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PH.D. THESIS

NUTRACEUTICALS IN ADIPOSE TISSUE DYSFUNCTION: LESSONS FROM *CITRUS AURANTIUM* L. DRY EXTRACT

UNIVERSITÀ DEGLI STUDI DI NAPOLI FEDERICO II - Dottorato Di Ricerca In Medicina Clinica Sperimentale - DIPARTIMENTO DI SCIENZE MEDICHE TRANSLAZIONALI
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Campitelli M, Desiderio A, Cacace G, Nigro C, Prevenzano I, Leone A, de Simone S, Campiglia P, Formisano P, Raciti GA, Beguinot F, Miele C. Citrus Aurantium L. Dry Extracts Ameliorate Adipocyte Differentiation of 3T3-L1 Cells Exposed to TNFα by Down-Regulating miR-155 Expression. Nutrients. 2020 May 28;12(6):1587. doi: 10.3390/nu12061587.

Desiderio A, Longo M, Parrillo L, **Campitelli M**, Cacace G, de Simone S, Spinelli R, Zatterale F, Cabaro S, Dolce P, Formisano P, Milone M, Miele C, Beguinot F, Raciti GA. Epigenetic silencing of the ANKRD26 gene correlates to the pro-inflammatory profile and increased cardio-metabolic risk factors in human obesity. Clin Epigenetics. 2019 Dec 4;11(1):181. doi: 10.1186/s13148-019-0768-0.

Raciti GA*, Fiory F*, **Campitelli M***, Desiderio A, Spinelli R, Longo M, Nigro C, Pepe G, Sommella E, Campiglia P, Formisano P, Beguinot F, Miele C. Citrus Aurantium L. dry extracts promote C/ebpβ expression and improve adipocyte differentiation in 3T3-L1 cells. PLoS One. 2018 Mar 29;13(3):e0193704. doi: 10.1371/journal.pone.0193704.

1. Abstract

Metabolic and/or endocrine dysfunction of the white adipose tissue contributes to the development of metabolic disorders, such as Type 2 Diabetes and other pathologies, like atherosclerosis and cardiovascular disease.
Therefore, the identification of products able to improve adipose tissue function represents a valuable strategy for the prevention and/or treatment of these disorders. The aim of this study was to investigate whether a nutraceutical compound obtained from *Citrus Aurantium* L. fruit juice (*CA*de) might regulate adipocyte differentiation and function *in vitro*, elucidate the underlying molecular mechanism through which *CA*de may exert its function and evaluate whether it might be effective against micro-environmental insults, which affect adipogenesis and adipose tissue function. The following results have been obtained in two separate studies.

Study 1: *CA*de enhances terminal adipocyte differentiation of 3T3-L1 cells raising the expression of *CCAAT/enhancer binding protein beta* (*C/ebpβ*), *peroxisome proliferator activated receptor gamma* (*Pparγ*), *glucose transporter type 4* (*Glut4*) and *fatty acid binding protein 4* (*Fabp4*). *CA*de improves insulin-induced glucose uptake of 3T3-L1 adipocytes, as well. A focused analysis of the phases occurring in the differentiation process from pre-adipocytes into mature adipocytes furthermore revealed that *CA*de promotes the early differentiation stage by anticipating the 3T3-L1 cell cycle entry and progression during mitotic clonal expansion.

Study 2: *CA*de enhanced the early adipogenesis phases by down-regulating *miR-155* expression and increasing both *C/ebpβ* and *cAMP response element-binding protein* (*Creb*) mRNA and protein levels. Also, *CA*de prevented the anti-adipogenic effects of the Tumor necrosis factor alpha (TNFα) by improving adipocyte differentiation and restoring *miR-155* and *C/ebpβ* and *Creb* gene expression respectively, at early time points in cells exposed to TNFα.

In conclusion, in my PhD thesis I revealed the nutraceutical properties of *CA*de on the fat cell functional capacity in terms of enhanced adipocyte differentiation and function *in vitro*. I also demonstrated that the down-regulation of the *miR-155*, which causes the up-regulation of the target genes, *C/ebpβ* and *Creb*, is part of the mechanism through which *CA*de enhances adipocyte differentiation of pre-adipocytes *in vitro*. Furthermore, I provided substantial evidence of the efficacy of *CA*de against micro-environment insults, which are harmful to adipose cell functionality.

These data suggest that the development of nutraceutical products derived from *CA*de may be an effective strategy for treating adipocyte dysfunction and its related disorders.

2. Introduction

2.1 Adipose tissue

Adipose tissue (AT) is a complex organ with heavy effects on physiology and pathophysiology. However, for long time AT has been considered a connective tissue, storing just lipids, and without any link with the metabolism in living organisms [1, 2]. Nevertheless, this notion gradually shifted and AT is now recognized as a tissue critical in the regulation of energy homeostasis and metabolic functions. AT is indeed a metabolically active tissue, consisting of lipid-filled cells, called adipocytes, that make-up 90% of the tissue volume [3]. In addition, to mature adipocytes, fat pads contain several metabolically active and inflammatory cells, including stromal vascular cells such as fibroblasts, leukocytes, macrophages, pericytes, endothelial cells, and pre-adipocytes, which take on a significant part in the integrity of AT [4].

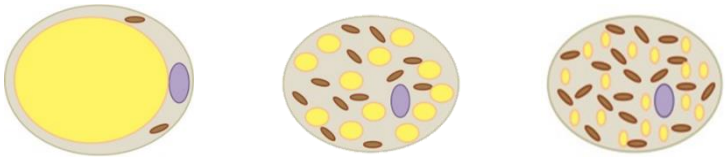
Three forms of AT can be distinguished in mammals: white, brown and beige, also called "brite" (brown in white). They differ from each other for morphology, location and function (Figure 1) [5].

White adipose tissue (WAT), the most abundant AT, is a heterogeneous tissue whose different anatomical locations determine its core functions and metabolic identity [6]. In humans, WAT consists of a central intra-abdominal portion (visceral adipose tissue, VAT), associated with metabolic disease risk, and a subcutaneous peripheral depot (subcutaneous adipose tissue, SAT) associated with protective effects on energy homeostasis [7]. WAT primary function is to preserve energy homeostasis by storing triglycerides and releasing fatty acids for energy synthesis. Nevertheless, WAT also regulates a wide range of functions, including immune and inflammatory regulation, glucose and lipid homeostasis, and control of food intake or metabolism by secreting adipokines [2]. The energy-storing white adipocytes exhibited a variable size (25-200 μm) and contained a single, large and unilocular droplet of lipid surrounded by a cytoplasm layer. Because of the single lipid droplet, in adipocytes the nucleus is situated in the periphery and the thin ring of cytoplasm contains few mitochondria and little but recognizable smooth endoplasmic reticulum (ER) [8]. White adipocytes are also distributed and subdivided into small lobules, which are well vascularized by the surrounding anatomical vessels and innervated by sympathetic and parasympathetic nervous systems [9].

Brown adipose tissue (BAT) is the leading site of mammalian non-shivering thermogenesis and energy expenditure. BAT is a remarkably plastic tissue, and its depots expand when thermogenesis is triggered by hypertrophic and hyperplastic processes [10]. Differently from the initial thoughts, BAT is not only restricted to neonates and young children in humans. Recent studies, using positron emission tomography (PET) imaging techniques, have indeed reported functional BAT also in adults, primarily in the ventral neck, supraclavicular area, mediastinum, paravertebral and suprarenal fat [11]. Furthermore, BAT has been inversely associated with body mass index (BMI) [12]. BAT is different from WAT due to a unique secretory profile and essentially opposite physiological

functions in energy metabolism [13]. The brown adipocytes are characterized by the expression of uncoupled protein 1 (UCP1), which dissipates energy as heat through the oxidative phosphorylation for ATP synthesis, constituting the primary thermogenesis regulator [14]. Energy-expended brown adipocytes are cells with tiny, multilocular lipid droplets. They also contain several highly oxidative mitochondria compared to white adipocytes. The more significant number of mitochondria supports the brownish colour of this tissue [15]. Also, BAT is highly vascularized and internalized by the sympathetic nervous system, which promotes heat dissipation through the blood vessels [16].

Recently, the presence of a third type of AT, the beige AT, has been proposed [17]. In particular, it has been reported that sustained thermogenic activation caused by prolonged cold exposure leads to WAT "browning", with brown adipocyte-like cells appearing in WAT depots. A dynamic hormonal interplay and several environmental factors such as prolonged cold exposure, exercise and environmental enrichment control the browning cycle [18]. The most concentrated beige adipocytes are in the inguinal WAT, a large subcutaneous depot in rodents, while it has not been determined how many beige adipocytes are present in human WAT depots [19]. Some beige cells show distinct gene expression patterns compared to those of white or brown adipocytes, and importantly, the thermogenic profile of beige adipocytes is reversible. Thus, former beige adipocytes can shift their morphology and gene expression profile into a white adipocyte upon warm adaptation [16].



	White Adipose Tissue	Beige Adipose Tissue	Brown Adipose Tissue
Localization	• Subcutaneous • Intra-abdominal • Epicardial • Gonadal	• Emerges in white adipose tissue depots with appropriate stimuli	• Interscapular • Paravertebral • Perirenal • Cervical • Supraclavicular
Morphology	• Spherical	• Spherical	• Elliptical and smaller than white
Cell composition	• Single lipid droplet • Few mitochondria • Flattened peripheral nucleus • Little endoplasmic reticulum	• Unilocular morphology but small lipid droplets after stimulation • Mitochondria appear after stimulation	• Multiple small lipid droplets • Large number of mitochondria • Oval central nucleus
Function	• Storing energy	• Thermogenic potential	• Expending energy and heat production • (non- shivering thermogenesis)
Uncoupling protein	• Undetectable	• Positive after stimulation	• Positive

= mitochondria = nucleus = lipid droplet

Figure 1. Characteristics of white, brown, and beige adipocytes. Modified from *El Hadi H et al. Front Physiol. 2019*

2.2 Cellular Heterogeneity of Adipose Tissue (AT)

As above mentioned, adipocytes are the primary cells of the AT and the main ones responsible for the energy homeostasis control and adipokines release. AT is however formed by other different cell types, including pre-adipocytes, immune cells, nerve cells, and vascular cells. The interplay among all of them contributes to the main functions of each AT depots [2]. Mature adipocytes are replaced during normal AT turnover by newly differentiated and mature adipocyte progenitors or pre-adipocytes. The adipocyte turnover in adult humans is observed around 10% yearly [20]. This number changes under conditions of nutrient challenge that cause WAT expansion, and it is likely also depot-specific [21]. Pre-adipocyte functions in fat depots also include modulation of the extracellular matrix (ECM) remodelling and secretion of endocrine factors [22]. Immune cells also constitute an essential cellular component within the varied AT cellularity. In particular, the first immune cells identified in AT were the adipose tissue-resident macrophages (ATM), which showed a close connection with obesity [23]. Moreover, AT is characterized by

a neuronal net, which controls processes such as lipolysis or thermogenesis. Nerve cells comprising the neuronal mesh stimulate the sympathetic neural system in the WAT parenchymal in cold environments, leading to the release of free fatty acids (FFAs) via lipolysis and activating BAT thermogenesis [24]. Likewise, WAT is linked by sensory nerve afferent networks to other organs, including the brain [25]. Moreover, vascularization of fat depots in adults proceeds through angiogenic expansion of the existing vasculature, as shown to occur during the formation of fat pads from implanted cells [26]. Interestingly, high AT areas rich in adipocyte precursor cells are highly vascularized, suggesting a relationship between mature adipocyte turnover and vascularization. Metabolic processes in adipocytes are also dependent on an adequate nutrient uptake or release, leading, therefore, to a central role of the blood vessels in this process [27].

2.3 Origin of Adipose Tissue (AT)

AT is formed at stereotypical times and locations in various organisms [28]. For each AT depot, gene signature, lipid storage ability, and adipokine profile follow a specific developmental origin [29]. Although white and brown adipocytes originate from the same mesenchymal stem cells, the paraxial mesodermal mesenchymal stem cells express the Myogenic transcription factor 5 (Myf5) and become brown adipocytes or myocytes [30]. On the other hand, the lateral mesodermal mesenchymal stem cells, which do not express Myf5, differentiate into white adipocytes or pericytes associated with the blood vessels.

White adipogenesis is a two-step, closely controlled process involving multiple factors [31]. First, pluripotent stem cells differentiate into unipotent adipocyte precursors or pre-adipocytes. Subsequently, these pre-adipocytes differentiate into mature adipocytes [32].

PPARγ, a nuclear-receptor superfamily member, has been identified as the master regulator of white adipogenesis [33]. Indeed, whereas PPARγ deficiency fails to promote adipogenesis, its overexpression is sufficient to induce adipocyte differentiation in fibroblasts [34]. Cold or 3-adrenergic stimulation are instead causes of a rise in brown adipocytes. WAT-derived progenitor cells have recently been shown to undergo brown adipogenesis *in vitro* in both mice [35, 36] and humans [37], but the underlying molecular mechanism remains unexplained.

Besides PPARγ, other factors or pathways, including pro-adipogenic factors, such as C/EBPs and Krüppel-like factors (KLFs), regulate adipogenesis [38]. In support of this, the bone morphogenetic proteins (BMPs), members of the transforming growth factor (TGFβ) superfamily, also play an essential role in this transformation, due to their regulatory function, by boosting the differentiation of pluripotent stem cells to chondrocytes, osteoblasts or adipocytes [39]. In particular, BMP-2 and BMP-4, through the induction of the small mothers against decapentaplegic (SMAD) pathways, promote the transcription of target genes involved in white adipogenesis [40]. BMPs also mediate their signals via SMAD-independent pathways, including the-p38

mitogen-activated protein kinase (MAPK) and MAP3K7, also known as TAK1**,** signalling pathways [41]. Nonetheless, other transcription factors, such as the anti-adipogenic GATA transcription factor, repress adipogenesis.

Adipogenesis of brown adipocytes is different from that of white adipocytes. The activation of both BMP-7 and PR domain 16 (PRDM16) is necessary to promote BAT adipogenesis. PRDM16 selectively initiates the switch of myoblasts to brown adipocytes by forming a transcriptional complex with C/EBPβ. It also suppresses white adipocyte-specific genes, forming complexes with C-terminal binding proteins, CTBP1 and CTBP2, and upregulates brown fat-specific markers, such as PRDM16, PPAR coactivator 1 (PGC-1) a/b and UCP1 [42].

2.4 White adipocyte adipogenesis

Adipogenesis is the differentiation process during which pre-adipocytes develop into mature fat cells (adipocytes) and accumulate lipid droplets [43] (Figure 2). During adipocyte differentiation, the adipocyte phenotype's acquisition is characterized by chronological changes in numerous genes expression. It is characterized by the expression of early, middle, and late mRNA/protein markers and the accumulation of triglycerides. Genes that are inhibitory to adipogenesis or simply unnecessary for adipose cell activity are repressed in addition to those that are activated. Changes in gene expression during the early and late adipocyte maturation stages have primarily been studied using pre-adipose cell lines. The different adipogenesis phases can be summed up in the following phases: growth arrest, mitotic clonal expansion, early differentiation and late differentiation [44].

