

Original Paper

# Adipose TSHB in Humans and Serum TSH in Hypothyroid Rats Inform About Cellular Senescence

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## Key Words

Thyroid-stimulating hormone • Euthyroidism • Hypothyroidism • Adipose tissue • Cellular senescence

## Abstract

**Background/Aims:** Thyroid hormones have been recently linked to senescence and longevity. Given the recent description of *TSHB* mRNA in human adipose tissue (AT), we aimed to investigate the relationship between local AT TSH and adipose tissue senescence. **Methods:** *TSHB* mRNA (measured by real-time PCR) and markers of adipose tissue senescence [*BAX*, *DBC1*, *TP53*, *TNF* (real-time PCR), telomere length (Telo TAGGG Telomere Length Assay) and lipidomics (liquid chromatography mass spectrometry)] were analysed in subcutaneous (SAT) and visceral (VAT) AT from euthyroid subjects. The chronic effects of TSH were also investigated in AT from hypothyroid rats and after recombinant human TSH (rhTSH) administration in human adipocytes. **Results:** Both VAT and SAT *TSHB* gene expression negatively correlated with markers of AT cellular senescence (*BAX*, *DBC1*, *TP53*, *TNF* gene expression and specific glucosylceramides) and positively associated with telomere length. Supporting these observations, both rhTSH administration in human adipocytes and increased TSH in hypothyroid rats resulted in decreased markers of cellular senescence (*Bax* and *Tp53* mRNA) in both gonadal and subcutaneous white adipose tissue. **Conclusion:** These data point to a possible role of TSH in AT cellular senescence.

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## Introduction

Thyroid function is well known to be involved in longevity and senescence [1-9] and in energy balance [10]. Different animal models characterized by increased longevity had in common the following hormonal and metabolic characteristics: 1) reduced thyroid function (absent TSH and severely reduced thyroid hormone in Ames and Snell dwarf mice, in naked mole-rat and calorie-restricted rats), 2) reduced basal metabolic rate, and 3) reduced core body temperature. A causal relationship between thyroid function and longevity was further demonstrated by experimental induction of hypothyroidism in rats, which resulted in extended life span. Conversely, inducing hyperthyroidism resulted in significantly shorter life span [4-6].

In humans, the relationship between hypothyroidism and increased life expectancy has also been observed [4-9]. Euthyroid 50 year-old participants with increased levels of TSH had a longer life expectancy than participants with decreased serum TSH levels [4]. Increased TSH levels were also associated with lower mortality in participants between ages 85 and 90 [5] and in their offspring [6], and even in centenarians [7, 8]. In fact, increased familial longevity is characterized by higher TSH secretion, without altering whole body energy metabolism [9].

Adipose tissue cellular senescence is characterized by increased expression of apoptotic and senescence markers (caspase induction leading to increased *BAX*, *TP53* and *DBC1* expression), decreased telomere length [11-15], and impaired capacity to generate new adipocytes and mitochondrial dysfunction [12-14].

We hypothesized that TSH might modulate AT senescence. Given the recent description of *TSHB* mRNA in human AT [16], the potential associations of *TSHB* mRNA with markers of AT senescence were explored. Then, the impact of recombinant human (rh)TSH in human adipocytes and of chronic serum TSH elevation (induced by aminotriazole) on AT senescence in rats was evaluated.

## Materials and Methods

### Patient recruitment

Forty-five paired SAT and VAT samples from euthyroid morbidly obese ( $\text{BMI} > 35 \text{ kg/m}^2$ ) subjects (Cohort 1) were studied. All these subjects were recruited at the Endocrinology Service of the Hospital of Girona “Dr Josep Trueta”, were of Caucasian origin and reported that their body weight had been stable for at least three months before the study. Subjects were studied in the post-absorptive state. BMI was calculated as weight (in kg) divided by height (in m) squared. Patients had no systemic disease other than obesity and all were free of any infections in the previous month before the study. Liver diseases (specifically tumoral disease and HCV infection) and thyroid dysfunction were specifically excluded by biochemical work-up. All subjects gave written informed consent, validated and approved by the Ethical Committee of the Hospital of Girona “Dr Josep Trueta”, after the purpose of the study was explained to them. Samples and data from patients included in this study were partially provided by the FATBANK platform promoted by the CIBEROBN and coordinated by the IDIBGI Biobank (Biobanc IDIBGI, B.0000872), integrated in the Spanish National Biobanks Network and they were processed following standard operating procedures with the appropriate approval of the Ethics, External Scientific and FATBANK Internal Scientific Committees.

