

# Triiodothyronine modulates the expression of leptin and adiponectin in 3T3-L1 adipocytes

Triiodotironina modula a expressão de leptina e adiponectina em adipócitos 3T3-L1

Miriane de Oliveira<sup>1</sup>, Maria Teresa De Sábio<sup>1</sup>, Regiane Marques Castro Olimpio<sup>1</sup>, Fernanda Cristina Fontes Moretto<sup>1</sup>, Renata de Azevedo Melo Luvizotto<sup>2</sup>, Celia Regina Nogueira<sup>1</sup>

## ABSTRACT

**Objective:** To study the effect of different doses of triiodothyronine on gene expression of the adipokines leptin and adiponectin, at different times, and to evaluate the difference in expression between the two adipokines in each group. **Methods:** 3T3-L1 adipocytes were incubated with triiodothyronine at physiological dose (10nM) and supraphysiological doses (100nM or 1,000nM), or without triiodothyronine (control, C) for 0.5, 6, or 24 hours. Leptin and adiponectin mRNA was detected using real-time polymerase chain reaction (RT-PCR). One-way analyses of variance, Tukey's test or Student's *t* test, were used to analyze data, and significance level was set at 5%. **Results:** Leptin levels decreased in the 1,000nM-dose group after 0.5 hour. Adiponectin levels dropped in the 10nM-dose group, but increased at the 100nM dose. After 6 hours, both genes were suppressed in all hormone concentrations. After 24 hours, leptin levels increased at 10, 100 and 1,000nM groups as compared to the control group; and adiponectin levels increased only in the 100nM group as compared to the control group. **Conclusion:** These results demonstrated fast actions of triiodothyronine on the leptin and adiponectin expression, starting at 0.5 hour, at a dose of 1,000nM for leptin and 100nM for adiponectin. Triiodothyronine stimulated or inhibited the expression of adipokines in adipocytes at different times and doses which may be useful to assist in the treatment of obesity, assuming that leptin is increased and adiponectin is decreased, in obesity cases.

**Keywords:** Leptin; Adiponectin; Triiodothyronine; Adipocytes

## RESUMO

**Objetivo:** Examinar o efeito de diferentes doses de triiodotironina sobre a expressão gênica das adipocinas leptina e adiponectina,

em diferentes períodos de tempo, além de avaliar a diferença de expressão entre as duas adipocinas em cada grupo. **Métodos:** Adipócitos 3T3-L1 foram incubados com triiodotironina nas doses fisiológica (10nM) e suprafisiológicas (100nM ou 1.000nM), ou na ausência de triiodotironina (controle, C) durante 0,5, 6 ou 24 horas. O mRNA das adipocinas foi analisado em tempo real, utilizando a reação em cadeia de polimerase. Para as análises dos dados, foi utilizada a análise de variância, complementada com o teste de Tukey, ou o teste *t* de Student com 5% de significância. **Resultados:** Os níveis de leptina diminuíram no grupo com dose de 1.000nM em 0,5 hora. A adiponectina também diminuiu no grupo com dose de 10nM, porém se elevou com a dose de 100nM. Após 6 horas, ambos os genes foram suprimidos em todas concentrações de hormônio. Em 24 horas, os níveis de leptina foram elevados em 10, 100 e 1.000nM, em relação ao grupo controle. No que concerne à adiponectina, observou-se aumento apenas no grupo cuja dose foi de 100nM, em comparação ao controle. **Conclusão:** Foram demonstradas ações rápidas da triiodotironina sobre a expressão da leptina e da adiponectina, iniciando em 0,5 hora na dose de 1.000nM, para a primeira, e na dose de 100nM, para a segunda. A triiodotironina estimulou ou inibiu a expressão de adipocinas em adipócitos em diferentes tempos e doses, o que pode auxiliar no tratamento da obesidade, levando em consideração que, nesta, a leptina está aumentada e adiponectina, diminuída.

