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Short Communication

Association between thyroid hormones and TRAIL

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A B S T R A C T

Introduction: Recent studies suggest that a circulating protein called TRAIL (TNF-related apoptosis-inducing ligand) might have a role in the regulation of body weight and metabolism. Interestingly, thyroid hormones seem to increase TRAIL tissue expression. This study aimed at evaluating whether overt thyroid disorders affected circulating TRAIL levels.

Methods: TRAIL circulating levels were measured in euthyroid, hyperthyroid, and hypothyroid patients before and after thyroid function normalization. Univariate and multivariate analyses were performed to evaluate the correlation between thyroid hormones and TRAIL. Then, the stimulatory effect of both triiodothyronine (T3) and thyroxine (T4) on TRAIL was evaluated *in vitro* on peripheral blood mononuclear cells.

Results: Circulating levels of TRAIL significantly increased in hyperthyroid and decreased in hypothyroid patients as compared to controls. Once thyroid function was restored, TRAIL levels normalized. There was an independent association between TRAIL and both fT3 and fT4. Consistent with these findings, T3 and T4 stimulated TRAIL release *in vitro*.

Conclusion: Here we show that thyroid hormones are associated with TRAIL expression *in vivo* and stimulate TRAIL expression *in vitro*. Given the overlap between the metabolic effects of thyroid hormones and TRAIL, this work sheds light on the possibility that TRAIL might be one of the molecules mediating thyroid hormones peripheral effects.

1. Introduction

TRAIL, which is an acronym for TNF-related apoptosis inducing ligand, is the name of a protein belonging to the TNF superfamily, which was discovered in 1995 on the basis of its high homology to other TNF family members, such as FasL/CD95L and TNF- α [1]. TRAIL is widely expressed in different cells and tissues [1,2] as a type 2 transmembrane protein, which is usually cleaved to form a circulating protein. Be it in the native or processed form, TRAIL's main function is to activate the extrinsic apoptotic pathway upon binding to its specific receptors (TRAIL-R1 and TRAIL-R2), and to cause cell death [3]. Interestingly, as compared to the other pro-apoptotic TNF family members, TRAIL has the unique ability to induce apoptosis preferen-

tially in transformed cells, such as tumor cells, while it spares the normal ones [3]. The discovery of this property has led to the current study of TRAIL as an anticancer therapy [4,5].

Beside its anticancer potential, recent studies suggest that TRAIL has also significant metabolic effects, such that it could be a potential candidate for the treatment of obesity and its associated diseases [6–9]. In particular, *in vivo* studies have shown that high-fat diet-fed mice put on more weight if they were TRAIL deficient [6], and that TRAIL delivery significantly reduced the amount of fat mass in high-fat diet-fed mice [7]. In addition, *in vitro* studies have shown that TRAIL-R2 activation blocked *de novo* lipogenesis in human adipocytes [10] and that TRAIL treatment inhibited adipocyte differentiation [9].

Interestingly, experimental evidence suggests that thyroid hor-

Abbreviation: BMI, body mass index; CLEIA, chemiluminescence enzyme immunoassay; CNT, controls; CRP, C-reactive protein; CV, coefficient of variation; ELISA, enzyme-linked immunosorbent assay; FFA, free fatty acids; fT3, free triiodothyronine; fT4, free thyroxine; HDL-C, high-density lipoprotein cholesterol; HYPER, hyperthyroid patients; HYPO, hypothyroid patients; OPG, osteoprotegerin; PBMC, peripheral blood mononuclear cells; PBS, phosphate buffered saline; TC, total cholesterol; TG, triglycerides; TG-Ab, anti-thyroglobulin antibodies; TPO-Ab, anti-thyropoxidase antibodies; TRAIL, TNF-related apoptosis-inducing ligand; TSH, thyroid-stimulating hormone; TSHr-Ab, anti-TSH receptor antibodies

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mones [11,12] influence TRAIL tissue expression. The group of Chi and colleagues has demonstrated that thyroid hormones induced TRAIL transcription in hepatoma cell lines [11]. In addition, it has also been reported that TRAIL gene expression was upregulated in the skeletal muscle by levothyroxine replacement therapy [12]. Nevertheless, it remains to be established whether thyroid hormones can affect circulating TRAIL levels. The aim of the present study was to evaluate the effect of overt thyroid disorders (hyperthyroidism and hypothyroidism) on circulating TRAIL levels.