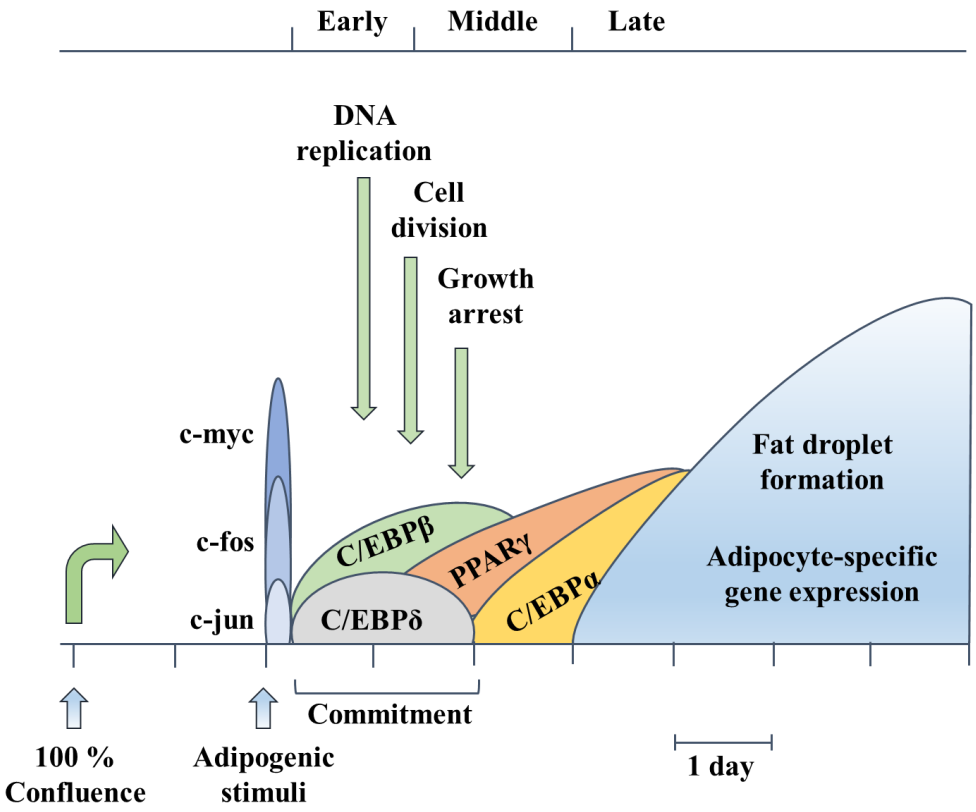


Figure 2. Adipocyte differentiation and gene expression. The major identified events of pre-adipocyte differentiation are presented chronologically. Areas labeled with gene names represent periods of gene expression in the differentiation program. The discrete levels of differentiation (early, middle and late) are likewise offered. Modified from *Ntambi JM et al. J Nutr. 2000*

Growth arrest. This process is required to withdraw committed pre-adipocytes from the cell cycle. Growth arrest can be induced through contact inhibition. However, contact inhibition is not essential for adipogenesis; indeed, pre-adipocytes cultivated at low density in medium without serum have been observed to differentiate in the absence of cell-cell contact, which indicates that growth arrest and lack of confluence are sufficient conditions for the initiation of adipogenesis. Two major transcription factors act cooperatively in growth arrest, PPARγ [45] and C/EBPs [46]. Low expression of these two adipocyte transcription factors is indeed sufficient to mediate growth arrest preceding differentiation.

Mitotic Clonal Expansion (MCE). After the growth arrest, to be determined to proceed to differentiation, committed pre-adipocytes must receive a combination of mitogenic and adipogenic signals [47]. In this phase, pre-adipocytes re-enter into the cell cycle and undergo about two rounds of division. The cell-cycle markers of S-phase entry are synchronously expressed concomitant with the initiation of DNA replication [48]. MCE is a required step in the adipocyte differentiation program. Thus, blocking DNA replication by various means, e.g., by inhibitors of DNA polymerase or by blocking

progression of the cell cycle [49], prevents differentiation. Also, the constitutive over-expression of cell-cycle inhibitor, such as p27, prevents cells from entering the S-phase of the cell cycle and thereby disrupts all subsequent steps of differentiation [48].

Changes in gene and protein expression during adipogenesis. Following growth arrest and clonal expansion, temporal and dynamic changes of gene and protein expression patterns occur during adipogenesis [50]. These modifications are reflected by the presence of early, intermediate and late markers of differentiation and by the accumulation of triglycerides, and are primarily consequences of specific regulation at a transcriptional level, though the regulation also occurs at a post-transcriptional level in some instances. Silencing of unnecessary genes for adipocyte differentiation and function also occurs in this phase [51].

The C/EBPs and PPAR transcription-factor families are mainly involved in the early and intermediate stage of adipogenesis. Three transcription factors from the C/EBPs family, C/EBPα, C/EBPβ, and C/EBPδ, have been related to adipocyte differentiation induction. These transcription factors have C-terminal basic region/leucine zipper (bZIP) domains that confer DNA binding ability and dimerization, either as homodimers or heterodimers, with other family members [52, 53]. The PPARs, a subgroup of nuclear hormone receptors, have been linked to adipocyte differentiation and the most adipose-specific is PPARγ, which is most abundant in AT and adipocyte cell lines and least abundant in other tissues and cell lines [54]. Two isoforms of PPARγ, *i.e.* PPARγ1 and PPARγ2, are generated by alternative splicing and alternate translation initiation [55]. Although PPARγ2 appears to be more adipose-specific than PPARγ1 [56], functional differences between the two isoforms have not been detected. Expression of PPARγ is induced concomitantly with (or perhaps preceding) and before the transcriptional activation of most adipocyte genes [57]. In this phase, morphological changes in cells after differentiation induction occur due to actin cytoskeleton remodelling, enable cells to lose their fibroblastic form and to take a spherical shape. The development of cortical actin structures in adipocytes progresses with filamentous actin accumulation near the cell membrane. The actin-related protein 2/3 (Arp2/3) complex controls actin cortical assembly and nucleation. Yang *et al.* reported that knocking out Arp2/3 severely inhibited adipocyte differentiation and that the cortical actin cytoskeleton was needed for the translocation of the glucose transporter type 4 (GLUT4) into cell membrane [58].

Terminal differentiation. During this last phase, for adipocytes in culture, there is a significant increase in lipogenesis protein levels and enzymes involved in triacylglycerol metabolisms such as ATP citrate lyase (ACLY), malic enzyme, acetyl CoA carboxylase (ACC), stearoyl CoA desaturase (SCD1), glycerol 3-phosphate acyltransferase (GPAT), glycerol 3-phosphate dehydrogenase (GPDH), fatty acid synthase (FAS) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [59]. Furthermore, adipocytes synthesize specific proteins for AT, such as the glucose transporter Glut4, the fatty acid-binding

protein 4 (Fabp4), the fatty acid transporter CD36, and perilipin, which coats the lipid droplets of adipocytes [60]. During terminal differentiation, the leptin level also increases with a much higher level *in vivo* than *in vitro* [61].

2.5 Other factors involved in white adipocyte adipogenesis

As described above, adipogenesis is regulated by several adipocyte-selective mRNAs and transcription factors that regulate the proliferation and differentiation of cells [2, 38, 62]. However, recent research indicates that even miRNAs are implicated in adipocyte differentiation and mature adipocyte function, including lipolysis, glucose-uptake, and insulin sensitivity. In detail, miRNAs are a class of short non-coding RNAs (19-22 nucleotides) involved in the post-transcriptional regulation of genes [63]. miRNAs can bind to complementary target sites in mRNAs, causing translation repression or cleavage, deadenylation and degradation of target mRNA genes [64]. Several studies have shown that miRNAs play an important role in adipogenesis [65]. For example, *miR-422b*, *-148a*, *-107*, *-103*, *-30c*, *-30a-5p*, and *-143* are induced during adipogenesis, but are downregulated in mature adipocytes. Conversely, *miR-222* and *-221* are decreased during adipogenesis, but are upregulated in mature adipocytes [66]. Although ectopic expression of *miR-103* or *miR-143* has little effect on adipocyte growth, they accelerate the rate of adipocyte differentiation, as measured by triglyceride accumulation. Expression of PPARγ2 is doubled by ectopic expression of either *miR-103* or *miR-143*. However, *miR-103* increases the expression level of FABP4 and adiponectin [66]. Each miRNA targets multiple mRNAs, which may coordinate or antagonize each other's functions. Several miRNAs are also dysregulated in other metabolic tissues during obesity-related diseases [67].

Among miRNAs, *miR-155* is of particular interest. *miR-155* is a typical multifunctional miRNA involved in numerous physiological and pathological processes such as haematopoietic lineage differentiation, immunity, inflammation, cancer, and cardiovascular diseases [68 , 69]. Furthermore, has also been described that *miR-155* is involved in the regulation of adipocyte differentiation [70]. Liu *et al.* indeed reported that *miR-155* expression is down-regulated at the early stage of 3T3-L1 adipogenesis and that its overexpression suppresses adipocyte differentiation by directly targeting the 3'-untranslated regions (3'-UTRs) of *C/ebpb* and *Creb* mRNAs [70]. Also, it has been identified as the most responsive miRNA to inflammatory stimuli [70, 71], and its expression is higher in the AT of obese compared to normal-weight subjects and correlates with both TNFα expression and BMI [71]. Thus, the modulation of the *miR-155* expression in adipocyte precursor cells may offer new ways to enhance AT homeostasis during obesity.

2.6 Adipose Tissue (AT) Dysfunction

The development of AT dysfunctions is, at least in part, due to the chronic availability of excess nutrients, that promotes the expansion of fat depots, and to altered secretion of bioactive mediators from AT. However, it largely remains unknown. Recently, it has been proposed a model in which genetic, environmental, and behavioural factors are involved both in decreased physical activity and excess energy intake, and in physiologic versus pathologic fat accumulation [72]. Based on this model, the physiologic response to excess energy intake and/or less energy expenditure increases fat cell number known as hyperplasia (recruitment of new adipocytes from a reservoir of resident progenitor cells of adipose tissue). These fat cells function normally and are insulin sensitive. On the contrary, the pathophysiologic response to excess energy intake and/or less energy expenditure cause adipocyte hypertrophy, which refers to expanding single adipocyte size [73].

AT ability to expand is essential for accommodating energy availability changes, but this capacity is not unlimited and varies between individuals [74]. Indeed, adipocytes can grow up to ~20 fold in diameter and several thousand-fold in volume [75]. However, when adipocytes become too large, several signals will be induced. For example, when vascularisation is inadequate for the expanded AT, hypoxia can occur. In addition, endoplasmatic reticulum (ER) stress [76], either induced by hypoxia or nutrient excess, leads to an unfolded protein response (UPR) [77].

Dysfunctional adipocytes produce more inflammatory adipokines and cytokines than functional adipocytes. Also, adipocyte hypertrophy attracts immune cells (among which macrophages), the physiological role of infiltrating macrophages is probably debris clearing due to premature adipocyte apoptosis [78]. Eventually, a positive feedback cycle is formed in which infiltrating macrophages recruit several immune cells, and a state of chronic inflammation is induced. Some of the adipokines and cytokines secreted interfere with insulin signalling, others with adipocyte differentiation. For example, TNFα and Interleukin-6 (IL-6) impair adipocyte differentiation, reduce lipid accumulation, and increase adipocyte lipolysis [79]. The latter is on top of the limited capacity of hypertrophic adipocytes to absorb and release FFAs. This induces a redirection of lipids in non-adipose organs, including the liver, skeletal muscle, heart and pancreas, forming ectopic fat deposition (Figure 3) [80, 81]. If lipid supply exceeds oxidative capacity in these tissues, intracellular lipid accumulation occurs, and organ function might be impaired. The consequences of ectopic fat accumulation depend on the specific organ. However, the mechanisms leading to organ function disruption are quite similar at the cellular level since lipid accumulation is associated with decreased insulin sensitivity [82]. Thus, antagonizing deleterious effects associated with the unhealthy expansion of the WAT is necessary to enhance AT homeostasis and may represent a source of new therapeutic agents.

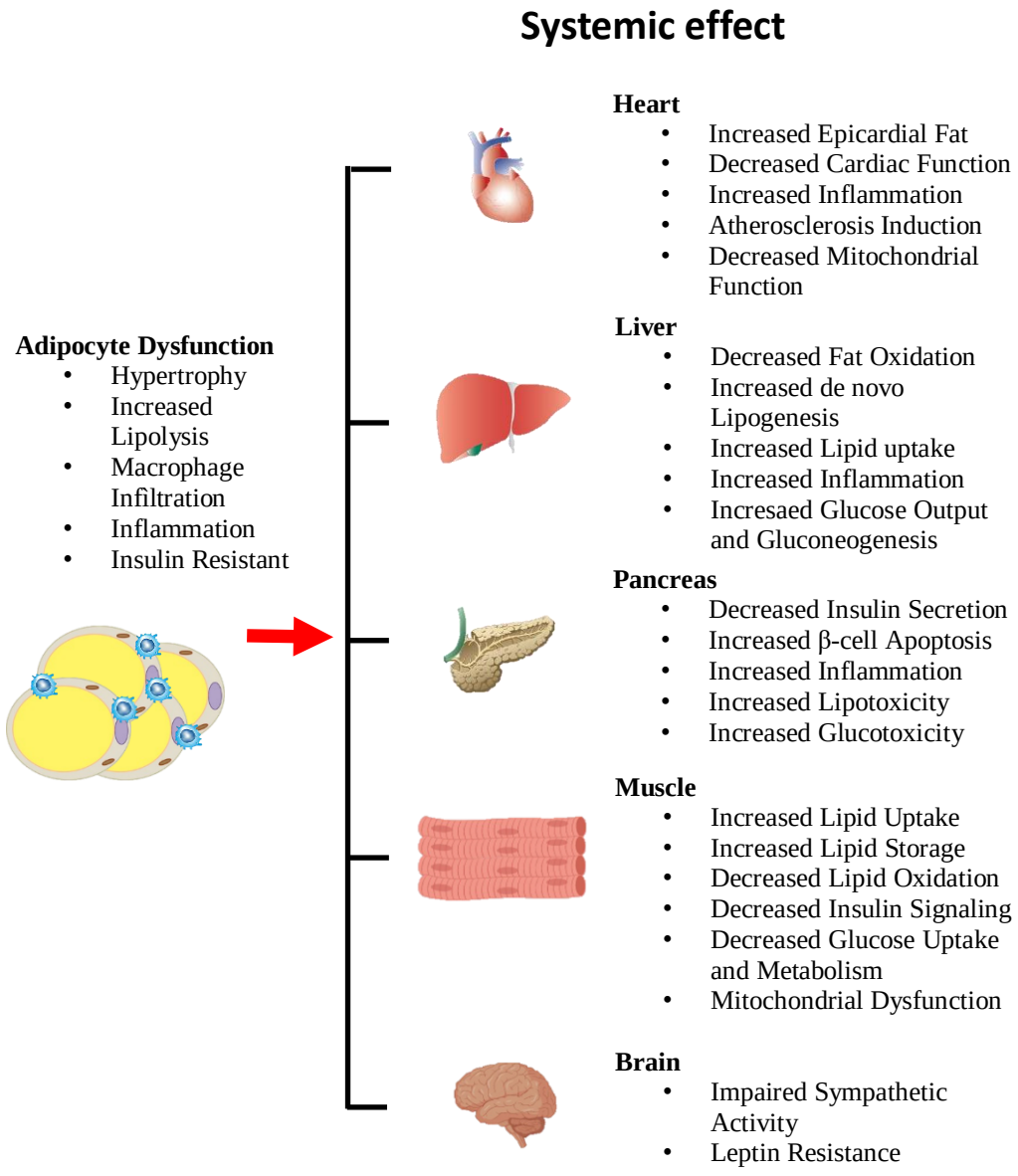


Figure 3. Adipose tissue dysfunction and systemic effects on peripheral tissues. Modified from *Jack BU et al, Biomed Pharmacother. 2019.*

2.7 Interventions affecting adipose tissue (AT) function

Increasing physical activity and weight reduction are two significant lifestyle changes to reduce insulin resistance and visceral obesity (mainly by decreasing the size of existing adipocytes), and they are central in modifying AT dysfunction. Several studies indicated that after 12 weeks of restricted caloric intake and increased exercise, TNFα, leptin and IL-6 levels decreased while anti-inflammatory cytokines (adiponectin and IL-10) were significantly increased in obese subjects with metabolic risk factors [83]. Even before weight reduction, insulin sensitivity improved, and adiponectin plasma levels increased, suggesting that adipocyte function has ameliorated [84, 85]. However, changes

in lifestyle are difficult to achieve and maintain in clinical practice, but are required for a constant improvement of a patient's metabolic profile [86].

Statins have anti-inflammatory properties, which may also influence adipocyte function [87]. Nevertheless, short-term treatment with simvastatin and ezetimibe alone or in combination did not result in changes in adiponectin, leptin, and resistin levels in healthy subjects and obese patients with metabolic syndrome [88, 89]. Moreover, lower (instead of higher) adiponectin levels have been observed after simvastatin treatment in patients with vascular diseases [90]. In randomized statin trials, a lower incidence of Type 2 Diabetes (T2D) has been also observed in patients on statin compared with the placebo group [91]. The underlying mechanism of this effects is unclear since insulin sensitivity and adiponectin levels did not improve after nine weeks of simvastatin treatment in subjects with metabolic syndrome [92].

Fibrates, which act as agonists of PPARα, stimulate cellular fatty acid uptake and β-oxidation pathways predominantly in the liver, kidney, heart, and skeletal muscle. Recent research shows that PPARα may also be involved in adipocyte gene regulation specific for fatty acid metabolism [93]. A decrease in very low-density lipoprotein production is observed in combination with a reduction in fatty acid and triglyceride synthesis [94].

Thiazolidinediones (TZDs) are ligands of the nuclear transcription factor PPARγ and have been shown to regulate the expression of numerous genes affecting glycaemic control, lipid metabolism, and inflammation in pre-adipocytes. TZDs improve insulin sensitivity in T2D patients and are proposed to have favourable effects on other metabolic alterations seen in metabolic syndrome and adipocyte dysfunction. Although currently available, TZDs (rosiglitazone and pioglitazone) have similar effects on insulin action, and they differ in their effects in the occurrence of vascular events and carotid artery intima-media thickening in patients with T2D [95].