**Descritores:** Leptina; Adiponectina; Tri-iodotironina; Adipócitos

## INTRODUCTION

The effects of thyroid hormones (THs) are especially important during development, since they regulate the growth and maturation of different organs and tissues during fetal and neonatal life.<sup>(1,2)</sup> Many tissues are

<sup>1</sup> Universidade Estadual Paulista "Júlio de Mesquita Filho", Botucatu, SP, Brazil.

<sup>2</sup> Universidade Federal de Mato Grosso, Cuiabá, MT, Brazil.

Corresponding author: Miriane de Oliveira – Distrito de Rubião Jr., s/n – Distrito Rubião Jr – Zip code: 18618-000 – Botucatu, SP, Brazil – Phone: (55 14) 3881-6213 – E-mail: miriane.deoliveira@yahoo.com.br

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regulated by THs to their full development, including effects on gene groups involved in the differentiation process. Like other tissues, the adipose tissue (AT) is an important target for THs,<sup>(3)</sup> because it specializes in transport, synthesis, storage and mobilization of lipids. Its primary function is the storage of energy in the form of triglycerides, providing a reserve of energy to be used in times of caloric deprivation.<sup>(4)</sup> The AT is the largest endocrine organ in the whole body, secreting hormones, chemokines and adipokines, which are important paracrine/endocrine regulators.<sup>(5)</sup>

THs, especially triiodothyronine (T3), modulate the proliferation and differentiation of adipocytes<sup>(6)</sup> and are involved in cellular processes such as signal transduction, apoptosis, and inflammatory response.

Leptin and adiponectin are adipokines synthesized and secreted by the AT. Leptin acts mainly on the central nervous system, producing anorectic effects and stimulating energy expenditure.<sup>(7)</sup> Adiponectin is involved in important metabolic effects, such as stimulating fatty acid oxidation, reducing gluconeogenesis and increasing thermogenesis.<sup>(8-11)</sup>

Obese humans have high serum levels of leptin. Leptin concentrations are directly proportional to body fat mass, specifically the volume of adipocytes.<sup>(12,13)</sup> Regarding THs, there is evidence indicating that human obesity is usually associated with increased levels of thyroid stimulating hormone (TSH) and T3.<sup>(14,15)</sup> As in mice, human studies came to conflicting results on the effect of THs on leptin concentrations. In subjects with hypothyroidism, leptin was found increased,<sup>(16,17)</sup> decreased<sup>(18,19)</sup> or unchanged<sup>(20,21)</sup> when compared with euthyroid individuals. The same controversial results are found in studies with hyperthyroidism patients.<sup>(16-22)</sup>

The administration of T3 in hypothyroid rats decreased the messenger RNA (mRNA) expression of leptin in the AT and circulating levels of leptin.<sup>(23)</sup> However, in other studies, THs increased leptin in adipocytes differentiated from 3T3-L1 cells.<sup>(24)</sup> Studies in humans did not show conclusive evidence on the relationship between THs and leptin levels.<sup>(25,26)</sup>

Adiponectin shares some physiological effects with THs, such as reducing body fat, increasing thermogenesis and promoting lipid oxidation.<sup>(27)</sup>

The interaction between THs and adiponectin concentrations is still undefined. Some studies suggest that thyroid function has influence on its serum levels. Some authors have reported that the concentration of this adipokine is higher in hyperthyroidism, when compared with hypothyroidism in patients with Graves' disease<sup>(28)</sup> and in euthyroid patients with Basedow's disease.<sup>(29)</sup>

Studies on a possible relation between adiponectin and deviations in lipid metabolism associated with thyroid dysfunction are scarce. Patients with hyperthyroidism showed increased body weight, cholesterol and body mass index after controlling for thyrotoxicosis. After adjusting adiponectin levels for body mass index, no significant change was observed in patients with hyper- and hypothyroidism, suggesting that THs play a small role in the modulation of adiponectin levels.<sup>(30)</sup>

THs act by increasing the metabolic rate and oxygen consumption, regulating heat production and energy supply; leptin and adiponectin are involved in the regulation of energy balance.<sup>(31)</sup>

Conflicting results may be explained by the existence of many factors influencing the levels of leptin, adiponectin and THs, and more studies are needed to fully explain the relation between leptin, adiponectin and THs.

