects against many diseases, appears to be important not only from a scientific point of view, but would also be an incentive to the daily consumption of foods rich in this fatty acid by

those sections of the population more exposed to the onset of diseases. The influence of dietary fatty acids on the endocannabinoid system (ECS) represents an emerging and interesting matter of study for nutrition research, in fact ECS is deeply involved in a variety of physiological mechanisms, including the control of feeding behavior, energy balance, and energy homeostasis.

There are no many studies and experimental data published in literature about the influence of CLA on endocannabinoid metabolism, and the aim of the present thesis is to investigate the impact of dietary CLA intake on fatty acid metabolism and endocannabinoid biosynthesis in an animal model of obesity, the fatty Zucker rat, and in healthy subjects.

1.1 Conjugated linoleic acid (CLA)

Conjugated linoleic acid (CLA) refers to a group of at least 28 positional and geometric isomers of linoleic acid (LA, 18:2 *c*9,*c*12). These fatty acids are characterized by a carbon chain containing conjugated double bonds. These double bonds may be found from position Δ6 to position Δ13, in *cis* (*c*) or *trans* (*t*) configuration. The different combinations lead to the formation of different isomers, however, in nature and in synthetic blends the most abundant are the *c*9,*t*11 CLA and *t*10,*c*12 CLA isomers (Fig.1), which showed different biological activities. The predominant isomer found in foods is the *c*9,*t*11 CLA, also called as rumenic acid (RA). CLA isomers are mainly found in the meat and dairy products derived from ruminants (1). The presence of CLA in dairy products is due to a biochemical process called biohydrogenation, which takes place in the rumen of ruminant animals by fermentative activity of the anaerobic bacterium *Butyrivibrio fibrisolvens* (2).

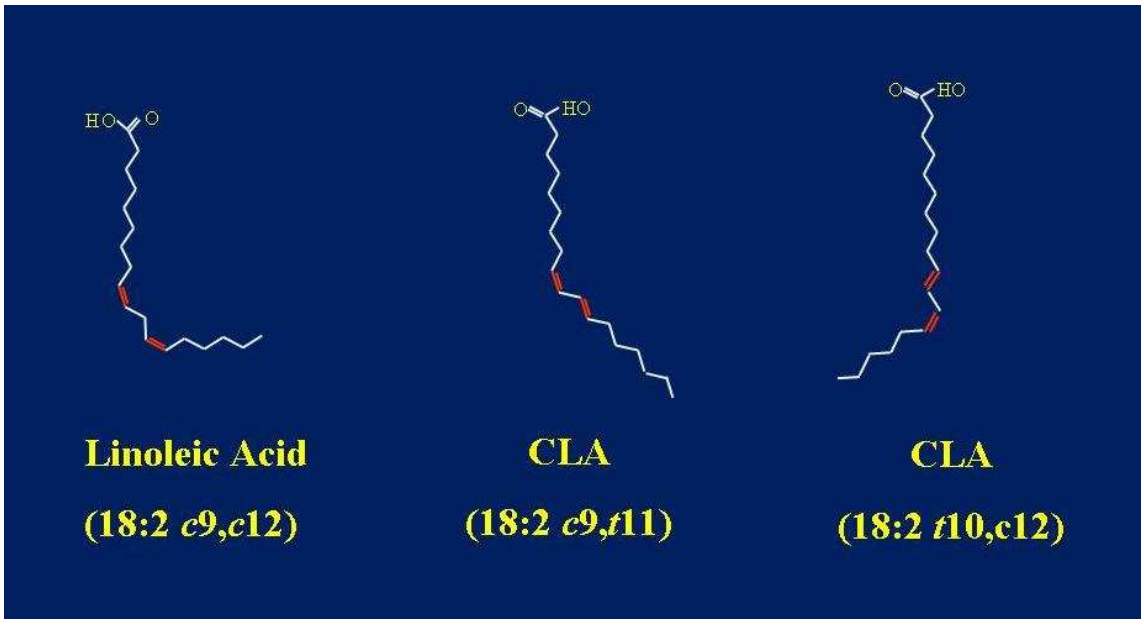


Figure 1. Chemical structure of the main CLA isomers c9,t11 and t10,c12, compared to linoleic acid (LA, 18:2 c9,c12).

The biohydrogenation (Fig.2) consists of several enzymatic reactions, one of them is catalyzed by the enzyme linoleic isomerase and implies an isomerization reaction where LA is directly converted into CLA isomers. Other fatty acids with 18 carbon atoms and double bonds in position Δ9 and Δ12 of the carbon chain, such as alpha linolenic acid (ALA, 18:3 *c*9,*c*12,*c*15) and gamma linolenic acid (GLA, 18:3 *c*6,*c*9,*c*12), can be converted into CLA through multiple reactions. These reactions involve the activity of enzymatic isomerases, and lead to the formation of conjugated double bond fatty acids. These intermediates are converted into vaccenic acid (VA, 18:1 *t*11) by other enzymes called reductases. VA can undergo further chemical reduction of the double bond in position Δ11, which determines the formation of the stearic acid (SA, 18:0). Alternatively, part of the produced VA can be absorbed in the intestine and transported into the tissues, where it is desaturated to *c*9,*t*11 CLA isomer. This biochemical conversion represents the main pathway responsible for the formation of *c*9,*t*11 CLA isomer in cow's milk (3). Several studies led to identify the endogenous synthesis of *c*9,*t*11 CLA from VA in animals, and were also definitely confirmed in humans (4,5), through the activity of the enzyme Δ9 desaturase (6,7). The presence of small amounts of CLA and other fatty acids with conjugated double bond in many foods of plant origin such as margarine, can be explained by a process of partial hydrogenation of industrial origin. In the absence of oxygen, unsaturated fatty acids (UFAs) undergo the addiction of hydrogen to the double bonds, and the subsequent recombination of them leads to the formation of conjugated double bonds (8).

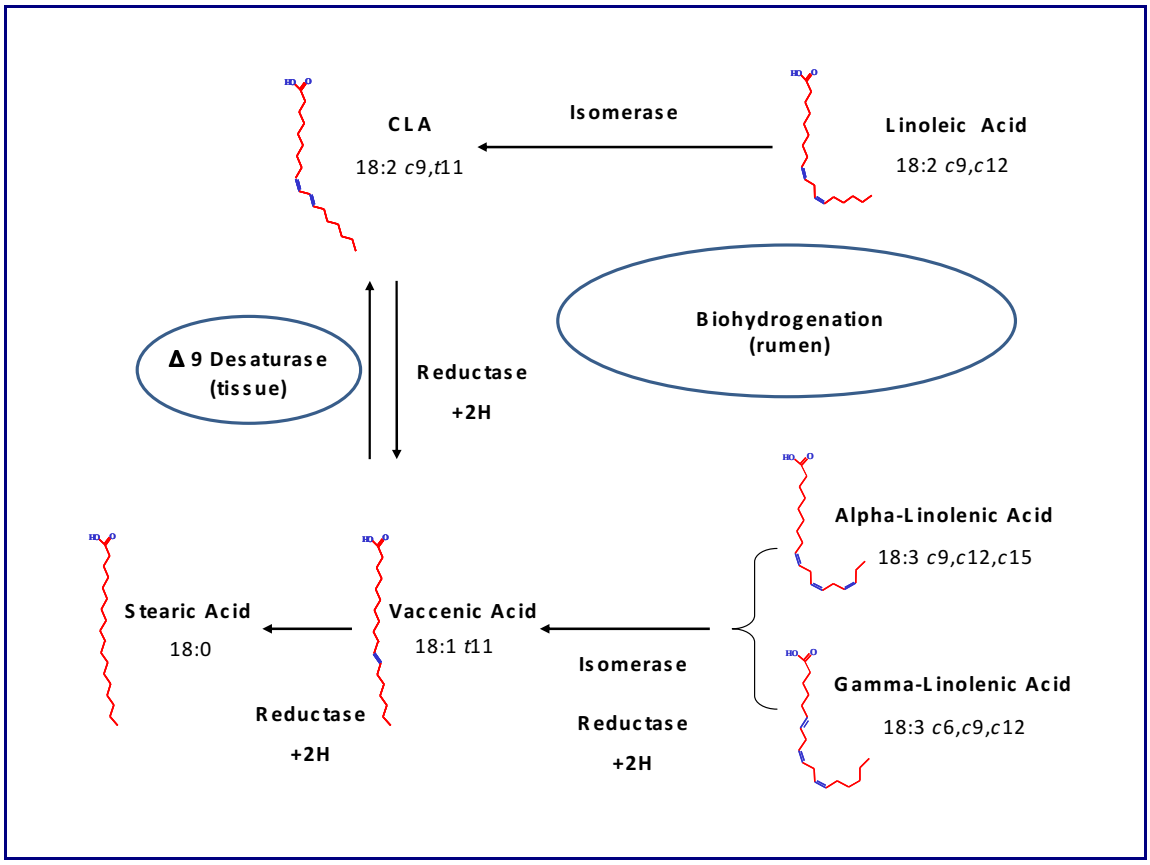


Figure 2. Synthesis of CLA and vaccenic acid (VA, 18:1 t11) from different polyunsaturated fatty acids by biohydrogenation.

1.2 Dietary sources of CLA

The main sources of CLA in the human diet are represented by dairy products like milk and cheese, and meat of ruminants (9), which provide about 0.4 g/day (10). The main isomer found in these foods is the *c*9,*t*11 CLA, whereas the *t*10,*c*12 isomer is present in trace amount (3). Concentration of CLA in dairy products typically ranges from 2.9 to 8.9 mg/g fat, with *c*9,*t*11 isomer representing from 73% to 93% of total CLA. Homogenized cow's milk typically contains 5.5 mg/g fat. Low-fat yogurt has 4.8 mg/g fat, butter 4.7 mg/g fat and cottage cheese 4.5 mg/g fat. Other types of cheese, as well as ice cream and sour cream, are also good sources of CLA. Grass-fed cows produce milk with the highest CLA content (11), but a grain diet supplemented with full-fat rapeseed or soybean concentrate increases CLA (12). Aged cheese generally has lower amounts than cheese with a shorter ripening period, while processed cheese contains higher amounts (12). Beef also has CLA in a similar range as dairy products, with the *c*9,*t*11 isomer contributing from 57% to 85% of total CLA (9,13,14). Ground beef has 4.3 mg/g fat of CLA. Lamb typically contains 5.6 mg. Meats from chickens and pigs, non-ruminants, contain small amounts under 1 mg per gram of fat. CLA in meat is not destroyed by cooking (12). Vegetable oils and margarine have small amounts of CLA, about the same as the meat of non-ruminant animals and birds. The amount typically ranges from 0.6 to 0.9 mg/g fat (9). CLA is claimed to cause weight loss and reduce fat mass (15-18), mixtures of *c*9,*t*11 and *t*10,*c*12 CLA are produced by industrial process and sold in health food stores as supplements, especially as muscle builder used by athletes in gyms. In these supplements, chemically synthesized CLA consists for most of *c*9,*t*11 and *t*10,*c*12 CLA isomers present in equal amounts (50:50), and in the form of either free fatty acids (FFAs) or triacylglycerols (TAGs).

1.3 Dietary intake in humans

The recommended daily amount of total CLA in human diet should be about 0.15 and 0.2 g for women and men, respectively (19,20). About 60% of the CLA intake is derived from dairy products and 37% from meat products. The *c*9,*t*11 CLA isomer that represents about 90% of the total CLA intake has been estimated to be about 190 and 140 mg/d for men and women, respectively (19). There is no relationship between body composition and total CLA or *c*9,*t*11 isomer intake, suggesting that dietary CLA has little effect on body composition in humans (19). The effects of CLA on body weight and body fat in humans were considerably less than those seen in mice, although the doses of CLA used in mouse and human were comparable (21).

It has been shown that diets rich in high-fat dairy foods (and thus containing *c*9,*t*11 CLA) significantly influence both lipid and *c*9,*t*11 CLA concentrations in human milk (22). In men, *c*9,*t*11 CLA intake correlates with TAG *c*9,*t*11 CLA content, suggesting that TAG *c*9,*t*11 CLA may serve as a biomarker for *c*9,*t*11 CLA intake. In females, there are no correlations between *c*9,*t*11 CLA intake and the *c*9,*t*11 CLA content of any esterified plasma lipid fraction. In neither sex there is a relation between dietary *c*9,*t*11 CLA intake or plasma *c*9,*t*11 CLA concentration and circulating lipoprotein cholesterol level (23). Although some adverse effects of CLA intake on liver function are reported in multiple studies, including different animal models (24-26), a very high daily intake of CLA did not produce clinically relevant effects on markers of liver and kidney function in healthy volunteers (27). High intake of an 80:20 mixture of *c*9,*t*11 CLA and *t*10,*c*12 CLA raised the total to high density lipoprotein (HDL) cholesterol ratio in healthy subjects (28).

1.4 CLA metabolism

The current knowledge regarding the CLA metabolism has been developed primarily through studies carried out in animal models. In the phospholipid (PL) fraction of lamb liver naturally exposed to a diet rich in CLA, were found different fatty acids with a structure of conjugated dienes (CD) such as conjugated linolenic acid (CD 18:3), conjugated eicosatrienoic acid (CD 20:3), and conjugated arachidonic acid (CD 20:4) (29). These experiments demonstrated that CLA is metabolized in a manner similar to LA, and competes for the same enzyme system (Fig.3). The CLA can undergo desaturation reaction by the enzyme named Δ6 desaturase, the subsequent elongation of the carbon chain by the elongase, and finally a further desaturation by the Δ5 desaturase, while preserving the structure of conjugated double bonds (25,30).

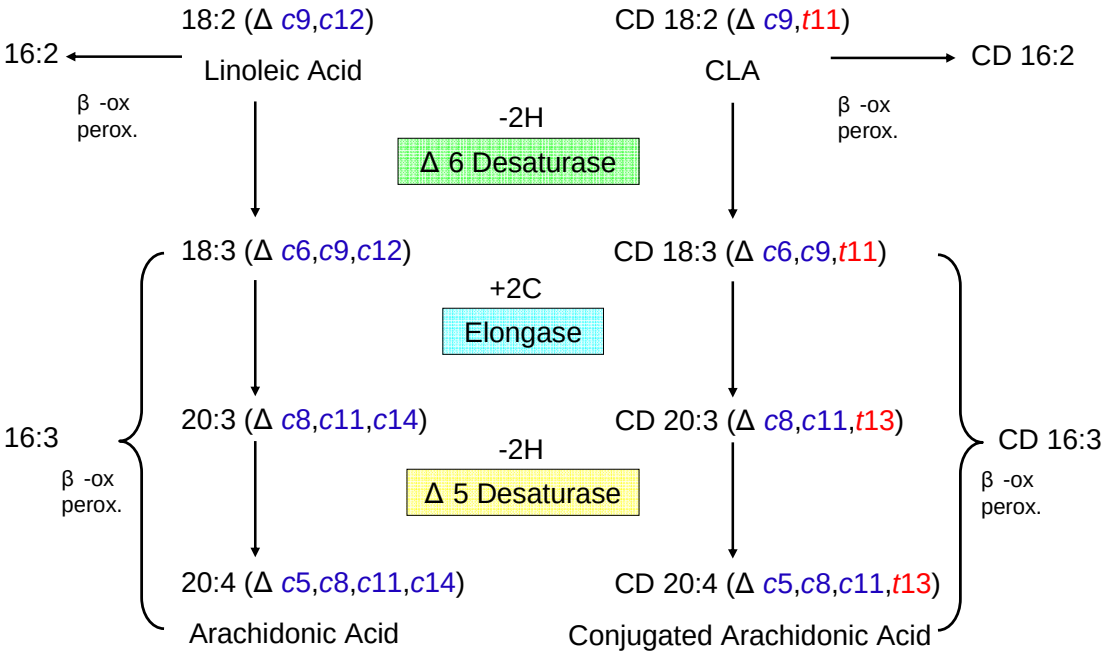


Figure 3. Metabolism of CLA (c9,t11) and linoleic acid (LA, 18:2 c9,c12).

These findings were also confirmed in other experimental models and in human studies (31). CLA and its metabolites CD 18:3 and CD 20:3 are mainly incorporated in the neutral lipids, while the CD 20:4 is predominantly found in the PLs, as well as the metabolites produced from LA, GLA, 20:3 *c*8,*c*11,*c*14, and arachidonic acid (AA, 20:4 *c5*,*c*8,*c*11,*c*14). The different incorporation into lipids can be explained by the increasing of double bonds with the *cis* configuration that facilitates the incorporation into PLs, while the conjugated diene structure favors the incorporation into neutral lipids. The presence of double bonds with *cis* configuration into CD 20:4 appears more decisive than the conjugated double bonds, making this CLA metabolite more suitable for incorporation into PLs. Significant changes in the levels of LA metabolites (GLA, 20:3 *c*8,*c*11,*c*14, and AA) were observed only in adipose tissue and in mammary gland, which are very rich in neutral lipids (32).

A crucial aspect of the CLA metabolism is to understand if the competition of CLA with LA occurs at the enzymatic level or as incorporation. It is likely that CLA competes across the two modes. Enzymatic competition takes place at the level of the liver, while in the extra hepatic tissues there is competition as incorporation. It has been shown that in those tissues rich in neutral lipids, CLA is able to decrease the concentration of the LA metabolites, but not in liver, where CD 20:4 has not been found (33). Also, since the presence of CD 20:4 and LA are correlated, it is likely that CLA modulates the formation and incorporation of the LA metabolites, such as GLA, eicosatrienoic acid (20:3), and AA (all substrates for the biosynthesis of eicosanoids), replacing them with its metabolites (CD 18:3 and CD 20:3), which are able to inhibit the metabolic pathways of cyclooxygenase (COX) and lipoxygenase (LOX), resulting in a reduction of the eicosanoid biosynthesis (34).

It has been demonstrated that CLA and its metabolites accumulate in rat tissues in a dose dependent manner, and AA significantly decreased, suggesting that the effects explicated by the CLA metabolism may interfere with the metabolism of eicosanoids (33). Fatty acid oxidation also occurs in peroxisomes, when the fatty acid chains are too long to be handled by the mitochondrial enzymes. Eicosanoids (35), isoprostanes (36), and AA (37) undergo β-oxidation at the level of peroxisomes. Even for the biosynthesis of docosahexaenoic acid (DHA, 22:6 *c*4,*c*7,*c*10,*c*13,*c*16,*c*19) is required the peroxisomal β-oxidation (38). CLA and CD 20:4 are converted to CD 16:2 and CD 16:3 through β-oxidation in peroxisomes (39).