### Adipose tissue handling

Adipose tissue samples were obtained from SAT and VAT depots during elective surgical procedures (cholecystectomy, surgery of abdominal hernia and gastric by-pass surgery). Samples of adipose tissue were immediately transported to the laboratory (5-10 min). The handling of tissue was carried out under strictly aseptic conditions. Adipose tissue samples were washed in PBS, cut off with forceps and scalpel into small pieces (100 mg), and immediately flash-frozen in liquid nitrogen before being stored at  $-80^\circ\text{C}$ .

## *In vitro experiments*

Isolated human visceral preadipocytes from obese subjects (BMI > 30) were purchased in a specialized company (Zen-Bio Inc., Research Triangle Park, NC, USA) and were cultured (~40,000 cells/cm<sup>2</sup>) with pre-adipocyte medium (Omental/Visceral Preadipocyte Medium, Item# OM-PM, Zen-Bio Inc.) composed of DMEM/Nutrient Mix F-12 medium (1:1, v/v), HEPES, FBS, Penicillin and Streptomycin in a humidified 37°C incubator with 5% CO<sub>2</sub>. Adipocyte differentiation was performed as described elsewhere [17]. Briefly, twenty-four hours after plating, cells were checked for confluence (day 0th) and differentiation was induced using differentiation medium (Omental/Visceral Preadipocyte Differentiation Medium, Item# OM-DM, Zen-Bio Inc) composed of OM-PM, human Insulin, dexamethasone (DXM), isobutylmethylxanthine (IBMX) and PPAR $\gamma$  agonists (rosiglitazone). After 7 days (day 7), OM-DM was replaced with fresh adipocyte medium (Omental/Visceral Adipocyte Maintenance Medium, Item# OM-AM, Zen-Bio Inc.) composed of DMEM/Nutrient Mix F-12 medium (1:1, v/v), HEPES, FBS, Biotin, panthothenate, human insulin, DXM, Penicillin, Streptomycin and amphotericin. Fourteen days after the initiation of differentiation, cells appeared rounded with large lipid droplets apparent in the cytoplasm. Cells were then considered mature adipocytes. Recombinant human TSH $\alpha/\beta$  (rhTSH, 1 and 10 mU/l) was administrated on differentiated visceral adipocytes during 2 and 7 days. Then, cells were harvested and stored at -80°C for RNA extraction to study gene expression levels. The experiment was performed in triplicate for each sample.

## *Experiments in rats*

Adult male Sprague-Dawley rats (250-300 g; *Animalario General USC*; Santiago de Compostela, Spain) were housed on a 12 h light (8:00 to 20:00), 12 h dark cycle, in a temperature- and humidity-controlled room. Animals were allowed free access to standard laboratory pellets of rat chow and tap water. Hypothyroidism was induced as previously described [18-20] by administration of 0.1% aminotriazole (Sigma, St Louis MO, USA) in drinking water for a period of one, two and three weeks. Eight rats per experimental group were used. Body weight and food intake were recorded twice per week. At the end of the treatment rats were killed by cervical dislocation and the tissues quickly harvested and stored at -80 °C until processing. The experiments were performed in agreement with the International Law on Animal Experimentation and were approved by the USC Ethical Committee (Project ID 15010/14/006).

## *RNA expression*

RNA purification, gene expression procedures and analyses were carried out, as previously described [13]. Briefly, RNA purification was performed using the RNeasy Lipid Tissue Mini Kit (QIAGEN, Izasa SA, Barcelona, Spain) and the integrity was checked by the Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA). Gene expression was assessed by real-time PCR using a LightCycler<sup>®</sup> 480 Real-Time PCR System (Roche Diagnostics SL, Barcelona, Spain), using TaqMan<sup>®</sup> and SYBRgreen technology suitable for relative genetic expression quantification.