2. Methods

2.1. Study population

A total of 39 euthyroid (CNT), 49 hyperthyroid (HYPER), and 28 hypothyroid (HYPO) patients were consecutively selected over a period of 12 months (January 2013–January 2014) from the subjects referring to the Endocrine Service of Cattinara Hospital (Azienda Ospedaliero-Universitaria di Trieste). Euthyroidism, hyperthyroidism, and hypothyroidism were defined biochemically. Euthyroid patients were patients who had undergone thyroid hormone measurement as a part of their medical workup carried out in the Endocrine Service, and who did not result affected by any thyroid dysfunction, such that they were selected as age and sex-matched controls. By contrast, hyperthyroid patients had lower TSH and higher fT3 and fT4, while hypothyroid patients had higher TSH and lower fT3 and fT4 values as compared to reference ranges. Exclusion criteria were: age below 18 or above 80 years, diabetes, dyslipidemia, cardiopulmonary, renal, and hepatic failure. After the first visit, all the subjects were asked to give their written informed consent to participate in this study, whose protocol had been previously approved by the Institutional Ethics Committee of AOUTS.

2.2. Study protocol

At presentation, and before starting any specific treatment, euthyroid (CNT), hyperthyroid (HYPER before), and hypothyroid patients (HYPO before) underwent a medical visit and a blood sampling. The clinical and laboratory parameters recorded are reported in Table 1. Then, the appropriate medical treatments were prescribed to the

patients with thyroid disorders, and subsequent follow-up visits were scheduled (generally monthly for hyperthyroid and quarterly for hypothyroid patients), to check on their thyroid function. Once euthyroidism was restored, patients underwent a second medical visit and blood sampling (HYPER after and HYPO after). Blood sampling was performed at 08.00 a.m., after an overnight fasting.

2.3. Clinical laboratory

TSH, fT3, fT4, TG-Ab, TPO-Ab were measured by chemiluminescence (CLEIA; tracer alkaline phosphatase Lumiphos 530) with a Dxl800 analyzer (Beckman Coulter, Fullerton, CA) [13]. TSHr-Ab were measured by ELISA (Alisei, Omnia Diagnostica, CT, Italy). Thyroid hormone assay precision (within-run and between-day) was calculated by daily quality controls (Bio-Rad Immunoassay Plus, Hercules, CA), as summarized in Supplementary material (Table 1). Thyroid hormone reference ranges were 0.4 to 4 μ IU/mL for TSH; 7.20 to 15.44 pmol/L for fT4; and 3.0 to 6.9 pmol/L for fT3. These reference ranges have been calculated by our central laboratory on our local population, as recommended by standard published procedures [14]. Glucose, TC, TG, and HDL-C (enzymatic colorimetric method) and CRP (immunoturbidimetric method) were measured with an AU5800 analyzer (Beckman Coulter, Fullerton, CA). All these laboratory data were verified by external proficiency testing (CRB-Centro Ricerca Biomedica, Padova, Italy and RIQAS, Randox Laboratories Ltd). As for TRAIL and OPG (which is the soluble decoy receptor for TRAIL), they were measured by a solid-phase sandwich ELISA (#DTRL00 and #DY805; R & D Systems, Minneapolis, MN) in the sera, as previously reported [15,16]. The CV of TRAIL assay is reported in Supplementary Table 1.

2.4. In vitro study

PBMC were isolated from 8 blood donors following their informed consent. Briefly, blood samples were collected at fasting and diluted with 2 volumes of cold PBS (Sigma-Aldrich, Milan, Italy), layered on Ficoll (Sigma-Aldrich), and centrifuged for 20 min at 400 \times g at 4 $^{\circ}$ C. The mononuclear cell layer obtained after centrifugation was harvested, washed with PBS, and PBMC (1×10^6 cells/mL) were cultured in RPMI 1640 (Sigma-Aldrich) supplemented with 10% FCS (Invitrogen) in 24-well plates at 37 $^{\circ}$ C in 5% CO₂. Cells were treated

Table 1
Patients' characteristics.