The metabolism of CLA has been ascertained only for the isomers *c*9,*t*11 and *t*10,*c*12, while for the other isomers is assumed a different metabolism (30). Compared to the *c*9,*t*11, the *t*10,*c*12 CLA isomer appears able to generate larger amounts of 18:3 *c*6,*t*10,*c*12 and less of 20:3 *c*8,*t*12,*c*14, this may depend on the difficulty in elongation of 18:3 *c*6,*t*10,*c*12 (40). In both rat and human, the *t*10,*c*12 CLA isomer is converted by Δ6 desaturase activity into CD 18:3, but it was not found CD 20:3. Opposite, the CD 20:3 produced from *c*9,*t*11 CLA isomer is found in high concentrations (40). The presence of the CD 20:4 seems instead to be correlated to low concentrations of LA in the diet (30).

CLA is able to influence lipid metabolism by altering the fatty acid composition of the cell membrane through the reduction of monounsaturated fatty acids (MUFAs). CLA directly reduces the activity of Δ9 desaturase enzyme rather than influencing its protein or mRNA syntheses (41). Such activity is estimated by the index of desaturation that is calculated by the palmitoleic acid (16:1 *c*9)/palmitic acid (PA, 16:0), and the oleic acid (OA, 18:1 *c*9)/SA ratios. A balanced ratio between MUFAs and saturated fatty acids

(SFAs) is important to preserve the fluidity of the cell membrane. Unbalanced MUFA/SFA ratio can lead to significant changes of insulin sensitivity (42), metabolic rate (43), and determine conditions such as obesity (44). A mixture of equal amounts of *c*9,*t*11 and *t*10,*c*12 CLA isomers added to a diet based on fat of vegetal origin, palm oil, compared to a diet made with fat of animal origin, ovine fat, and enriched with the same CLA mixture, improved serum profile of adipokines and inflammatory markers in obese Zucker rats (45). Rats fed with diets enriched in CLA exhibited lower daily feed intake, final body and liver weights, and hepatic lipid content. Total and low density lipoprotein (LDL) cholesterol levels were increased in CLA supplemented groups. CLA also promoted higher adiponectin and lower plasminogen activator inhibitor-1 (PAI-1) serum concentrations. In contrast to palm oil diets, ovine fat increased insulin resistance and serum levels of leptin, tumor necrosis factor alpha (TNFα) and interleukin-1beta (IL-1β). Adipose tissue from epydidimis and retroperitoneum showed similar deposition of individual fatty acids. The analysis showed that *t*10,*c*12 CLA isomer was highly associated with adiponectin and PAI-1 levels.

1.5 Biological activities of CLA isomers

At the end of the 80s, the anti-carcinogenic properties of CLA were identified (46,47), since then, more and more scientists and studies investigated on the biological properties exerted by this group of unusual fatty acids.

The physiological effects and the benefits on human health exerted by CLA isomers can be summarized in five main biological activities:

- Effects on body weight and composition;
- Influence on lipid metabolism, beneficial effects for prevention and treatment of obesity and diabetes;
- Anti-atherosclerosis activity;
- Anti-carcinogenic properties;
- Immune response modulation and anti-inflammatory activity.

The various biological effects reported for CLA cannot be explained by a single biochemical mechanism. A major direct line of support for this conclusion is that the *t*10,*c*12 and *c*9,*t*11 CLA isomers appear to produce different effects (48). For example, the *t*10,*c*12 isomer of CLA shows at the same time anti-carcinogenic, anti-obese and anti-diabetic effects, whereas the *c*9,*t*11 CLA isomer essentially exerts an anticancer effect (49).

Even those effects that are clearly attributed to a single isomer may not all be caused by the same biochemical mechanism. For example, it has been reported evidence indicating that *t*10,*c*12 CLA directly inhibits the activity of stearoyl-CoA desaturase (SCD) (48). Moreover, it is difficult to imagine how this finding alone could explain the range of physiological effects associated to this CLA isomer. Of the two major isomers of CLA, *t*10,*c*12 is specifically responsible for the antiobesity effects (50-52).

1.5.1 Effects of CLA on body weight, influence on energy and lipid metabolism, adipogenesis, inflammation and apoptosis.

In the first study that has shown the capacity of CLA to influence body composition in animals, male and female mice fed with a 0.5% (w/w) CLA mixture had 57% and 60% lower body fat mass (BFM), respectively, compared to controls (53). Subsequently, other studies showed as CLA supplementation was able to reduce BMF in mice, rats, and pigs (54,55).

There are some contradictory results about the ability of CLA to reduce BFM in humans. For example, supplementation of CLA in overweight and obese people (3–4 g/day for 24 weeks) decreased BFM and increased lean body mass (LBM) (56), while healthy adults supplemented with a CLA mixture in yogurt (3.76 g/day for 14 weeks) had no effect on body composition (57).

CLA mixture composition, individual CLA isomer used, dose, time of treatment, gender, weight, age and metabolic status of the subjects, are all factors that can explain the contradictory results obtained in human trials. Primarily, the dose of CLA administered in animal and human studies may underlie the differences of the obtained results.

Overweight subjects supplemented with a mixture of CLA isomers (3.76 g/day for 14 weeks) had not decreased body weight, body mass index (BMI), or BFM (57), in contrast, mice supplemented with 1.5% (w/w) CLA mixture for 4 weeks weighed significantly less and had reduced adiposity, compared to controls (58). Referring to body weight, the administered dose of CLA in mice was 20 times than that administered to humans. The biochemical mechanisms underlying the anti-obesity effect of CLA are multiple. CLA decreases energy intake in animal models (53,59,60) but this has not

been demonstrated in humans (61,62). CLA could exerts a direct effect on hypothalamic appetite-regulating genes (60,63).

It has been proposed that CLA might reduce adiposity by elevating energy expenditure via increased basal metabolic rate (BMR), thermogenesis, or lipid oxidation in animals (64,65). Mice fed *t*10,*c*12 CLA isomer also showed increased β-oxidation in differentiating mouse preadipocytes (66), and expression of the mitochondrial protein responsible for transferring fatty acids into mitochondria, carnitine palmitoyltransferase 1 (CPT1) in white adipose tissue (67). The regulation of the energy expenditure in human studies by CLA, showed different results, with no reported changes in BMR or BMF (62), or higher basal metabolic rate although body weight was not affected (57). Only one study reported both increased energy expenditure and decreased body weight in subjects supplemented with a CLA mixture (68).

Other studies have demonstrated that supplementation with mixed CLA isomers increases LBM associated with higher levels of energy expenditure, in healthy obese humans (69). CLA could enhance LBM by increasing bone or muscle mass (70). Some studies reported that CLA suppresses preadipocyte differentiation in animal (50,66,71) and human (72). It has been shown that *t*10,*c*12 CLA isomer treatment suppressed adipogenesis and lipogenesis, reduced preadipocyte differentiation and promoted maintenance of mature adipocytes, specifically by reducing sterol regulatory element binding protein 1c (SREBP-1c), peroxisome proliferator-activated receptor gamma (PPARγ), liver X receptor (LXRα), CCAAT-enhancer-binding protein alpha (C/EBPα), and adipocyte fatty acid binding protein (aP2) expression (73-75).

*t*10,*c*12 CLA isomer supplementation decreased the expression of PPARγ and its target genes in animal models (67,76,77) and *in vitro* studies (50,78).

In adipogenesis, PPARγ activity may be regulated by phosphorylation, which can be mediated by the mitogen activated protein kinase (MAPK) (79). Phosphorylation of PPARγ2, that leads to adipocyte differentiation, may decrease its activity by ubiquination and proteasome degradation (80), and by reducing both its ligand-dependent and ligand-independent transactivating functions (81). *t*10,*c*12 CLA increases PPARγ phosphorylation (78) without significantly decrease its protein levels, suggesting that the subsequent downregulation of PPARγ target genes is due to decreased transactivating function.

After CLA stimulation, extracellular signal regulated kinase (ERK) phosphorylation occurs and leads to inactivation of PPARγ, suppression of adipogenic gene expression and insulin-stimulated glucose uptake (82). *t*10,*c*12 CLA increased expression of proinflammatory cytokines (83) by nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) activation in adipocytes. NFκB may also repress PPARγ activity by the interaction with the DNA-bound retinoid X receptor (RXR)-PPARγ heterodimer.

Pro-inflammatory cytokines produced by adipose tissue, can cause insulin resistance, suppressed lipid synthesis and increased lipolysis in adipocytes. It has been reported that *t*10,*c*12 CLA supplementation increased the expression or secretion of interleukins IL-6 and IL-8 in adipocyte cultures (76,67,82), TNFα and IL-1β, suppressing PPARγ activity and insulin sensitivity (58,77,78,84).

In human subjects, *t*10,*c*12 CLA increases the levels of inflammatory prostaglandins (PGs) (69), and the expression of cyclooxygenase 2 (COX-2), an enzyme involved in the synthesis of PGs in differentiated human adipocytes (71). Also, *t*10,*c*12 CLA increased PGF2α secretion in human adipocytes (83), a prostaglandin able to inhibit

adipogenesis via phosphorylation of PPARγ by MAPKs (85), and through activation of the hypoxia inducible factor-1 (HIF-1) responsible for decreasing PPARγ and C/EBPα expression.

Supplementation with mixed isomers of CLA or *t*10,*c*12 CLA alone reduced various proteins involved in lipogenesis: acetyl-CoA carboxylase (ACC), lipoprotein lipase (LPL), SCD, and fatty acid synthase (FAS) (67,72,82,86). *t*10,*c*12 CLA may exert its anti-lipogenic effects by the repression of the transcription factor SREBP-1 and its target genes.

It has been reported hyperinsulinemia associated with insulin resistance in animals (67) and humans (87,88) after supplementation with a CLA mixture or *t*10,*c*12 CLA isomer alone. CLA can determine inhibition of insulin signaling by activation of inflammatory pathways and stress kinases, and down regulating the expression of some genes involved in the insulin signaling and glucose uptake pathways (suppression of glucose transporter type 4 or GLUT4) (71,82). In addition, some *in vitro* studies (71,76) suggested that CLA may inhibit insulin signaling by increasing the expression of the suppressor of cytokine signaling (SOCS)-3. SOCS-3 impairs insulin signaling and glucose uptake by promoting the phosphorylation of the inhibitory serine 307 on insulin receptor substrate 1 (IRS-1), leading to its ubiquination and proteasome degradation (89). *t*10,*c*12 CLA treatment also showed to decrease the protein levels of important signaling proteins for insulin sensitivity such as insulin receptor (IR) β and IRS-1 (76). *t*10,*c*12 CLA may cause insulin resistance decreasing expression of the insulin-sensitizing hormone adiponectin (76,82). Adiponectin is a target gene of PPARγ (90), so, its suppression may be conducted, almost in part, to the activity of *t*10,*c*12 CLA in antagonizing PPARγ.

In mice and human adipocytes, acute treatment with mixed CLA isomers or *t*10,*c*12 CLA alone increased lipolysis, releasing FFAs and glycerol from stored TAGs through the action of the hormone-sensitive lipase (53,86,91). On the other hand, studies carried out by animal models have demonstrated that chronic supplementation with a mixture of CLA had no effect on lipolysis (92,93). In contrast, chronic treatment with mixed CLA isomers reduced glycerol release from isolated rat adipocytes (94). FFA levels have been reported to be lower in serum of OLETF rats supplemented with a CLA mixture [1.0% (w/w) for 4 weeks], compared to controls (95).

CLA may be able to reduce BFM by apoptosis. Supplementation with *t*10,*c*12 CLA or CLA mixture showed to stimulate apoptosis in mice (64,67) and murine adipocytes (96). CLA may exert its effect on apoptosis enhancing TNFα gene expression in white adipose tissue, and increasing the ratio of BAX relative to Bcl2, an inducer and suppressor of apoptosis in the mitochondrial apoptotic pathway, respectively (77). *t*10,*c*12 CLA treatment in mice and murine adipocytes increased the mRNA levels of genes involved in the integrated stress response (ISR), such as activating transcription factor 3 (ATF3), C/EBP homologous protein (CHOP), pseudokinase tribbles 3/SKIP 3 (TRIB3), X-box binding protein (XBP-1), and growth arrest and DNA damage inducible protein (GADD34) (74).

1.5.2 Anti-atherosclerosis activity.

Several studies have attributed to dietary CLA an antiatherogenic activity with a reduction of the atherosclerotic plaque in animal models.

CLA affects initiation and progression of the atherosclerotic lesions in rabbits through its effect on lipid peroxidation (97). Feeding rabbits with an atherogenic diet and supplemented with 0.5g CLA/day for 22 weeks, significantly lowered plasma levels of triglyceride, LDL-cholesterol, and LDL-cholesterol /HDL-cholesterol ratio, compared to control animals. CLA feeding also resulted in fewer aortic fatty lesions (97). Subsequent studies using other animal models of atherosclerosis, such as the ApoE−/− mouse, have also demonstrated that an 80:20 isomeric CLA mixture induced regression of pre-established atherosclerosis (98,99). The study with ApoE−/− animal model involved the administration of a 1% cholesterol diet for 8 weeks to induce atherosclerosis, followed by a further administration for 8 weeks of 1% cholesterol alone or 1% cholesterol supplemented with isomeric 1% CLA mixture.

ApoE−/− mice fed CLA blend supplemented diet showed a 30% decrease of lesion area. CLA reduced the levels of plasma total cholesterol, LDL- and very-low density lipoprotein (VLDL) cholesterol, and TAGs in hamsters. There was no effect on HDL-cholesterol, compared to controls (100). CLA fed hamsters also developed 45% fewer aortic fatty streaks than control animals. CLA reduced the development of early aortic atherosclerosis to a greater degree than LA did, possibly through changes in LDL oxidative susceptibility in hypercholesterolemic hamsters.

Rats fed with CLA (2.3 energy % level) did not show a cholesterol lowering effect on serum and liver in contrast to the hypocholesterolemic observation in rabbits (101). LA in liver cardiolipin was reduced by CLA. The decrease of LA in cardiolipin of heart

mitochondrial membrane diminished heart cytochrome C oxidase activity, that required cardiolipin as an activator. These results (101) suggested that modified fatty acid composition in liver cardiolipin might influence mitochondrial respiratory function in liver. The antiatherogenic effect of CLA could be explained by lowering hepatic cholesterol (102), reducing synthesis of cholesterol by the liver (103), or through its ability to inhibit the production of PGE2 (101), thromboxanes (104), and platelet aggregation (105). It has been hypothesized that the ability of CLA to reduce the formation of atherosclerotic plaque could be due to changes in the susceptibility of LDL to be oxidized (106). CLA may also act by altering size and lipid composition of lipoproteins (107,108). Some studies showed that *c*9,*t*11 CLA was the key isomer involved in the impediment of the atherosclerosis development, with *c*9,*t*11 CLA fed mice exhibiting reduced plasma cholesterol and glucose, and reduced lesional area of the aorta as well as increased expression of markers of plaque stability (109).

Cluster of differentiation 68 (CD68), a glycoprotein which binds LDL and expressed on monocytes and macrophages, has been identified as a possible target for anti-atherosclerosis activity of CLA, in fact in the lesions of ApoE−/− mice fed high cholesterol diet, it has been shown increased expression of CD68 (98). In contrast, the expression of CD68 was significantly decreased in the lesions of mice fed CLA supplemented diet, suggesting that CLA intake decreased the infiltration of macrophages into the atherosclerotic plaque. Furthermore, CLA inhibited monocyte migration through at least two differential mechanisms, one that is PPARγ dependent and the other one is PPARγ independent, depending on the chemoattractant used (110). Pre-treatment of RAW macrophages with either 50 μM *t*10,*c*12 or *c*9,*t*11 CLA isomers decreased foam cell formation in response to acetylated LDL (ac-LDL) loading (111).

Dietary supplementation of CLA did not affect atherosclerosis in mice fed high-cholesterol (16%, w/w fat and 1.25%, w/w cholesterol) atherogenic diet (112). In this study, ApoE−/− mice were fed with a dietary treatment supplemented or not in LA, *c*9,*t*11 CLA, *t*10,*c*12 CLA, or an equimolar mixture of the two CLA isomers, at a concentration of 0.5% (w/w). Treatment lasted 12 weeks. *t*10,*c*12 CLA caused adverse changes in adipocyte function, and plasma and liver lipid metabolism, which are partially ameliorated by the administration of *c*9,*t*11 CLA.

There are other studies reporting contradictory results for the potential ant-iatherogenic property of CLA, then, further investigations are needed to better elucidate the mechanism through CLA may determine regression of atherosclerosis.

1.5.3 Anti-carcinogenic properties.

One of the first studies aimed at assessing the anticancer activity of CLA reported the ability of this group of fatty acids to inhibit the initiation phase of carcinogenesis in forestomach induced by benzo[a]pyrene (BP), in mice (113).

Subsequent studies conducted using mixtures of CLA isomers showed inhibition of chemically-induced tumors in mammary gland, skin and colon, in different animal models. The inhibition of tumorigenesis was dependent upon the dietary concentrations of the CLA mixtures used (0.05 to 1% of the diet), and the timing and duration of CLA feeding (114,115). Also, the mechanisms of modulation of CLA were related to the stages of initiation, promotion, progression, and metastasis of malignant tumors. CLA administered before or after treatment with the carcinogen, prevents tumor evolution. A single administration of CLA in the period immediately following weaning and during maturation of the mammary gland, seems to be sufficient to protect against chemically-induced tumorigenesis (116). Also, CLA is active when administered after the onset of the tumor, in this case, however, requires its own continuous supply to achieve the maximum inhibition of tumor progression. CLA might act causing changes during the development of the mammary gland and making it less susceptible to the neoplastic transformation. For example, it has been reported as CLA may act by inhibition of the expansion and proliferation of mammary epithelial cells normally susceptible to carcinogen-induced transformation (117). Different types of tumors from various organs probably respond differently to CLA treatments. The ability of CLA in the prevention of tumor process is independent of the amount and type of other fatty acids in the diet (118). Few studies conducted in humans have investigated the relationship between CLA intake or CLA concentration in tissues and tumor incidence. Female subjects

between 55 and 69 years of age, answered questionnaires regarding the amount of their dietary CLA intake, familial incidence of cancer, and other risk factors (119). Unexpectedly, this study showed a weak positive relationship between CLA intake and breast cancer incidence. A study reported as the levels of serum and dietary CLA were significantly lower in breast cancer patients than in control subjects (120). In contrast, there was no direct correlation between levels of CLA in breast adipose tissue and women with and without breast cancer (121).