The commercially available and pre-validated TaqMan<sup>®</sup> primer/probe sets were used as follows: Peptidylprolyl isomerase A (cyclophilin A) (4333763, *PPIA* as endogenous control), thyroid stimulating hormone beta (*TSHB*, Hs02759015\_s1), tumor necrosis factor (*TNF*, Hs00174128\_m1), BCL2-associated X protein (*BAX*, Hs00180269\_m1), tumor protein p53 (*TP53*, Hs01034249\_m1) and cell cycle activator and apoptosis regulator 2 (*CCAR2* or *DBC1*, Hs00368356\_m1), and in rats, Bcl2-associated X protein (Bax, Rn01480161\_g1) and tumor protein p53 (Tp53, Rn00755717\_m1).

## *Telomere length measurement*

In a subgroup of participants (n=22) in that adipose tissue DNA was available telomere length was measured as described elsewhere [14]. Briefly, total DNA purification from subcutaneous adipose tissue was performed using the phenol-chloroform method. Frozen adipose subcutaneous tissue was homogenized with lysis buffer containing proteinase K for 30 s. The homogenate was incubated at 56 °C for 1 h and centrifuged at 13,000g at 4 °C for 15 min. The infranatant fraction was added and mixed in phenol:chloroform:isoamil solution, and centrifuged at 5,000g at 4 °C for 15 min. Total DNA was quantified by means of spectrophotometer (GeneQuant; GE Healthcare, Piscataway, NJ). Telomere length was measured by Telo TAGGG Telomere Length Assay (Roche, Penzberg, Germany), according to manufacturer's instructions. Purified genomic DNA was digested by an optimized mixture of frequently cutting restriction enzymes, which digested to low molecular weight fragments nontelomeric DNA. Following DNA digestion,

the DNA fragments were separated by gel electrophoresis and transferred to a nylon membrane by Southern blotting. The blotted DNA fragments were hybridized to a digoxigenin-labeled probe-specific for telomeric repeats and incubated with a digoxigenin-specific antibody covalently coupled to alkaline phosphatase. Finally, the immobilized telomere probe was visualized by virtue of alkaline phosphatase metabolizing CDP-Star, a highly sensitive chemiluminescent substrate. The average terminal restriction fragments length was determined by comparing the signals relative to a molecular weight standard.

#### Lipidomic analyses

Lipidomic analyses were performed as described elsewhere [16], in a subsample of 12 consecutive subjects from Cohort 1. Briefly, 35–50 mg samples of SAT (n=12) were homogenized in 20 volumes (v/w) cold methanol (with BHT 1  $\mu$ M) (1:20 w:v) at 4°C. Thereafter, we added 20 volumes of lysis buffer (containing 80 mM KCl, 5 mM EDTA, and 1 mM diethylenetriaminepentaacetic acid; pH 7.3) and 40 volumes of chloroform containing representative internal standards class lipids. The characteristics of these subjects did not differ significantly from the whole cohort. The chloroform phase was injected in a LC-MS system (UPLC 1290 couple to QTOF 6520 Agilent Technologies, Santa Clara, CA, USA). This method allows the orthogonal characterization [based on exact mass (<10 ppm) and on retention time] of lipids. When combined with internal standards, this strategy is useful for attributing potential identities with low uncertainty. Data were collected in both positive and negative electrospray ionization-Q-TOF operated in full-scan mode at 100–3000 m/z in an extended dynamic range (2 GHz), using N<sub>2</sub> as nebulizer gas (5 L/min, 300°C). The capillary voltage was 3500 V with a scan rate of 1 scan/s. Targeted lipidomic approach was chosen to detect the abundance of specific senescence-associated glucosylceramides [21].

#### Analytical methods

Serum glucose concentrations were measured in duplicate by the glucose oxidase method using a Beckman glucose analyser II (Beckman Instruments, Brea, California). Roche Hitachi Cobas c711 instrument (Roche, Barcelona, Spain) was used to perform HDL-cholesterol and total serum triglycerides determinations. HDL-cholesterol was quantified by a homogeneous enzymatic colorimetric assay through the cholesterol esterase /cholesterol oxidase /peroxidase reaction (Cobas HDLC3). Serum fasting triglycerides were measured by an enzymatic, colorimetric method with glycerol phosphate oxidase and peroxidase (Cobas TRIGL). LDL-cholesterol was calculated using the Friedewald formula.