In patients with abdominal obesity, there is evidence that the endocannabinoid system is hyperactive, leading to increased food intake and weight gain, offering a new therapeutic option [96]. Rimonabant is an antagonist of the endocannabinoid-1 receptor (CB1), a receptor present on cells of the central nervous system, the liver, and adipocytes. Treatment with rimonabant leads to a reduction in body weight and waist circumference, and a 15% reduction in plasma triglycerides and a 25% increase in High-density lipoprotein cholesterol levels have been reported [97]. As a marker of AT dysfunction, Adiponectin increased 58% when compared with that at the start of the treatment. This increase may not only be explained by changes in body weight, leaving the suggestion that direct CB1 receptor blockade in adipocytes results in changes in adipocytes function [97].

2.8 Nutraceuticals and effects on adipose tissue (AT) function

In the last decades, scientific research has also focused on producing beneficial compounds that can play an essential role in future therapeutic development (dietary supplements, functional food, nutraceuticals and conventional drugs) from medicinal and food plants for human healthy [98]. It is important to emphasise that the terms "nutraceuticals", "functional foods" and "dietary supplements" are still vague and are often confused. These concepts deserve to be distinguished. "Functional food" has been coined to identify foods or nutrients whose intake contributes to essential biochemical changes within the body that are different from those associated with their function as nutrients [99]. "Dietary supplements", instead, are products intended to supplement the diet, contains one or more dietary ingredients (including vitamins, minerals, herbs or other botanicals, amino acids, and other substances) with a more defined health role to supplement the total dietary intake of these ingredients [100]. Ultimately, "nutraceuticals" focus more on disease prevention and/or treatment. Stephen De Felice coined this term from the words "nutrition" and "pharmaceutical" in 1989. According to De Felice, nutraceuticals can be described as "a food (or part of a food) that provides medical or health benefits, including the prevention and/or treatment of a disease" [101].

Nutraceuticals provide various advantages and are believed to have physiological benefit or protection from CVD, obesity, diabetes, cancer, stress, Parkinson and Alzheimer diseases, pulmonary and respiratory disorders [102]. For example, in recent years, scientific results have shown that nutraceuticals composed of minerals, dietary fibres, and omega-3 polyunsaturated fatty acids (PUFAs) are recommended for the prevention and treatment of CVD [103]. Recent evidence from human and animal studies strengthens also their use as complementary strategy in support to the pharmacological treatment of several diseases, including T2D and obesity [104]. Some of them are, indeed, being used as good co-adjuvants along with the balanced diets and with the currently used drugs for the management of the blood glycaemia and for the prevention and treatment of T2D [105, 106]. Oral administration of the high molecular weight *Gymnema sylvestre* R. Br. leaf extract, Om Santal Adivasi, increases circulating serum insulin and reduces both fasting and post-prandial blood glucose in humans [107]. Also, oral assumption of hydroalcoholic extracts of *Trigonella foenum-graecum* L. seeds improves glycemic control and decreases insulin resistance in T2D [108]. Further, it has been demonstrated that nutraceuticals composed of capsaicin-conjugated linoleic acid, *Momordica charantia*, and *Psyllium* fibre have potential anti-obesity properties [102]. Caffeine and ephedrine have been proposed as treatments for weight loss and weight maintenance for a long time, as well [109]. Caffeine indeed increases energy expenditure by inhibiting the phosphodiesterase (PDE)-induced degradation of intracellular cyclic adenosine monophosphate (cAMP) and decreases energy intake by reducing food intake [110].

Nutraceuticals are also involved in regulating AT functions. For example, supplementation of dietary polyphenols, epigallocatechin-3-gallate and

resveratrol (RES) for 12 weeks induces suppression of gene sets related to adipocyte turnover (adipogenesis and apoptosis/autophagy), inflammation and the immune system in AT in overweight and obese men and women [111]. Moreover, curcumin treatment increased Sirtuin 1 mediated adiponectin transcription and reduced ER stress in adipocytes by activating forkhead transcription factor O1 (Foxo1) and enhancing Foxo1 and C/EBPα interaction [112, 113]. Also, curcumin treatment blocked inflammatory response in the epididymal AT through inhibition of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) expression and c-Jun N-terminal kinases signaling pathway activation in diet induced obese mice [114]. Nobiletin, a polymethoxylated flavone, present in some citrus fruits such as *Citrus Depressa Hayata* and *Citrus Sinensis* L., has shown to regulate AT function in diet induced obese mice, as well [115]. Nobiletin treatment indeed increased energy expenditure related genes in WAT and improved insulin signalling through the inhibition of the inflammatory cytokines TNFα and IκB kinase/ NFκB activation. Furthermore, in these mice it also reduced significantly the body and total WAT weight, plasma TG and increased plasma adiponectin levels. Finally, quercetin, a flavonoid present in citrus fruits, has been reported to improve obesity-associated inflammation acting on multiple targets [116]. Indeed, quercetin supplementation significantly suppressed the pro-inflammatory cytokines TNFα, IL-6, and monocyte chemoattractant protein-1 (MCP-1) levels in epididymal WAT and sera, suggesting its ability to reduce obesity-induced AT and systemic inflammation [117].

2.9 *Citrus Aurantium* Linnaeus

Citrus Aurantium L., also known as "bitter orange", is a common plant present in the Mediterranean basin, whose health properties have been described since the time of the ancient Greeks and Romans [118]. *Citrus Aurantium* L., indeed, contains several bioactive compounds, including alkaloids, flavonoids, and polyphenols [119]. In particular, the predominant components of *Citrus Aurantium* L. are flavonoids, including flavones, flavanones, flavonols, and anthocyanins (present only in blood oranges) [120, 121].

Several studies have been performed investigating the effects of different *Citrus Aurantium* L. parts, including flowers, fruits and essential oils, demonstrating that compounds in *Citrus Aurantium* L. have beneficial properties [122, 123]. For example, extracts obtained from the immature fruits of *Citrus Aurantium* L. are commonly used in weight management due to effects on thermogenesis regulation [124]. Also, specific bioactive components present in *Citrus Aurantium* L., such as the alkaloid *p*-synephrine and its metabolite *p*-octopamine, exhibit sympathomimetic actions on the α- and β-adrenergic receptors thus modulating lipolysis of adipocytes [125]. The use of *Citrus Aurantium* L. extract and of its constituent *p*-synephrine ($C_9H_{13}NO_2$) for the treatment of obesity in 360 subjects was also reviewed. *Citrus Aurantium* L. extracts, alone or combined with other ingredients, enhance energy expenditure and metabolic rate and promote weight loss when given for 6-12 weeks in these

subjects without causing any significant adverse effects [119]. However, to date, due to substantial qualitative and quantitative differences in the composition among *Citrus Aurantium* L. extract preparations, several studies conducted both at pre-clinical and clinical level have reported conflicting findings on the effective role of *Citrus Aurantium* L. extracts as thermogenic agents [119, 124].

Several *in vitro* and *in vivo* studies have supplied evidence to support the protective effect of *Citrus Aurantium* L flavonoids against AT inflammation associated with obesity [126]. Naringenin and hesperetin have shown to inhibit TNFα-stimulated FFAs secretion both in 3T3-L1 adipocytes and in mouse epididymal primary adipocytes, through the inhibition of NF-κB and ERK pathways [127]. In addition, naringenin treatment for 14 days, suppresses macrophage infiltration into epididymal AT in diet-induced obese mice. In these mice, this effect was mediated by the suppression of MCP-1 expression, through inhibition of JNK phosphorylation in AT [128].

Based on the above, it is thus plausible that *Citrus Aurantium* L. extracts may exert beneficial properties on adipose function. However, up today the potential nutraceutical effects of *Citrus Aurantium* L. on metabolic health targeting of AT has not been clearly addressed [129].

3. Aim of the study

Metabolic and/or endocrine dysfunction of the WAT contributes to the development of metabolic disorders, such as T2D and other pathologies, like atherosclerosis and CVD. Therefore, the identification of products able to improve AT function represents a valuable strategy for the prevention and/or treatment of WAT dysfunction.

Nutraceuticals, which are nutritional products derived from plants and food sources with health or medical benefits, might be used as a complementary strategy to support the pharmacological treatment of these disorders. E.g., some of them are being used as good co-adjuvants along with balanced diets and with the currently used drugs for weight loss or management of blood glycaemia.

In my PhD thesis, disclosed the nutraceutical properties of preparation of *Citrus Aurantium* L. dry extracts (*CA*de) obtained from its fruit juice on adipocyte function, by *i.* investigating the potential effects of this compound on the regulation of 3T3-L1 cells adipocyte differentiation and function *in vitro*, and by *ii.* exploring the molecular mechanisms at the basis of the compound action. I also investigated whether *CA*de might be effective against micro-environmental insults, which affect adipogenesis and adipose tissue function.

4. Materials and Methods

4.1 *Citrus Aurantium* L. Dry Extract (*CA*de)

Dry extracts from *Citrus Aurantium* L. (*CA*de) fruit juice were obtained as previously described [130]. Briefly, *Citrus Aurantium* L. fruit juice was provided by the company "Agrumaria Corleone" (Palermo, Italy), which used fruits harvested from *Citrus Aurantium* L. plants cultivated in Eastern Sicily, Italy. To remove fibers, 100 mL of hand-squeezed juice were centrifuged at 12000 rpm for 15 min at 25°C, and then lyophilized for 24 h by setting the condenser temperature at -52°C and the vacuum value at 0.100 mBar. The powder was extracted with MeOH, and the procedure was repeated three times for the complete recovery of polyphenolic compounds. The extract was filtered through a 0.45 μm nylon membrane (Merck Millipore, Billerica, MA, USA), evaporated under vacuum to dryness, and stored at 4°C until used. The lyophilized dried extracts were then re-hydrated with distilled H_2O to a final concentration of 10 mg/mL. Quantitative analysis of compounds in *CA*de was also performed through advanced processes of extraction and refining, details are reported in Table 1. Treatments with *CA*de were made to concentrations and at the time indicated in the following paragraphs of the section "Materials and Methods".

Compound	**Extract flavonoids**
Narirutin	67.51 ± 1.98 mg/g
Vicenin-2	55.56 ± 2.47 mg/g
Hesperidin	39.05 ± 1.94 mg/g
Lucenin-2 4'-methyl ether	23.58 ± 2.55 mg/g
Neohesperidin	10.45 ± 2.32 mg/g
Nobiletin	19.48 ± 0.88 mg/g
Sinensetin	8.57 ± 0.86 mg/g
Heptamethoxyflavone	3.96 ± 0.89 mg/g
Tetramethyl-*o*-isoscutellarein	3.77 ± 0.47 mg/g
Isosinensetin	3.53 ± 1.14 mg/g
Eriocitrin	3.06 ± 0.21 mg/g
Neodiosmin	3.01 ± 0.41 mg/g
Tangeretin	2.91 ± 0.49 mg/g
Isoquercitrin	2.86 ± 0.37 mg/g
Hexamethoxyflavone *isomer*	1.86 ± 0.54 mg/g
Didymin	1.75 ± 0.19 mg/g
Hexamethoxyflavone	1.25 ± 0.57 mg/g

Table 1. Quantitative flavonoid profile of CAde. The amount of the compounds is expressed as milligram per gram of extract. Data are reported as mean ± relative standard deviation (RSD%) values of at least three independent experiments.

4.2 Cell culture, adipocyte differentiation and treatments

Murine embryonic fibroblast 3T3-L1 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in expansion medium containing Dulbecco's modified Eagle's medium (DMEM, 4.5 g/L glucose, Sigma Aldrich, St Louis, MO, USA) supplemented with 10% fetal calf serum (FCS, Thermo Fisher Scientific, Waltham, MA, USA), 100U/mL penicillin (Lonza Walkersville, MD, USA), and 100mg/mL streptomycin (Lonza Walkersville, MD, USA), at 37°C in a humidified atmosphere of 5% CO_2 [131].

3T3-L1 cells were differentiated as previously described [132]. In details, to induce differentiation, 3T3-L1 pre-adipocytes were seeded in a 6-well culture plate at a density of 8.0 x 10^4 cells per well and grown in expansion medium. Two days after 100% confluence (Day 0, D0), the pre-adipocyte expansion medium (DMEM 10% FCS) was removed. Adipocyte differentiation was initiated by culturing growth-arrested 3T3-L1 pre-adipocytes for 48 h with the differentiation medium (AS) containing DMEM (4.5 g/L glucose) 10% fetal bovine serum (FBS, Thermo Fisher Scientific) supplemented with 3-isobutyl-1-methylxanthine (0.5 mM, Sigma Aldrich, St Louis, MO, USA), dexamethasone (1 µM, Sigma Aldrich) and insulin (1 µg/mL, Sigma-Aldrich, St. Louis, MO, USA). Starting from day 2 (D2), cells were cultured in an adipocyte maintenance medium containing DMEM (4.5 g/L glucose) 10% FBS supplemented with 1 µg/mL insulin, and the medium was changed every 48 h until day 8 (D8). *CA*de (100 µg/mL) was added in every medium replacement except on experiments where it was added only from D0 to D2 or from D2 to D8.

For the experiments with specific flavonoids metabolites, 3T3-L1 pre-adipocytes were seeded in a 6-well culture plate at a density of 8.0 x 10^4 cells per well. Two days after confluence, growth-arrested 3T3-L1 pre-adipocytes were cultured in AS for 2, 4 and 8 h in the presence of 6.7 µg/mL narirutin or 3.9µg/mL hesperidin or 5.5 µg/mL vicenin-2. Compounds were from Extrasynthese (Genay, France).

For long-term experiments with TNFα, cells were differentiated, as above described, in the presence of *CA*de (100 µg/mL), or TNFα (1 ng/mL; EMD Millipore, Burlington, MA) or both. *CA*de was added at D0 and in every medium replacement. TNFα was added only at day 0. For short-term experiments with TNFα, 3T3-L1 cells at D0 were stimulated for 15, 30, 60, 120, and 240 min in AS or in AS supplemented with *CA*de (100 µg/mL), or TNFα (1 ng/mL) or both.

4.3 Sulforhodamine B (SRB) assay

SRB assay is a colourimetric assay frequently used in cytotoxicity screening [133]. It relies on the ability of SRB, a bright-pink aminoxanthene dye with two sulfonic groups, to stoichiometrically bind to protein components of cells [134]. SRB assay was performed as described [135]. The method was optimized for the toxicity screening of *CA*de in a 96-well plate. After 24 h of treatment, cells were fixed with 50% trichloroacetic acid at 4°C (100 µl/well, final concentration 10%) for 1 h, and then stained with 0.4% SRB (Sigma-Aldrich) dissolved in 1% acetic

acid (50 µl/well) for 30 min. Excess of dye was removed by washing with l% (vol/vol) acetic acid. Plates were air-dried, and protein-bound dye was solubilized in 100 mM Tris base solution. Optical density was determined at 490 nm using a microplate reader.

4.4 Image Acquisition, Oil-Red O Staining, and Triglyceride (TG) quantification assay

Images of 3T3-L1 cells at day eight post-induction were taken using an Olympus microscope system (Olympus, Center Valley, PA, USA). Microphotographs are shown (×10 magnifications); scale bars, 30µm. Oil-Red O staining was performed as described in [136]. Briefly, 3T3-L1 cells at day eight post-induction were fixed with 10% formaldehyde for 5 min at room temperature, washed with PBS 1X and then dried thoroughly. Fixed cells were stained for 30 min at room temperature with Oil-Red O staining solution (Sigma-Aldrich), consisting of 0.5g Oil Red O dissolved in 60% isopropyl alcohol solution. Excess dye was washed with 60% isopropyl alcohol. The Oil Red O dye was then extracted from the stained cells using 100% isopropyl alcohol (1mL). Intracellular lipid content was then quantified by measuring the optical density of the dissolved Oil-Red O staining at 490nm by a spectrophotometer.

Cellular TG concentration was determined according to [137]. Briefly, 3T3-L1 cells at day eight post-induction were lysed into PBS 1X by sonication. TG content per sample was measured using a TG assay kit from Sigma-Aldrich. Per sample DNA was also isolated using the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Hilden, Germany), and DNA concentration was quantified and used to normalize data. The values were expressed as µg Triglyceride (TG)·µg Deoxyribonucleic acid $(DNA)^{-1}$.