Parameter	CNT (n = 39)	HYP before (n = 49)	HYP after (n = 49)	HYP before (n = 28)	HYP after (n = 28)
Age (years)	54.8 \pm 2.3	50.1 \pm 2.5	=	61.9 \pm 3.0 [#]	=
Sex (F/M)	29/10	39/10	39/10	23/5	23/5
BMI (kg/m ²)	24.6 \pm 0.8	24.4 \pm 0.8	26.1 \pm 1.0	26.8 \pm 0.9	25.6 \pm 1.1
TSH (μ IU/mL)	1.4 \pm 0.1	0.0 \pm 0.0 [*]	2.4 \pm 0.3 [#]	68.2 \pm 5.7 ^{*,#}	2.8 \pm 0.6 [†]
fT3 (pmol/L)	4.6 \pm 0.1	16.7 \pm 13.1 [*]	4.3 \pm 0.1 [#]	2.8 \pm 0.2 ^{*,#}	4.2 \pm 0.1 [†]
fT4 (pmol/L)	11.5 \pm 0.5	43.6 \pm 2.5 [*]	11.1 \pm 0.8 [#]	4.1 \pm 0.5 ^{*,#}	13.6 \pm 1.0 [†]
TSHr-Ab (U/L)	0.7 \pm 0.5	10.9 \pm 3.2	4.6 \pm 2.0	4.2 \pm 3.3	3.8 \pm 3.7
TG-Ab (U/L)	33.4 \pm 14.4	237.4 \pm 42.1	55.2 \pm 51.0	938.8 \pm 240.1 ^{*,#}	203.7 \pm 123.6
TPO-Ab (U/L)	127.7 \pm 69.5	216.1 \pm 41.9	203.4 \pm 125.5	550 \pm 101.1 ^{*,#}	420.8 \pm 195.1
CRP (mg/L)	4.6 \pm 1.6	2.9 \pm 1.3	1.9 \pm 0.5	2.9 \pm 1.3	3.1 \pm 1.2
Glucose (mM)	5.2 \pm 0.2	5.5 \pm 0.1	5.3 \pm 0.2	5.3 \pm 0.3	5.4 \pm 0.1
TC (mg/dL)	5.5 \pm 0.1	4.3 \pm 0.2 [*]	5.5 \pm 0.2 [#]	6.4 \pm 0.3 ^{*,#}	5.1 \pm 0.2 [†]
HDL-C (mg/dL)	1.6 \pm 0.1	1.3 \pm 0.0 [*]	1.6 \pm 0.1 [#]	1.6 \pm 0.1 [#]	1.7 \pm 0.1
TG (mg/dL)	1.0 \pm 0.1	1.0 \pm 0.1	1.2 \pm 0.1	1.7 \pm 0.2 ^{*,#}	1.3 \pm 0.1
OPG (pg/mL)	541.5 \pm 60.1	900.4 \pm 72 [*]	514.8 \pm 69.3 [#]	926.5 \pm 133.9 [*]	461.2 \pm 41.3 [†]

Data are expressed as mean \pm SEM. CNT is for control; HYP before is for hyperthyroid patients before treatment; HYP after is for hyperthyroid patients after treatment; HYP before is for hypothyroid patients before treatment; HYP after is for hypothyroid patients after treatment; BMI is for body mass index; TSH is for thyroid stimulating hormone; fT3 is for free triiodothyronine; fT4 is for free thyroxine; TSHr-Ab is for anti-TSH receptor antibodies; TG-Ab is for anti-thyroglobulin antibodies; TPO-Ab is for anti-thyroperoxidase antibodies; CRP is for C-reactive protein; TC is for total cholesterol; HDL-C is for high-density lipoprotein cholesterol; TG is for triglycerides.

^{*} $p < 0.05$ vs CNT.

[#] $p < 0.05$ vs HYP before.

[†] $p < 0.05$ vs HYP after.

with IFN- γ (200 IU/mL, Peprotech), T3 (from 10^{-6} M to 10^{-10} M), T4 (from 10^{-6} M to 10^{-10} M), or T3 and T4 (10^{-9} M) in addition to IFN- γ for 24 h. Cell viability of PBMC was monitored by Trypan blue-dye exclusion. As before, TRAIL was quantified by ELISA (#DTRL00; R & D Systems, Minneapolis, MN) in the cell-free supernatant.