The biological roles of THs, leptin and adiponectin intersect at regulation of energy expenditure, and this provides a way to study the response of the AT to T3 without the interference of systemic factors. Therefore, we evaluated the effects of different doses of T3 at different timepoints on the levels of gene expression of leptin and adiponectin, *in vitro*, in 3T3-L1 cells differentiated into adipocytes. Both adipokines are modulated by T3 over short or long periods, increasing or decreasing, according to the dose of this hormone.

Our study aims to demonstrate that physiological doses of T3 act by decreasing gene expression of adiponectin and increasing that of leptin. However, supraphysiological doses of T3 act over longer time frames, increasing the concentration of leptin and decreasing that of adiponectin. Our results confirm the action of T3 on the AT at the physiological dose and allow for further studies on the use of T3 in the treatment of obese patients. As shown in the literature, after losing 5-10% of weight, weight loss becomes more difficult,<sup>(32)</sup> and patients show low serum T3 levels.<sup>(33)</sup> Therefore, our findings could warrant the administration of physiological doses of the hormone at this stage of the treatment.

## OBJECTIVE

To study the effect of different doses of triiodothyronine on gene expression of the adipokines leptin and adiponectin over different time periods, and assess the difference in expression between the two adipokines in each group.

## METHODS

### Cell culture and differentiation

The experimental protocol was approved by the Ethics Committee for Animal Studies of the *Faculdade de Medicina de Botucatu da Universidade Estadual Paulista "Júlio de Mesquita Filho"*, under number 752.

For the *in vitro* study, we used the cell line 3T3-L1. These cells were obtained from the Cell Bank of the *Universidade Federal do Rio de Janeiro* (UFRJ) and cultured as described in the literature,<sup>(34)</sup> in Dulbecco's modified medium (DMEM; Gibco®) supplemented with 10% fetal bovine serum (FBS; Gibco®), 1% antibiotic/antimycotic (Sigma®), under an atmosphere of 5% carbon dioxide (CO<sub>2</sub>) at 37°C. The cells were kept under culture in said conditions until reaching a confluence of approximately 100%, and then were transferred to six-well plates for the experiments. After reaching 100% confluence in the wells, the cells were subjected to differentiation. They were kept for 3 days in DMEM containing 10% FBS, 100mM 1-methyl-3-isobutylxanthine (IBMX; Sigma®), 1mM dexamethasone (Sigma®) and 5mg/L insulin (Sigma®). After this period, the cells were left for 7 days in DMEM containing 10% FBS and 5mg/mL insulin. After cell differentiation, adipocytes were subjected to TH depletion for 36 hours in DMEM supplemented with Charcoal-Stripped Fetal Bovine Serum (Sigma®). After TH depletion, the cells were treated with T3 at the physiological dose (10nM, designated F) or supraphysiological doses (100nM and 1,000nM, designated SI and SII, respectively) for 0.5, 6 and 24 hours. The group not treated with T3 was used as control (C).

### Oil Red O Staining

After 10 days of differentiation, the culture medium was removed from the cells and they were washed twice with phosphate-buffered saline (PBS). Thereafter, we added 1mL formaldehyde, in which the cells were left for 30 minutes at room temperature. After this time, the cells were washed three times with PBS. Then we added 300μL *Oil Red O* (Sigma®), and the cells were incubated for 2 hours at 37°C. After this period, they were again washed three times with distilled water and placed in oven to dry. The cells were observed under a microscope for verification of the differentiation by red staining of adipose cells.

### Gene expression

Total RNA was extracted from 3T3-L1 cells using TRIzol® (Invitrogen®) as reagent, according to the manufacturer's

instructions. The High Capacity cDNA kit for reverse transcription in real-time polymerase chain reaction (RT-PCR, Invitrogen, São Paulo, Brazil) was used for the synthesis of 20μL complementary DNA (cDNA) from 1,000ng total RNA.