4.5 Total RNA and miRNA Purification, Reverse Transcription, and qPCR

Total RNA, including miRNA, was isolated from 3T3-L1 cells using AllPrep DNA/RNA/miRNA Universal Kit (Qiagen), according to the manufacturer's instructions. Total RNA concentration was quantified with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Gene expression was determined as described [138]. Total RNA (1000 ng) was reverse-transcribed using the SuperScript III Reverse Transcriptase (Qiagen). Gene expression was analyzed by quantitative real-time PCR using the iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and quantified as relative expression units. Cyclophilin was used as a housekeeping gene. Primer sequences used are the following: *C/ebpβ*: F, 5'-cgcccgccgcctttagac-3'; R, 5'-cgctcgtgctcgccaatgg-3'; *Creb*: F, 5'-agctgccactcagccgggta-3'; R, 5'-tggtgctcgtgggtgctgtg-3'; *Pparγ*: F, 5'-ggaagccctttggtgactttatgg-3'; R, 5'-gcagcaggttgtcttggatgtc-3'; *Glut4*: F, 5'-cctggaatgctgtctctgg-3'; R, 5'-tggctctgtcttaatgttgatg-3'; *Fabp4*: F, 5'-aatcaccgcagacgacag-3'; R, 5'-acgcctttcataacacattcc-3'; *Cyclophilin*: F, 5'-gcaagcatgtggtctttggg-3'; R, 5'-gggtaaaatgcccgcaagtc-3'.

miRNA expression was determined as described in [139]. Total RNA (500 ng) was reverse-transcribed using the miScript II RT Kit (Qiagen). miRNA expression was analyzed by quantitative real time-PCR using the miScript SYBR Green PCR Kit (Qiagen) and quantified as relative expression units. U6 small nuclear RNA (snRNA) was used as housekeeping small RNA. Primer sequences were from Qiagen: Mm_miR-155_1 miScript Primer Assay, MS00001701; Mm_miR-130a_1 miScript Primer Assay MS00001547; Mm_miR-375_2 miScript Primer Assay MS00032774; RNU6B_13 miScript Primer Assay, MS00014000.

4.6 2-Deoxy glucose (2-DG) uptake

2-DG uptake was performed as previously described [140]. In details, 3T3-L1 cells differentiated for eight days in mature adipocytes treated or not with *CA*de (100 μg/mL) from D0 to D8, or from D0 to D2 or from D2 to D8, were starved for 24 h in DMEM supplemented with 0.1% BSA. Adipocytes were then washed twice with KRH buffer (HEPES 50 mM, NaCl 137 mM, KCl 4.7 mM, MgSO4 1.3 mM, CaCl2 1.85 mM, BSA 0.1%) and stimulated or not with insulin (100 nmol/L) for 30 min. Glucose uptake was determined by the addition of 2-DG mix containing 1 mM of cold 2-DG and 0.25 μCi of 2-[14C]-DG for 5 min. Cells were then washed with KRH buffer and lysed with NaOH 0.05 M. The 2-DG uptake was quantified by liquid scintillation counting and normalized for protein content by Bradford protein assay.

4.7 Cell growth and flow cytometry analysis

Cell growth analysis was performed as previously described [141]. Briefly, the mouse embryonic 3T3-L1 pre-adipocytes and the mouse embryonic fibroblasts NIH-3T3 cells were seeded in 6-well culture plates at a density of 8.0 x 10^4 cells per well. The day after (day 0), 3T3-L1 pre-adipocytes were cultured in complete medium for the following 72 h in the presence or absence of *CA*de (100 μg/mL). Cell growth was analysed by counting cells at day 0 and every 24 h for three days using the TC10™ Automated Cell Counter (Bio-Rad Laboratories).

Flow cytometry analysis was performed as previously described [47]. 3T3-L1 and NIH-3T3 cells were seeded in a 6-well culture plate at a density of 8.0 x 10^4 cells per well. After two days post-confluence, cells were incubated in complete medium for the following 16 h in the presence or absence of *CA*de (100 μg/mL) or DMEM differentiation medium in the presence or absence of *CA*de (100 μg/mL) for 12, 14 and 16 h. The 3T3-L1 cells were harvested and fixed with ethanol 70% a 4°C overnight. Fixed 3T3-L1 cells were stained with propidium iodide 50 μg/mL (PI; Sigma Aldrich) and incubated with RNase 10 μg/mL (Sigma Aldrich) for 30 min at 37°C in the dark. Fluorescence emitted from cells was measured by flow cytometry (BD FACSAria III, Becton, Dickinson and Company, Franklin Lakes, NJ) using BD FACSDiva software. A total of 10.0 x 10^3 cells in each sample were analyzed.

4.8 Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation (ChIP) assays are used to evaluate transcription factor-DNA interactions and are critical for advancing gene expression regulation and epigenetic modifications studies. ChIP can detect and relatively quantify specific protein-DNA and protein-protein interactions at a single locus or multiple loci. ChIP involves chemically cross-linking proteins to DNA sequences, which is followed by immunoprecipitation of the cross-linked complexes, and analysis of the resultant DNA by endpoint.

ChIP assays were performed as described [135]. For the ChIP assay, sonicated chromatin was immune-precipitated with the anti-CREB antibody (48H2; #9197; Cell Signaling Technology) and anti-rabbit IgG from Sigma-Aldrich. Relative protein binding to the *C/ebpβ* gene was evaluated on recovered DNA by qPCR. Primers used are the following: CREB bs: F, 5'-gccctctcgcgctc-3'; R, 5'-ggctccgctgcgtc-3'. Samples were normalized to their respective input using the $2^{-\Delta CT}$ method.

4.9 Western blot (WB) analysis

WB analysis was performed as described in [131]. 3T3-L1 cell lysates were obtained by lysing cells in buffer containing 20 mm Tris-HCl, pH 7.5; 5 mm Ethylenediaminetetraacetic acid (EDTA); 150 mm NaCl; 1% Nonidet P40 (NP40), 10 μm phenylmethylsulfonyl fluoride (PMSF); 5 μg/mL aprotinin; and 5 μg/mL leupeptin. Reagents used for cell lysates were from Sigma-Aldrich. Protein concentration was determined by Coomassie blue protein assay (Bio-Rad Laboratories). Equal amounts of proteins lysates were then analyzed by SDS-PAGE, and then electrophoretically transferred to a Polyvinylidene fluoride (PVDF) membrane. Membranes were probed with antibodies to phospho-CREB Ser133 (1B6; #9196 Cell Signaling Technology, Danvers, MA, USA), total CREB (48H2, #9197; Cell Signaling Technology, Danvers, MA, USA), C/EBPβ (C-19, sc-150; Santa Cruz Biotechnology, Dallas, TX, USA), VINCULIN (7F9, sc-73614; Santa Cruz Biotechnology, Dallas, TX, USA), and β-ACTIN (I-19; sc-1616 Santa Cruz Biotechnology), and successively re-probed with secondary mouse or rabbit antibodies (Bio-Rad Laboratories) before signal detection with Enhanced chemiluminescence (ECL) plus (GE Healthcare, Chicago, IL, USA).

4.10 miRNA Mimic and Inhibitor Transfection

miRNA mimic and inhibitor transfection were performed according to [138]. Briefly, 100% confluent 3T3-L1 cells (day 2) were transfected with 5 $nmol \cdot l^{-1}$ of miRIDIAN mimic *mmu-miR-155-5p* (C-310430-07-0005, Dharmacon Inc., Lafayette, CO, USA) or 5 $nmol \cdot L^{-1}$ of the miRIDIAN Hairpin Inhibitor *mmu-miR-155-5p* (IH-310430-08-0005, Dharmacon Inc.) using Lipofectamine 3000 Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. The non-targeting control oligonucleotide miRIDIAN microRNA Mimic negative control #1 (5 $nmol \cdot L^{-1}$; CN-001000-01-05, Dharmacon Inc.) and the non-targeting control oligonucleotide miRIDIAN

microRNA Hairpin Inhibitor negative control #1 (5 nmol·L^{-1}; IN-001005-01-05, Dharmacon Inc.) were used as a negative control of miRNA mimic and inhibitor transfection, respectively. Forty-eight hours after the transfection (D0), adipogenesis was induced into cells and cells were left to differentiate into mature adipocytes for a further 8 days, as described above in this section.

4.11 Statistical procedures

For experiments with only two groups, the comparison was made using a two-tailed, unpaired Student's t-test. For experiments with three or more groups, comparison between groups was determined by one-way analysis of variance (ANOVA) and Bonferroni correction post hoc test was carried out to determine significant differences between specific groups. GraphPad Software was used to analyse data (GraphPad Software, version 6.00 for Windows, La Jolla, CA).

5. Results

To establish whether and how our *Citrus Aurantium* L. fruit juice *CA*de preparation exerts beneficial properties on adipose cells, different aspects of adipocyte function of 3T3-L1 cells, including differentiation, lipogenesis, glucose uptake and response to micro-environmental insults have been investigated. In the first part of these "results" section (5.1 - 5.7), data relative to *CA*de effects on early and late phases of differentiation process, gene expression and glucose uptake have been reported. In the second part of this section (5.8-5.10), data relative to *CA*de effects on miRNA regulation of specific target genes and evaluation of protective properties of *CA*de on microenvironment insults are reported.

5.1 *CA*de does not influence 3T3-L1 cell viability

Firstly, cell viability of the 3T3-L1 cells in response to *CA*de at the concentrations of 1, 10, 100 and 1000 μg/mL was investigated. SRB assays showed a 10% reduction of viability in cells exposed to the highest *CA*de concentration (1000 μg/mL) compared with untreated cells, whereas *CA*de did not affect 3T3-L1 cell viability at lower doses (1, 10 and 100 μg/mL; Table 2). The concentration of *CA*de (1000 μg/mL) was therefore excluded for the subsequent experiments.

Cells	*CA*de (μg/mL)	Cell viability (% over Ctrl)
3T3-L1 pre-adipocytes	/	100.0 ± 0.0
	1000 μg/mL	92.6 ± 2.7***
	100 μg/mL	99.1 ± 3.3
	10 μg/mL	103.9 ± 5.4
	1 μg/mL	103.8 ± 3.7

Table 2. Effects of CAde on 3T3-L1 pre-adipocytes cell viability. 3T3-L1 pre-adipocytes cultured in a 96-well plate were treated with scalar concentrations of *CA*de for 24h. Cytotoxicity was then determined by SRB assay as described under "Materials and Methods". Data are mean ± SD of determinations from three independent experiments. Statistical analysis was performed using one-way ANOVA. ***$p<0.001$, vs untreated 3T3-L1 pre-adipocytes.

5.2 *CA*de treatment promotes adipogenesis in 3T3-L1 cells

To explore the adipogenic effects of *CA*de, 3T3-L1 pre-adipocytes were differentiated into mature adipocytes with an adipogenic stimuli (AS) in the presence or absence of various doses of *CA*de (1, 10 and 100 μg/mL) for eight days. Lipid accumulation of cells and TG deposition were measured at the end of the treatment, as events associated with terminal adipocyte differentiation.

As shown by Oil-Red O staining, in the presence of *CA*de at a dose of 10 or 100 μg/mL for eight days, but not at 1 μg/mL, cells displayed a dose-dependent increase of the intracellular lipid accumulation compared to control adipocytes (Figures 4A and 4B). Concurrently, treatment with *CA*de at the doses of 10 and 100 μg/mL, but not at the lower concentration of 1 μg/mL, enhanced about 40 and 70%, respectively, the TG deposition of treated 3T3-L1 adipocytes compared with the TG deposition of control adipocytes (Figure 4C). DNA content of 3T3-L1 cells treated with all three concentrations of *CA*de (1, 10 and 100 μg/mL) was unchanged compared with the untreated control cells (data not shown). Altogether, these results indicate that treatment with *CA*de facilitates 3T3-L1 adipogenesis in terms of lipid accumulation and TG. From now on, the dose of 100 μg/mL *CA*de, which showed the more adipogenic effect on the 3T3-L1 cell, was used for all subsequent experiments.

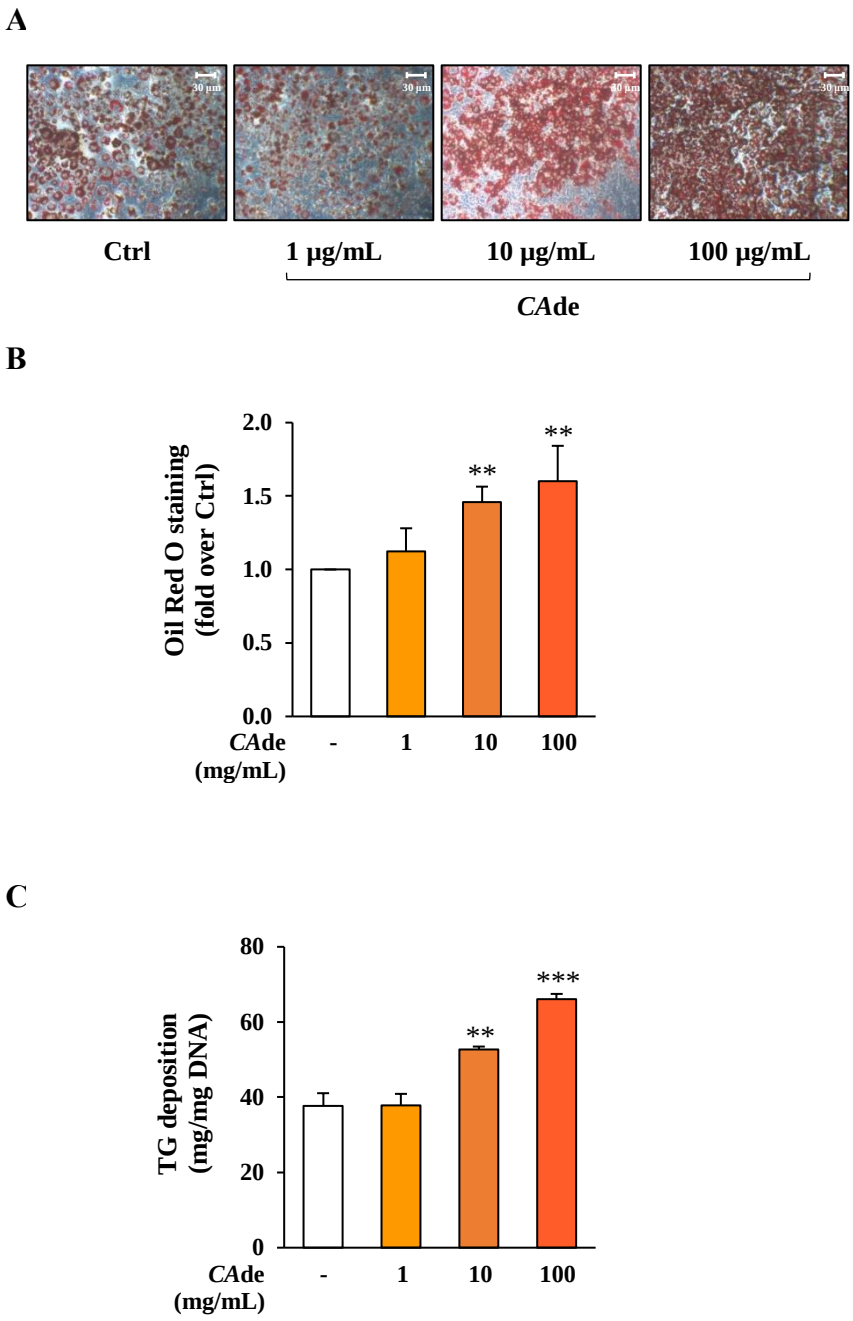


Figure 4. The effects of CAde on TG deposition and intracellular lipid accumulation in 3T3-L1 adipocytes. 3T3-L1 pre-adipocytes were differentiated into mature adipocytes for eight days, as described under "Materials and Methods", in the absence (Ctrl) or presence of *CA*de (1, 10 or 100 µg/mL). Microscopic images (10X magnification) **(A)**, and lipid quantization **(B)** of mature adipocytes stained with Oil-Red O. The results are means ± SD of the Oil-Red O absorbance values measured at 490 nm from three independent experiments and are expressed as fold changes over control. Statistical analysis was performed using one-way ANOVA. **p<0.01 vs Ctrl. **C)** Total TG deposition of mature adipocytes. Data are mean ± SD of determinations from three independent experiments. Statistical analysis was performed using one-way ANOVA. **p<0.01, and ***p<0.001, vs Ctrl.