Several studies regarding the growth inhibition of numerous types of tumors, indicate as the different CLA isomers have distinct effects on tumorigenesis and lipid metabolism. One of these studies (122), showed that purified *c*9,*t*11 CLA isomer administered to diet either as butter fat or as purified isomer, was as effective as a mixture of CLA isomers (0.8%) in reducing mammary epithelial mass, size of the terminal end buds, and mammary tumor development induced by *N*-Nitroso-*N*-methylurean (MNU). Rats fed with diets containing 1% *c*9,*t*11 CLA or 2% VA (which can be metabolized to *c*9,*t*11 CLA) for 6 weeks, reduced the MNU-induced tumor by 50%, compared with a control group fed regular butter (123). The concentrations of CLA in the tissues and the concentration of *c*9,*t*11 CLA in the mammary gland were 4-fold greater in the VA group than the control group. This means that the rats that were able to convert VA into *c*9,*t*11 CLA, were able to decrease tumorigenesis. Results from other studies carried out in rodents showed that *c*9,*t*11 and *t*10,*c*12 CLA isomers alone were as effective as the CLA isomeric mixture in reducing mammary tumors induced by MNU (124,125).

Compared to *c*9,*t*11 CLA, *t*10,*c*12 CLA was more effective in lowering incidence of BP-induced forestomach tumors in mice, but the tumor size did not differ between the

two isomers (126). Another study conducted in intestinal tumors of mice showed opposite results for *t*10,*c*12 CLA (127).

Mice with mutation of the APC gene that leads to intestine tumors were fed either a diet with sunflower and rapeseed oils or a diet supplemented in 1% *c*9,*t*11 CLA or *t*10,*c*12 CLA. The sizes of the adenomas were significantly greater in the distal part of small intestine in mice fed with the diet containing *t*10,*c*12 CLA isomer, compared to the control group, suggesting that *t*10,*c*12 CLA may act as a growth promoter in small intestine carcinogenesis. *In vitro* studies reported the effects of CLA purified isomers on growth, viability, and apoptosis of tumor cell lines from mammary gland, prostate, and digestive tract or their metastasis. In most of the studies with mouse and human mammary tumor cell lines, *c*9,*t*11 CLA did not inhibit tumor cell growth (128), and *t*10,*c*12 CLA showed a greater growth inhibitory capacity than *c*9,*t*11 CLA. Culture conditions including concentration of CLA, duration of the treatment, and tumor type as well as the cell lines used, seem to determine whether CLA isomers would affect cell growth. Also, CLA isomers may reduce tumor cell numbers through different mechanisms, one of them involves the estrogen receptor (ER) (129). *t*10,*c*12 CLA and *c*9,*t*11 CLA differentially influence the expression of many genes in mouse liver (130) and adipose tissue (131). CLA isomers showed differential effects on fatty acid composition into PLs of several tissues, including liver, adipose tissue, heart, spleen, and mammary gland (132), leading to alterations in AA availability and therefore influencing the metabolism of eicosanoids. In particular, the prostaglandins PGE_2 and $PGF_{2\alpha}$ by influencing inflammation, cell proliferation, immunity, platelet aggregation, and cell differentiation in key tissues (prostate, mammary gland, colon, and skin), may modulate tumorigenesis (133). CLA could reduce biosynthesis of eicosanoids through a

competition for AA incorporation in PLs (134), as shown in the colonic mucosa (135), or inhibition of the expression/activity of the enzymes COX-1 and COX-2. COX-1 is the constitutive enzyme in different cell types and produces small amounts of PGE_2 for the regulation of the cellular homeostasis, while COX-2 is expressed in response to inflammatory signals and generates large quantities of PGE_2 inducing inflammation. It has been shown *in vitro* studies as CLA decreased the conversation of AA into eicosanoid metabolites in the presence of COX-1 (136), and in cultured macrophages reduced mRNA and protein levels of COX-2 (137). The association between high levels of COX-2 with tumor progression and inhibition of apoptosis (138,139), could explain the benefic role of CLA in tumorigenesis.

Other studies conducted using purified isomers showed as *c*9,*t*11 CLA decreased the incorporation of AA into phosphatidylcholine and increased AA uptake into phosphatidylethanolamine in human breast and colon cell lines (140). In contrast, *t*10,*c*12 CLA had no effect on AA uptake into breast cells (140). Both isomers were able to inhibit cell growth, but only *c*9,*t*11 CLA decreased PGE2 production. *c*9,*t*11 CLA and *t*10,*c*12 CLA were both effective in blocking 12-otetradecanoylphorbol-13-acetate (TPA)-induced expression of COX-2, compared to cell cultures treated with LA (141).

In human prostate cancer cell lines, *c*9,*t*11 CLA but not *t*10,*c*12 CLA increased TNFα-induced apoptosis (142), decreased prostate cancer cell proliferation, and reduced mRNA expression of 5-lipoxygenase (5-LOX) (143). Oppositely, in breast cancer cells *t*10,*c*12 CLA but not *c*9,*t*11 CLA, decreased cell growth and production of hydroxyeicosatetraenoic acid (5-HETE) while increasing apoptosis (144).

These studies suggest that the inhibitory effects on cell growth in tumor cells by *c*9,*t*11 CLA are associated to changes in COX-2 expression, while alternative mechanisms

may mediate the effects of *t*10,*c*12 CLA isomer. Regarding cell proliferation and apoptosis, CLA has shown to enhance accumulation of tumor suppressive proteins such as p21, p27, and p53, which interrupt the transition from G1 to the S phase of the cell cycle (145,146), and to reduce expression of cyclins A and D, and cyclin-dependent kinases (CDKs), all protein kinases with a key role in the regulation of the cell cycle (147,148).

In most of the study carried out using purified isomers and tumor cell lines, compared to *c*9,*t*11 CLA, the *t*10,*c*12 CLA isomer appears to be more inhibitory of the genes that regulate cell cycle and growth. Also, increased apoptosis has been associated to decreased phosphorylation of Akt and ErbB3 proteins (149). *t*10,*c*12 CLA increased apoptosis by enhancing caspase-3 activity and p21 mRNA, but decreasing cell death regulator protein B-cell lymphoma 2 (bcl-2) mRNA (150,143), and by activating both caspases-3 and 9 (151). FAS protein and mRNA levels are over expressed in many carcinomas (152). It has been shown that the pharmacological inhibition of FAS expression/activity in cancer cells resulted in apoptosis suggesting that FAS expression is important for cell proliferation and cancer development (153). In estrogen receptor-negative breast cancer cell line MDA-MB-231, treatment with 25 mM of *c*9,*t*11 CLA and *t*10,*c*12 CLA, co-ordinately decreased expression of FAS and its controlling transcription factor SREBP-1c, while in estrogen receptor-positive breast cancer MCF-7 cells, the decrease of SREBP-1c and FAS expressions were dependant on the concentration of CLA used. (154). In both types of breast cancer cell lines, this inhibition was concurrent with an increased apoptosis in these cells.

1.5.4 Immune response and inflammatory cell signalling modulation.

CLA is able to influence the modulation of the immune response, primarily by interfering with the production of eicosanoids. CLA has shown the property to decrease tissue levels of AA, the main substrate for the synthesis of PGE_2. In turn this prostaglandin is a regulator of the cytokine TNFα, a key factor in several pathological conditions such as carcinogenesis (155), atherosclerosis (156), inflammation (157), and obesity (158).

Many experimental studies have demonstrated the ability of CLA to inhibit COX, and interact with PPAR receptors, widely expressed in the cells of the immune system and determinants for the regulation of several genes involved in the proliferation of lymphocytes, monocytes and macrophages. Dietary CLA supplementation in rats showed a positive influence on the immune response, such as increased production of immunoglobulin IgA, IgG and IgM (159), increased phagocytosis, and proliferation of cytotoxic CD8 T and helper T lymphocytes (160).

Synthetic and natural sources of CLA isomers may exert beneficial effects in many inflammatory diseases including colitis, atherosclerosis, metabolic syndrome and rheumatoid arthritis.

In bone marrow derived dendritic cells (BMDC) stimulated with lipopolysaccharide (LPS), *c*9,*t*11 CLA reduced IL-12p70 and IL-12p40 while increased concentrations of IL-10 and surface expression of the IL-10 receptor (161). *c*9,*t*11 CLA also reduced NF-kB signalling proteins (161-163), and TNFα production and mRNA expression in pigs fed with CLA after LPS treatment (164).

In a study conducted in an animal model of inflammatory bowel disease (IBD) (165), CLA-fed mice were protected from weight loss, rectal bleeding, and colonic tissue

damage inducted by colitis, by up regulation of PPARγ and PPARδ, and a subsequent reduction of inflammation mediated by NF-kB. High fat diet enriched in *c*9,*t*11 CLA significantly reduced the insulin resistance indicators fasting plasma glucose, insulin and TAG concentrations in obese mice, compared to LA enriched diet (166). This study showed as *c*9,*t*11 CLA increased insulin regulated glucose transporter GLUT-4 and IRS-1, reduced macrophage infiltration into the adipose tissue of mice, and decreased mRNA expression of the pro-inflammatory molecules monocyte chemoattractant protein 1 (MCP-1), CD68, IL-6 and TNFα in adipose tissue. All these experimental evidences demonstrate that *c*9,*t*11 CLA promotes insulin sensitivity by reducing adipose tissue inflammation. In contrast, many studies clearly highlighted the activity of *t*10,*c*12 CLA in inducing liver steatosis and insulin resistance (167,168).

Only few studies evaluated the effects of CLA isomers on inflammation in humans. Riserus *et al.* (168) showed that 3 months supplementation with *t*10,*c*12 CLA isomer in obese men decreased insulin sensitivity, increased pro-insulin, lipid peroxidation, C-reactive protein, and fasting glucose. An analogue study, reported that *c*9,*t*11 CLA supplementation over 3 month in obese men (169) decreased insulin sensitivity, but in a less intense manner than *t*10,*c*12 CLA isomer did. Supplementation with mixtures of CLA isomers did not improve markers of insulin resistance in risk prone patients and showed inconsistent effects on healthy volunteers (170,171).

1.6 The endocannabinoid system (ECS)

The endocannabinoid system (ECS) refers to the cannabinoid receptors 1 (CB1) and 2 (CB2), and their specific endogenous ligands, a group of neuromodulatory which are involved in a variety of physiological processes.

These ligands for cannabinoid receptors are also called endocannabinoids, and the best characterized are 2-arachidonoyl-glycerol (2-AG), and *N*-arachidonoylethanolamine (AEA or anandamide). The endocannabinoids are synthesized from AA (172), and modulate energy balance, feeding behavior, glucose homeostasis, hepatic lipogenesis, pain sensation, mood, and memory.

The name of this system is derived from the discovery of the terpenoid derivative Δ 9-tetrahydrocannabinol (Δ 9-THC), the major psychoactive component of *Cannabis sativa* and marijuana (173). The milestones in the history of ECS research that led to today's knowledge may be briefly summarized in Fig. 4.

CB1 are located in several areas of the brain and in a variety of tissues such as adipose tissue, liver, muscle, the gastrointestinal tract, pancreas, gonad and sensory neurons (174-181). CB2 receptors are located in immune cells such as T and B-cells, monocytes, and also in spleen and tonsils (182,183).

Unlike classical neurotransmitters, endocannabinoids are not stored within the synaptic vesicle in cells; rather, they are synthesized on demand from phospholipid-derived precursors in the cell membrane. The biosynthesis of the long chain *N*-acylethanolamine (NAE) compound AEA starts with the transfer of AA from the sn-1 position of PLs to the nitrogen atom of phosphatidylethanolamine by the acyl-transferase Ca^{2+}-dependent enzyme (184,185). This trans-acylase-catalyzed reaction leads to the formation of *N*-arachidonoyl-phosphatidylethanolamine (NArPE), which is

2600BC	**The Chinese emperor Huangdi advised taking *Cannabis sativa* for the relief of cramps, and rheumatic and menstrual pain**
1839	**Publication by physician O'Shaughnessy who publicized therapeutic potential of Cannabis sativa to Western world**
1964	**Isolation of TETRAHYDROCANNABINOL (THC), the main psychoactive constituent of Cannabis sativa**
1988	**First identification of cannabinoid receptors in rat brain**
1990	**Cloning of the first G protein-coupled cannabinoid receptor CB1 in rat**
1991	**Cloning of the human CB1 receptor**
1992	**Discovery of anandamide, the first endogenous cannabinoid**
1993	**Cloning of the CB2 receptor**
1994	**Characterization of the first CB1 receptor inhibitor, SR141716 (rimonabant).**
1995	**The term of "Endocannabinoid " was coined**
1995	**Isolation of a second endocannabinoid, 2-AG**
2000s	**Wide tissue distribution of CB1 receptors; Characterization of peripheral endocannabinoid system (ECS); Role of ECS in human obesity and obesity-related metabolic disorders**

Figure 4. Timeline of endocannabinoid system (ECS) discoveries.

subsequently hydrolyzed at the phosphoester bond site by the NAPE-specific ''phospholipase D'' (NAPE-PLD) (186). Diacylglycerols (DAGs) derived from the hydrolysis of phosphatidylinositol or phosphatidic acid (187) and containing AA esterified in sn-2 position, represent the main precursor for 2-AG biosynthesis.

The specific enzyme responsible for 2-AG biosynthesis in cells and tissues is named DAG lipase (188), and its activity is stimulated by Ca^{2+} and glutathione, and selectively inhibited by two agents, RHC80267 and tetrahydrolipstatin (189). Two separate genes encode for the two DAG lipases, termed DGLα and DGLβ (188). They share around 30% of sequence identity and aren't specific for any of the acyl groups in sn-1 and sn-2 position of DAGs. The endocannabinoids are synthesized and released in response to physiologic (neuronal depolarization) and pathologic stimuli which trigger the opening

of calcium channels. After their biosynthesis, these lipophilic compounds are immediately released and diffuse freely across cell membranes spilling out into the synaptic cleft where they act locally (190). The transport mechanism is currently unknown. Endocannabinoids then bind to and activate CB1 receptors located presynaptically. This reverse path is referred to as retrograde signaling. After binding to the CB1 and CB2 receptors the G-proteins are stimulated to relay the signals to regulate a number of cellular processes. Various different signaling pathways are affected by this G protein stimulation. CB1 but not CB2 receptor stimulation of G-proteins is directly coupled to inhibition of voltage-activated Ca^{2+} channels. Inhibition of Ca^{2+} channels and stimulation of K^{+} channels both inhibit neurotransmitter release. Follows the inhibition of adenylate cyclase (AC) with corresponding inactivation of the protein kinase A (PKA) phosphorylation pathway. This determines the stimulation of mitogen-activated protein kinase (MAPK). These two intracellular events lead to, among other effects, the regulation of expression of several genes.

After endocannabinoids act locally at the site of synthesis, they are immediately metabolized by reuptake and subsequent hydrolysis into cells by the enzyme fatty acid amide hydrolase (FAAH), which predominantly catabolises AEA and 2-AG to AA and ethanolamine or glycerol, respectively (191,192). 2-AG can also be inactivated by the monoacyl-glycerol lipase (MAGL) (193), a cytosolic enzyme which is associated to the plasma membrane. In brain most of the 2-AG (around 85%) is hydrolyzed by MAGL while the remaining part is catalyzed by two enzymes, named ABHD6 and ABHD12 (194), and this estimation has been confirmed by the selective MAGL inhibitor JZL184 (195). The degradation of AEA and 2-AG generates AA, ethanolamine and glycerol, all

precursors available into the membrane PLs for a new round of endocannabinoid biosynthesis (196).

Other molecules belonging to the NAEs family have been identified. The naturally occurring amide of PA and ethanolamine, *N*-palmitoylethanolamide (PEA), and the amide of OA and ethanolamine, *N*-oleoylethanolamide (OEA). These represent the most important congeners or endocannabinoid related compounds (ERC) that act mainly by influencing AEA metabolism and binding the PPARα (197,198).

PEA and OEA have practically no effect on classical CB1 and CB2, but exert their biological actions targeting other types of receptors. It has been observed that topic application on mouse skin with PEA, this NAE-derived amide exerts anti-inflammatory action by direct activation of PPARα and stimulating PPARα gene expression (199). These effects were replicated by treatment with the synthetic PPARα agonists GW7647 and Wy-14643 (199). Other mechanisms for the anti-inflammatory activity of PEA have been reported, such as enhancement of AEA actions at CB1, transient receptor potential of vanilloid type-1 (TRPV1) and PPARγ receptors (200).

Conversely to AEA, OEA decreases food intake and body weight gain (201) by binding and increasing the transcriptional activity of PPARα (202). In wild-type mice, OEA stimulated lipolysis and fatty acid oxidation, but not in mutant mice deficient in PPARα (203). The anorectic properties of OEA were confirmed after sub-chronic treatment in diet-induced obese rats and wild-type mice, where OEA reduced body weight gain and TAG content in liver and adipose tissue, but not in obese PPARα$^{-/-}$ mice (203). Other implications have been observed for PPARα mediated responses of OEA. These include neuromodulatory properties (204), and memory consolidation through noradrenergic activation of the basolateral complex of the amygdala (205).

1.7 Role of ECS in feeding behavior and energy homeostasis

It has been well known for a long time that exposure to cannabis produces an increase of appetite (a phenomenon referred to as the 'munchies'). Marihuana use is associated with increased caloric intake and weight gain (206). This observation led to an exploration of the role of the ECS in the regulation of feeding behavior. It has been shown that central stimulation of the CB1 receptor by AEA (207) and 2-AG (208) increases food intake through increased hunger and decreased satiety.

In the first study that demonstrated the central CB1 receptor-mediated overeating effect of AEA (207), all doses of AEA increased significantly food intake in pre-satiated male rats, and 1.0 mg/kg resulted the most potent dose. Hyperphagia induced by AEA was dose-dependently attenuated by the CB1 receptor inhibitor SR141716 (rimonabant).

Similarly, injection of 2-AG into the nucleus accumbens shell of rats, stimulated feeding dose-dependently (208), and this effect was attenuated by rimonabant. In the same study, fasting increased levels of AEA and 2-AG in the limbic forebrain and 2-AG in the hypothalamus. Hypothalamic 2-AG level declined as animals ate. No changes were detected in satiated rats. If the hyperphagic actions of endocannabinoids were selectively blocked by rimonabant, but not by the antagonist of peripherally expressed CB2 receptors, SR144258, indicating that the overeating actions of AEA and 2-AG are mediated specifically by CB1 receptors (208,209).

ECS has been proposed as power regulatory controller of the hedonic evaluation of palatable foods in central (209,210) and peripheral areas of the body (211). ECS is deeply involved in the motivation to eat at the level of nucleus accumbens and hunger in hypothalamus by orexigenic (appetite-stimulating) and anorexigenic (appetite-reducing) mediators (189).