The levels of adiponectin (Applied Biosystems assay Mm00456425\_m1) and leptin (Applied Biosystems assay Mm00434759\_m1) were analyzed by real-time PCR (RT-PCR). Analyzes were performed on *Applied Biosystems StepOne Plus*, a detection system that uses the Taqman qPCR commercial kit (Invitrogen) according to the manufacturer's instructions. The amplification conditions were as follows: enzyme activation at 50°C for 2 minutes; denaturation at 95°C for 10 minutes; cDNA products amplified with 40 denaturation cycles at 95°C for 15 seconds; and annealing/extension at 60°C for 1 minute.

After normalization to the internal control, cyclophilin (assay Mm00434759\_m1),<sup>(34)</sup> using the 2<sup>-ΔΔCt</sup> method as previously described,<sup>(35)</sup> the mRNA expression of leptin or adiponectin was evaluated for comparison between the values of Group C and the treatment groups (F, SI, SII), or comparison of the difference between expression of leptin and adiponectin within the same group. Relative quantification of gene expression was performed with the comparative Cq method.<sup>(35)</sup>

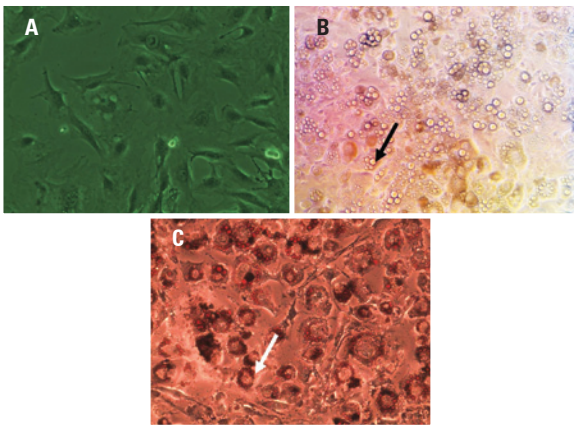
### Statistical analysis

The differences between mRNA levels of leptin and adiponectin in each group, whether or not treated, were analyzed by the Student *t* test. The differences in expression of the gene for leptin or adiponectin, at different T3 doses in each timepoint were assessed by analysis of variance (ANOVA) followed by Tukey's test. Data were expressed as mean ± standard deviation. The level of significance was set at 5%.

## RESULTS

### 3T3-L1 Cell culture and differentiation

Figure 1A shows 3T3-L1 cells prior to differentiation. In the presence of the differentiation solution (insulin, dexamethasone and IBMX), preadipocytes developed the morphology of mature adipocytes (Figures 1B and 1C), with primary features, including a large number of cytoplasmic lipid droplets. Staining with *Oil Red O* highlighted the lipid droplets in red (Figure 1C).



**Figure 1.** 3T3-L1 cells before and after differentiation into adipocytes. (A) Non-differentiated cells. (B) Cells after 10 days of differentiation. (C) Cells stained with *Oil Red O* after 10 days of differentiation. The arrows show the adipocyte with cytoplasmic lipid droplets

### Level of expression of adipokines in 3T3-L1 adipocytes

Table 1 shows the difference in expression levels between adipokines leptin and adiponectin for all groups at different timepoints. Adiponectin, whether with or without treatment, showed higher expression levels than leptin.