#### Statistical analyses

Statistical analyses were performed using SPSS 12.0 software. Unless otherwise stated, descriptive results of continuous variables are expressed as mean and SD for Gaussian variables or median and interquartile range. Parameters that did not fulfil normal distribution criteria were Log transformed to improve symmetry for subsequent analyses. The relation between variables was analyzed by simple correlation (Pearson's test and Spearman's test). Levels of statistical significance were set at  $p < 0.05$ .

## Results

#### *AT TSHB mRNA is linked to AT cellular senescence in humans*

In euthyroid morbidly obese subjects (Table 1), both SAT and VAT *TSHB* gene expression was negatively associated with markers of AT senescence (*TNF*, *TP53*, *BAX* and *DBC1*) (Table 2, Fig. 1A–D). Confirming these findings, SAT *TSHB* mRNA was positively linked to telomere

**Table 1.** Anthropometric and clinical parameters and VAT and SAT *TSHB* gene expression in human cohort. <sup>a</sup>Mean  $\pm$  SD; <sup>b</sup>Median (interquartile range); VAT, Visceral Adipose Tissue; SAT, Subcutaneous Adipose Tissue

COHORT 1	All participants
N	45
Sex (men/women)	10/35
Age (years) <sup>a</sup>	47.5 $\pm$ 8.6
BMI (kg/m <sup>2</sup> ) <sup>a</sup>	45.1 $\pm$ 7.1
Fasting glucose (mg/dl) <sup>b</sup>	98 (90–107)
Total cholesterol (mg/dl) <sup>a</sup>	190 (165–213)
LDL cholesterol (mg/dl) <sup>a</sup>	122 (100.5–145.5)
HDL cholesterol (mg/dl) <sup>a</sup>	46 (40–50.5)
Fasting triglycerides (mg/dl) <sup>b</sup>	120 (80–153.5)
Serum TSH (mU/l) <sup>b</sup>	2.05 (1.39–2.92)
VAT <i>TSHB</i> (RU) <sup>b</sup>	0.00043 (0.00013–0.00641)
SAT <i>TSHB</i> (RU) <sup>b</sup>	0.00227 (0.00012–0.00552)

length in a subgroup of consecutive subjects ( $r = 0.43$ ,  $p = 0.04$ ,  $n = 22$ ; Fig. 2A), suggesting decreased senescence with less exposure to thyroid hormones. Furthermore, SAT *TSHB* mRNA was negatively correlated to specific senescence-associated glucosylceramides (Fig. 2B-D).

A positive correlation between adipose tissue *TSHB* and *PPARGC1A* mRNA has been recently reported [22]. Interestingly, in both SAT and VAT, *PPARGC1A* was negatively correlated with *TP53* ( $r = -0.44$ ,  $p = 0.01$  in SAT and  $r = -0.42$ ,  $p = 0.02$  in VAT) and *BAX* ( $r = -0.55$ ,  $p = 0.001$  in SAT and  $r = -0.39$ ,  $p = 0.04$  in VAT) gene expression.