Leptin might influence biosynthesis of AEA and 2-AG in hypothalamus, and it is believed that this hormone signals the nutritional status to the brain areas that control food intake and energy balance (212). Leptin reduces food intake at two levels; 1) by upregulating α-melanocyte-stimulating hormone, a neuropeptide with an anorexigenic action, and 2) downregulating orexigenic factors, such as neuropeptide Y. Acute treatment of leptin in normal rats reduced hypothalamic endocannabinoid levels; while genetically obese, chronically hyperphagic Zucker rats and mice express elevated, leptin-reversible, hypothalamic anandamide or 2-AG levels (174). Then, endocannabinoids in the hypothalamus through activation of CB1 receptors maintain food intake and take part of the neural control of appetite regulated by leptin. Plasma levels of leptin and endocannabinoids are inversely correlated. Leptin peripherally acts on taste function, and in wild-type mice selectively suppresses sweet taste responses, but not in leptin receptor-deficient mice (213-215).

It has been shown that endocannabinoids act enhancing sweet taste, opposing to the action of leptin. Administration of AEA or 2-AG increases gustatory nerve responses to sweeteners in a concentration-dependent manner without affecting responses to salty, sour, bitter, and umami compounds (216).

Endocannabinoids are not able to increase sweet taste responses at cellular, nerve, or behavioural levels in CB1 receptor knock-out mice, and are reduced in taste cells by CB1 receptor antagonist and not by CB2 receptor antagonist (216). Type II taste cells express CB1 and TAS1R3 sweet taste receptors (216), suggesting that the taste organ is a peripheral target of endocannabinoids. A recent study confirmed the involvement of the ECS with taste in normal-weight PROP supertasters and PROP non-tasters (217). The genetically determined ability to taste 6-*n*-propylthiouracil (PROP), is a property

that has been linked with lowered acceptance of some bitter foods, and increased energy intake and BMI. This study revealed as in normal weight subjects, taste sensitivity is associated with AEA and 2-AG plasma levels. In the hypothalamus, the ECS not only acts influencing appetite signalling, but it has been shown to interact with pathways known to be involved in the regulation of energy balance, and lipid and glucose metabolism (174,175).

The peripheral action of the ECS is associated with fat accumulation in adipose tissue, and impaired and decreased glucose uptake in skeletal muscle (181,218). Also, CB1 receptors present in liver, contribute to the peripheral metabolic activity of the ECS.

High fat feeding increases expression of the CB1 receptor and hepatic AEA levels. In liver, dietary LA and pharmacological stimulation of CB1 receptor enhance gene expression of the lipogenic transcription factor SREBP-1c (180). Through induction of hepatic acetyl-CoA carboxylase-1 (ACC1) and FAS, leading to an increased hepatic accumulation of fat, which would be expected to enhance VLDL production and TAG flux, and to increase hepatic insulin resistance.

Prior to any significant difference in body weight, high fat feeding leads the hepatic metabolic shift to fatty acid synthesis and reduced oxidation. This represents a direct effect of up regulation ECS-mediated on hepatic metabolism, rather than a secondary consequence of body weight change. In a correlated study (219), genetically modified mice for CB1 [CB1(-/-)] and liver CB1 receptors [LCB1(-/-)] fed with a high fat diet developed a reduced degree of dyslipidemia, hyperglycemia, steatosis, insulin and leptin resistance, compared to wild-type mice fed with the same diet. Also, LCB1(-/-) mice showed obesity in a similar manner to wild-type mice. These findings highlighted the

role of the endocannabinoids and their target receptors in the development of high fat diet-induced steatosis without increase of adipose tissue.

It has been shown in mouse 3T3-F442A adipocytes that ECS takes part to adipocyte differentiation and lipogenesis, and causes depression of adiponectin expression in mature adipocytes (220,221).

The reduced expression of adiponectin due to the overstimulation of CB1 receptors by high levels of 2-AG in adipocytes during hyperinsulinemia (condition typical of obesity), contributes to the hormonal and metabolic changes characterizing obesity (222). The CB1 receptor antagonist rimonabant restores in diet-induced obesity mice the normal expression of the adiponectin-dependent enzymes involved in lipid and glucose metabolism (223). However, the role of the ECS in adiponectin regulation still remains unclear, and some studies showed mixed results. Rimonabant treatment in rats (224) and human adipocytes (225) failed to influence adiponectin levels. No association has been reported among CB1 mRNA expression in adipose tissue, circulating CB1 levels, and adipose tissue expression of adiponectin in lean and obese subjects (226).

In mature adipocytes through the secretion of leptin, levels of AEA and 2-AG were efficaciously reduced (220). Matias *et al.* (220) also revealed as the endocanabinoid/CB1 signalling stimulates insulin secretion in RIN-m5F rat β-cells (pancreatic cells), and it was overactive also in pancreas of mice with obesity induced by diet, and in the visceral adipose tissue of obese individuals.

In pathological conditions characterized by hyperglycemia, such as prediabetes, type 2 diabetes and obesity, the ECS in β-cells instead of remain under insulin control, becomes dysregulated. The hyperinsulinemia might be reinforced by overstimulation of endocannabinoid receptors with the CB1/CB2 HU-210 agonist, triggering to permanent

hyperinsulinemia (220). This condition would increase the levels of endocannabinoids in β-cells, leading to endocannabinoid hyperactivity and increased lipogenesis in adipocytes, accompanied by decreased levels of adiponectin. Some studies have suggested the hyperactivity of the ECS in human obesity, and in genetic and diet-induced obesity in animal models. The positive stimulation of the ECS in the brain and peripheral tissues drives robust physiologic and metabolic changes characteristic not only of obesity but also in conditions of insulin resistance and potentially diabetes (227, 228). Comparison of lean and obese female human subjects showed that circulating AEA levels were positively correlated with BMI, while 2-AG with waist circumference (229). Also, obese subjects had both increased plasma AEA (35%) and 2-AG levels (52%) ana reduced FAAH activity (around 59%). This evidence further supports the role of the ECS in human obesity. Circulating 2-AG levels positively correlated to visceral adipose tissue, HDL-cholesterol, and high TAG concentrations in plasma of viscerally obese men (BMI around 31 kg/m2) (230). Reduction of body weight and waist circumference following a 1 year of lifestyle programme was accompanied by declined levels of 2-AG in plasma. In a similar study (231), a significant correlation between BMI and plasma AEA concentration was observed in human subjects with varying BMI.

1.8 Influence of dietary fats on endocannabinoids

The endocannabinoids AEA and 2-AG are biosynthesized “on demand” from AA bound to the membrane PLs in position sn-1 and sn-2, respectively (232).

Being an essential fatty acid, the tissue content of AA esterified to PLs is primarily influenced by the diet. Moreover, the content of the n-6 precursor of AA, LA, and the competition with other fatty acids such as eicosapentaenoic acid (EPA, 20:5 *c*5,*c*8,*c*11,*c*14,*c*17) and DHA, contribute to affect AA concentrations in PLs (233,234). AEA and 2-AG levels were elevated in liver and erythrocytes along with food intake, feed efficiency, and adiposity in mice fed with a LA enriched diet (233). The addition of EPA and DHA to the highest level of LA in the diet reversed the adipogenic effect of LA and normalize endocannabinoid tone in mice.

Some studies reported the positive correlation between dietary AA and AEA and 2-AG levels in the hypothalamic regions of piglet brain (235), and the inverse correlation between a prolonged intake in n-3 PUFA-enriched diet and endocannabinoids in mouse brain (236), as well as in small intestine and liver (237).

After 14 weeks administration of a high-fat diet (60 % energy), levels of AEA in mouse liver were increased and the authors of this study (180) suggested the potential role for CB1 receptor activation in contributing to diet-induced obesity by increased fatty acid synthesis. Artmann *et al*. (237) compared the short-term effects of five different dietary fats on 2-AG and AEA concentrations, in rat brain, liver and duodenum. The five diets included: palm oil (rich in PA), olive oil (rich in OA), safflower oil (rich in LA), fish oil (FO), and a diet rich in AA. The authors also examined the amounts of the ERC, PEA and OEA, and the NAE compounds *N*-stearoylethanolamine, *N*-linoleoylethanolamine, 22:5 n-3 NAE, and 22:6 n-3 NAE. After 1 week feeding, rats showed increased *N*-

linoleoylethanolamine in brain, jejunum and liver by LA diet. The OA diet increased brain levels of AEA and OEA. OA diet also increased OEA in liver. The diet rich in AA elevated AEA and 2-AG in jejunum but none effect in liver was observed. FO diet decreased all NAE levels in liver. The AA and FO diets had no effect on NAEs, endocannabinoids or precursor lipids in brain.

It has been shown that feeding mice with different high fat diets containing various amounts of SFAs, MUFAs, and PUFAs, increased levels of AEA or 2-AG, or both, in brown adipose tissue, heart, skeletal muscle and kidney (238). On the other hand, a diet higher in n-3 PUFA content reduced AEA and 2-AG levels. Another study reported the effects of dietary n-3 long chain polyunsaturated fatty acids (LCPUFAs) compared to a control diet containing no EPA and DHA (239). After 4 weeks of treatment, n-3 PUFA supplemented diets lowered liver and heart TAGs, and the peritoneal macrophage response to an inflammatory stimulus in Zucker rats. These effects were associated with reduced levels of AEA and 2-AG in the visceral adipose tissue, and of AEA in liver and heart, which, in turn, was associated with lower levels of AA in membrane PLs. The enzymatic activities of the AEA-hydrolyzing enzyme FAAH and the MAGL specific for 2-AG, did not differ.

Experiments *in vitro* definitely confirmed as dietary fatty acids influence endocannabinoid biosynthesis and then might modulate the ECS (240,241). Treating mouse pre-adipocyte 3T3- F442A with 100 μM of different n-6 and n-3 PUFAs, Matias *et al*. (240) reported that AA strongly increased 2-AG levels; DHA decreased 2-AG; and no effect on 2-AG levels was observed after EPA treatment. If DHA decreased AEA levels, AA and EPA treatments did not. 3T3-L1 adipocytes were able to convert DHA and EPA into their NAE derivatives docosahexaenoyl ethanolamine (DHEA) and

eicosapentaenoyl ethanolamine (EPEA), respectively, in a dose-dependent response manner (241). DHA and EPA did not affect AEA level in the culture medium.

The anti-inflammatory molecules DHEA and EPEA have been recently identified as alternative agonists of CB1 and CB2 receptors in human and mouse leukocytes (242,243). In human polymorphonuclear leukocytes, DHEA-derived products showed to be poorer activators of CB1 compared to AEA, but preferentially activate CB2 than AEA (242). The concentration of DHEA required to activate CB1 receptors was much higher, compared to AEA. It has been shown that DHEA possesses anti-inflammatory properties in both mouse peritoneal and RAW264.7 macrophages (244). Activation of CB2 receptors by n-3 PUFA derived metabolites, resulted in a reduction of inflammatory cytokines production in leukocytes (242), which finally could lead to a decreased state of chronic inflammation that may be associated with obesity.

In a study conducted in humans, 2g/day of dietary krill oil for 4 weeks decreased plasma 2-AG levels in obese subjects (BMI around 30 kg/m2), and no changes were detected in BMI, waist circumference, glycemia and insulinemia after any of the treatments (245). The decrease of 2-AG was correlated to the decrease of plasma n-6/n-3 phospholipid LCPUFAs ratio, with the reduced 2-AG biosynthesis probably caused by the replacement of 2-AG precursor, AA, with n-3 PUFAs contained in krill oil. Krill oil more efficiently than fish oil was able to reduce endocannabinoids in different tissues of obese Zucker rats (239) and in plasma of obese subjects (245). The effects of dietary krill oil on peripheral endocannabinoid overactivity were recently evaluated in mildly obese men (BMI of 32.3 kg/m2) by Berge *et al.* (246). After 12 and 24 weeks of dietary supplementation, 4 g/day of krill powder (34% proteins, 61.8% krill oil) reduced AEA levels in 59 and 84% subjects, respectively, compared to six control subjects that

had not taken any supplements. The treatment also produced a significant decrease in waist/hip ratio and visceral fat/skeletal muscle mass ratio at 24 weeks, but no change in body weight. DHA and EPA significantly increased with a reduction of TAG plasma levels.

Only few data reported the possible effects of CLA on ECS. This group of unusual fatty acids has been found to be linked with changes of endocannabinoids levels in mouse brain. Mice fed with diets containing 3% of either LA or CLA for 4 weeks, showed significantly reduced amounts of 2-AG in the cerebral cortex, compared with LA treatment (247). CLA supplementation did not affect AEA, OEA, and PEA levels. The 2-AG variations measured in the cerebral cortex were not detected in the hypothalamic region of the brain. In a recent study conducted on forty-two adult volunteers with diagnosed mildly hypercholesterolemia (248), Pintus *et al.* compared the effects of 90 g/day consumption of a naturally enriched sheep cheese in some fatty acids and a control cheese, on plasma lipid and endocannabinoid profiles. In this 3 weeks cross-over study, the enriched cheese in ALA, CLA and VA, decreased AEA levels. Plasma of volunteers also revealed significantly decreased LDL-cholesterol level and increased concentrations of CLA, VA, ALA and EPA. None of the parameters measured were modified by the control cheese.

All these findings strongly support the significant role of dietary fatty acids in the physiological control of the ECS and their beneficial effects by reducing biosynthesis of endocannabinoids and their possible role in promoting metabolic syndrome.

2. Aims of the study

CLA influencing different biochemical pathways determines alteration of body composition in experimental animal and impaired lipid accumulation in adipocytes, as well as a whole range of beneficial effects such as anti-inflammatory and anti-tumor activity. The central role in feeding behavior and energy homeostasis of the ECS and its lipid derived neuromodulatory molecules, point the need to investigate possible effects of CLA on the levels of endocannabinoids AEA and 2AG, along with their congeners OEA and PEA.

The first objective of this thesis is to study the effects of CLA enriched diets on fatty acid metabolism, examining hepatic fatty acid profile, including total lipids, UFAs, SFAs, and the CLA derived metabolites in obese Zucker rats. In the same experimental animal, we also want to assess the influence of dietary CLA on the levels of endocannabinoids and congeners. Another issue was to evaluate and compare the influence of CLA supplementation in combination with fat from vegetable origin versus fat from animal origin on fatty acid deposition.

Only few data resulting from trials in humans evaluating the effect of very high dose of CLA on biochemical and physiological parameters are available (27,28), and no studies have been conducted on the influence of endocannabinoid levels and biosynthesis.

The other aim of the present study was then to measure total fatty acid profile, CLA metabolites, and endocannabinoids and congeners in subjects fed with a CLA enriched diet (mainly *c*9,*t*11 CLA isomer), for three weeks. The safety of a short term treatment with very high doses of CLA in humans has been already proved (27,28), but the influence on whole fatty acid metabolism and endocannabinoid synthesis represents an intriguing and unexplored issue to be investigated.

The effects of *c*9,*t*11 CLA on serum fatty acid and endocannabinoid profiles were compared to those generated by other two identical diets that differed only for the presence of OA or industrial trans fatty acids, instead of CLA.

3. Experimental design

3.1 Study in animal model: fatty Zucker rats

To start gaining insights on the effect of CLA on fatty acid metabolism and endocannabinoid biosynthesis, we have primarily performed this study evaluating fatty acid deposition and endocannabinoid levels in liver of Zucker rats supplemented with CLA isomers mixture in combination with fat from different dietary origins. These rodents represent a genetic model for research on obesity and hypertension (249), in particular the characteristically obese (or fatty) Zucker rat, which is a recessive trait (*fa/fa*) of the leptin receptor, determines hyperphagia resulting in visceral obesity and ectopic fat accumulation. Liver samples from Zucker rats were kindly provided by Prof. Jose´ AM Prates and his research team. All the detailed information regarding animals, CLA oil, and experimental diets is reported in reference n. 250 of the present thesis. Briefly, rodents were purchased from Harlan Interfauna Iberia (Barcelona, Spain) at 5 weeks old. Upon arrival, animals were housed individually and maintained on a 12 h light:dark cycle at 22 ± 2 °C. After 1 week adaptation period, thirty-two obese male *fa/fa* Zucker rats were randomly divided into four groups with eight rats each (n = 8), and assigned to one of four diets containing palm oil (PO) or ovine fat (OF), supplemented or not with 1% of 1:1 *c*9,*t*11 and *t*10,*c*12 CLA isomers mixture, and fed for 13-14 weeks. The differences of the four experimental diets are reported in Table 1.

The CLA oil purchased from PharmaNutrients Inc. (Gurnee, IL,USA) had a purity of 80%. Peritoneal ovine fat collected from lambs raised with forage enriched in seed oils, was finally melted and filtered to subsequent mixture with the remaining components of the experimental diets (purchased from Provimi Kliba SA, Kaiseraugst, Switzerland).

Diets	Composition
PO	11.25% of palm oil plus 3.75% of sunflower oil
POCLA	11.25% of palm oil plus 2.53% of sunflower oil and 1.22% of CLA oil
OF	11.25% of ovine fat plus 3.75% of sunflower oil
OFCLA	11.25% of ovine fat plus 2.53% of sunflower oil and 1.22% of CLA oil

Table 1. Fat composition of the experimental diets.

Diets were prepared following the AIN-93G formulation, with some modifications to obtain high fat diets. Diets had a dry matter concentration of 928 g/kg feed and the following crude composition (g/100 g dry matter) was 20.0 proteins, 16.6 fats, 3.9 ash, 3.9 fibers, and 55.6 carbohydrates. The four experimental diets differed in their fat composition. Two groups received fat from vegetable origin: group PO 11.25% of palm oil plus 3.75% of sunflower oil; group POCLA 11.25% of palm oil plus 2.53% of sunflower oil and 1.22% of CLA oil. The other two groups, OF and OFCLA, had the same fat composition of PO and POCLA, respectively, but received ovine fat instead of palm oil. Animals had free access to water and food. Body weight and food intake were monitored twice a week. At the end of the scheduled feeding time with experimental diets, rats were killed by decapitation, under light isofluorane anesthesia. Liver was dissected, weighed and stored at -80 °C. The experimental protocol of this study was reviewed by the Ethics Commission of CIISA/FMV and approved by the Animal Care Committee of the National Veterinary Authority of Portugal, following the appropriated European Union guidelines (N. 86/609/EEC).

3.2 Randomized cross-over study in humans

In order to extend the study of nutritional influence of CLA on fatty acid metabolism, we analyzed through a lipidomic approach total lipid profile, and plasma levels of endocannabinoids and congeners in healthy subjects fed with a very high dose of dietary CLA.

In this controlled single blind randomized multiple crossover trial with three consecutive periods of 21 days, the effects of CLA (mainly *c*9,*t*11 CLA isomer) were compared to OA and a diet rich in industrial trans fatty acids.

Samples of human plasma were provided by Prof. Ingeborg Brouwer and Prof. Martijn B. Katan, Department of Health Sciences, University of Amsterdam. The approved protocol for this clinical trial, the characteristics of enrolled volunteers into the study, and the fatty acid composition of the oils and fats used to produce the experimental diets, can be found in a paper previously published (28).

We screened plasma from 24 healthy men and women (aged 18 to 65) which consumed each of three diets for three weeks, in random order.