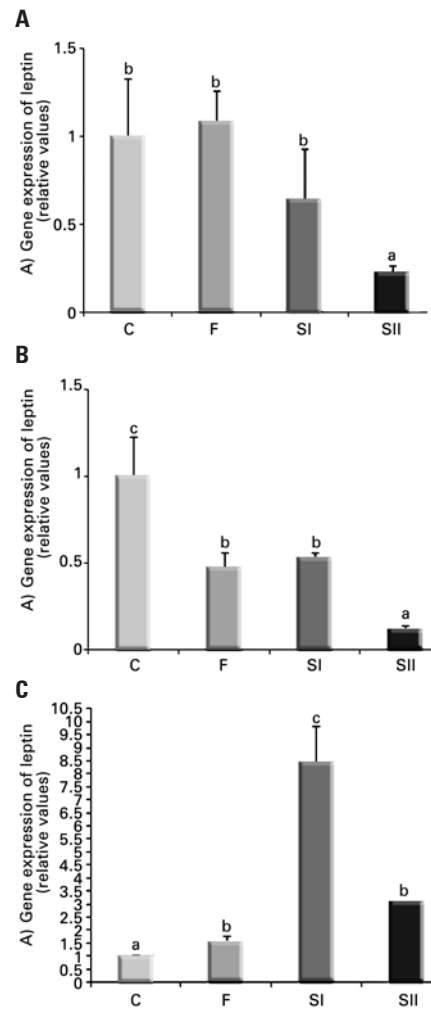
**Table 1.** Adiponectin versus leptin at different timepoints within each group in the absence (control) or presence (10nM, 100nM and 1,000nM) of triiodothyronine

	Groups according to timepoints	Adiponectin M ± SD	Leptin M ± DP	p value
0.5hr	C	1.00±0.18	0.000034±0.000003	<0.001
	F	0.28±0.08	0.000032±0.000005	0.005
	SI	3.08±0.24	0.000017±0.000003	<0.001
	SII	1.35±0.10	0.000007±0.000001	<0.001
6hr	C	1.00±0.15	0.000033±0.000008	<0.001
	F	0.23±0.05	0.000016±0.000003	0.002
	SI	0.27±0.03	0.000018±0.000001	<0.001
	SII	0.28±0.05	0.000004±0.000008	<0.001
24hr	C	1.00±0.17	0.000029±0.000001	<0.001
	F	1.18±0.12	0.000045±0.000007	<0.001
	SI	1.75±0.45	0.000248±0.000042	0.003
	SII	0.71±0.14	0.000089±0.000001	<0.001

The mRNAs for adiponectin and leptin were analyzed by real-time polymerase chain reaction. Data expressed as mean ± standard deviation. Comparison of adiponectin versus leptin levels, within each group, with the Student t test. All assays were performed in triplicate (n = 3 for each treatment). M: mean; SD: standard deviation; C: control; F: triiodothyronine at 10nM; SI: triiodothyronine at 100nM; SII: triiodothyronine at 1,000nM.

### Different doses of triiodothyronine suppress mRNA levels for leptin at 0.5 and 6 hours, but supraphysiological doses increase levels at 24 hours

Figure 2 shows the modulation of mRNA levels for leptin in 3T3-L1 adipocytes, in the absence (C) or presence (F, SI, SII, respectively) of T3 at different timepoints (0.5, 6 and 24 hours) by RT-PCR.