5.3 *CA*de up-regulates the expression of master regulators of adipogenesis in 3T3-L1 cells

To investigate how *CA*de promotes adipogenesis in 3T3-L1 cells, we measured the expression of adipogenic markers at day 2 (D2), D4 and D8 of the differentiation process. The mRNA levels of the *CCAAT/enhancer-binding protein beta* (*C/ebpβ*) at D2 were increased by 2-fold (Figure 5A), the expression levels of the *Peroxisome proliferator-activated receptor gamma* (*Pparγ*) at D4 were increased by 1.8-fold (Figure 5B), and *glucose transporter type 4* (*Glut4)* and the *Fatty acid-binding protein 4* (*Fabp4*) at D8 were increased by 2.3- and 1.9-fold, respectively, (Figures 5C and 5D) in 3T3-L1 cells differentiated in the presence of *CA*de compared with control cells. Differently, no changes in the mRNA expression levels of *C/ebpδ* at day 2 and *C/ebpα* at day 4 were found among cells differentiated in the presence or absence of *CA*de (data not shown). These findings suggest that *CA*de enhances the expression of specific master regulator genes of adipogenesis as early as 2 days upon induction of differentiation.

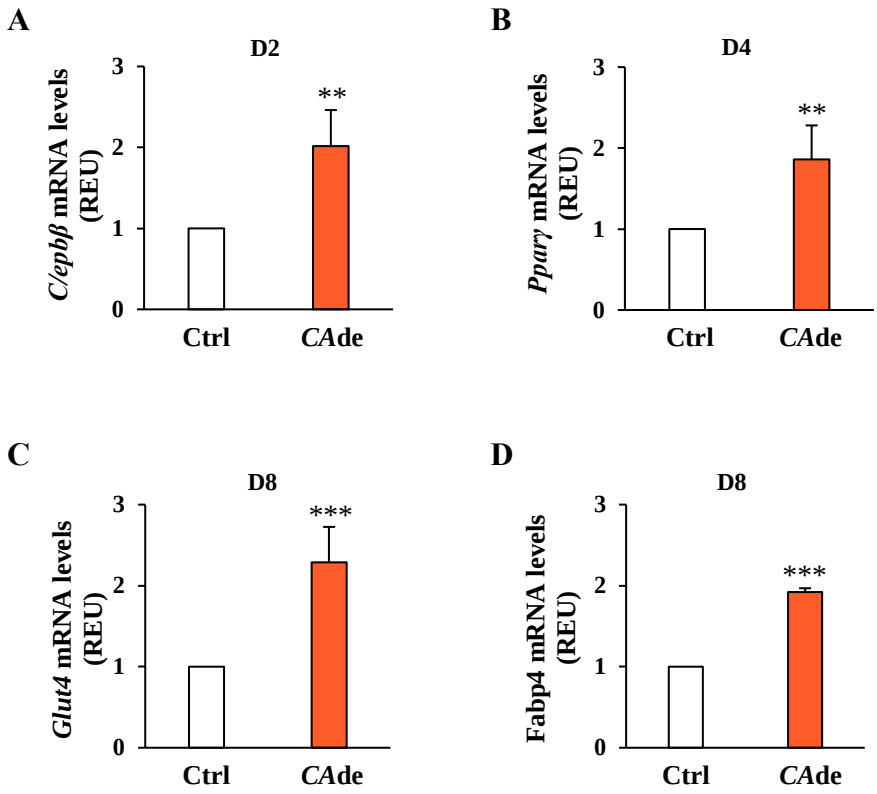


Figure 5. The effects of CAde on gene expression during adipogenesis in 3T3-L1 cells. 3T3-L1 pre-adipocytes were differentiated into mature adipocytes for eight days, as described under "Materials and Methods", in the absence (Ctrl) or presence of *CA*de (100 µg/mL). At the indicated time points, cells were collected for the extraction of RNA. qPCR was performed to detect the mRNA expression of **(A)** *C/ebpβ* at D2, **(B)** *Pparγ* at D4, and **(C)** *Glut4* and **(D)** *Fapb4* at D8 of the adipogenesis. Results are means ± SD of three independent experiments and are expressed as relative changes over control. Statistical analysis was performed using Student's t-test. **p<0.01, and ***p<0.001 vs Ctrl at D2, D4 or D8.

5.4 *CA*de promotes the early stages of adipocyte differentiation and enhances insulin-mediated glucose uptake in 3T3-L1 cells

The pre-adipocyte differentiation into mature adipocytes requires cell commitment and mitotic clonal expansion (MCE), occurring within the first 48 h upon adipogenic induction, and intermediate and late cell differentiation phases, proceeding from D2 to D8 [142]. To evaluate whether *CA*de modulates specific phases of adipose cell differentiation, 3T3-L1 cells were differentiated in the presence of *CA*de from D0 to D2 or from D2 to D8 of adipocyte differentiation. At the end of the differentiation process, similarly to adipocytes matured in the presence of *CA*de for eight days (D0-D8), cells exposed early to *CA*de (D0-D2) showed a significant increase of both TG deposition and intracellular lipid accumulation compared with control mature adipocytes (Figures 6A, 6B and 6C). Also, gene expression of *Pparγ* at D4, and *Glut4* and *Fabp4* at D8 was augmented in these conditions (Figures 6D, 6E and 6F).
On the other hand, in cells treated with *CA*de from D2 to D8, no increases of TG levels and lipid droplet formation at D8 (Figures 6A, 6B and 6C) and of the mRNA expression of *Pparγ*, *Glut4* and *Fabp4* of adipocyte differentiation (Figures 7A, 7B and 7C), were observed compared with control adipocytes. These data indicate that the effectiveness of treatment with *CA*de is restricted within the first 48 h of the differentiation process in 3T3-L1 cells. Then, to determine whether the treatment with *CA*de may also enhance adipocyte functionality, we investigated the basal and the insulin-mediated 2-DG uptake in mature 3T3-L1 adipocytes differentiated in the presence or absence of *CA*de from D0 to D8, from D0 to D2 or from D2 to D8. As expected, in adipocytes differentiated without *CA*de, 100 nmol/l insulin for 30 min induced by 4.5-fold the 2-DG uptake compared with the basal 2-DG uptake (Figure 8A). On the other hand, cells differentiated in the presence of *CA*de from D0 to D8 showed a 5.7-fold increase of the insulin-mediated 2-DG uptake compared with the basal 2-DG uptake (Figure 8A). Interestingly, the cells treated with *CA*de from D0 to D2, similarly to the cells exposed to *CA*de from D0 to D8, also increased the insulin-mediated 2-DG uptake compared with control cells (Figure 8A). On the contrary, the insulin-mediated 2-DG uptake of cells treated with *CA*de from D2 to D8 was similar to the glucose uptake of unexposed control cells (Figure 8A). These data suggest that the treatment with *CA*de, even restricted within the first 48 h of the differentiation process, also promotes glucose uptake in response to insulin stimulation.

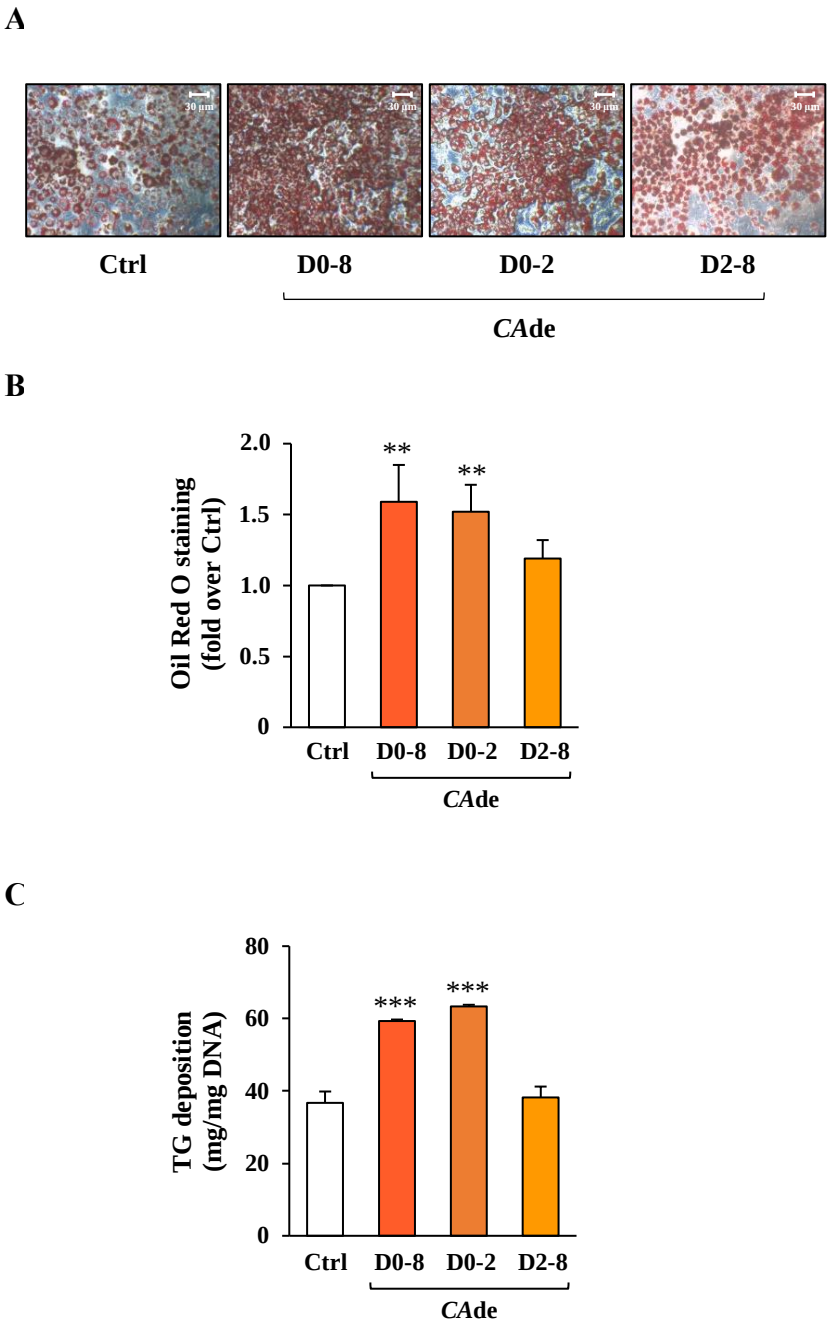


Figure 6. The effects of CAde on the early stage of adipogenesis in 3T3-L1 cells. 3T3-L1 pre-adipocytes were differentiated into mature adipocytes for eight days, as described under "Materials and Methods", in the absence (Ctrl) or presence of *CA*de (100 µg/mL) from D0 to D2 (D0-D2), from D2 to D8 (D2-D8) and from D0 to D8 (D0-D8). **A)** Total TG deposition of mature adipocytes. Data are mean ± SD of determinations from three independent experiments. Statistical analysis was performed using one-way ANOVA. ***p<0.001, vs Ctrl. Microscopic images (10X magnification) **(B)** and lipid quantization **(C)** of mature adipocytes stained with Oil-Red O. The results are means ± SD of the Oil-red O absorbance values measured at 490 nm from three independent experiments and are expressed as fold changes over control. Statistical analysis was performed using one-way ANOVA. **p<0.01 vs Ctrl.

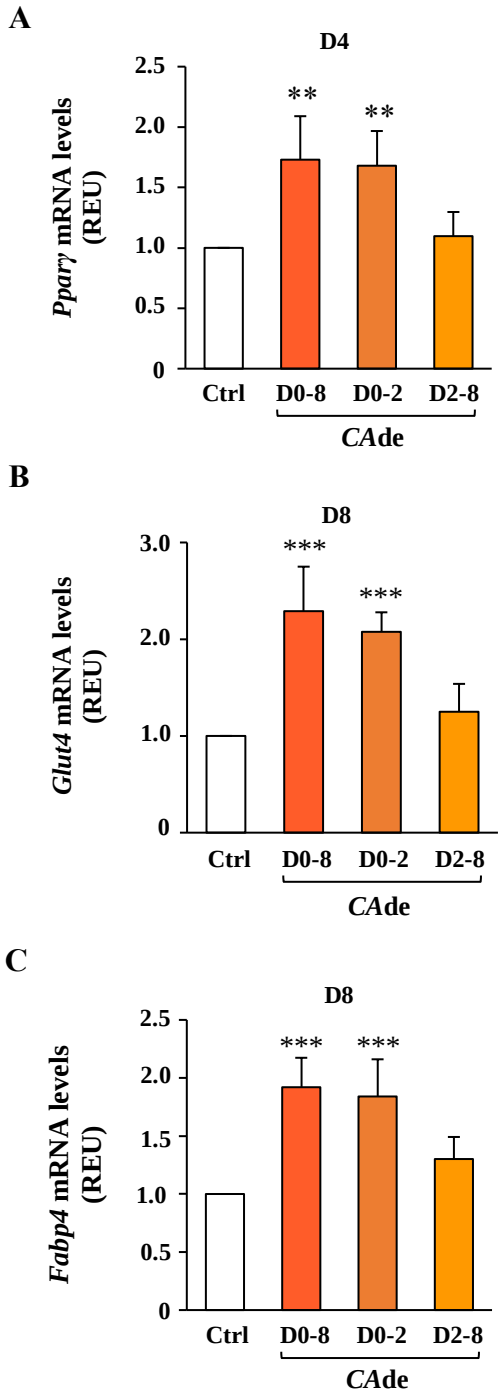


Figure 7. The effects of CAde on the early stage of adipogenesis in 3T3-L1 cells. 3T3-L1 pre-adipocytes were differentiated into mature adipocytes for eight days, as described under "Materials and Methods", in the absence (Ctrl) or presence of *CA*de (100 µg/mL) from D0 to D2 (D0-D2), from D2 to D8 (D2-D8) and from D0 to D8 (D0-D8). qPCR was performed to detect the mRNA expression of **(A)** *Pparγ* at D4, and **(B)** *Glut4* and **(C)** *Fapb4* at D8 of the adipogenesis. Results are means ± SD of three independent experiments and are expressed as relative changes over control. Statistical analysis was performed using one-way ANOVA. *p<0.05, **p<0.01, and ***p<0.001 vs Ctrl at D2, D4 or D8.

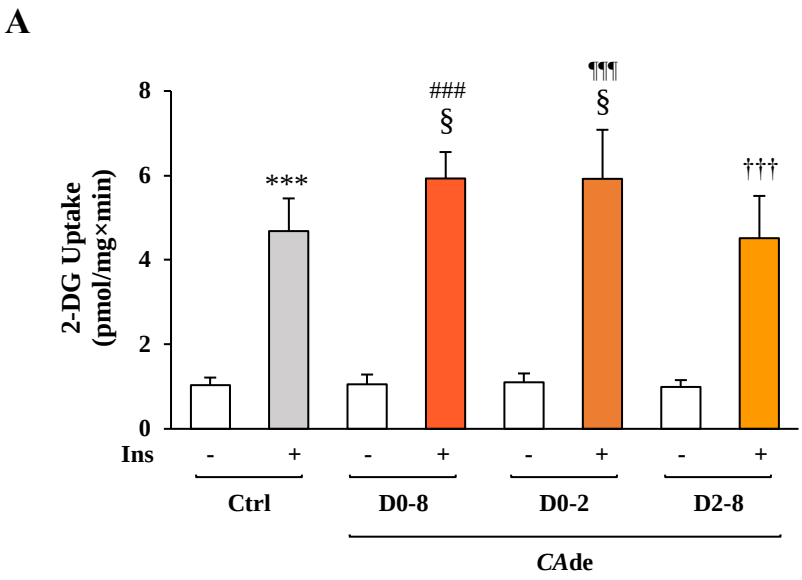


Figure 8. The effects of CAde on the early stage of adipogenesis in 3T3-L1 cells. 3T3-L1 pre-adipocytes were differentiated into mature adipocytes for eight days, as described under "Materials and Methods", in the absence (Ctrl) or presence of CAde (100 µg/mL) from D0 to D2 (D0-D2), from D2 to D8 (D2-D8) and from D0 to D8 (D0-D8). **A)** The uptake of 2-DG was then evaluated in mature adipocytes upon stimulation with insulin (100 nmol/l; Ins) for 30 min. The results are means ± SD of three independent experiments. Statistical analysis was performed using one-way ANOVA. $^{***}p<0.001$ vs Ctrl -Ins; $^{###}p<0.001$, *CA*de D0-D8 + Ins vs *CA*de D0-D8 -Ins; $^{¶¶¶}p<0.001$, *CA*de D0-D2 + Ins vs *CA*de D0-D2 -Ins; $^{†††}p<0.001$, *CA*de D2-D8 + Ins vs *CA*de D2-D8 -Ins; $^{§}p<0.05$ vs Ctrl + Ins.