Diets were identical except for 7% of total energy (in a 2500 Kcal diet, approximately 20 g/day) which was provided either by:

- 80% *c*9,*t*11 CLA and 20% *t*10,*c*12 CLA (**CLA diet**);
- Industrial trans fatty acids (**TFA diet**);
- Oleic acid (**OA diet**).

4. Material and methods

Acetonitrile (CH_3CN), methanol (CH_3OH), chloroform ($CHCl_3$), *n*-hexane (C_6H_{14}), ethanol (C_2H_5OH), acetic acid (CH_3COOH) were HPLC grade and purchased from Sigma Chemicals Co., St. Louis, MO, USA. All standards of SFAs, UFAs, and *c*9,*t*11 CLA and *t*10,*c*12 CLA isomers, 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 ($[^2H]_8$AEA, $[^2H]_5$2AG, $[^2H]_4$ PEA, $[^2H]_4$ OEA) were purchased from Cayman Chemicals, MI, USA.

4.1 Extraction of total lipids

Total lipids were extracted by the method of Folch (251). Briefly, samples of human plasma (1 ml) and rat liver (about 0.3 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 (ddH_2O) 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 different aliquots for subsequent analyses and evaporated under vacuum by a rotator evaporator at room temperature.

4.2 Quantitative determination of total lipids

Total lipid quantification was performed by the method of Chiang (252) on evaporated aliquots of chloroform phase containing lipids, collected after initial lipid extraction.

1.5 ml of the Chiang reagent (2 g of $K_2Cr_2O_7$ into 4 ml ddH_2O, make up to a final volume of 100 ml adding H_2SO_4) was added, and the samples incubated for 30 min. at 100 °C. Finally, 1.5 ml of ddH_2O was added, and the absorbance measured at the wavelength of 600 nm by a colorimeter. 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.

4.3 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 FFAs for HPLC analysis. Lipid extracts were dissolved in 5 ml of ethanol, 100 µl of desferal (25 mg/ml ddH_2O), 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 ddH_2O were added, then the samples were acidified with 0.35 ml of 37% HCI, to a pH 3-4. Samples were centrifuged for 1h at 900 x *g*. The hexane phase containing FFAs was collected, the solvent evaporated, and the residue was dissolved in 0.5 ml of CH_3CN/0.14% of CH_3COOH (v/v).

Separation of fatty acids, including CLA and its metabolites, 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 $CH_3CN/H_2O/CH_3COOH$ (70/30/0.12, v/v/v) at a flow rate of 1.5

ml/min (253). 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 (254).

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. H_2 served as carrier gas (1 ml/min), and the FID gases were H_2 (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.

4.4 Analysis of the endocannabinoids and their congeners

Aliquots of organic phase (chloroform) containing extracted lipids were evaporated to dryness under vacuum, and reconstituted with 0.3 ml and 0.4 ml of 100% methanol for human plasma and rat liver samples, respectively.

Quantification of AEA, 2-AG, PEA and OEA, was carried out by liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS), and using selected ion monitoring (SIM) at M+1 values for the four compounds and their deuterated homologues. A C-18 Zorbax Eclipse Plus column (Agilent, Palo Alto, CA) 5 μm particle size, 50 x 4.6 mm, was used with a mobile phase of $CH_3OH/H_2O/CH_3COOH$ (80/20/0.3, v/v/v) at a flow rate of 0.5 ml/min.

4.5 Statistical analysis

To calculate mean, standard deviation, and standard error of the measurements, InStat software (GraphPad Software, San Diego, CA, USA) was used. Statistical differences among different experimental dietary treatments in human plasma and rat liver experiments were evaluated by one-way ANOVA and the Bonferroni test for post hoc analyses. CLA dose response curve in human was assessed by Pearson simple correlation analysis. We assumed statistical significance for p-value <0.05.

5. Summary of results

In the first part of this thesis, we aimed to evaluate the CLA activity on fatty acid metabolism in liver of Zucker rats with two very different background diets (Fig.5). One of vegetable origin, PO, characterized by high levels of PA and OA and low in ALA, while the other one, OF, characterized by high levels of trans fatty acids and RA. The fatty acid characterization of the four experimental diets is presented in Table 2.

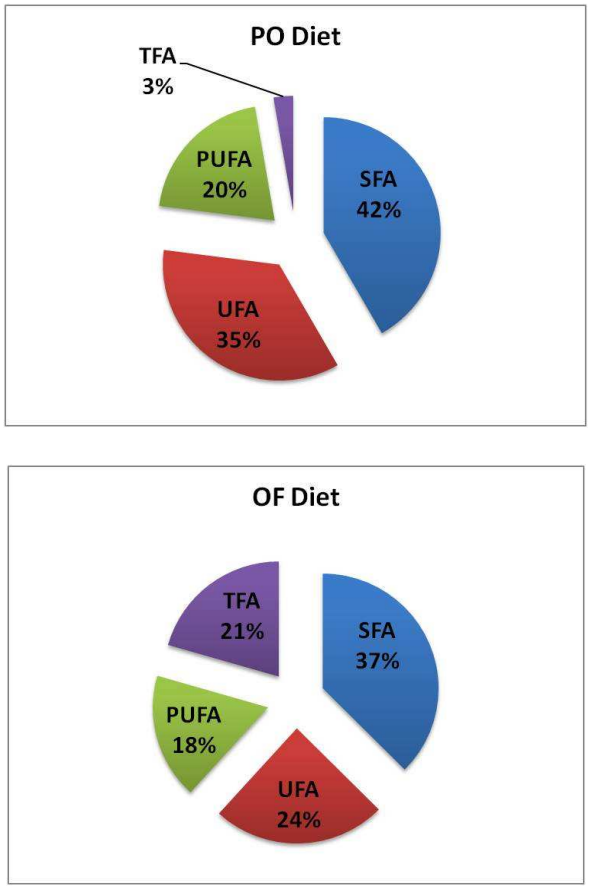


Figure 5. Background of vegetable (PO) and animal origin (OF) diets.

	PO	POCLA	OF	OFCLA
Fatty acids				
12:0	0.020	0.019	0.011	0.011
14:0	0.113	0.105	0.162	0.169
15:0	0.006	0.006	0.038	0.040
16:0	4.354	3.938	1.512	1.550
c7-16:1	0.004	0.003	0.029	0.030
c9-16:1	0.025	0.015	0.068	0.073
c9-17:1	0.004	0.002	0.018	0.016
18:0	0.520	0.428	2.520	2.709
c9-18:1	4.293	3.392	2.676	2.646
18:2n-6	2.460	1.680	1.884	1.537
18:3n-3	0.016	0.012	0.148	0.156
20:0	0.042	0.033	0.026	0.028
20:2n-6	0.001	n.d.	0.002	0.004
22:0	0.027	0.016	0.023	0.019
24:0	0.020	0.013	0.012	0.010
18:1 isomers				
t6 + t7 + t8	0.009	0.007	0.072	0.077
t9	0.011	0.009	0.061	0.067
t10	0.011	0.008	0.113	0.105
t11	0.007	0.006	1.106	1.187
t12	n.d.	n.d.	0.128	0.142
c11	0.173	0.117	0.131	0.132
c12	0.017	0.004	0.136	0.149
c13	0.002	0.003	0.019	0.018
t16 + c14	0.002	0.001	0.053	0.057
c15	n.d.	n.d.	0.023	0.024
Non-conjugated 18:2 isomers				
t9,t12	n.d.	n.d.	0.053	0.059
t8,c12/c9,t12	n.d.	n.d.	0.035	0.052
t8,c13/c9,t13	0.047	0.038	0.022	0.023
t9,c12	0.044	0.037	0.016	0.018
t11,c15	n.d.	n.d.	0.191	0.204
c9,c15	n.d.	n.d.	0.022	0.024
c12,c15	n.d.	n.d.	0.017	0.018
Conjugated (CLAs)				
t12,t14	n.d.	n.d.	<0.001	0.005
t11,t13	n.d.	n.d.	0.013	0.015
t10,t12	0.002	0.008	0.004	0.011
t9,t11	0.002	0.008	0.008	0.016
t8,t10	<0.001	0.005	0.001	0.001
t7,t9	<0.001	<0.001	0.001	0.001
c/t11,13	0.001	n.d.	0.049	0.058
t10,c12	n.d.	0.260	n.d.	0.270
c9,t11	0.002	0.268	0.149	0.430
t8,c10	<0.001	0.003	0.004	0.006
t7,c9	<0.001	<0.001	0.005	0.006

n.d.= not detected

Table 2. Fatty acid composition (g/100 g diet) of experimental diets.

5.1 Modulation of body composition and fat deposition in liver of Zucker rats by dietary CLA.

CLA feeding for 3 months decreased body and liver weight irrespective of the background lipid diets (Fig.6). Data about body composition variables were previously published by Martins SV *et al.* (255). The decrease of liver weight was mainly due to a reduced deposition of lipids (Fig.7).

						Significance level		
Growth and body composition	**PO**	**POCLA**	**OF**	**OFCLA**	SEM	CLA	Fat	CLA x Fat
Initial body weight (g)	240.0	234.0	234.0	234.0	11.2	NS	NS	NS
Final body weight (g)	577.0	552.0	584.0	511.0	12.7	***	NS	NS
Daily feed intake (g/d)	23.7	23.0	23.9	21.0	0.5	**	NS	NS
Retroperitoneal fat weight (g)	24.0	24.7	23.7	20.7	1.6	NS	NS	NS
Epididymal fat weight (g)	13.7	15.2	14.7	14.6	0.6	NS	NS	NS
Liver weight (g)	62.2	54.7	60.6	48.1	1.9	***	*	NS

Figure 6. Body composition variables.
*Dietary treatments: PO, palm oil diet; POCLA, palm oil diet + 1 % of conjugated linoleic acid; OF, ovine fat diet; OFCLA, ovine fat diet + 1 % of conjugated linoleic acid. Mean values were significant: NS P>0.05, * P<0.05, ** P<0.01, *** P<0.001.*

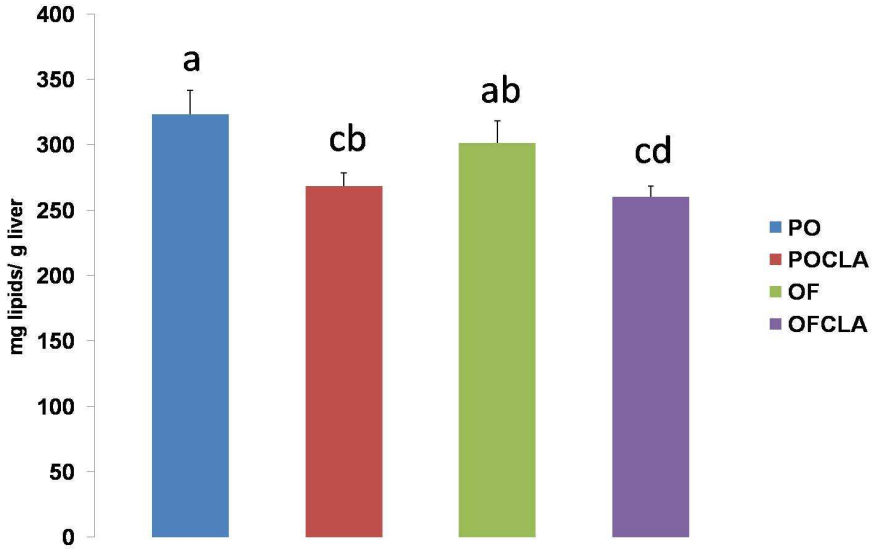


Figure 7. Lipid deposition in liver.
Dietary treatments: PO, palm oil diet; POCLA, palm oil diet + 1 % of conjugated linoleic acid; OF, ovine fat diet; OFCLA, ovine fat diet + 1 % of conjugated linoleic acid. Statistical significance: p-value <0.05.

5.2 Modulation of liver fatty acid profile by dietary CLA in Zucker rats.

CLA intake modified AA (20:4) concentration with a slight increase of about 15% with both palm oil (POCLA) or ovine fat (OFCLA) diets (Fig.8). While DHA (22:6) increased (about 25%) when CLA was included in the OF diet (OFCLA), compared to PO diet enriched in CLA (Fig.9). DHA increase resulted in a higher n-3 highly unsaturated fatty acid (HUFA) score (Fig.10). Δ9 desaturase index (or Δ9 desaturase activity) decreases with CLA intake with both diets (Fig.11). Interestingly, only in the OF diets, the increase in n-3 HUFA score was inversely correlated to Δ9 desaturase index (Fig.12).

As expected, CLA levels were higher after CLA feeding, but also with OF diet not supplemented in CLA which, containing RA, increased CLA liver content with respect to both PO and POCLA diets (Fig.13).

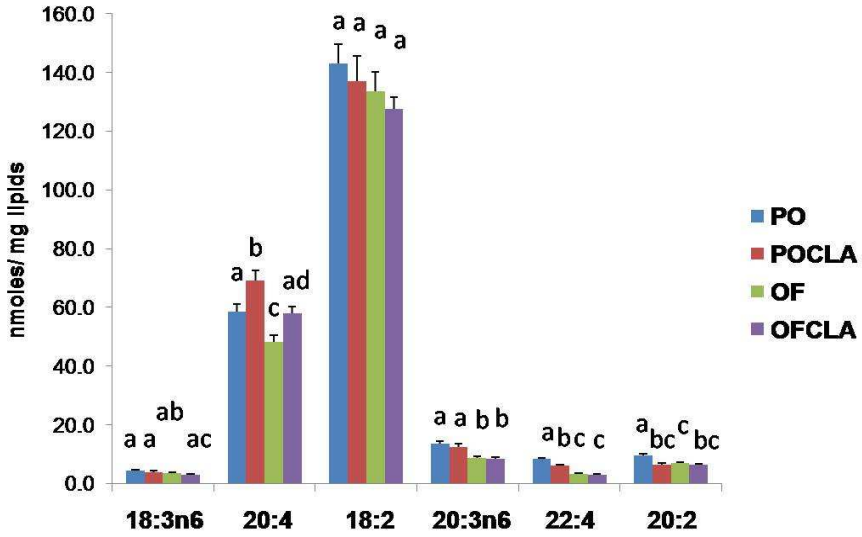


Figure 8. Main n-6 PUFAs.

Dietary treatments: PO, palm oil diet; POCLA, palm oil diet + 1 % of conjugated linoleic acid; OF, ovine fat diet; OFCLA, ovine fat diet + 1 % of conjugated linoleic acid. Statistical significance: p-value <0.05.

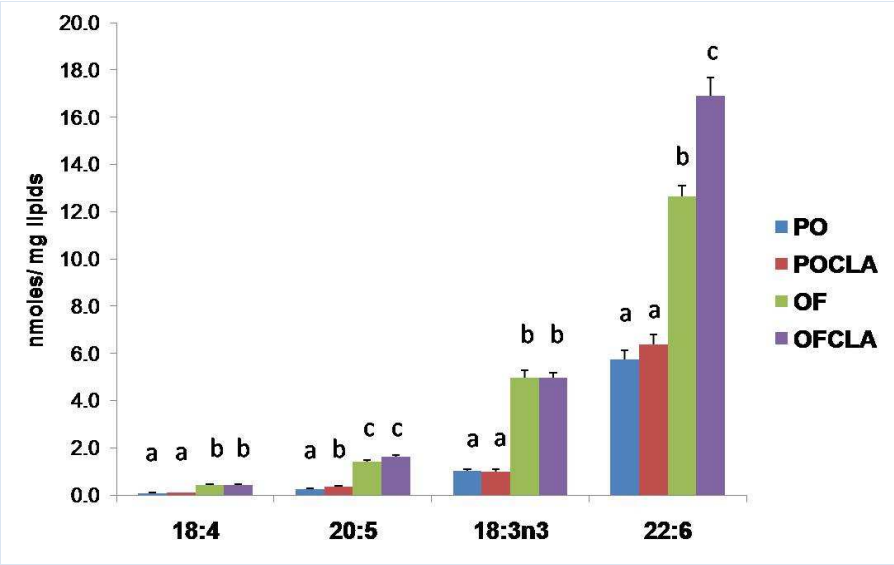


Figure 9. Main n-3 PUFAs.

Dietary treatments: PO, palm oil diet; POCLA, palm oil diet + 1 % of conjugated linoleic acid; OF, ovine fat diet; OFCLA, ovine fat diet + 1 % of conjugated linoleic acid. Statistical significance: p-value <0.05.

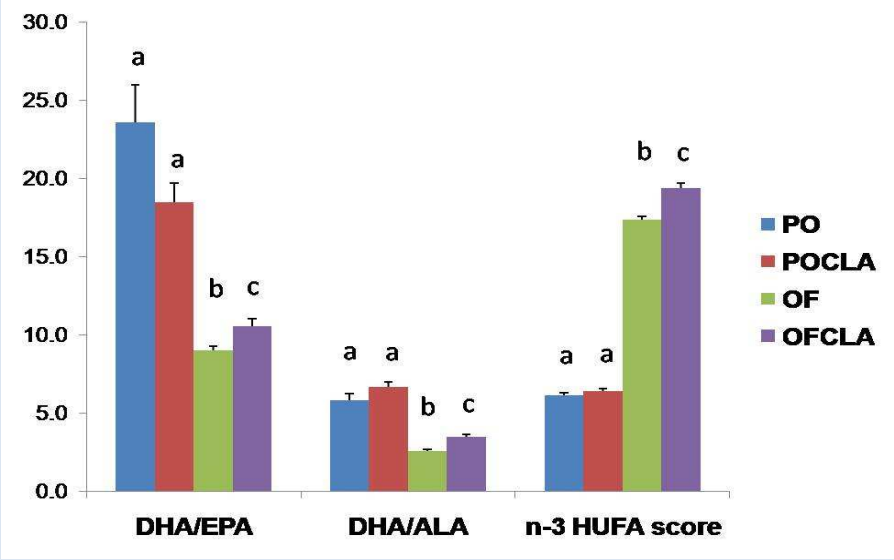


Figure 10. n-3 HUFA score, DHA/EPA and DHA/ALA ratios.

Dietary treatments: PO, palm oil diet; POCLA, palm oil diet + 1 % of conjugated linoleic acid; OF, ovine fat diet; OFCLA, ovine fat diet + 1 % of conjugated linoleic acid. Statistical significance: p-value <0.05. n-3 HUFA score was calculated by the ratio 20:5n-3+22:6n-3/20:5n-3+22:6n-3+ 20:4n-6+22:5+20:3n-6+20:3n-9+22:4n-6.