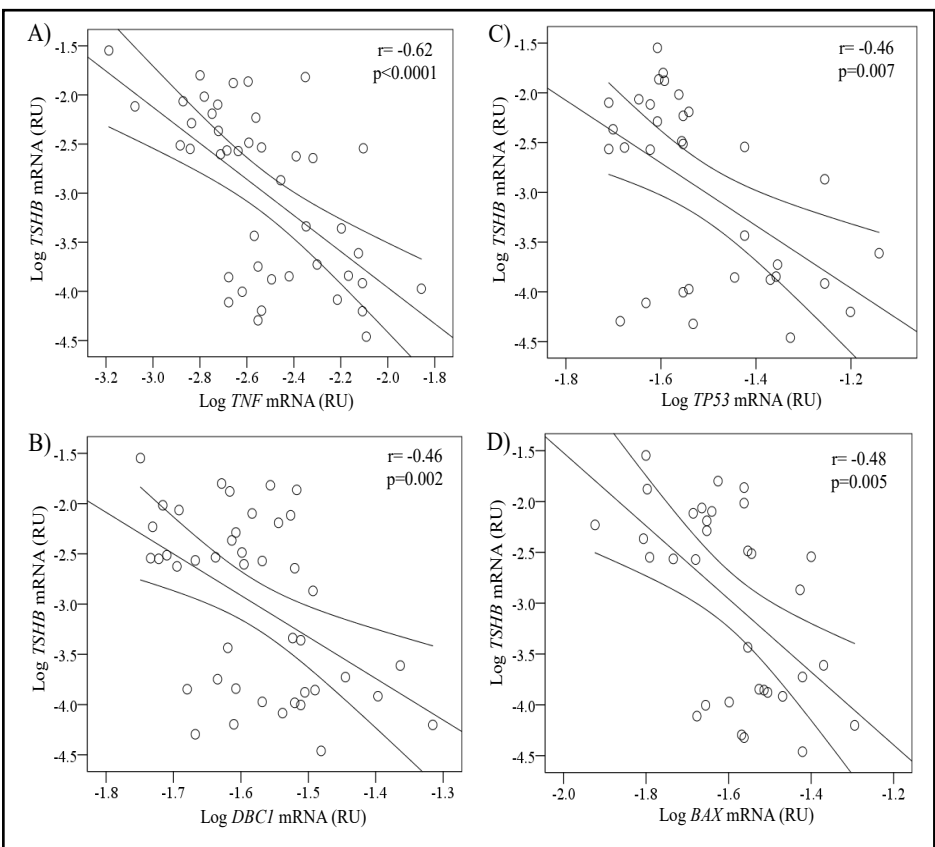
#### In vitro experiments

In vitro experiments in differentiated visceral adipocytes indicated that while long-term (7 days) rhTSH (1 and 10 mU/l) administration resulted in a significant decreased cellular senescence-related (*DBC1*, *TNF*, *BAX* and *TP53*) gene expression (Fig. 3A-D), no significant effects