2.5. Statistical analysis

Statistical analysis was performed by using the statistical software R version 3.0.3 (2014). Results were expressed as mean \pm standard error of the mean and as median and 25th and 75th percentiles for skewed variables where appropriate. A p value < 0.05 was considered statistically significant. Shapiro-Wilk test was applied to continuous variables to check for normality distribution. Comparisons of data between the groups at baseline (CNT, HYPER before, HYPO before) were performed by one-way analysis of variance (ANOVA) or Kruskal-Wallis test (in cases of non-parametric data) followed by the post-hoc multiple comparison (Holm-Bonferroni method). TRAIL levels before and after treatment in HYPER and HYPO patients were compared by Wilcoxon-test for paired data (TRAIL), as they didn't have normal Gaussian distributions. The Spearman rank correlation coefficient for continuous variables was used to assess the correlation between baseline TRAIL and age, fT3, fT4, BMI, waist circumference, CRP, glucose, TC, TG, HDL-C, one at a time. Then, a stepwise multiple linear regression analysis was performed in order to determine the independent variables affecting TRAIL.

3. Results

3.1. Patients' characteristics

Clinical and biochemical parameters of hyperthyroid, hypothyroid patients, and their controls are shown in Table 1. Hyperthyroid and hypothyroid patients presented with overt thyroid disease. Hypothyroid patients were a decade older than hyperthyroid patients. They were overweight, and had higher levels of total cholesterol and triglycerides as compared to controls (Table 1). On the contrary, hyperthyroidism was associated with a decrease in total and HDL cholesterol. Hyperthyroid patients were treated with methimazole (18.1 ± 1.3 mg/day) and euthyroidism was restored after 5.6 ± 0.8 months of therapy. Hypothyroid patients were treated with levothyroxine (102.9 ± 5.2 mcg/day) and euthyroidism was restored after 9.2 ± 0.8 months of therapy. By restoring normal thyroid hormone levels, the clinical differences seen at baseline disappeared (Table 1).

3.2. TRAIL circulating levels in patients with hyperthyroidism and hypothyroidism

At baseline, hyperthyroid patients displayed significantly higher levels of circulating TRAIL as compared to controls, while in hypothyroid patients they were significantly lower (Fig. 1A). After thyroid function restoration, TRAIL levels normalized in both groups (Fig. 1A). In order to exclude that what we observed depended on changes in OPG, which is TRAIL decoy receptor, we measured OPG levels (Table 1) and found that OPG variations did not explain TRAIL changes.

3.3. Association between thyroid hormones and TRAIL

At baseline, there was a significant direct correlation between thyroid hormones and TRAIL ($r = 0.62$ and $p < 0.0001$ for fT3, $r = 0.65$ and $p < 0.0001$ for fT4, Fig. 1B), and a significant inverse correlation between TSH and TRAIL ($r = -0.61$ and $p < 0.0001$). We did not find any correlation between TRAIL and the other variables (age, sex, BMI, waist circumference, CRP, glucose, insulin, TC, TG, HDL-C). The multiple linear regression analysis showed that fT3 and BMI were significantly associated with TRAIL independently from the

other covariates (Supplementary Table 2) However, only fT3 turned out to significantly influence TRAIL after stepwise linear regression analysis (β -estimate = 1.16, standard error 0.47663, $p < 0.001$), as shown in Supplementary Table 3.

3.4. Effect of T3 and T4 on TRAIL release in vitro

In order to evaluate whether T3 or T4 could stimulate TRAIL release, purified PBMC were cultured for 24 h in the presence of either T3 or T4 at different concentrations. IFN- γ was used as a positive control [17,18]. None of the treatments affected cell viability. As expected, protein expression analysis showed that IFN- γ significantly increased TRAIL to 23.17 ± 0.44 pg/mL as compared to CNT, where TRAIL was 11.10 ± 0.23 pg/mL ($p < 0.0001$). T3 and T4 significantly increased TRAIL release (Fig. 1C), but T3 had a greater effect than T4 on TRAIL release. In the cells treated with the highest dose of T3 (10^{-6} M), TRAIL levels went up to 26.95 ± 0.10 pg/mL ($p < 0.0001$ vs CNT), while the highest dose of T4 (10^{-6} M) increased TRAIL up to 13.80 ± 0.19 pg/mL ($p < 0.01$ vs CNT). These treatments did not induce any OPG release (data not shown).