5.5 *CA*de modulates cell cycle progression during MCE in 3T3-L1 cells

After exposure to AS, post-confluent growth-arrested pre-adipocytes, undergo two successive mitoses over two days, indicated as MCE [143]. Therefore, to evaluate whether *CA*de promotes adipocyte differentiation in 3T3-L1 cells by inducing changes in cell cycle progression during MCE, cell cycle distribution was analysed by flow cytometry. At 12 h post-induction, no differences of the cell distribution in the G0/G1, S and G2/M phases were observed among 3T3-L1 pre-adipocytes exposed or not to *CA*de (Figures 9A and 9B). At 14 h post-induction, treatment with *CA*de decreased the number of cells in the G0/G1 phase and concomitantly increased the number of cells in the S phase (S phase: Ctrl, 26.5% ± 5.7% vs *CA*de, 38.0% ± 1.0%; $p<0.05$; Figures 9A and 9B). At 16 h post-induction, *CA*de not only decreased the number of cells in the G0/G1 phase but also increased the number of cells in the S and G2/M phases (S phase: Ctrl, 40.2% ± 1.5% vs *CA*de, 52.2 ± 0.9%; $p<0.001$; G2/M phase: Ctrl, 11.1% ± 2.5% vs *CA*de, 17.0 ± 2.2%; $p<0.05$; Figures 9A and 9B). Interestingly, *CA*de did not induce enhancement of MCE in the uncommitted mouse embryonic fibroblasts NIH-3T3 cells (data not shown). Furthermore, the cell cycle distribution of both post-confluent growth-arrested 3T3-L1 and NIH-3T3 cells just treated with *CA*de for 16 h in the absence of the adipogenic cocktail did not differ compared with the untreated control cells (data

not shown). In addition to this, treatment with *CA*de did not change both 3T3-L1 and NIH-3T3 cell proliferation (data not shown). Thus, these results suggest that *CA*de exhibits pro-adipogenic properties specifically in 3T3-L1 cells by modulating and promoting their cell cycle progression in the early stage of the differentiation process.

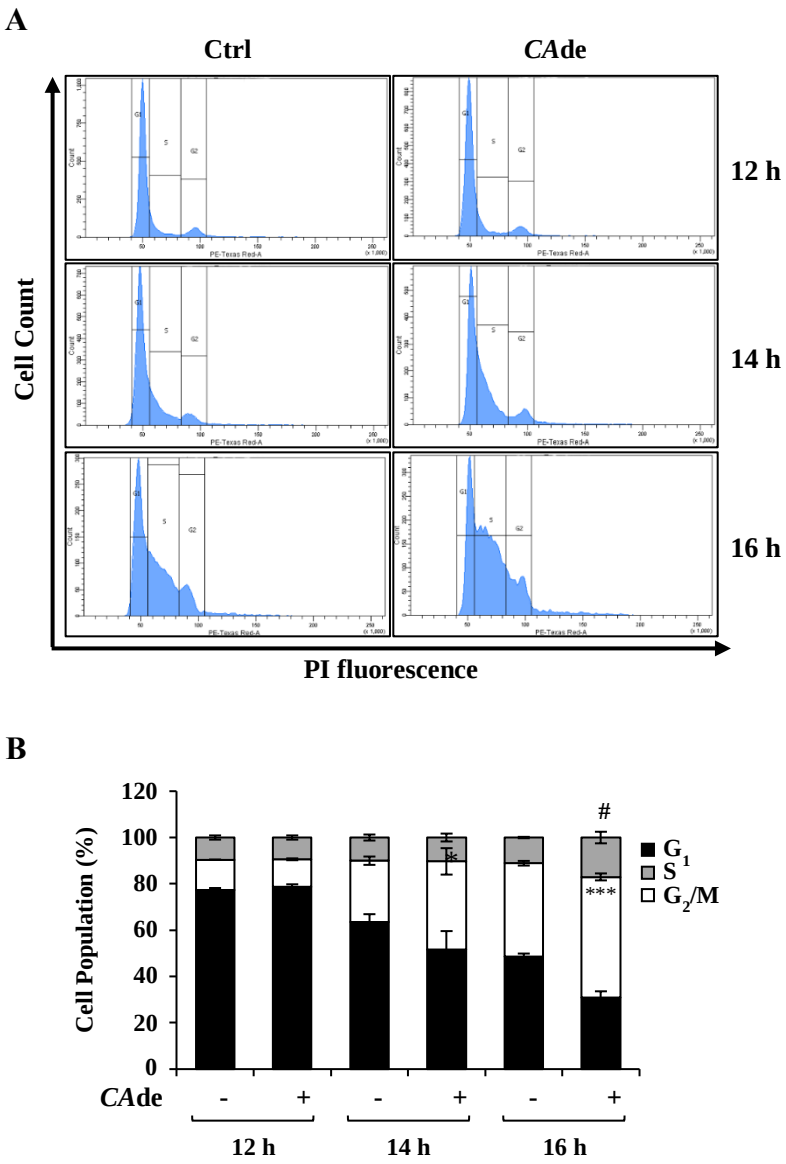


Figure 9. The effects of CAde on cell cycle progression during adipogenesis in 3T3-L1. MCE was induced in 3T3-L1 pre-adipocytes with differentiation medium, as described under "Materials and Methods". Cells were then harvested at 12, 14, and 16 h after the initiation of differentiation in the absence (Ctrl) or presence of *CA*de (100 µg/mL) and stained with the PI solution for flow cytometer cell cycle analysis. **A)** Histograms of cell cycle distribution in G0/G1, S or G2/M phases. **B)** Quantitative analysis of cell cycle distribution. The results are means ± SD of three independent experiments. Statistical analysis was performed using Student's t-test *p<0.05, and ***p<0.001, *CA*de S Phase vs Ctrl S Phase; $^{\#}p$<0.05, *CA*de G2/M Phase vs Ctrl G2/M Phase.

5.6 *CA*de early enhances *C/ebpβ* expression through the activation of CREB in 3T3-L1 cells

C/ebpβ plays a basic role in regulating adipogenesis and is necessary to initiate the MCE in 3T3-L1 cells [144-149]. To investigate whether and how *CA*de directly modulates *C/ebpβ*, we measured both the *C/ebpβ* gene expression and the activation state of its transcriptional regulator CREB [146] in the early times post the adipogenic induction of 3T3-L1 cells with the AS. As expected, in the control cells, AS induced already upon 1 h a 1.7-fold increase of the mRNA expression of *C/ebpβ*, which levels furtherly raised at 2 and 4 h and reached its maximal expression at 8 h post-treatment (Figure 10A). Concurrently, in these cells, AS also led to CREB activation, measured as phosphorylation of the Ser133, which was evident as early as 1 h and persisted up to 8 h following the adipogenic induction (Figure 10B). Interestingly, treatment with *CA*de also enhances the effect of AS on *C/ebpβ* expression and CREB activation (Figures 10A and 10B). Indeed, in cells treated with *CA*de, the *C/ebpβ* gene expression resulted in to be increased of about 30, 40 and 45% at 2, 4 and 8 h upon AS and *CA*de induction, respectively, compared with its expression at the same time points in the AS treated control cells (Figure 10A). These data were also paralleled by a strong induction of the CREB activation, in which phosphorylation levels were higher as early as 1 h after the combined AS and treatments with *CA*de and remained more elevated up to 8 h following the treatments compared with the phosphorylation levels of the AS treated control cells (Figure 10B). Furthermore, the specific recruitment of CREB to its binding sites (Site 1, -111/-101 bp from the *C/ebpβ* gene transcription starting site, TSS, and Site 2, -65/-55 bp from the TSS) on the *C/ebpβ* promoter investigated by ChIP analysis showed a 2-fold increase in CREB binding to the C/ebpβ promoter at 4 h in 3T3-L1 cells differentiated in the presence of *CA*de compared with the AS treated cells (Figure 10C). These data indicate that *CA*de enhances the adipogenic induction effect in 3T3-L1 cells at least in part by early promoting and sustaining the *C/ebpβ* expression through the increased activation of the transcription factor CREB.

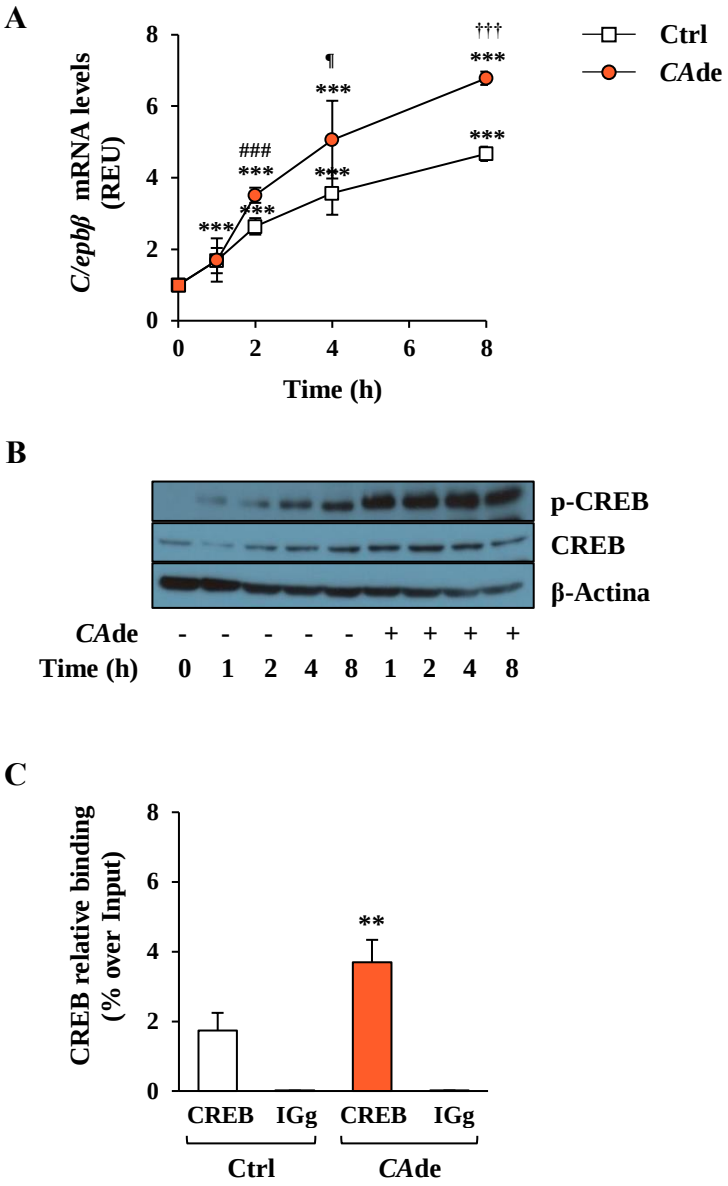


Figure 10. The effects of CAde on C/ebpβ gene expression and CREB activation during the early stage of adipogenesis in 3T3-L1. Adipogenesis was induced in 3T3-L1 pre-adipocytes with the differentiation medium (AS), as described under "Materials and Methods". Cells were then harvested at 1, 2, 4 and 8 h after the initiation of differentiation in the absence (Ctrl) or presence of *CA*de (100 µg/mL) and processed for qPCR and western blot analysis. **A)** qPCR of C/ebpβ mRNA expression. Results are means ± SD of three independent experiments and are expressed as relative changes over control. Statistical analysis was performed using one-way ANOVA. ***p<0.01, vs 3T3-L1 cells at 0 h; $^{###}p$<0.001, *CA*de 2 h vs Ctrl 2 h; $^{¶}p$<0.05, *CA*de 4 h vs Ctrl 4 h; $^{†††}p$<0.001, *CA*de 8 h vs Ctrl 8 h. **B)** The representative western blot show levels of the total and Ser133 phosphorylated form of the cAMP response element-binding protein (CREB) and the β-Actin protein. **C)** CREB protein binding on *C/ebpβ* promoter was evaluated by ChIP analysis on 3T3-L1 cells harvested at 4 h after the initiation of differentiation in the absence (Ctrl) or presence of *CA*de (100 µg/mL). ChIP enrichment is relative to input chromatin. Data are expressed as mean ± SD of values from at least three independent experiments. Statistical analysis was performed using Student's t-test. **p<0.01, *CA*de 4 h vs Ctrl 4 h.

5.7 Effects of single compounds on *C/ebpβ* gene expression in 3T3-L1 cells

Finally, to investigate the hypothesis that the effects of *CA*de on *C/ebpβ* gene expression may be dependent on the activity of specific molecules within *CA*de, a qualitative and quantitative analysis of compounds in *CA*de was performed. From this analysis resulted that among 17 identified metabolites, the O-glycoside flavanones narirutin and hesperidin, which concentration for gram of *CA*de is 67.51 and 39.05 mg, respectively, and the C-glycoside flavone vicenin-2 (55.56 mg/g of *CA*de) were the most abundant (Table 1). The effects of these three flavonoids on *C/ebpβ* gene expression in the early times post the adipogenic induction of 3T3-L1 cells with AS was thus investigated. Interestingly, similarly to cells treated with *CA*de, cells exposed to narirutin showed a stronger increase of the mRNA expression of *C/ebpβ* at 2 h and 4 h post adipogenic induction compared to control cells (Figures 11A and 11B), but at 8 h the *C/ebpβ* levels were comparable among control, and narirutin treated cells (Figure 11C). Differently from narirutin, hesperidin significantly enhanced *C/ebpβ* expression of 3T3-L1 cells only at 2 h post adipogenic induction, but not at 4 and 8 h, compared to control cells (Figures 11A, 11B and 11C), while vicenin-2 did not affect *C/ebpβ* gene expression levels of 3T3-L1 cells neither at 2, 4 or 8 h upon AS (Figures 11A, 11B and 11C).

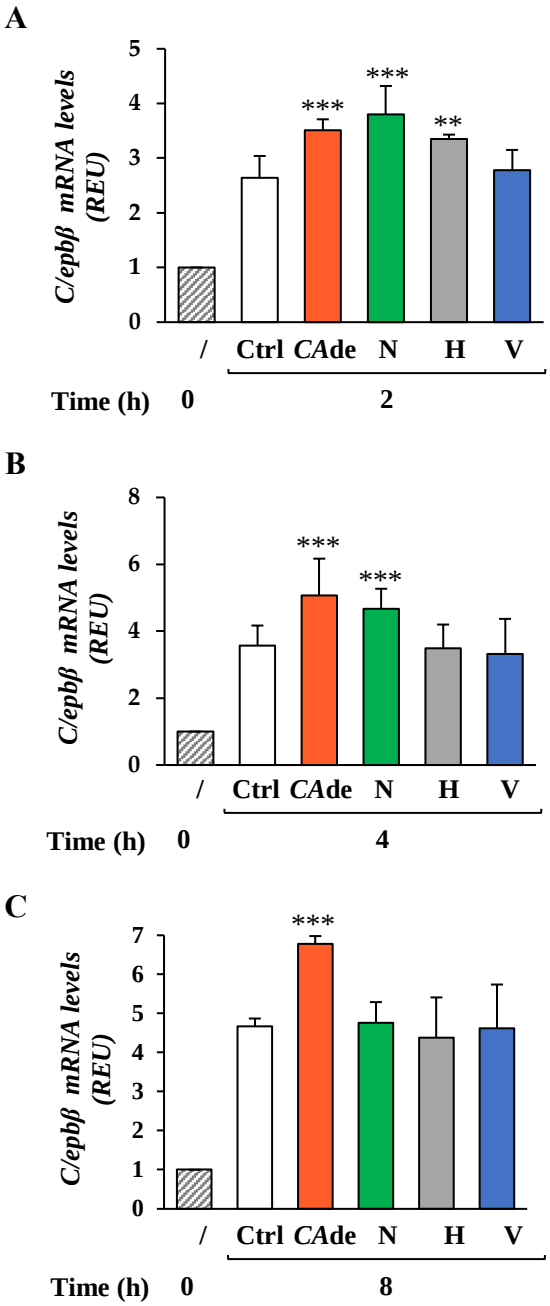


Figure 11. The effects of single flavonoids on C/ebpβ gene expression during the early stage of adipogenesis in 3T3-L1. Adipogenesis was induced in 3T3-L1 pre-adipocytes with adipogenic differentiation medium (AS). Cells were then harvested at 2, 4 and 8 h after the initiation of differentiation in the absence (Ctrl) or presence of 6.7 µg/mL narirutin (N) or 3.9 µg/mL hesperidin (H) or 5.5 µg/mL vicenin-2 (V). Cells treated with *CA*de (100 µg/mL) were also used. At the indicated time points, cells were collected for the extraction of RNA. qPCR was performed to detect the mRNA expression of *C/ebpβ* at 2h **(A)**, 4h **(B)**, and 8h **(C)** upon adipogenesis. Results are means ± SD of three independent experiments and are expressed as relative changes over control cells at time 0. Statistical analysis was performed using one-way ANOVA. **p<0.01, and ***p<0.001 vs Ctrl at 2, 4 or 8 h.