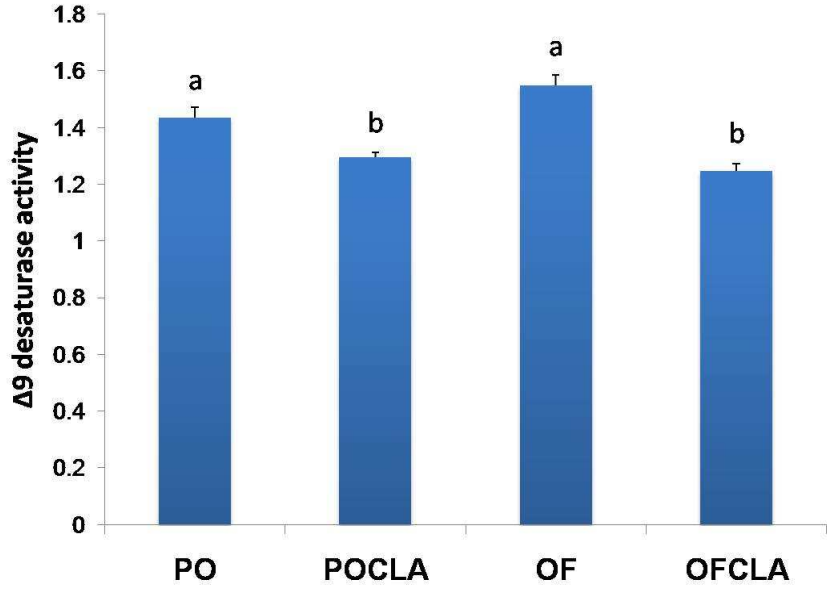


Figure 11. Δ9 desaturase activity.

Dietary treatments: PO, palm oil diet; POCLA, palm oil diet + 1 % of conjugated linoleic acid; OF, ovine fat diet; OFCLA, ovine fat diet + 1 % of conjugated linoleic acid. Statistical significance: p-value <0.05. Δ9 desaturase activity was calculated by the 16:1+18:1/16:0 + 18:0 ratio. 16:1, palmitoleic acid; 18:1, oleic acid; 16:0, palmitic acid; 18:0, stearic acid.

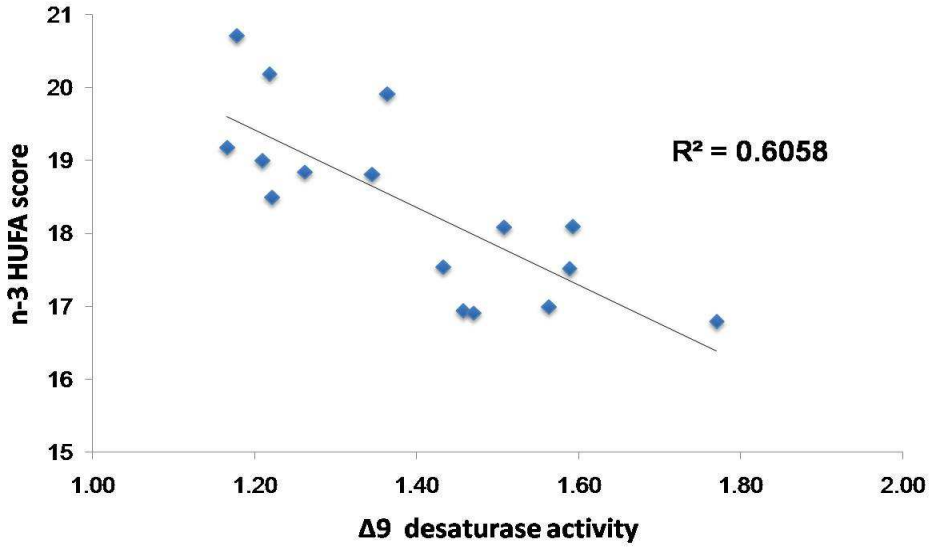


Figure 12. Correlation between n-3 HUFA score and Δ9 desaturase index in OF diets with or without CLA supplementation.

Δ9 desaturase activity was calculated by the 16:1+18:1/16:0 + 18:0 ratio. 16:1, palmitoleic acid; 18:1, oleic acid; 16:0, palmitic acid; 18:0, stearic acid.

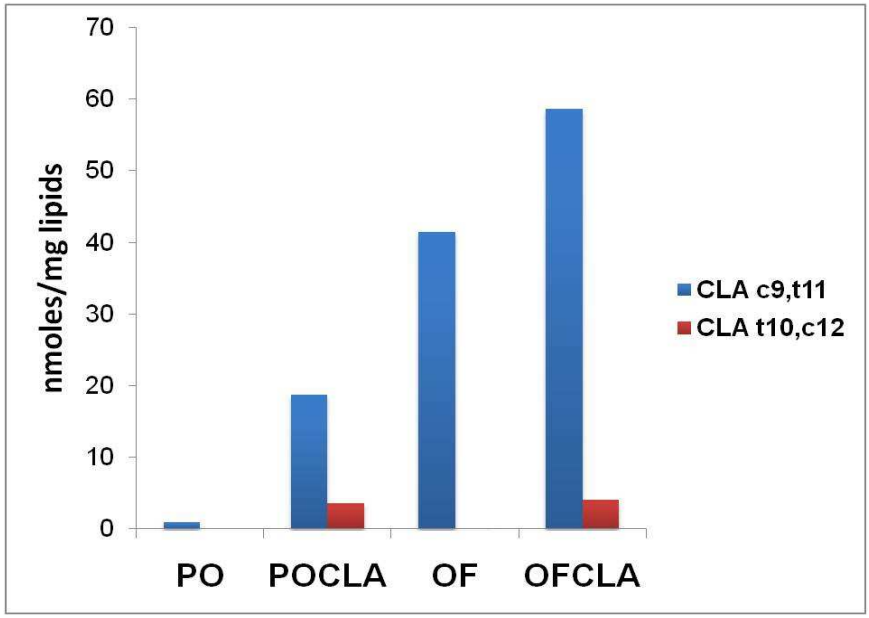


Figure 13. Cis9,trans11 and trans10,cis12 CLA amounts.

Dietary treatments: PO, palm oil diet; POCLA, palm oil diet + 1 % of conjugated linoleic acid; OF, ovine fat diet; OFCLA, ovine fat diet + 1 % of conjugated linoleic acid. Statistical significance: p-value <0.05.

5.3 Modulation of liver retinol concentration by dietary CLA in Zucker rats.

CLA feeding increased retinol level, but was significantly only versus PO diet (Fig.14).

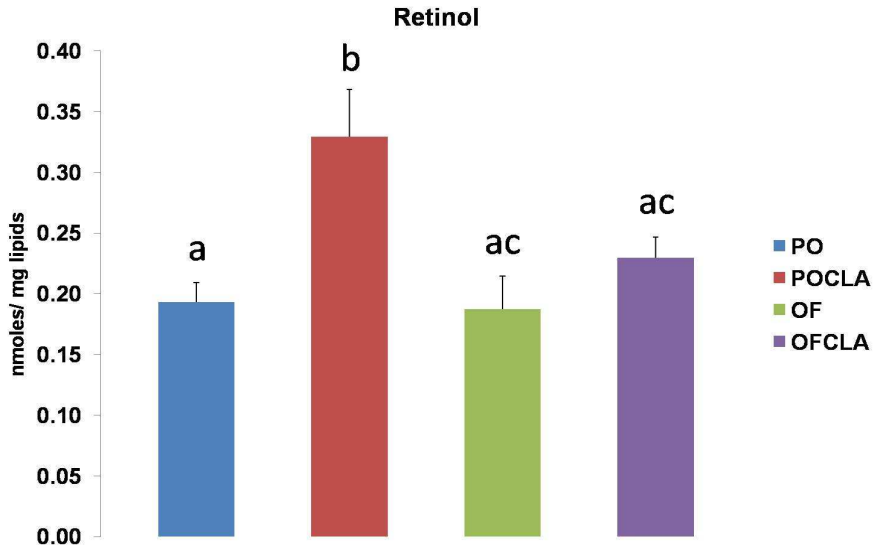


Figure 14. Retinol concentration in liver of Zucker rats.
Dietary treatments: PO, palm oil diet; POCLA, palm oil diet + 1 % of conjugated linoleic acid; OF, ovine fat diet; OFCLA, ovine fat diet + 1 % of conjugated linoleic acid. Statistical significance: p-value <0.05.

5.4 Influence of dietary CLA on endocannabinoid levels in Zucker rat liver.

The results of the experiments show as CLA does not alter the levels of endocannabinoids in the liver of Zucker rats. We found only a non-statistical significant decrease in the levels of AEA in the OF diet, compared with the same enriched in 1% CLA. The most interesting result for those anorectic properties ascribed to OEA comes out by the CLA action on the levels of this NAE compound, even in the presence of different background diets. CLA feeding significantly enhances OEA levels in both diets POCLA and OFCLA (Fig.15).

Interestingly, despite the PO diet is rich in OA (Table 2), the intake of CLA increases the levels of OEA. We also found between the two dietary treatments based on ovine fat, a statistically significant increase of PEA in favor of the OFCLA diet.

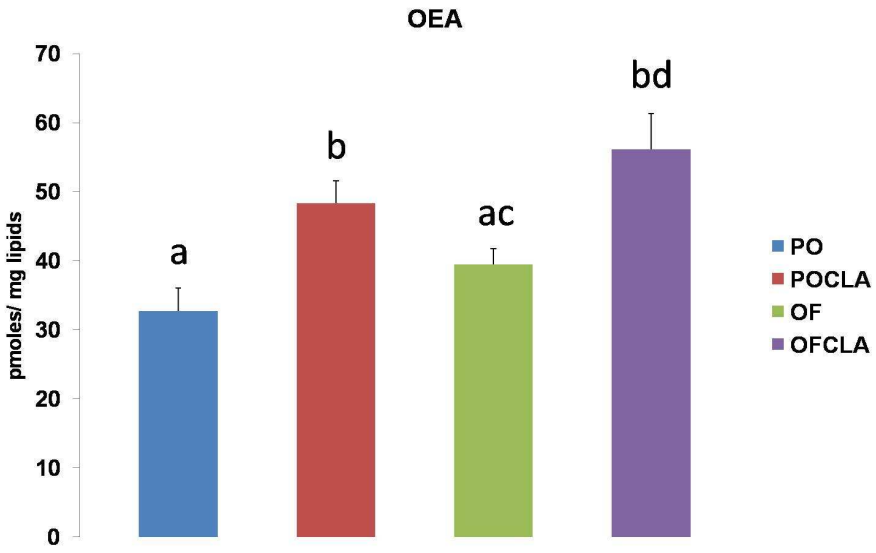


Figure 15. OEA concentration in liver of Zucker rats.

Dietary treatments: PO, palm oil diet; POCLA, palm oil diet + 1 % of conjugated linoleic acid; OF, ovine fat diet; OFCLA, ovine fat diet + 1 % of conjugated linoleic acid. Statistical significance: p-value <0.05.

5.5 Modulation of polyunsaturated fatty acids (PUFAs) and CLA metabolism by very high dose of dietary CLA in human plasma.

After having evaluated the effects of dietary CLA on fatty acid metabolism and endocannabinoid levels in the liver of Zucker rats, we analyzed plasma samples from volunteers who have had taken very high doses of dietary CLA, and compared its effects on lipid metabolism with a diet enriched in trans fatty acids of industrial origin, and a control diet characterized by the presence of OA. TFA and CLA diets increased serum total lipids relative to OA control diet (Fig.16).

Calculating the percentage of the produced ALA metabolites (fatty acids belonging to the n-3 family) with respect to their ALA precursor for the three different diets, CLA and trans fatty acids are more efficient than OA in reducing metabolism/incorporation of ALA metabolites (Fig.17).

With regard to the n-6 fatty acids, CLA compared to OA significantly decreases the metabolism and the incorporation of the LA metabolites (Fig.18). CLA competes with LA for the Δ6 desaturase. Besides, TFA compared to the OA diet, significantly decreased metabolism/incorporation of LA metabolites.

Regarding the n-9 fatty acids family, compared to OA diet, TFA decreases in a significant manner the metabolism/incorporation of the OA metabolites, while CLA diet doesn't (Fig.19). Considering the metabolic influence of the CLA dietary treatment only, CLA is desaturated and elongated less than LA but more than OA, and competes with LA for metabolism/incorporation of metabolites (Fig.20). CLA increased serum level of ALA (Fig.21) and LA (Fig.22), and reduced that of AA (Fig.22), relative to OA control diet. We can conclude that compared to both TFA and OA diets, CLA significantly reduces n-6 HUFAs (Fig.23), which resulted in an increased n-3 HUFA score (Fig.24).

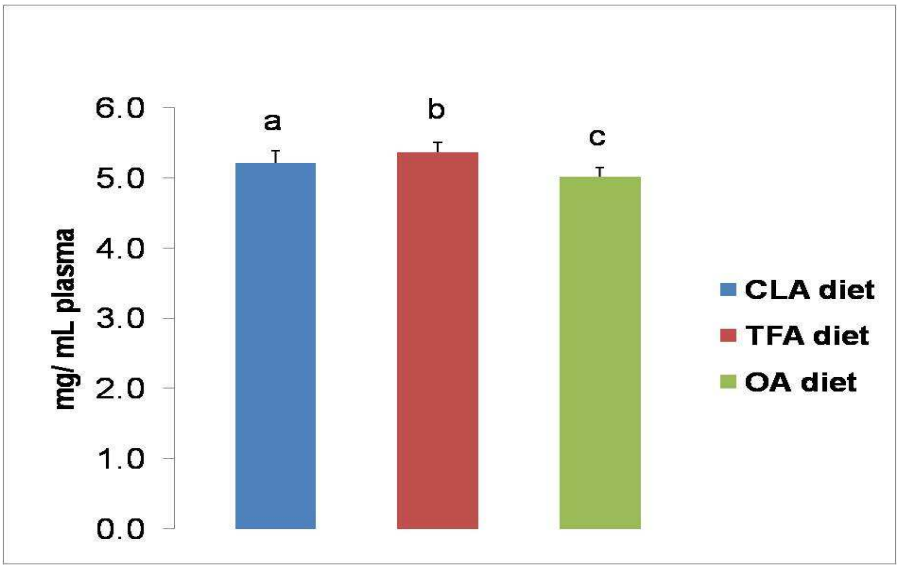


Figure 16. Total lipids in human plasma.

Dietary treatments: CLA diet, 80% c9,t11 CLA and 20% t10,c12 CLA; TFA diet, industrial trans fatty acids; OA diet, oleic acid. Statistical significance: p-value <0.05.

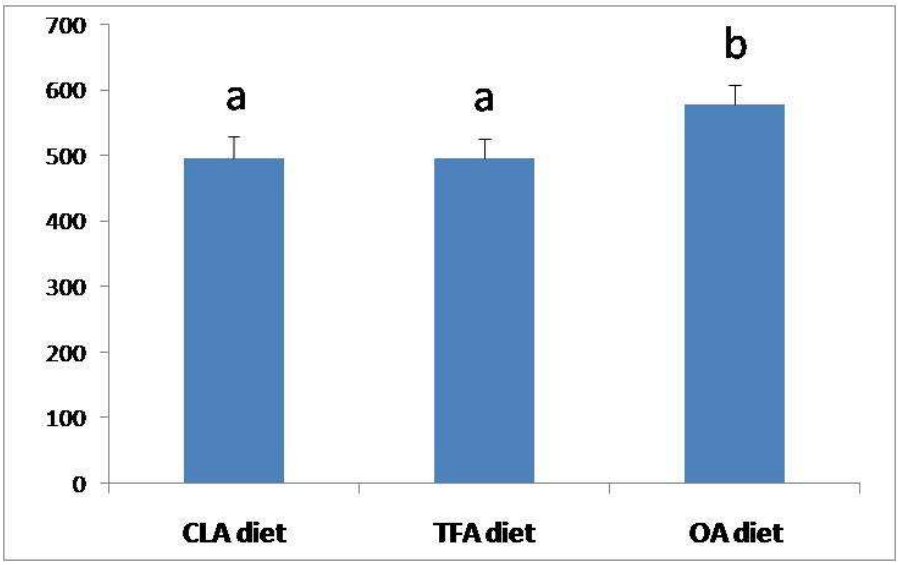


Figure 17. Alpha linolenic acid (ALA) metabolism.

*ALA metabolism was calculated as (18:4+EPA+DHA)*100/ALA. Dietary treatments: CLA diet, 80% c9,t11 CLA and 20% t10,c12 CLA; TFA diet, industrial trans fatty acids; OA diet, oleic acid. Statistical significance: p-value <0.05*

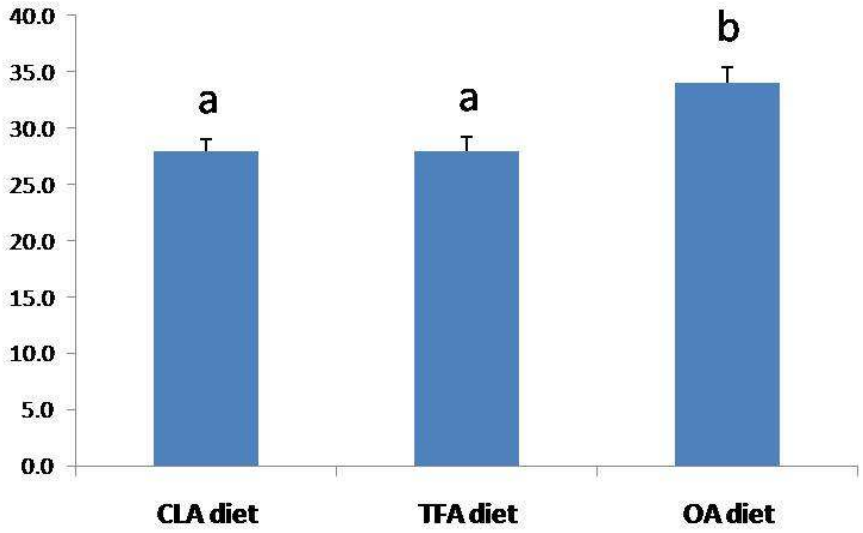


Figure 18. Linoleic acid (LA) metabolism.