4. Discussion

This is the first study where the relationship between thyroid hormones and the circulating levels of TRAIL has been analyzed. In particular, here we found that hyperthyroidism significantly increased circulating TRAIL levels, which, on the contrary were significantly decreased in patients with hypothyroidism, and -in both conditions- normalized after euthyroidism restoration. Overall, there was a significant association between TRAIL circulating levels and both fT3 and fT4. These changes could be ascribed to a stimulatory effect of T3 and T4 on TRAIL expression, which we demonstrated *in vitro* and is consistent with previous reports [11]. In our *in vitro* experiments, T3 had a greater effect than T4 on TRAIL release, which is consistent with the concept that T3 is the physiologically active thyroid hormone and that T4 is its precursor [19]. Nevertheless, also T4 elicited a small but significant cellular response, and it induced TRAIL release. This might be explained by the affinity of T4 for the T3 receptor. Cell culture experiments have demonstrated that T4 behaves as a T3 receptor agonist with a potency that is about 10% of that of T3 [20].

If thyroid hormones stimulate TRAIL expression, it could be speculated that TRAIL is a molecule mediating T3 peripheral effects. In support of this hypothesis, some metabolic effects of thyroid hormones overlap with those of TRAIL. For instance, it has been demonstrated that mutations in thyroid hormone receptors induce an increase in body fat and visceral adiposity [21]. Likewise, TRAIL deficiency was associated with an increase in body weight as compared to wild-type mice [6]. Moreover, experimental studies have shown that T3 influences peripheral metabolism by modulating substrate utilization [22], as T3 promotes FFA oxidation in the skeletal muscle [23]. Likewise, we have recently reported that TRAIL treatment significantly increased [$1-^{14}$ C]-palmitate oxidation in the skeletal muscle [7], which is considered to be a direct and accurate measure of FFA oxidation [24].

Based on these observations, further studies are needed to evaluate if TRAIL mediates T3 metabolic effects in the periphery. Additional future directions based on these findings might include the measurement of circulating TRAIL in patients who have undergone total thyroidectomy and are taking conventional thyroid replacement therapy [25]. After surgery, levothyroxine (LT4) is the standard of care. Nevertheless, a substantial amount of patients taking LT4 suffers from an impairment of their psychological well-being [25]. Lower circulating levels of TRAIL in these patients could be an additional mechanism underlying the impairment of their quality of life.

In conclusion, here we show for the first time that thyroid hormones are associated with TRAIL expression *in vivo* and stimulate TRAIL expression *in vitro*. In particular, given the overlap between the