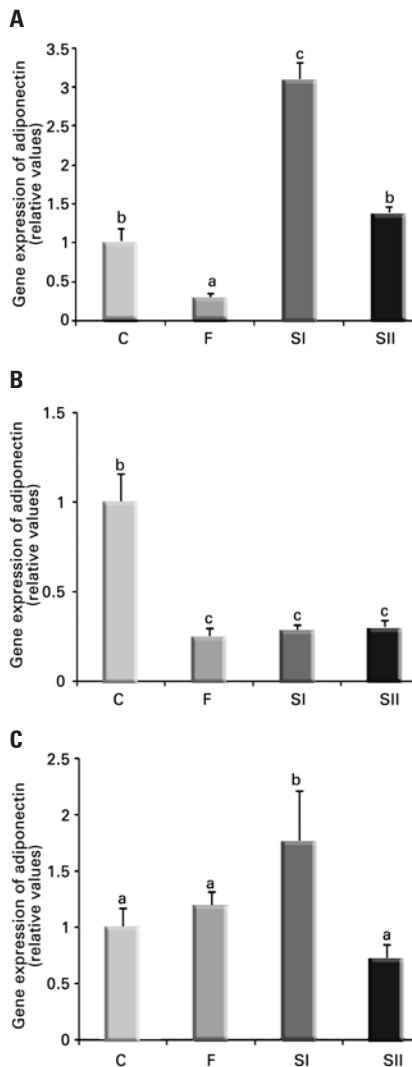


**Figure 2.** Effect of different doses of triiodothyronine on gene expression of leptin at 0.5 hour (A), 6 hours (B) and 24 hours (C). Data expressed as mean ± standard deviation. We used analysis of variance, followed by Tukey's test. Same letters represent  $p > 0.05$ ; different letters represent  $p < 0.05$ . Comparisons of Groups F, SI or SII versus C, and F versus SII; F versus SII; SI versus SII. n=3 for each treatment. C: Control; F: triiodothyronine at 10nM; SI: triiodothyronine at 100nM; SII: triiodothyronine at 1,000 nM

Figure 2A shows the suppression of mRNA levels for leptin by T3 starting at 0.5 hour in SII compared with Groups C, F and SI. At 6 hours of incubation, there was a decrease in leptin mRNA in all treatment groups and this suppression was more pronounced in SII, compared with F and SI (Figure 2B). At 24 hours of incubation, leptin was increased in F, SI and SII compared with C, and the increase in SI was more pronounced than in F or SII (Figure 2C).

### T3 modulates mRNA levels for adiponectin starting at 0.5 hour at different doses

Figure 3 shows the modulation of mRNA levels for adiponectin in 3T3-L1 adipocytes, in the absence (C) or presence (F, SI, SII, respectively) of T3 at different



**Figure 3.** Effect of different doses of triiodothyronine on gene expression of adiponectin at 0.5 hour (A), 6 hours (B) and 24 hours (C). Data expressed as mean  $\pm$  standard deviation. We used analysis of variance, followed by Tukey's test. Same letters represent  $p > 0.05$ ; different letters represent  $p < 0.05$ . Comparisons of Groups F, SI or SII versus C, and F versus SI; F versus SII; SI versus SII.  $n = 3$  for each treatment. C: Control; F: triiodothyronine at 10nM; SI: triiodothyronine at 100nM; SII: triiodothyronine at 1,000nM

timepoints (0.5, 6 and 24 hours) by RT-PCR. At 0.5 hour, there was suppression of adiponectin expression in F and increase in SI when compared to C (Figure 3A). At 6 hours of incubation, the adiponectin gene expression was decreased by all doses (F, SI and SII) when compared with the control group (Figure 3B). At 24 hours, adiponectin returned to Group C levels in groups F and SII, and was increased in SI (Figure 3C).

## DISCUSSION

Leptin and adiponectin differ from most other adipokines, because they are secreted exclusively by adipocytes. Although the details on all factors regulating

their synthesis, secretion and clearance are not entirely known, plasma leptin levels increase in proportion to fat mass, while adiponectin levels decrease with weight gain.<sup>(36)</sup> Leptin and adiponectin are involved in the regulation of energy balance, just like THs.<sup>(31)</sup>

We observed that adiponectin had higher expression than leptin in 3T3-L1 adipocytes, irrespective of the presence or absence of T3. This result can be explained by the fact that the 3T3-L1 adipocytes in this study did not represent a state of obesity. Obesity is based on an inflammatory process beginning with the increase of AT reserves, with hypertrophy and hyperplasia of this tissue, leading to the production of different proinflammatory molecules by both adipocytes themselves and the extracellular matrix that surrounds them. This, in turn, leads to the development of systemic low-grade inflammation.<sup>(37)</sup> In obesity, leptin production is also increased<sup>(38,39)</sup> while adiponectin expression is decreased, and consequently so are its anti-inflammatory, anti-atherogenic and insulin-sensitivity-promoting properties.<sup>(40,41)</sup>

Some studies have found a negative correlation between THs and leptin<sup>(42,43)</sup> while others have found an association.<sup>(44)</sup> Wang et al.<sup>(45)</sup> reported that although leptin and THs can act through the same pathways to regulate energy metabolism, the effects of leptin on metabolism do not depend on the presence of THs. However, leptin and THs may share some target sites and, therefore, have additive effects.