5.8 *CA*de down-regulated the expression of *miR-155* and enhanced *C/ebpβ* and *Creb* levels during the early stage of adipogenesis in 3T3-L1 cells

To test the hypothesis that *CA*de may potentially exert its pro-adipogenic effects *in vitro* by modulating *miR-155* expression, the early stage of fat cell differentiation was investigated in 3T3-L1 pre-adipocytes. In control cells, the AS induced at 15 min a 10% reduction of the *miR-155* expression, whose levels remained steadily lowered to about 30%-35% at 30 min, 1, 2, and 4 h upon the adipogenic induction (Figure 12A). In cells treated with *CA*de, the expression of *miR-155* resulted in levels being decreased by 31% already by 15 min upon the adipogenic induction, and its levels further declined by 58% at 30 min, 51% at 1 h, 54% at 2 h, and 54% at 4 h upon induction compared to control cells at time 0 (Figure 12A). Interestingly, compared to control cells, treatment with *CA*de enhanced the effect of the adipogenic stimuli on *miR-155* down-regulation by 25%-40% at each time point (Figure 12A).

Additionally, we evaluated *CA*de effect on *miR-130a* and *miR-375*, whose role in adipocyte fate determination has already been demonstrated [150]. In control 3T3-L1 cells, the induction of fat cell differentiation with AS caused a time-dependent down-regulation of the anti-adipogenic *miR-130a* (Figure 11B) and up-regulation of the pro-adipogenic *miR-375* (Figure 12C) compared to control cells at time 0. Of note, *CA*de treatment did not affect the expression of these two miRNAs. Indeed, in cells treated with *CA*de and AS, the expression levels of both *miR-130a* (Figure 12B) and *miR-375* (Figure 12C) at each time point were comparable to those levels observed in control cells. Altogether, these findings suggest that *CA*de specifically modulated the expression of *miR-155* in the early stage of fat cell differentiation.

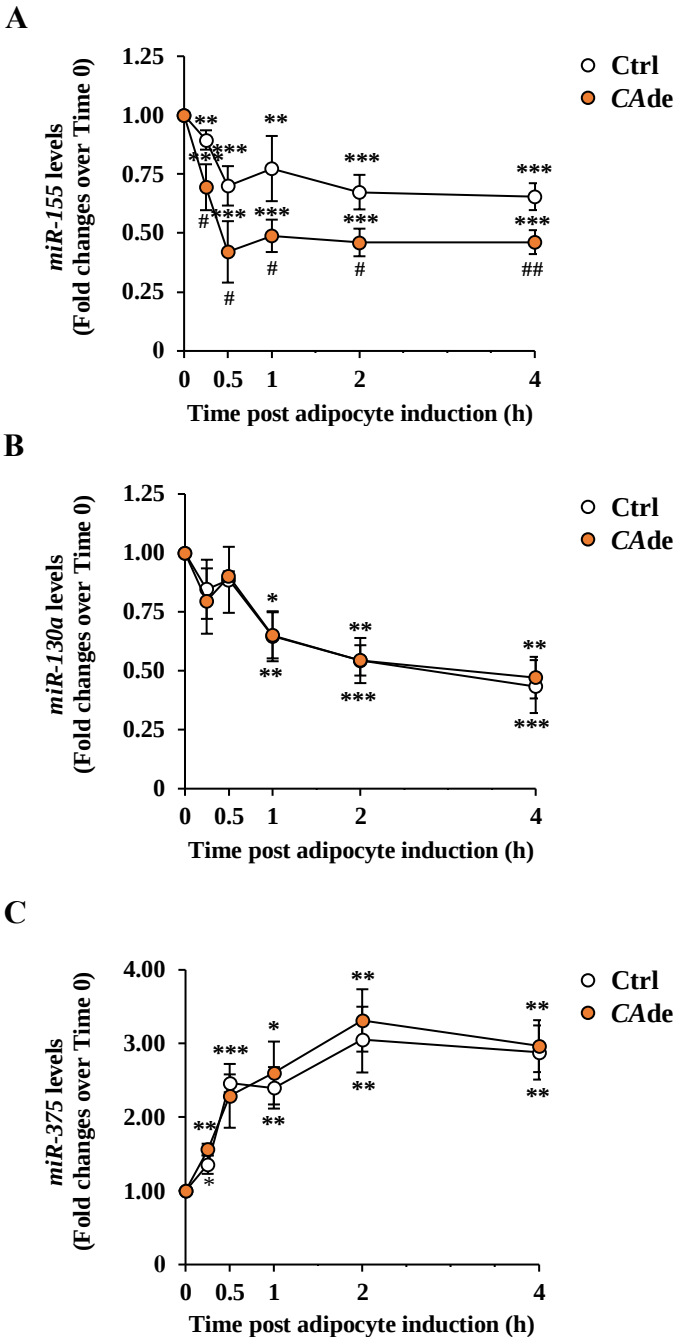


Figure 12. Effect of CAde on the expression of miR-155 during the early stage of adipogenesis in 3T3-L1 cells. 3T3-L1 pre-adipocytes were cultured for 0.25, 0.5, 1, 2, and 4 h with adipogenic differentiation medium (AS) (Ctrl) or AS and *CA*de (100 µg/mL). Time course of *miR-155* **(A)**, *miR-130a* **(B)**, and *miR-375* **(C)** levels, evaluated by quantitative real-time PCR, in Ctrl and *CA*de-treated cells relative to untreated 3T3-L1 cells at time 0. Values are means ± Standard Error of the Mean (SEM) of three independent experiments. Control value at time 0 was set as 1.00. Statistical significances among groups were tested by one-way ANOVA followed by Bonferroni correction post-hoc test (*p<0.05, **p<0.01, and ***p<0.001 vs untreated 3T3-L1 cells at time 0; $^{\#}p$<0.05, and $^{\#\#}p$<0.01 vs control 3T3-L1 cells).

Subsequently, it was investigated whether *CA*de could influence the *miR-155* target genes *C/ebpβ* and *Creb* [151]. In the control cells, AS led to a time-dependent up-regulation of both *C/ebpβ* (Figure 13A) and *Creb* (Figure 13B) mRNA levels compared to control cells at time 0. It is worth noting that, compared to control cells, treatment with *CA*de furtherly up-regulated the expression of *C/ebpβ* by about 65% and 45% at 2 and 4 h, respectively (Figure 13A), while the combined stimulation of AS and *CA*de up-regulated *Creb* mRNA levels by about 40%, 55%, and 25% at 1, 2, and 4 h, respectively, as compared to treated control cells (Figure 13B). Coherently with the gene expression data, the protein levels of C/EBPβ isoform liver-enriched activating protein (LAP) resulted up-regulated at 2 and 4 h from AS in the cells treated with *CA*de compared to control cells (Figure 13C). Additionally, the protein levels of CREB were increased, starting from 0.25 h from AS in cells treated with *CA*de (Figure 13C). Altogether, these findings at the early stage of fat cell differentiation indicated that *CA*de might exert its function on the adipogenic induction in 3T3-L1 cells, at least in part, by specifically lowering the expression of *miR-155* at the early time points and thus up-regulating the mRNA and protein expression of its target genes *C/ebpβ* and *Creb*.

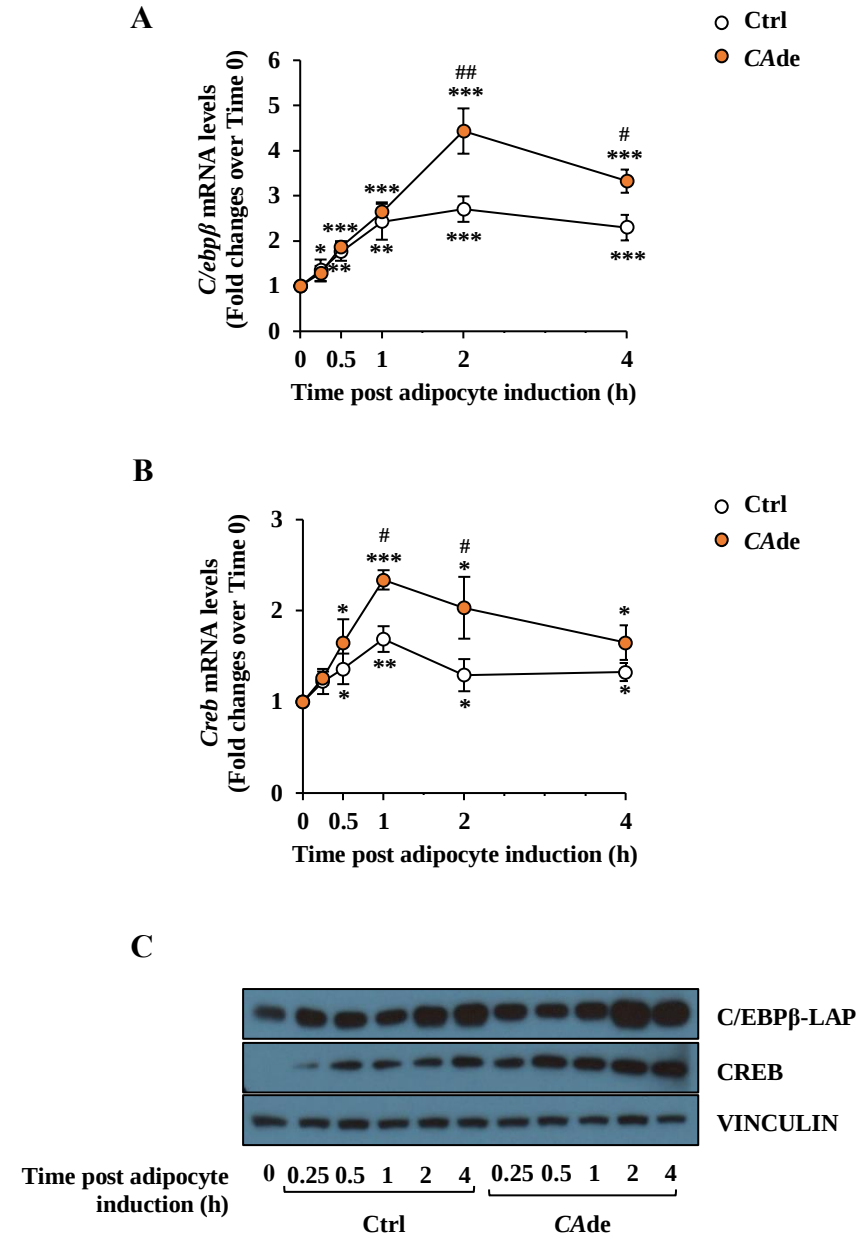
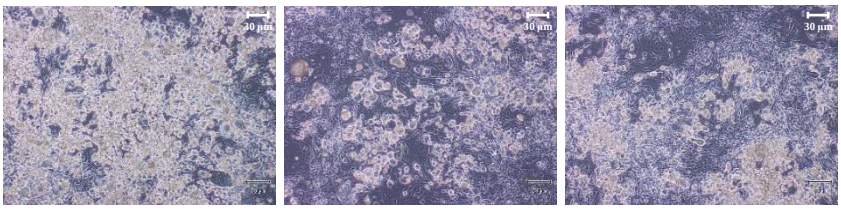


Figure 13. CAde effects on the expression of the miR-155 target genes, C/ebpβ and Creb. Pre-adipocytes were cultured for 0.25, 0.5, 1, 2, and 4 h with AS (Ctrl) or AS + *CA*de (100 µg/mL) *CCAAT/enhancer-binding protein beta* (*C/ebpβ*) and *cyclic Adenosine Monophosphate (cAMP) response element-binding protein* (*Creb*) levels were evaluated by quantitative real-time PCR. Time course of *C/ebpβ* **(A)** and *Creb* **(B)** levels in Ctrl and *CA*de-treated cells relative to untreated 3T3-L1 cells at time 0. Values are means ± SEM of three independent experiments. Control value at time 0 was set as 1.00. Statistical significances among groups were tested by one-way ANOVA followed by Bonferroni correction post-hoc test (*p<0.05, **p<0.01, and ***p<0.001 vs untreated 3T3-L1 cells at time 0; $^{\#}p$< 0.05 and $^{\#\#}p$<0.01 vs control 3T3-L1 cells). **(C)** Representative Western blots of the total form of the C/ebpβ-liver enriched activating protein (LAP), CREB, and VINCULIN proteins in control cells and CAde-treated cells at 0.25, 0.5, 1, 2, and 4 h after adipogenic induction. C/ebpβ-LAP and CREB protein expression levels at the basal (0) are also reported.

5.9 *CA*de improved terminal adipocyte differentiation of 3T3-L1 cells over-expressing *miR-155* by mimic transfection

Over-expression of *miR-155*, by a gain of function approach or by pro-inflammatory cytokine induction [70, 71], has been reported to impair *in vitro* adipocyte differentiation of 3T3-L1 cells. We then hypothesized that *CA*de might preserve the adipogenesis of 3T3-L1 cells, where *miR-155* was over-expressed by mimic transfection or induced by the pro-inflammatory cytokine TNFα. Fat cell differentiation of 3T3-L1 cells was firstly investigated in cells transfected with the *miR-155 mimic* or the *miR-155* inhibitor. The specific over-overexpression of mimic *miR-155* reduced the number of 3T3-L1 cells able to differentiate into adipocytes by about 50%, as shown by light microscopy images (Figure 14A) and Oil-Red O lipid accumulation (Figure 14B). In cells treated with *CA*de, the intracellular lipid accumulation was increased by about 1.4-fold compared to *miR-155* over-overexpressing cells (Figure 14A and 13B). On the other hand, the specific loss of function of *miR-155* by hairpin inhibitor transfection increased adipogenesis of 3T3-L1 cells by about 90% (data not shown); no further increase of adipogenesis was observed in 3T3-L1 cells transfected with miR-155 hairpin inhibitor upon *CA*de treatment (data not shown). These findings indicated that *CA*de treatment partially prevents the inhibitory effects of *miR-155* on adipocyte differentiation of 3T3-L1 cells.

A



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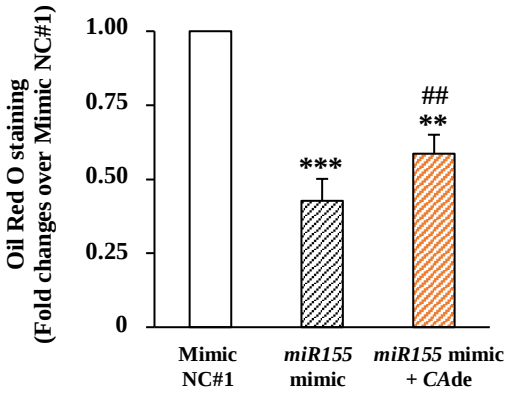

Figure 14. Effect of CAde on adipocyte differentiation and lipid accumulation of 3T3-L1 adipocytes over-overexpressing miR-155. 3T3-L1 pre-adipocytes were transfected with the mimic negative control (NC) #1 or with the *miR-155* mimic and cultured to reach adipocyte differentiation for eight days AS. Cells transfected with the *miR-155* mimic were also differentiated with AS supplemented with *CA*de (100 μg/mL). **(A)** Representative microphotographs of adipocytes transfected with mimic negative control (NC) #1 or with the *miR-155* mimic ± *CA*de are shown (X10 magnifications); scale bars, 30 μm. **(B)** Oil-Red O staining of adipocytes transfected with mimic negative control (NC) #1, or with the *miR-155* mimic ± *CA*de. Values are mean ± SEM of determinations from three independent experiments. Statistical significances among groups were tested by one-way ANOVA followed by Bonferroni correction post-hoc test (**p<0.01, and ***p<0.001 vs mimic negative control (NC) #1; $^{\#\#}p$<0.01, *miR-155* mimic + *CA*de vs *miR-155* mimic).

Secondly, we investigated whether *CA*de may preserve the adipogenesis of cells exposed to the pro-inflammatory cytokine TNFα, which exerts profound inhibition of adipocyte differentiation by *miR-155* induction [70, 151]. As expected, the number of 3T3-L1 pre-adipocytes able to achieve full differentiation was strongly reduced by TNFα treatment, as shown by the light microscopy images (Figure 15A). Consistent with this, TNFα reduced the intracellular lipid accumulation (Figure 15B) by about 60% and the TG deposition by about 70% (Ctrl, 41.5 ± 0.3; TNFα, 11.2 ± 0.3, μg TG · μg DNA^{-1}; p<0.001) compared to control adipocytes. It is worth noting that in cells co-treated with TNFα and *CA*de, the number of adipocytes was increased compared

to TNFα-treated cells (Figure 15A). In addition, upon *CA*de treatment, the intracellular lipid accumulation (Figure 15B) and TG deposition were increased by 1.5- and about 2.2-fold, respectively, compared to TNFα-treated adipocytes (TNFα + *CA*de, 26.4 ± 5.8; TNFα, 11.2 ± 0.3, μg TG · μg DNA^{-1}; $p<0.05$). Altogether, these data suggest that *CA*de treatment partially protected the adipogenesis of 3T3-L1 cells from the TNFα inhibitory effect.