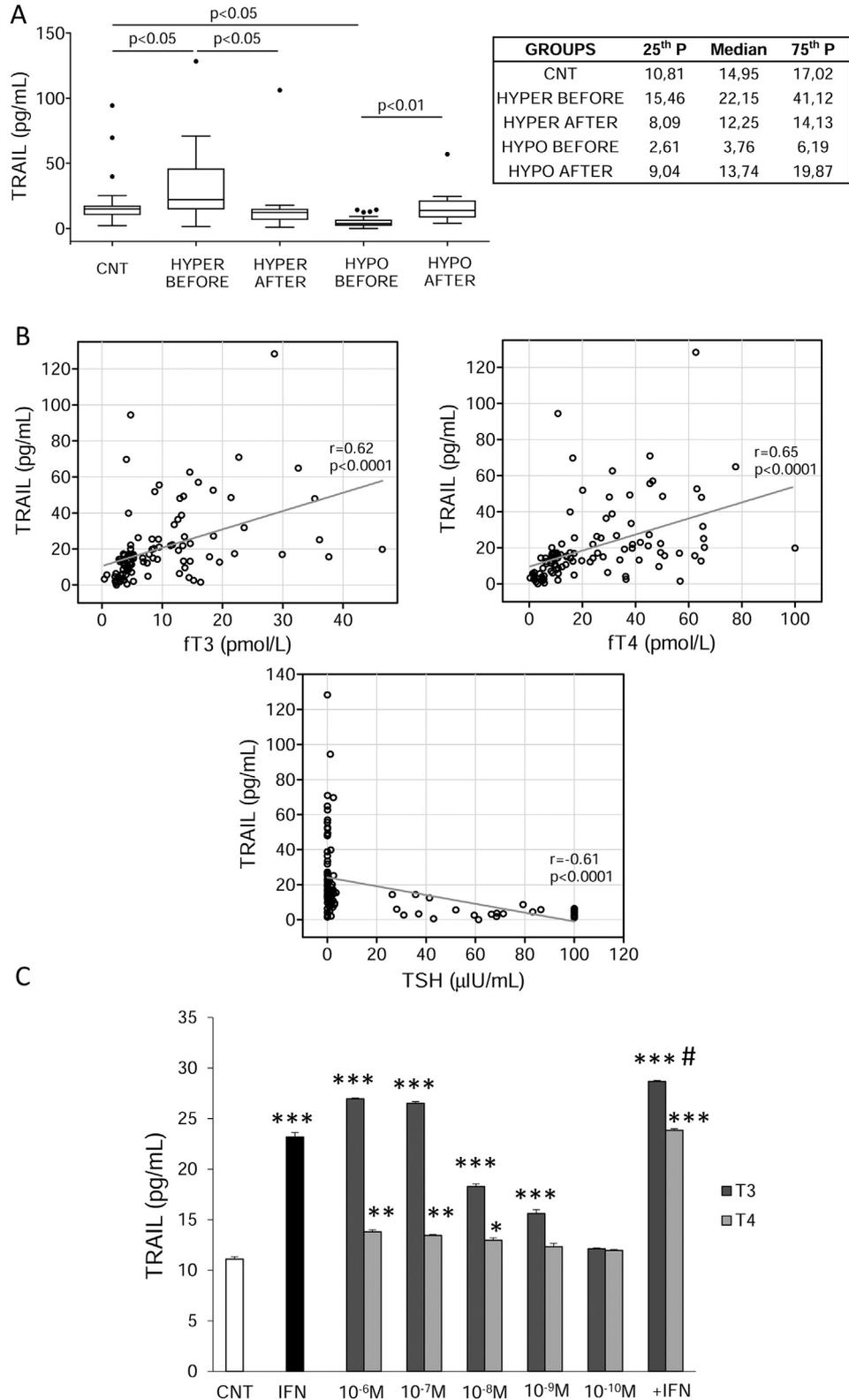


Fig. 1. A) Box plots of circulating TRAIL in control, hyperthyroid, and hypothyroid patients. CNT is for controls, HYPER is for hyperthyroid patients before and after treatment, HYPO is for hypothyroid patients before and after treatment, TRAIL is for TNF-related apoptosis-inducing ligand. Kruskal-Wallis Test was used for comparisons between the groups at baseline (CNT, HYPER before and HYPO before). Wilcoxon Test was used for comparisons between before and after (HYPER before vs HYPER after, and HYPO before vs HYPO after). The table on the right shows median, 25th, and 75th percentile (P) values. B) Correlation between thyroid hormones and TRAIL. On the upper left, correlation between FT3 and TRAIL (Spearman test, $r = 0.62$ and $p < 0.0001$); on the upper right, correlation between FT4 and TRAIL (Spearman test, $r = 0.65$ and $p < 0.0001$). Below, correlation between TSH and TRAIL (Spearman test, $r = -0.61$ and $p < 0.0001$). C) Effect of T3 and T4 on TRAIL expression *in vitro*. Isolated PBMC (peripheral blood mononuclear cells) were exposed to IFN- γ (200 IU/mL), T3 (from 10^{-6} M to 10^{-10} M), T4 (from 10^{-6} M to 10^{-10} M), and either T3 (10^{-10} M) + IFN- γ or T4 (10^{-10} M) + IFN- γ . TRAIL protein expression was measured by ELISA after a 24-hour stimulation. Data are expressed as mean \pm SEM. * $p < 0.05$ vs CNT; ** $p < 0.01$ vs CNT; *** $p < 0.0001$ vs CNT; # $p < 0.0001$ vs IFN.

metabolic effects of thyroid hormones and TRAIL, this work suggests that TRAIL might be one of the molecules mediating thyroid hormone peripheral effects.

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Disclosure statement

Conflicts of interest: none.

Author contributions

S.B. selected and followed the patients, designed and performed experiments, interpreted data, and drafted the manuscript. F.B. performed and designed experiments. B.T. interpreted data, drafted and revised the manuscript for important intellectual content. F.G. performed statistical analyses. A.B. carried out immunoassays. G.F. followed the patients. E.S. contributed to the data collection and revision of the manuscript for important intellectual content. R.C., P.S., G.Z. interpreted data, edited the manuscript. B.F. conceived the idea of the study, selected and followed the patients, and edited the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.clinbiochem.2017.05.011>.

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