of short-term (2 days) rhTSH administration on expression of these genes were observed (Fig. 3E-H).

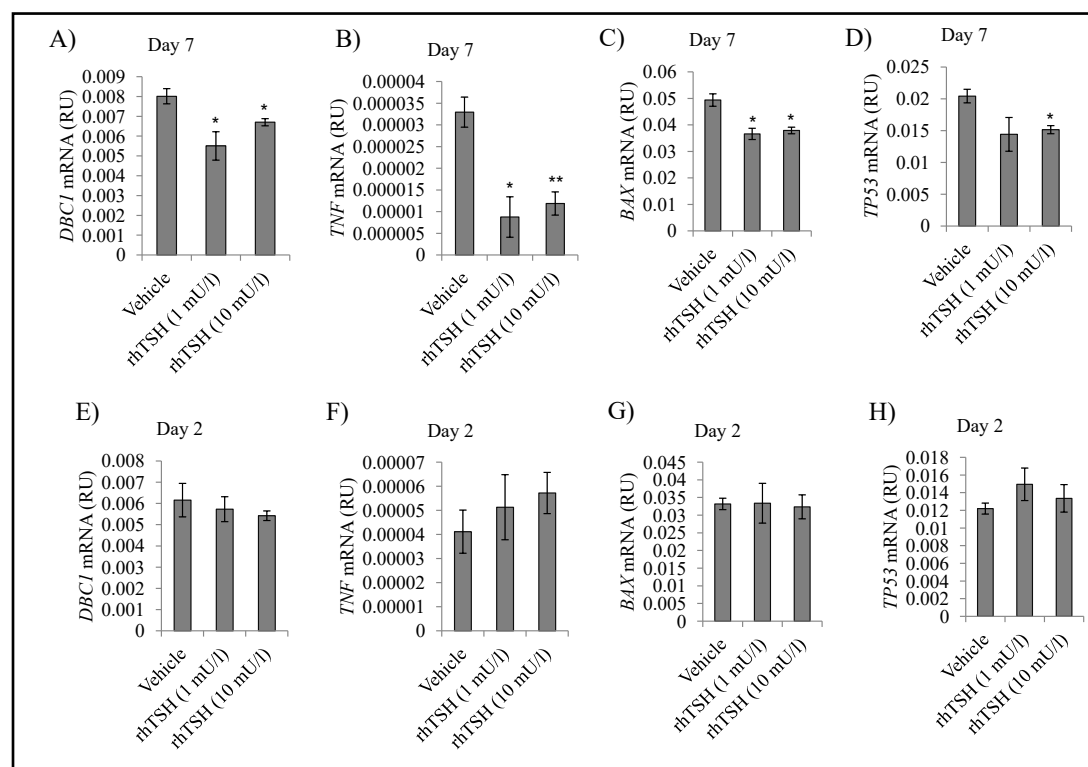
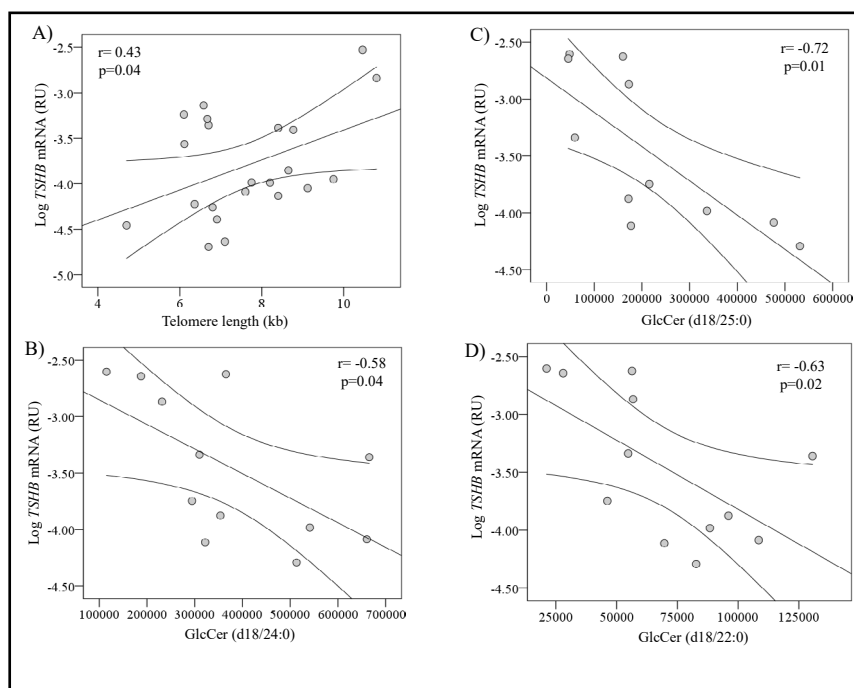
**Table 2.** Bivariate correlations between VAT and SAT *TSHB* gene expression, clinical parameters and expression of adipose tissue-related genes in human cohort