*LA metabolism was calculated as (22:5+22:4+AA+20:3n-6+GLA)*100/ LA. Dietary treatments: CLA diet, 80% c9,t11 CLA and 20% t10,c12 CLA; TFA diet, industrial trans fatty acids; OA diet, oleic acid. Statistical significance: p-value <0.05.*

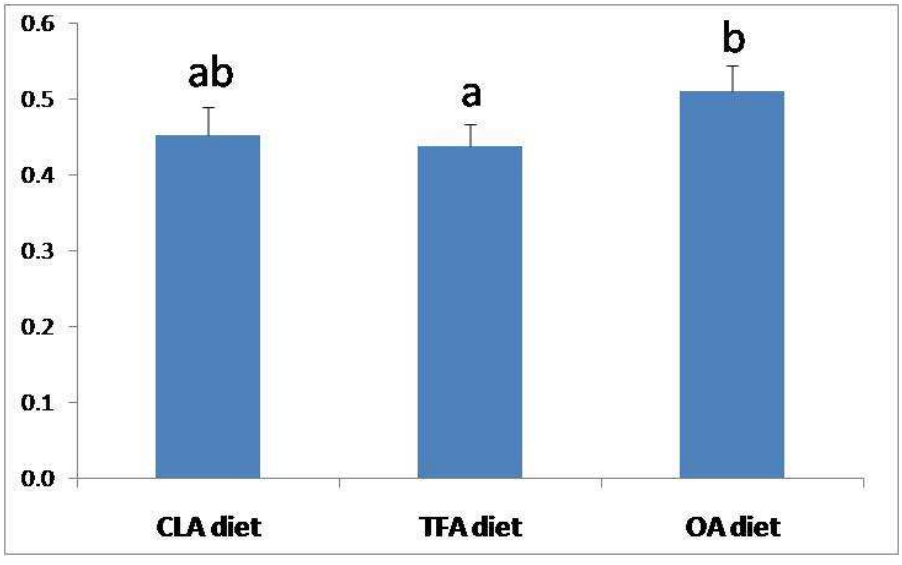


Figure 19. Oleic acid (OA) metabolism.

*OA metabolism was calculated as 20:3n-9*100/OA. Dietary treatments: CLA diet, 80% c9,t11 CLA and 20% t10,c12 CLA; TFA diet, industrial trans fatty acids; OA diet, oleic acid. Statistical significance: p-value <0.05.*

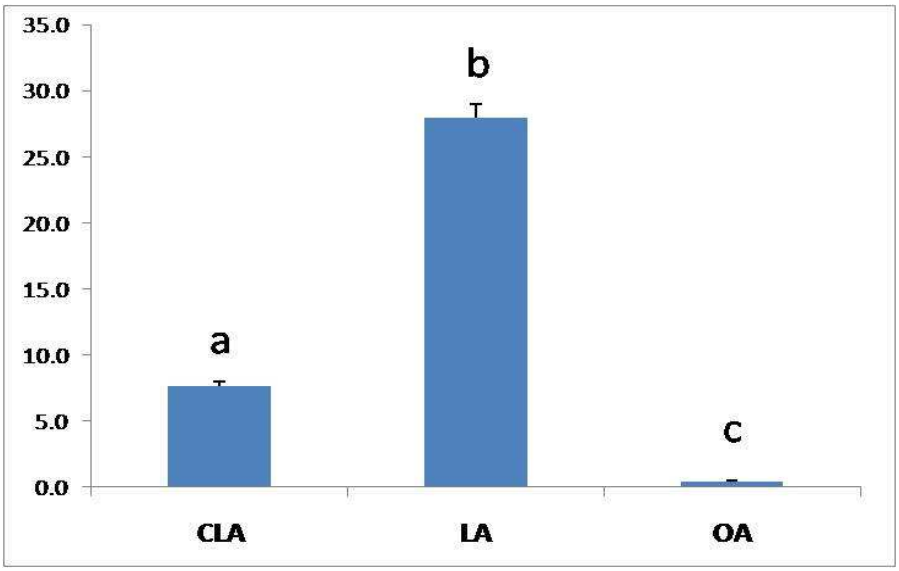


Figure 20. Effect of CLA diet on conjugated linoleic acid (CLA), linoleic acid (LA), and oleic acid (OA) metabolism in human plasma.

*CLA metabolism was calculated as (CD 20:4+CD 20:3+CD 18:3+CD 16:2)*100/CLA; LA metabolism was calculated as (22:5+22:4+AA+20:3n-6+GLA)*100/ LA; OA metabolism was calculated as 20:3n-9*100/OA. Statistical significance: p-value <0.05.*

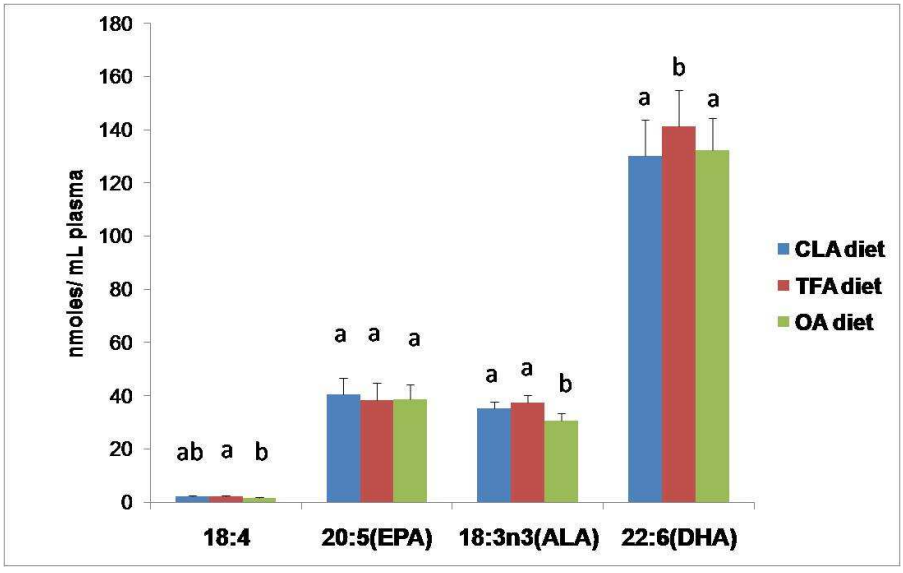


Figure 21. Main n-3 PUFAs.

Dietary treatments: CLA diet, 80% c9,t11 CLA and 20% t10,c12 CLA; TFA diet, industrial trans fatty acids; OA diet, oleic acid. Statistical significance: p-value <0.05.

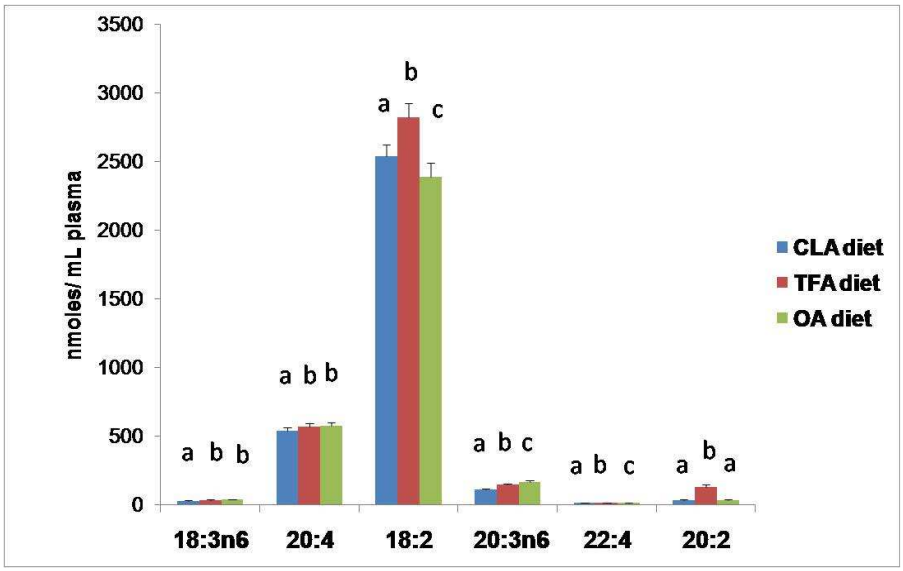


Figure 22. Main n-6 PUFAs.

Dietary treatments: CLA diet, 80% c9,t11 CLA and 20% t10,c12 CLA; TFA diet, industrial trans fatty acids; OA diet, oleic acid. Statistical significance: p-value <0.05.

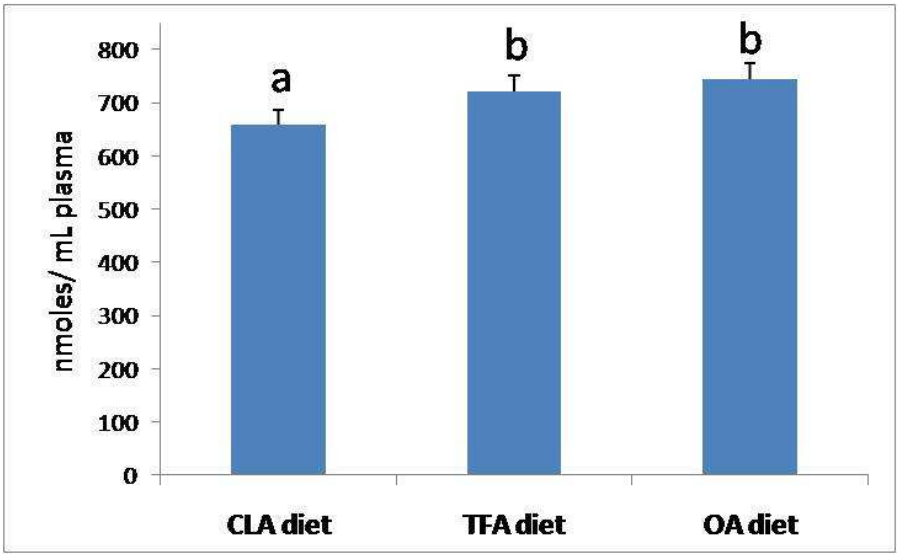


Figure 23. n-6 HUFAs.

Dietary treatments: CLA diet, 80% c9,t11 CLA and 20% t10,c12 CLA; TFA diet, industrial trans fatty acids; OA diet, oleic acid. Statistical significance: p-value <0.05. n-6 HUFAs refers to the sum of 20:3n-6, 20:4n-6, and 22:5n-6.

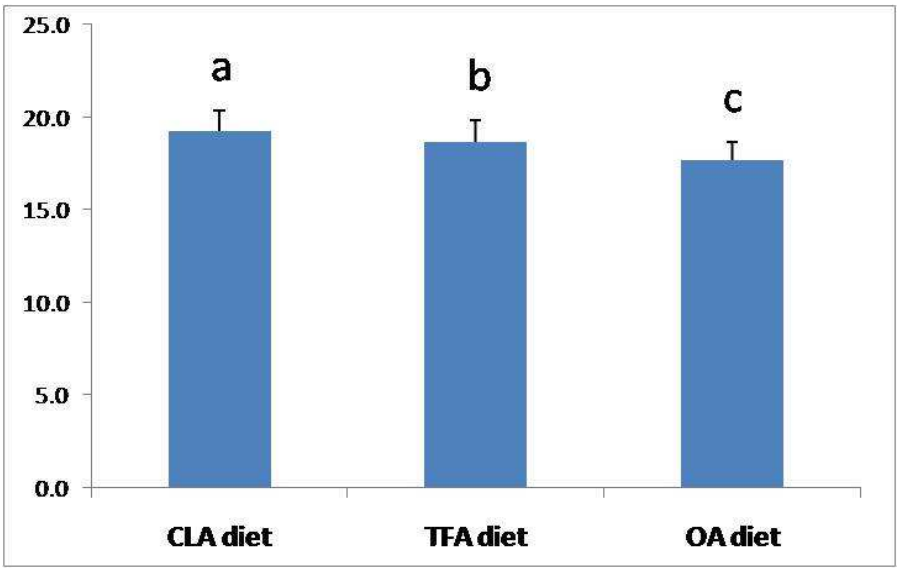


Figure 24. n-3 HUFA score.

Dietary treatments: CLA diet, 80% c9,t11 CLA and 20% t10,c12 CLA; TFA diet, industrial trans fatty acids; OA diet, oleic acid. Statistical significance: p-value <0.05. n-3 HUFA score was calculated by the ratio 20:5n-3+22:6n-3/20:5n-3+22:6n-3+20:4n-6+22:5+20:3n-6+20:3n-9+22:4n-6.

As might be expected, the amount of CLA and its metabolic derivatives are much greater in subjects who took high doses of CLA, compared to the other dietary treatments, TFA and OA. The desaturation-elongation index has been used to estimate the degree of metabolization of CLA into its diene conjugated metabolites. This index is given by the ratio of the metabolic derivatives of CLA (CD 16:2, CD 18:3, CD 20:3, CD 20:4) and CLA itself. CLA diet compared to OA shows less efficient conversion of CLA into its CD metabolites through saturation of enzymatic active sites (Fig.25). Also, the index of peroxisomal β-oxidation, given by the ratio between CD 16:2 and CLA, is significantly lower for the individuals who have taken high doses of CLA, compared to TFA and OA dietary treatments (not differences have been observed between them).

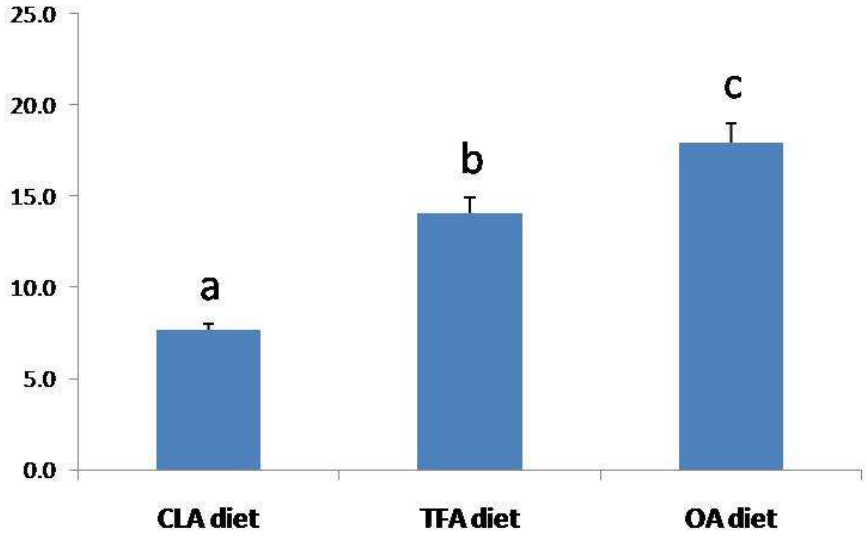


Figure 25. CLA metabolism in human plasma.
*CLA metabolism was calculated as (CD 20:4+CD 20:3+CD 18:3+CD 16:2)*100/CLA. Dietary treatments: CLA diet, 80% c9,t11 CLA and 20% t10,c12 CLA; TFA diet, industrial trans fatty acids; OA diet, oleic acid. Statistical significance: p-value <0.05.*

5.6 Effect of very high dose of dietary CLA on saturated fatty acids (SFAs) metabolism.

With regard to the measurement of serum SFA levels in samples from subjects who took the three different diets, an interesting result comes out from the calculation of the 22:0/20:0 fatty acids ratio, an index of fatty acid elongation. In fact, high dose of dietary CLA significantly decreases the 22:0/20:0 ratio, compared to OA control diet (Fig.26). The fatty acid elongation index in the TFA dietary treatment is intermediate to those calculated for the other two diets.

In terms of total amount of SFAs per ml of plasma, only OA and TFA showed statistical difference, and the values obtained were of 3.47, 3.67, and 3.91 μmoles/ml for OA, CLA, and TFA diets, respectively.

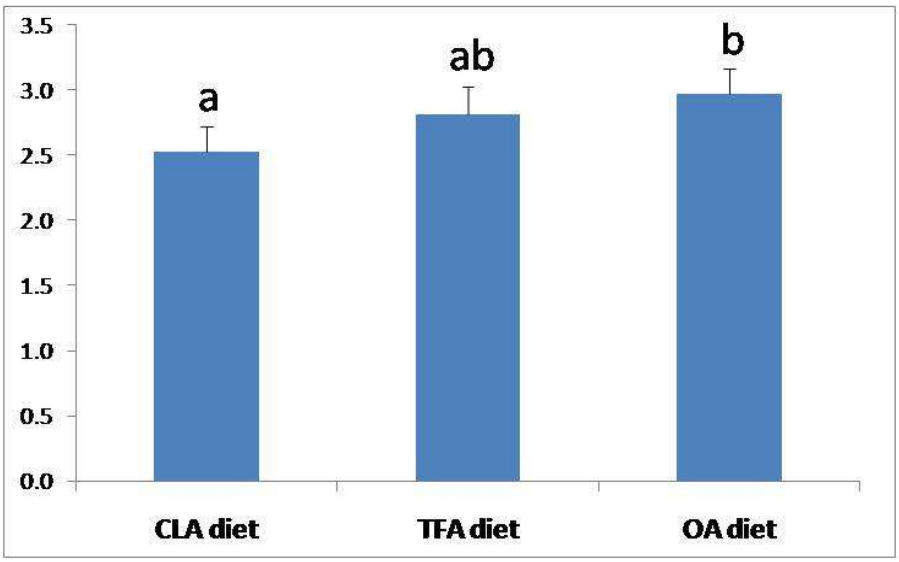


Figure 26. 22:0/20:0 ratio.
Dietary treatments: CLA diet, 80% c9,t11 CLA and 20% t10,c12 CLA; TFA diet, industrial trans fatty acids; OA diet, oleic acid. Statistical significance: p-value <0.05.

5.7 Modulation of retinol concentration by very high dose of dietary CLA in human plasma.

CLA compared to TFA and OA diets doesn't modify significantly total retinol level in human plasma (Fig.27). Only the difference found between TFA and OA dietary treatments was statistically significant.