Our findings are in agreement with Yoshida et al.,<sup>(24)</sup> who observed modulation of leptin by T3 at 24 hours in 3T3-L1 adipocytes for all doses tested; however, our results showed that starting at 0.5 hour, T3 was already affecting the mRNA levels of this gene, which was suppressed by supraphysiological doses in SII. Previous studies by our group demonstrated an increase in leptin, both mRNA and protein, at 1 hour of incubation.<sup>(34)</sup> Interestingly, at 6 hours of incubation, leptin is suppressed by the different doses of T3 administered to Groups F, SI and SII. This decrease is more pronounced for the 1,000nM dose, at which leptin expression decreases as the T3 concentration increases, just as observed in studies by Zabrocka et al.<sup>(43)</sup> and Pinkney et al.<sup>(16)</sup>

In previous studies we demonstrated in rats subjected to food restriction that the physiological dose of T3 (0.5 $\mu$ g/100g) increased leptin expression, which translates into this being the dose required for proper expression of leptin in obese animals treated with caloric restriction.<sup>(46)</sup> However, obese animals in a state of hyperthyroidism (25 $\mu$ g T3 per 100g weight) showed decreased leptin levels, as well as animals subjected to

food restriction in the same hyperthyroid conditions.<sup>(47)</sup> These results are in line with the findings of this study for the SII dose (1,000nM T3) at 0.5 hour, and for the tested treatments at 6 hours.

As for adiponectin, some studies suggest that thyroid function may influence its serum levels. Some authors reported that the concentration of this adipokine was higher in hyperthyroidism, compared to euthyroid and hypothyroid conditions.<sup>(28,29)</sup> This corroborates the results of our study, demonstrating that T3 affects the levels of adiponectin starting at 0.5 hour, with an increase in SI and decrease in F.

Just like leptin at 6 hours, adiponectin was suppressed by all doses tested and, at 24 hours, levels returned to C values or exceeded normal values, such as in Group SI. Fasshauer et al.,<sup>(48)</sup> using the same experimental model, observed no change in adiponectin levels at 16 hours of incubation with the same dose administered to Group SII, which demonstrated that T3 effects fluctuate over time.

Our results corroborate those of Saito et al.<sup>(29)</sup> and Yaturu et al.<sup>(28)</sup> Adiponectin was found increased in the groups receiving supraphysiological doses of T3, mimicking hyperthyroidism. Cabanelas et al.<sup>(49)</sup> concluded in their study that THs regulate mRNA expression of adiponectin in a tissue-specific fashion, without changing its secretion in the white AT.

Previous studies by our group showed that obese animals had decreased serum adiponectin and AT mRNA, and decreased leptin when compared with the control. Interestingly, the administration of supraphysiological T3 (25µg/100g) decreased body fat mass, serum levels of adiponectin and leptin, and mRNA.<sup>(50)</sup> However, in contrast, an experimental study in rats with hyperthyroidism showed a significant increase in serum adiponectin.<sup>(51)</sup> The findings of this *in vitro* study indicated that increase or decrease in leptin or adiponectin expression by T3 are dose- and time-dependent.

The results presented are important for understanding at which dose (physiological or supraphysiological) and over which period of time the modulation of adipokines leptin and adiponectin is triggered by T3 in mature adipocytes. It is now possible to aim for further studies focusing on the treatment of obesity with TH analogs, considering their ability to modulate the genes approached in this study.

## CONCLUSION

Our results demonstrated rapid effects of triiodothyronine on the expression of leptin and adiponectin, starting at 0.5 hour at 1,000nM for leptin, and 100nM for adiponectin.

Triiodothyronine stimulated or inhibited the expression of adipokines in adipocytes at different timepoints and doses, which can be helpful to assist in the treatment of obesity, considering that in this condition, leptin is increased and adiponectin decreased.

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