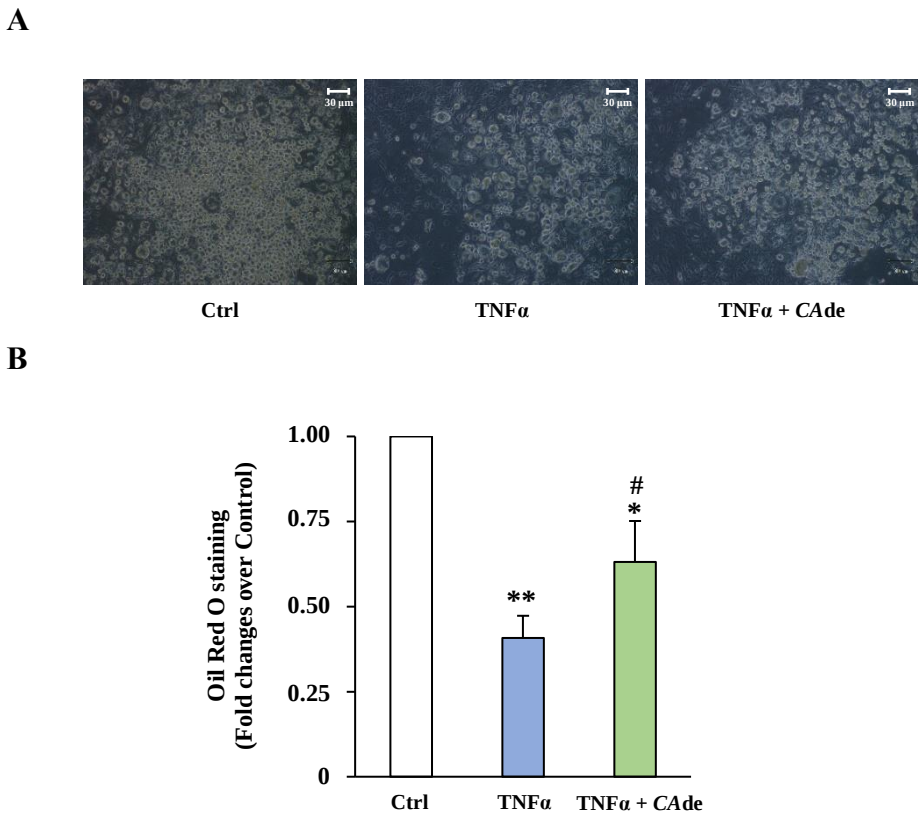

Figure 15. Effect of CAde on adipocyte differentiation and lipid accumulation of 3T3-L1 adipocytes treated with TNFα. 3T3-L1 pre-adipocytes were cultured to reach adipocyte differentiation for eight days with AS (Ctrl) or AS supplemented with TNFα (1 ng/mL) ± *CA*de (100 μg/mL). **(A)** Representative microphotographs of Ctrl, TNFα-, and TNFα + *CA*de-treated adipocytes are shown (X10 magnifications); scale bars, 30 μm. **(B)** Oil-Red O staining of Ctrl, TNFα-, and TNFα + *CA*de-treated adipocytes. Values are mean ± SEM of determinations from three independent experiments. Statistical significances among groups were tested by one-way ANOVA followed by Bonferroni correction post-hoc test. (**p*<0.05, and ***p*<0.01 vs Ctrl; $^{\#}$p<0.05, TNFα + *CA*de vs TNFα).

5.10 *CA*de prevented TNFα-induced dysregulation of *miR-155*, *C/ebpβ*, and *Creb* expression during the early stage of adipogenesis in 3T3-L1 cells

Up-regulation of *miR-155* and suppression of *C/ebpβ* and *Creb* expression were identified as one of the mediators of the TNFα-dependent inhibition of adipogenesis [70, 71]. We thus evaluated whether *CA*de may counteract these anti-adipogenic effects of TNFα during the early time points upon adipogenic induction of 3T3-L1 pre-adipocytes. As expected, TNFα already at 15 min from adipogenic induction induced a 30% increase in *miR-155* expression, whose level remained elevated at 30 min, 1, 2, and 4 h upon AS stimulation compared to control 3T3-L1 cells at time 0 (Figure 16A). Consistent with this, TNFα treatment also decreased the expression of *C/ebpβ* by about 50% at 30 min, 42% at 1 h, 66% at 2 h, and 35% at 4 h upon AS stimulation (Figure 16B) and *Creb* by about 52% at 30 min and 62% at 1 h upon AS stimulation (Figure 16C). Interestingly, the co-treatment with TNFα and *CA*de down-regulated *miR-155* expression during the early time points of adipogenic induction to levels comparable to AS-treated control cells (Figure 16A). At the same time points upon adipogenic induction, an up-regulation of both *C/ebpβ* (Figure 16B) and *Creb* (Figure 16C) mRNA expression, to levels comparable to those in AS-treated control cells, was observed in the TNFα + *CA*de-treated cells. Altogether, these findings suggest that *CA*de weakened the inhibitory effect of TNFα on the adipogenesis of 3T3-L1 cells by restoring the AS effects on *miR-155* down-regulation and *C/ebpβ* and *Creb* gene up-regulation at the early differentiation time points.

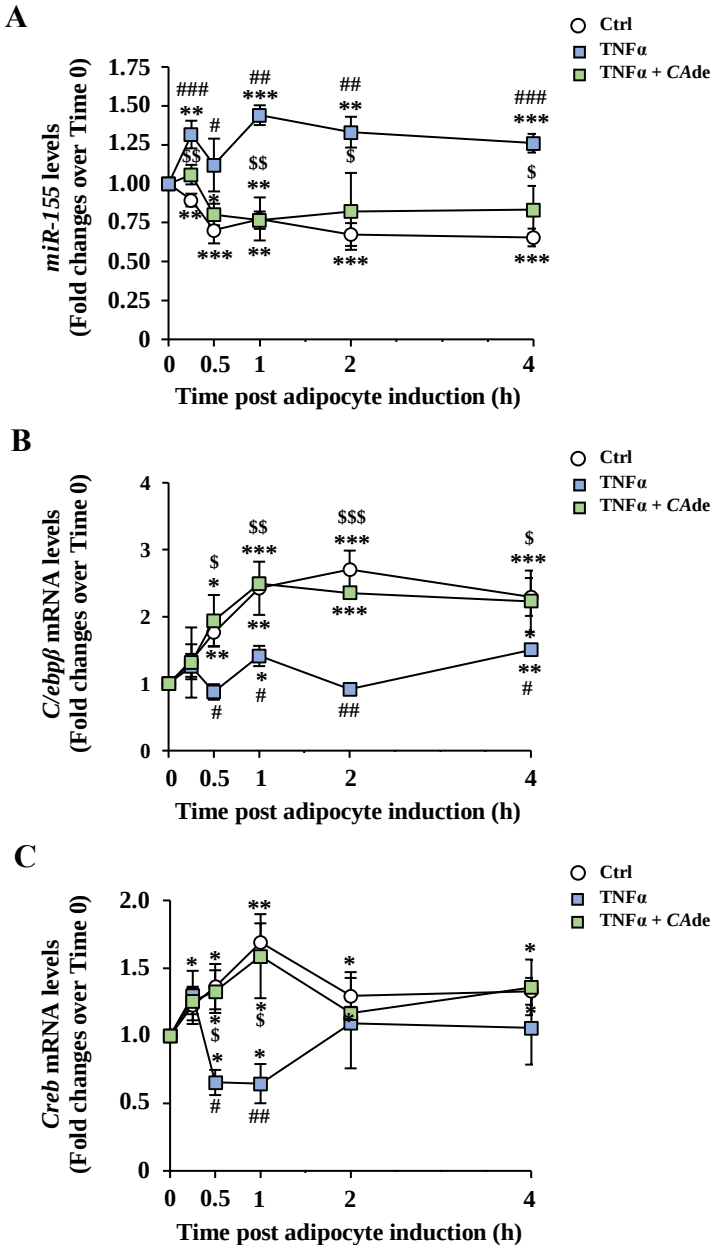

Figure 16. Effect of CAde on the expression of miR-155 and its target genes, C/ebpβ and Creb, during the early stage of adipogenesis in 3T3-L1 cells exposed to TNFα. 3T3-L1 pre-adipocytes were cultured for 0.25, 0.5, 1, 2, and 4 h with AS (Ctrl) or AS supplemented with TNFα (1 ng/mL) ± *CA*de (100 µg/mL). The miRNA and gene expressions were evaluated by quantitative real-time PCR. Time course of *miR-155* **(A)**, *C/ebpβ* **(B)**, and *Creb* **(C)** levels in Ctrl, TNFα-, and TNFα + *CA*de-treated cells are shown relative to untreated 3T3-L1 cells at time 0. Values are means ± SEM of three independent experiments. Control value at time 0 was set as 1.00. Statistical significances among groups were tested by one-way ANOVA followed by Bonferroni correction post-hoc test (*p<0.05, **p<0.01, and ***p<0.001 vs untreated 3T3-L1 cells at time 0; $^{\#}p$<0.05, $^{\#\#}p$<0.01 and $^{\#\#\#}p$<0.001 vs control 3T3-L1 cells; $^{\$}p$<0.05, $^{\$\$}p$< 0.01, $^{\$\$\$}p$<0.001 vs TNFα-treated 3T3-L1 cells).

6. Discussion and Conclusions

WAT, consisting of several cell types including mature adipocytes, is one of the largest organs in humans which mainly works as reserve of lipids to store and mobilize according to the energy needs, and as endocrine organ contributing to the complex homeostatic regulation of the energy intake, and of the glucose and lipid metabolisms [2, 154]. Several evidence sustains that its dysfunction contributed to the development of metabolic disorders, such as T2D, as well as, other pathologies, like atherosclerosis and cardiovascular disease [38, 155, 156]. Therefore, the generation of pharmaceutical and/or nutraceutical compounds targeted to improve WAT functional capacity represents an alternative and complementary strategy for the prevention and/or treatment of its related disorders.

In this context, in the first part of my PhD program I have intensely studied the potential nutraceutical effects of *CA*de, a preparation of dry extracts obtained from *Citrus Aurantium* L. fruit juice *in vitro*, on the regulation of 3T3-L1 cells adipocyte differentiation and function. My study revealed that the treatment of 3T3-L1 pre-adipocytes with *CA*de enhances adipogenesis *in vitro*, as shown by the increased TG and lipid accumulation and by the increased gene expression of the adipocyte-specific markers, *C/ebpβ*, *Pparγ*, *Glut4* and *Fabp4*. Furthermore, I also disclosed that *CA*de treatment is able to robustly improve the 2-DG uptake upon insulin stimulation. I have furthermore demonstrated that *CA*de improves adipocyte differentiation and function of the 3T3-L1 cells by acting on the early stages of adipogenesis. Indeed, when adipogenesis was investigated culturing pre-adipocytes with *CA*de at the temporal windows, from D0 to D2 and from D2 to D8, only the treatment with dry extracts for the first 48 h was sufficient to increase the expression of *Pparγ*, *Glut4* and *Fabp4*, to sustain the terminal adipocyte differentiation and to nurture the glucose uptake to levels comparable to that observed into cells exposed to a continuous treatment with *CA*de. Several evidence nowadays indicates that the transcription factor *C/ebpβ* plays a pivotal role in the regulation of the adipogenesis and its activation is a prerequisite for the initiation of the MCE in the adipocyte-differentiation program [155-160]. Accordingly with the latter observations, here I also demonstrated in 3T3-L1 pre-adipocytes that *CA*de strongly up-regulates, simultaneously with the CREB activation and binding to the *C/ebpβ* gene promoter, the *C/ebpβ* expression at 2, 4 and 8 h post the adipogenic induction and drives cell cycle progression during MCE at 14-16 h post AS treatment. Therefore, based on these findings the effects of *CA*de during early adipogenesis is, at least in part, due or associated with the specific activation of *C/ebpβ*. Finally, I have also evaluated the hypothesis that the effects of *CA*de on *C/ebpβ* gene expression may be dependent on the activity of specific molecules largely abundant within *CA*de, such as the O-glycoside flavanones narirutin and hesperidin and the C-glycoside flavone vicenin-2. However, when the effect of each one of these three flavonoids on *C/ebpβ* gene regulation was investigated, none of them reached effects comparable to *CA*de, supporting the concept that

the beneficial effects of *CA*de on adipocyte differentiation capacity are attributable to the specific and unique distribution of bioactive compounds within *CA*de.

In the second part of my PhD program, I have reported new evidence that sustains nutraceutical beneficial effects of *CA*de on the regulation of miRNA expression and function *in vitro* in 3T3-L1 pre-adipocytes. miRNAs have indeed emerged to contribute to the metabolic abnormalities associated with obesity and obesity-related complications by particularly affecting the function of the white adipose tissue (WAT) [161, 162]. Also, growing evidence sustains the hypothesis that dietary modulation of miRNA expression may explain in part some of the beneficial effects of nutraceuticals on health [161-165]. In particular, I demonstrated that the treatment of cells with *CA*de enhanced the down-regulation of the adipogenic suppressor *miR-155* [70, 71], as early as 15 min upon induction of adipogenesis in 3T3-L1 pre-adipocytes. This results in an up-regulation of the mRNA and protein levels of the *miR-155* target genes, *C/ebpβ* and *Creb* [70]. Specifically, *CA*de treatment further increased the expression of *C/ebpβ* mRNA and of the pro-adipogenic C/EBPβ-LAP protein isoform upon 2 h from the adipogenic induction [164]. Additionally, *CA*de further up-regulated *Creb* mRNA and protein levels. It is worth noting that *CA*de specifically modulated *miR-155* expression during the early stage of adipogenesis. Indeed, *CA*de treatment during the first 4 h post-adipogenic induction with AS did not affect the expression of other miRNAs, such as *miR-130a* and *miR-375*, whose role in adipocyte fate determination has been already demonstrated [165, 166]. I have also reported that *CA*de partially preserved adipogenesis of 3T3-L1 cells, where the expression of *miR-155* was up-regulated by mimic transfection. *CA*de did not further enhance adipogenesis of 3T3-L1 cells, where *miR-155* activity was abolished by specific hairpin inhibitor. These findings led us to suppose that the treatment with *CA*de may be effective against any micro-environmental insults, which impair adipocyte differentiation by up-regulation of *miR-155*. In accordance with this, we indeed found that the *CA*de treatment counteracted the detrimental effects of TNFα on adipogenesis. Indeed, terminal adipocyte differentiation of 3T3-L1 cells exposed to TNFα was improved by almost 50% by *CA*de and was associated with a restoring of the expression of *miR-155*, *C/ebpβ*, and *Creb* during the early stage of adipogenesis. TNFα is a pleiotropic cytokine that exerts homeostatic and pathogenic bioactivities [167]. High TNFα levels are observed in the WAT during obesity, and they have profound effects on adipocyte metabolism by impairing triglyceride synthesis and storage and inhibiting adipocyte differentiation [70]. Liu et al. have also demonstrated in 3T3-L1 pre-adipocytes that *miR-155*, whose expression is up-regulated by TNFα as early as 5 min via NFκB-p65 (nuclear factor kappa-light-chain-enhancer of activated B cells) binding to the *miR-155* promoter, mediates at least in part the TNFα-induced suppression of adipogenesis by down-regulating early adipogenic transcription factors [70]. These findings, therefore, provide the first piece of evidence for the efficacy of *CA*de treatment *in vitro* against micro-environment insults deleterious for the functional capacity of adipose cells. In

this scenario, *CA*de may ameliorate, in the early stage, the differentiation process by blocking NFκB-p65 into the cytosol and thus preventing the NFkB-p65–mediated transcription of *miR-155*. This hypothesis was indeed sustained by our preliminary data in 3T3-L1 pre-adipocytes short-term treated with TNFα, where the TNFα-induced NFκB-p65 translocation from cytosol to the nucleus was prevented by *CA*de treatment (data not shown).

In conclusion, my work reveals the nutraceutical properties of preparation of *Citrus Aurantium* L. fruit juice dry extracts (*CA*de) on the fat cell functional capacity in terms of enhanced adipocyte differentiation and function *in vitro*. Also, this study demonstrated that the down-regulation of the *miR-155*, which causes the up-regulation of the target genes, *C/ebpβ* and *Creb*, is part of the mechanism through which *CA*de enhances adipocyte differentiation of pre-adipocytes *in vitro*. Furthermore, I provide substantial evidence of this nutraceutical compound's efficacy against micro-environment insults, which are harmful to adipose cell functionality and affect *miR-155* expression, such as TNFα. The last data suggest that the development of nutraceutical products derived from *CA*de may be an effective strategy for treating adipocyte dysfunction and its related disorders.

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