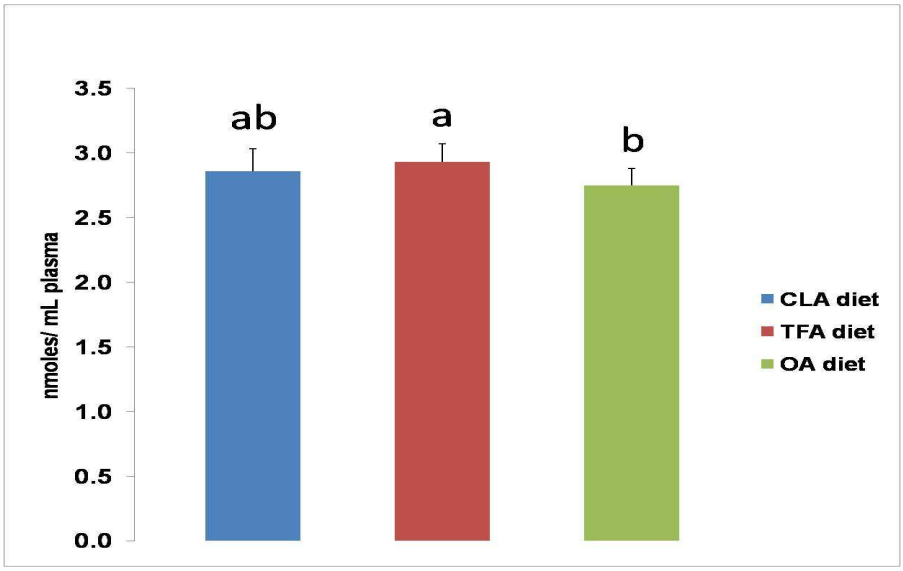
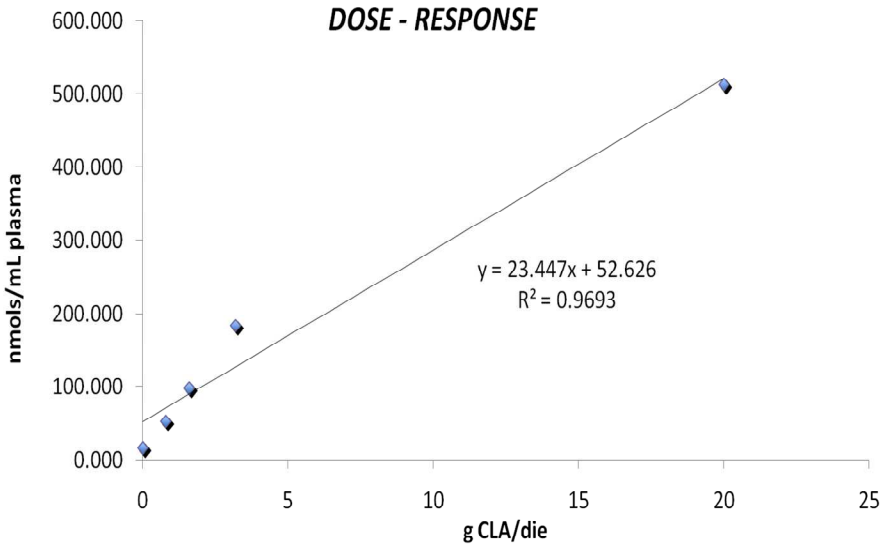


Figure 27. Retinol concentration in human plasma.
Dietary treatments: CLA diet, 80% c9,t11 CLA and 20% t10,c12 CLA; TFA diet, industrial trans fatty acids; OA diet, oleic acid. Statistical significance: p-value <0.05.

5.8 CLA dose-response curve in human plasma.

The result of a dose-response curve for CLA in humans is presented in Fig.28. The curve was made by measuring the amount of CLA in plasma following administration of specific quantities of CLA by a number of individuals (N) belonging to five different groups (0, 1, 2, 3, and 20). Administered CLA was 0, 1, 2, 3, and 20 g per day, respectively, while CLA plasma levels are reported in the y-axis of the Cartesian coordinate system, and expressed as nmols/ml plasma. The relationship of proportionality expressed by the linear curve is valid until the amount of 20 g of CLA taken daily by volunteers.



Descriptive Statistics

Group	Mean	Std Dev	Std Err	N
0	16.322	7.444	2.245	11
1	52.716	22.843	7.614	9
2	98.152	20.364	7.200	8
3	183.434	79.211	26.404	9
20	512.748	170.442	85.221	4

Figure 28. CLA dose-response curve in human plasma.

5.9 Influence on endocannabinoid levels by very high dose of dietary CLA in human plasma.

Results from the randomized cross-over study conducted in humans on the influence of CLA on endocannabinoids and their congeners are presented in Fig.29 and Fig.30, respectively. High dose of CLA, does not cause significant changes in the concentration of AEA, and instead slightly increases 2-AG level (Fig.29). Even not statistically significant, we note that high doses of CLA raised plasma concentrations of OEA and PEA (Fig.30), compared to the OA control diet. No particular influence has been observed about the dietary treatment with industrial trans fatty acids.

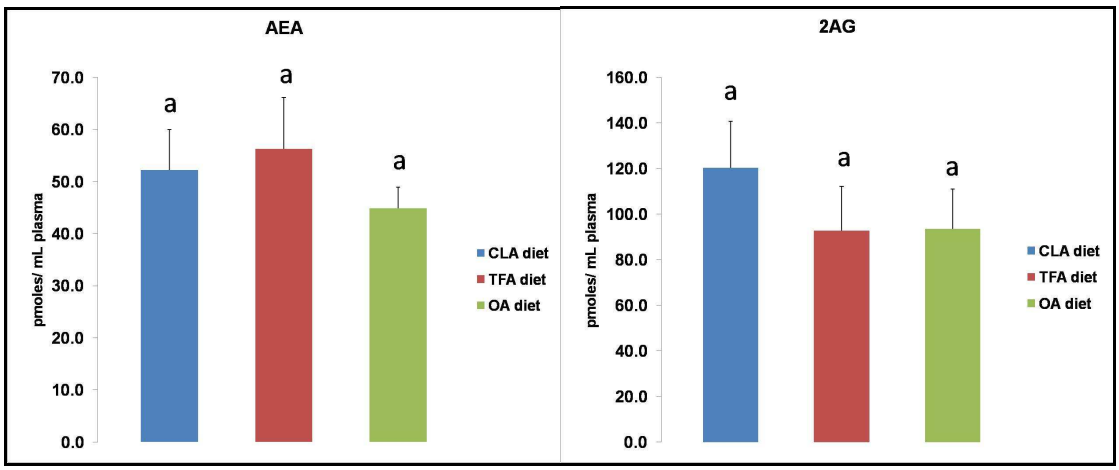


Figure 29. Levels of endocannabinoids in human plasma.
Dietary treatments: CLA diet, 80% c9,t11 CLA and 20% t10,c12 CLA; TFA diet, industrial trans fatty acids; OA diet, oleic acid. Statistical significance: p-value <0.05.

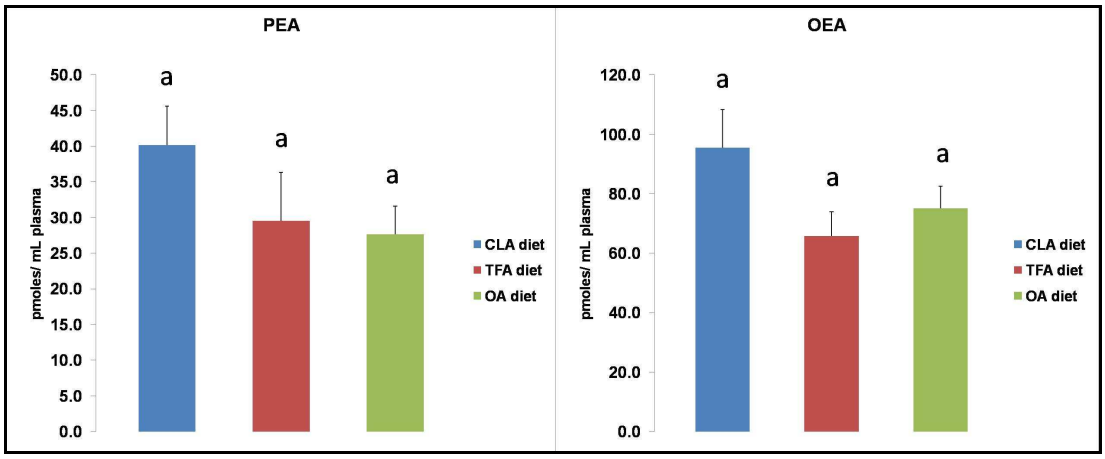


Figure 30. PEA and OEA concentrations in human plasma.

Dietary treatments: CLA diet, 80% c9,t11 CLA and 20% t10,c12 CLA; TFA diet, industrial trans fatty acids; OA diet, oleic acid. Statistical significance: p-value <0.05.

6. Discussion

6.1 Effect of dietary CLA on fatty acid metabolism.

The aim of the present work was to evaluate the effects of dietary CLA on fatty acid metabolism, tissue fatty acid incorporation and endocannabinoid biosynthesis in an experimental model of obesity and in healthy volunteers. The study carried out in liver of obese Zucker rats, compared the effect of an equimolar mix of *c*9,*t*11 and *t*10,*c*12 CLA isomers (about 1%) with two different background diets, one based on fat of vegetal origin, PO, and the other made with a fat content of animal origin, OF. Treatment lasted 3 months.

We found reduced lipid deposition in liver and decrease of body weight with both dietary treatments supplemented with CLA, as shown in previous studies (250), more evident with OF background diet. Retroperitoneal fat also decreased, even though not significantly, only in rats treated with CLA and OF diet. Interestingly, if we calculate the ratio between the two fat compartments retroperitoneal and epididymal, which respectively represent the visceral and the subcutaneous fats, there is a clear effect of OF diet (1.6 *vs*. 1.4). This data, even though not significant, merits further studies to evaluate the capacity of CLA with different background diet to modify adipose tissue compartmentalization probably by influencing insulin sensitivity and/or PPARγ activity. This effect may be mediated by the changes in PUFA metabolism exerted differently by CLA feeding according to background diet. In fact, even though rats fed both diets POCLA and OFCLA showed increased concentration of AA, which confirms previous data in the literature (250), only with OFCLA diet, liver of Zucker rats had elevated levels of DHA which results in a higher n-3 HUFA score. Interestingly, it has been shown that dietary n-3 PUFAs ameliorate insulin resistance (256) and enhanced PPARγ

activity (257). Therefore, the trend to improve adipose tissue compartmentalization in rats fed CLA with OF diets may be due to the improved n-3 HUFA score.

It has been shown that dietary intake of CLA modulates SFAs, particularly PA and SA, as well as MUFA palmitoleic acid and OA (258). In the present study, we found a reduced Δ9 desaturase index in the liver, after feeding CLA in combination with OF diet, and in a lesser extent with PO diet. Interestingly, in rats fed OF diets this index was strongly correlated to n-3 HUFA score, suggesting that n-3 PUFAs may downregulate SREBP-1 activity and thereby decreasing TAG biosynthesis, as already shown in different experimental models (257). In fact, fat deposition in the liver is reduced significantly by dietary CLA, more pronounced with OF diet.

Retinoids (vitamin A and its analogs) play an essential role in mammals for maintaining health and are involved in many physiological functions (259) including vision (260), regulation of cell proliferation and differentiation (261), immune function (262), and growth of bone tissue (263). Mammals obtain vitamin A in the form of retinyl esters and retinol (from animal products), or as provitamin A carotenoids such as β-carotene (from vegetables and fruits). It is demonstrated the capacity of CLA to alter the metabolism of retinoids in rats (264) and mice (265). In particular, it has been shown that chronic intake of CLA in rodents influences the maintenance of serum, liver, and adipose retinoid metabolism (265). *t*10,*c*12 and CLA *c*9,*t*11 CLA isomers enhance uptake of dietary vitamin A in liver and its resecretion into the bloodstream in the form of retinol bound to retinol binding protein (RBP) (265). Experimental data in mice have led to hypothesize that CLA might also increase the absorption of dietary vitamin A (266). Our data confirmed that dietary CLA intake increases retinol level in liver of Zucker rats, but the increase is significant only when CLA was in combination with PO

diet and not with OF diet. It might be possible that the marginal increase by feeding CLA with OF diet is due to a higher secretion of retinol bound to RBP to serum. Unfortunately, we don't have data on circulating retinol and/or RBP levels, however, we may speculate that OF by increasing n-3 PUFA levels, in combination with CLA may induce liver PPARα which increases RBP protein expression (267) and favor the exportation of retinol from liver to the blood. More specific studies are needed to unravel this important issue, which may explain some of the effects of CLA in particular as anti-carcinogenic activity (113-115). In fact, CLA and retinol share biological activities on suppressing mammary gland development (117), inhibit terminal duct and alveolar cell proliferation (117) and block mammary tumorigenesis induced by carcinogens (116,118).

We also evaluated the effects of very high dose of CLA in humans, measuring lipid profile in plasma of healthy subjects who consumed each three distinct diets for three weeks. The daily dietary treatments consisted in supplementation with 20g of CLA isomers (80% *c*9,*t*11 and 20% *t*10,*c*12), or industrial trans fatty acids, or OA. Using the dose-response curve of CLA intake in humans previously published (268), we found that daily intake of 20g CLA fits in the curve showing proportionality up to 20g/d. Since both CLA and LA are likely to share the same enzyme system for chain desaturation and elongation, increasing CLA intake may interfere with the further metabolism of LA. Fatty acid analysis of total lipids showed that CLA decreases the metabolism and the incorporation of LA metabolites. High dose of dietary CLA increases plasma levels of LA, compared to OA diet, while LA metabolites (including GLA, n-6 20:3, and AA) were significantly reduced. Of particular interest is the consistent drop in AA, which is the substrate for the COX and LOX pathways of

eicosanoid biosynthesis. Thus the CLA effect on AA suppression might represent a crucial mechanism for beneficial actions of CLA on human health and protection against some pathological conditions (33). We found that CLA also influence ALA metabolism and increased n-3 HUFA score, but this effect may be a consequence of the action of CLA in decreasing n-6 HUFAs.

Also, we confirmed the effect of CLA in reducing elongation of long chain fatty acids (LCFAs), another beneficial property that can be exploited in addition to a specific mixture of TAGs, the so called Lorenzo's oil, for the treatment of metabolic disease such as adrenoleukodystrophy (ALD) (269). This is a rare metabolic disease, transmitted through the maternal X chromosome to male children (270). This metabolic deficiency prevents the very long chain fatty acids (VLCFAs) to undergo the process of β-oxidation in the peroxisomes, resulting in their accumulation in plasma and tissues. High levels of VLCFAs may impair myelin biosynthesis, with the loss of neuronal function in different brain areas.. However, it has been recently proposed (269) that rather than accumulation of VLCFAs, impaired peroxisomal β-oxidation may affect eicosanoid catabolism and thereby sustaining inflammation, which has been shown to be a key factor in the pathogenesis of ALD and other neurodegenerative diseases, and CLA may enhance this process via activation of PPARα (269). Unlike the results obtained in liver of Zucker rats, our measurements show that high doses of dietary CLA do not influence retinol levels in human plasma. The reason could be due to the different source of vitamin A in humans, mainly as β-carotene, with respect to experimental animals mainly consisting of retinyl esters. Therefore, the hypothesis according to which CLA by enhancing RBP (265) influences retinol absorption and metabolism, may not be valid if the dietary source of vitamin A is β-carotene.

6.2 Impact of dietary CLA on endocannabinoids and their congeners.

Few studies have examined the effects of dietary CLA supplementation on endocannabinoids AEA and 2AG, and their congeners PEA and OEA. Tsuyama *et al*. (247) reported reduced amounts of 2-AG in the cerebral cortex of mice fed with a 3% CLA rich diet, compared with a diet containing the same amount of LA instead CLA. The Authors didn't find any significant variations of AEA, OEA, and PEA levels after 4 weeks of CLA treatment. The reduction of 2 AG levels in the cerebral cortex of the brain were not found in the hypothalamus.

Dietary CLA has been shown to exert peculiar biological effects in a concentration range of 0.5–1% (158). The fatty acid used as control in the paper of Tsuyama *et al*. (247) was LA, the biosynthetic precursor of AA. As a consequence, the CLA diet resulted 3% lower in LA than the control diet, probably influencing both brain AA and 2-AG levels, independently of dietary CLA. The levels of endocannabinoids and their congeners are affected by the dietary content of PUFAs or by their essential biosynthetic precursors, as shown by Artman *et al*. (237).

In the present work the possible effects of CLA on endocannabinoids have been investigated comparing different background diets in Zucker rats and in humans with high dietary CLA intake.

Our experiments conducted on Zucker rats showed that CLA does not significantly alter the concentration of endocannabinoids in liver, however, we detected changes in the concentrations of OEA and PEA. Diets containing 1% CLA increased OEA levels in both POCLA and OFCLA treatments. The increase of OEA doesn't seem due to a change in the fatty acid precursor OA, in fact PO and OF diet are rich in OA, and incorporation of CLA in the diet, doesn't modify the dietary content of OA in the OF

and actually decrease it in the PO diet. The increase of OEA levels in the liver, may be explained by activation of PPARα (271) by CLA, and in the OF diet may further contribute the higher levels of n-3 PUFAs. It has been shown that OEA possesses anti-lipogenic activity in hepatocytes, decreases food intake and body weight gain (201) through direct activation of PPARα. Therefore, this increase may also mediate the reduced daily food intake. On the other hand, PEA increased only with CLA feeding with ovine fat background diet.

The action of CLA on PEA deserves further scrutiny for its anti-inflammatory properties via several molecular mechanisms, including direct action of PPARα (197), or increasing AEA activity at CB1, TRPV1 or PPARγ receptors (200).

In humans, CLA influences fatty acid metabolism of n-3, n-6, and n-9 UFAs, while does not cause significant changes in the profile of endocannabinoids in human plasma compared to OA diet. This finding partly confirmed another study where 0.8g/d of CLA did not modify endocannabinoid profile (248). However, it has been shown that administration of cheese naturally enriched in CLA, ALA and VA in hypercholesterolemic and overweight individuals, decreased the levels of AEA, while CLA alone did not (248). We found however an increase, even though not significant, of OEA and PEA, which may have some implication for OEA anorectic properties making it a possible target in the strategies for the treatment of obesity, and for PEA as potential candidate in the prevention of inflammatory states characteristic of many chronic diseases.

Conclusions

The main conclusions from the present work can be summarized as follows:

- CLA affected body and liver weight irrespective of the background diets in Zucker rats. The decrease of liver weight was mainly due to a reduced deposition of lipids.
- CLA intake perturbed liver fatty acid and retinol metabolism, with increased concentration of AA with both PO or OF diets, and DHA (about 25%) only when CLA was included in the OF diet. CLA intake resulted in a decrease of Δ9 desaturase index in Zucker rat liver. In addition, CLA feeding in combination with PO diet increases retinol level in liver of Zucker rats.
- In human plasma, CLA compared to OA significantly decrease the metabolism and the incorporation of the LA metabolites. CLA is less efficiently desaturated and elongated than LA but more than OA, and competes with LA for metabolism/incorporation of metabolites. CLA reducing n-6 HUFAs, which resulted in an increased n-3 HUFA score. Also, we confirmed that high doses of CLA reduce elongation of LCFAs.
- CLA does not cause significant changes in the profile of endocannabinoids in liver of Zucker rats and human plasma, with only a small decrease in the levels of AEA in the OF diet, compared with the same enriched in 1% CLA in Zucker rat liver. Remarkable for the anorectic properties ascribed to OEA, CLA feeding in Zucker rats enhances its levels in both POCLA and OFCLA diets. Data in humans also show an increase, but not significantly, of OEA and PEA levels in plasma, compared to OA diet.
- Based on the results obtained from this work, we demonstrated that the nutritional effect of CLA is strongly influenced by the background diet, which may also

explain the differences found between experimental animals and humans. Dietary CLA may exert its beneficial actions on human health and protection against some diseases, by improving n-6/n-3 HPUFA balance and sustaining PPARα activity, directly and indirectly through OEA and PEA, especially those characterized by chronic inflammation due to an impaired body fat deposition.

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