	N	SAT		VAT	
		r	p	r	p
Age (years)	45	-0.10	0.5	-0.09	0.6
BMI (kg/m <sup>2</sup> )	45	-0.05	0.7	0.19	0.2
Fasting glucose (mg/dl)	45	-0.35	0.01	-0.26	0.1
Total cholesterol (mg/dl)	45	0.15	0.3	0.36	0.02
LDL cholesterol (mg/dl)	45	0.13	0.4	0.43	0.006
HDL cholesterol (mg/dl)	45	-0.07	0.6	-0.16	0.3
Fasting triglycerides (mg/dl)	45	0.15	0.3	0.11	0.5
Serum TSH (mU/l)	45	0.11	0.4	-0.09	0.6
<i>DBC1</i> (RU)	43	-0.46	0.002	-0.59	<0.0001
<i>TNF</i> (RU)	43	-0.62	<0.0001	-0.47	0.005
<i>BAX</i> (RU)	33	-0.48	0.005	-0.54	0.001
<i>TP53</i> (RU)	33	-0.46	0.007	-0.45	0.009

**Fig. 1.** A-F) Bivariate correlations between SAT *TSHB* and *TNF* (A), *DBC1* (B), *TP53* (C) and *BAX* (D) mRNA levels.

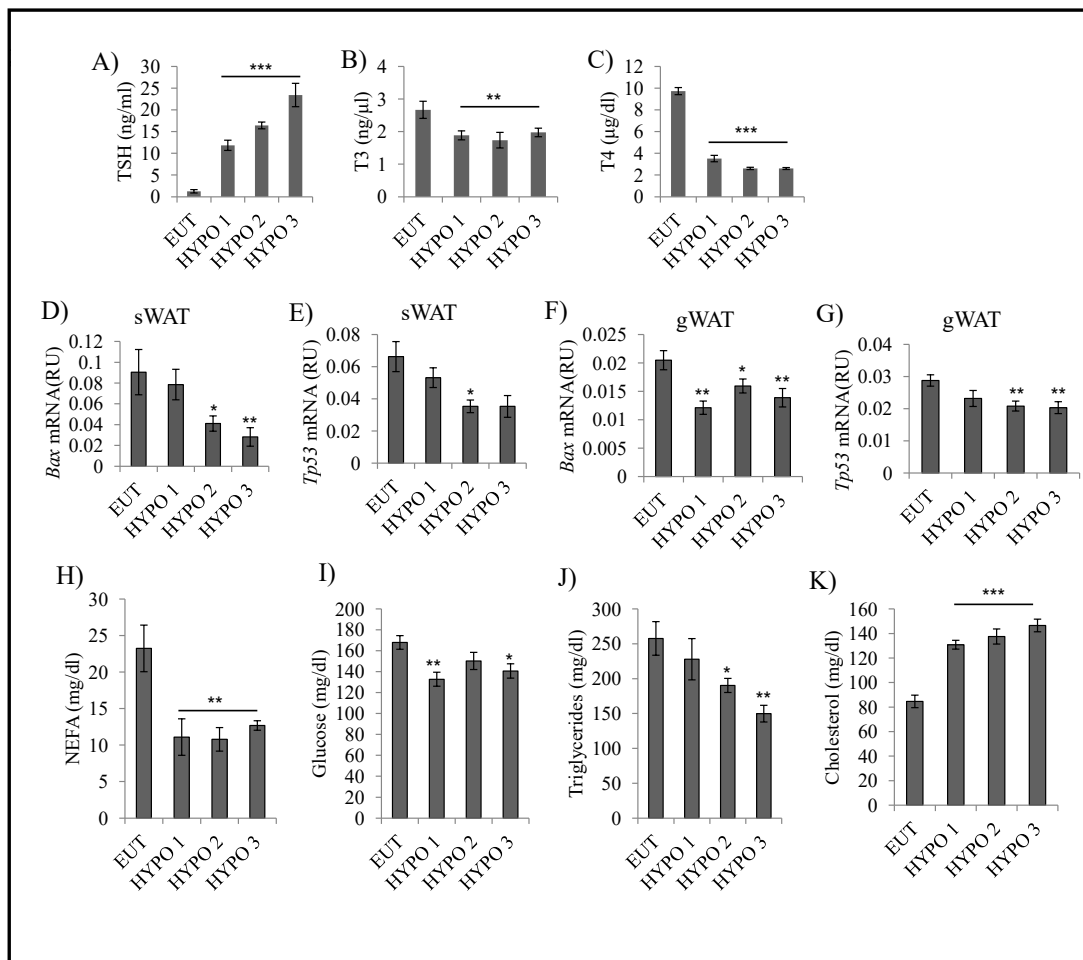


**Fig. 2.** Bivariate correlations between SAT *TSHB* and telomere length (n=22) (A) and specific senescence-associated glucosylceramides [N=12, except GlcCer(d18/25:0), which was not detected in one participant] (B-D).



**Fig. 3.** A-D) Effects of rhTSH (1 and 10 mU/l) administration during 7 (A-D) or 2 (E-H) days on *DBC1* (A,E), *TNF* (B,F), *BAX* (C,G) and *TP53* (D,H) mRNA levels in visceral adipocytes. \* $p < 0.05$  and \*\* $p < 0.01$  vs. vehicle.





**Fig. 4.** A-K) Effects of amino-triazole administration during 1 (HYPO1), 2 (HYPO2) and 3 (HYPO3) weeks on serum TSH (A), T3 (B), T4 (C), on sWAT and gWAT Bax and Tp53 mRNA levels (D-G) and on serum NEFA (H), glucose (I), triglycerides (J) and cholesterol (K) levels. EUT were non-treated or control rats. \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$  vs EUT rats.

#### Chronically increased TSH levels impact on AT cellular senescence in rats

Next, to explore the impact of increased serum TSH levels on AT cellular senescence, we evaluated the effects of chronic increased TSH levels exposure in an aminotriazole-induced hypothyroidism rat model. Of note, both increased circulating TSH levels (Fig. 4A) and decreased T4 and T3 levels (Fig. 4B-C), were associated with decreased markers of cellular senescence (Bax and Tp53 mRNA) in gonadal and subcutaneous white adipose tissue (Fig. 4D-G). Aminotriazole-induced hypothyroidism also resulted in decreased circulating glucose, NEFAs and triglycerides, as well as elevated cholesterol (Fig. 4H-K).

#### Discussion

Increased TSH mRNA levels and circulating TSH were significantly associated with reduced markers of cellular senescence (including BAX, DBC1, TP53, TNF gene expression [12, 13] and specific glucosylceramides [21, 23]) and increased telomere length in both human and rat AT. These data agree with the positive association between serum TSH levels and longevity reported in rodents [1-3] and euthyroid humans [3-9]. In line with these observations, rhTSH administration in human visceral adipocytes during 7 days, but not

during 2, resulted in decreased expression of cellular senescence-related gene markers. In parallel with these findings, a recent publication demonstrated that TSH administration (48h) in fully differentiated human adipocytes resulted in a significant improvement of mitochondrial biogenesis and function, increasing basal respiration, ATP production and spare respiratory capacity in parallel to increased SIRT1 and mitochondrial biogenesis-related gene markers [22]. Furthermore, this study also demonstrated that *TSHB* mRNA was positively correlated with mitochondrial function-related (*PPARGC1A* and *SIRT1*) gene expression in human adipose tissue [22]. *PPARGC1A* is a transcriptional coactivator that modulates the genes involved in energy metabolism, being a central regulator of mitochondria biogenesis [24-27]. *SIRT1*, which is another important mediator of mitochondrial biogenesis and energy expenditure in AT [27-29], is widely known as a negative regulator of cellular senescence [30, 31]. In humans, increased AT *SIRT1* mRNA levels and activity associates with mitochondrial function [28, 29] and with reduced markers of cellular senescence [32, 33]. Furthermore, DBC1 interacts and inhibits the activity of Sirt1 [34], a crucial component for the prevention of cellular senescence [35, 36].

Different studies have shown a negative role of p53 in adipose tissue physiology, decreasing adipocyte differentiation [37-40] and increasing the expression of proinflammatory cytokines via the NF- $\kappa$ B signaling pathway [41], leading to obesity-associated insulin resistance and adipose tissue dysfunction [13, 42-46]. In fact, p53 stimulation in dysfunctional adipose tissue enhanced adiposity-associated metabolic disturbances (such as glucose intolerance) [47], whereas its inhibition has therapeutic effects [48]. Interestingly, a recent study demonstrated that adipose tissue-specific ablation of ACSL4 mice were protected against high fat diet-induced metabolic disturbances and adipose tissue dysfunction and senescence, in parallel to reduced p53 activation and increased adipocyte oxygen consumption [49]. Sen et al. reported in HepG2 cells cultured in conditions of metabolic stresses (such as glucose/serum starvation or cold shock) that PGC1 $\alpha$  interacts with p53, increasing its transcriptional activity of proarrest and metabolic target genes in the first 36 h of metabolic stresses [50]. At extended periods of metabolic stresses (48 and 72h), PGC1 $\alpha$  protein levels drastically decreased, the interaction between PGC1 $\alpha$  and p53 was lost, and p53 transactivates its proapoptotic targets, resulting in cell death [50]. In the present study, *PPARGC1A* was negatively associated with *TP53* and *BAX* gene expression in both SAT and VAT, supporting that decreased PGC1 $\alpha$  levels enhanced the p53 transcriptional activity of proapoptotic target genes, and in consequence, promoted cellular senescence in adipose tissue. In fact, the contribution of mitochondrial dysfunction to cellular senescence in AT and the negative effects of aging on mitochondrial function are well known [51-53].

Current data and previous study [22] suggested that, mechanistically, the anti-senescence effects of TSH could be mediated through the improvement of adipocyte mitochondrial function.

Supporting all these findings, chronically elevated circulating TSH resulted in protection from AT senescence in rats. Aminotriazole-induced hypothyroidism resulted in decreased serum NEFAs. This data are in agreement with our metabolic characterization of this model showing, decreased fatty acid synthase expression in liver and WAT (the main sources of circulating NEFAs) of hypothyroid rats [20]. In keeping with this, severe hypothyroidism has been very recently described to result in suppression of adipose tissue lipolysis and decreased NEFAs in contrast to mild hypothyroidism [54].

## Conclusion

In conclusion, altogether these data suggest a possible role of adipose tissue *TSHB* endogenous biosynthesis in the regulation of adipose tissue cellular senescence, possibly through the maintenance of mitochondrial function.



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## Disclosure Statement

The authors declared no conflicts of interest.

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