Thyroid hormone contributes to the hypolipidemic effect of polyunsaturated fatty acids from fish oil: in vivo evidence for cross talking mechanisms

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Abstract

n-3 polyunsaturated fatty acids (n-3 PUFA) from fish oil (FO) exert important lipid-lowering effects, an effect also ascribed to thyroid hormones (TH) and TH receptor β1 (TRβ1)-specific agonists. n-3 PUFA effects are mediated by nuclear receptors, such as peroxisome proliferator-activated receptors (PPAR) and others. In this study, we investigated a role for TH signaling in n-3 PUFA effects. Euthyroid and hypothyroid adult rats (methimazole-treated for 5 weeks) received FO or soybean oil (control) by oral administration for 3 weeks. In euthyroid rats, FO treatment reduced serum triglycerides and cholesterol, diminished body fat, and increased protein content of the animals. In addition, FO-treated rats exhibited higher liver expression of TRβ1 and mitochondrial α-glycerophosphate dehydrogenase (mGPD), at protein and mRNA levels, but no alteration of glutathione S-transferase or type 1 deiodinase. In hypothyroid condition, FO induced reduction in serum cholesterol and increase in body protein content, but lost the ability to reduce triglycerides and body fat, and to induce TRβ1 and mGDP expression. FO did not change PPARα liver abundance regardless of thyroid state; however, hypothyroidism led to a marked increase in PPARα liver content but did not alter TRβ1 or TRα expression. The data suggest that part of the effect of n-3 PUFA from FO on lipid metabolism is dependent on TH signaling in specific steps and together with the marked upregulation of PPARα in liver of hypothyroid rats suggest important in vivo consequences of the cross-talking between those fatty acids and TH pathways in liver metabolism.

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Introduction

Thyroid hormones (TH) are potent modulators of lipid metabolism (Abrams et al. 1981, Erem et al. 1999, Hashimoto et al. 2006), and hypothyroidism is associated with higher serum lipids (Erem et al. 1999). Most of the hypolipidemic effects of TH are due to their action in the liver via the TH receptor (TR). The interaction of triiodothyronine (T3) with TR promotes the recruitment of cofactors, resulting in regulation of the transcriptional activity of genes encoding key enzymes, and other factors, involved in lipid metabolism (Yen 2001, Araki et al. 2009). TRβ is the most abundant hepatic TR isoform and mediates the majority of the hypolipidemic actions of TH, as demonstrated in animal models using selective TRβ agonists (Johansson et al. 2005) or transgenic models with mutations of TRβ (Gullberg et al. 2000, 2002, Hashimoto et al. 2006).

TR belongs to the superfamily of nuclear receptors and TR functional interactions with such nuclear receptors have been reported, especially nutrient-sensing receptors, such as peroxisome proliferator-activated receptors (PPAR) and liver X receptor (LXR) (Liu & Brent 2010). Currently, it is clear that the effects of nuclear receptors are not a consequence of the binding of their specific ligands alone, but also reflect interactions with other signaling pathways (Hashimoto et al. 2006, Liu et al. 2007). Knowing the consequences of these interactions for the in vivo effects of nuclear receptors, agonists have important implications, both at physiological and pharmacological levels.

Fatty acids are described as PPAR endogenous agonists, being polyunsaturated fatty acids of the omega 3 family (n-3 PUFA) high-affinity agonists (Bordoni et al. 2006, Gani & Sylte 2008). Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are long-chain n-3 PUFA found in fish oil (FO), which have an important hypolipidemic effect and act predominantly through the modulation of the transcription of hepatic metabolism genes (Jump 2008, Jump et al. 2008). The hypolipidemic effect of the FO has been explained by several
mechanisms. Most of the FO effects were exerted via PPARα (Sugiyama et al. 2008) and other hepatic transcriptional factors such as sterol regulatory element–binding protein–1 (Yahagi et al. 1999). In addition, the involvement of other nuclear receptors, such as retinoic X receptor (Suzuki et al. 2009) and LXR (Howell et al. 2009), has also been reported. However, the existence of other, still unknown, mechanisms has been suggested (Duplus & Forest 2002).

Recently, we described the ability of n-3 PUFA from FO to increase hepatic TRβ1 expression in animals that received a diet enriched with FO from lactation to adult age (Souza et al. 2010), and concomitantly, those animals presented an increase in the activity of mitochondrial α-glycerophosphate dehydrogenase (mGPD), a classical target of TH action. Considering the hypolipidemic action of TH, it is possible that TH signaling is another route that could contribute to n-3 PUFA actions. In order to test this hypothesis, we investigated the effects of oral administration of FO to adult rats with depleted TH compared to euthyroid rats. This approach also brought to light important in vivo interactions between fatty acids and TH signaling.

**Materials and Methods**

*Animal care and experimental design*

Adult Wistar male rats, 10 weeks of age, were kept in a room with controlled temperature and artificial darkness–light cycle (lights on from 0700 to 1900 h). Our protocol was approved by the institutional Animal Care Committee.

Experiments were conducted in euthyroid and hypothyroid rats. For 3 weeks, euthyroid rats received daily, by gavage, FO (0.5 ml/animal; Manna et al. 2010; ROPUFA –75, Roche/DSM) containing n-3 PUFA in a minimum of 42% EPA and 22% DHA. Control animals received soybean oil (Liza, Cargill, São Paulo, SP, Brazil). n-3 PUFA constitute 55% of total fatty acids in FO and only 4% in soybean oil, while n-6 PUFA constitute approximately 56% of total fatty acids in soybean oil. The choice of soybean oil as a control relies on the fact that it is a source of n-6 PUFA, which represents the major PUFA present in western diets (Spector 1999) and, as recommended by the American Institute of Nutrition (AIN-93), should be the major lipid source in rodent diets (Reeves et al. 1997). In addition, both groups received the same amount of calories from lipids. This is important since it has been shown that excess of calories, per se, can influence TH signaling (Redonnet et al. 2001).

In order to induce hypothyroidism, rats received methimazole (0.03%) in the drinking water, for 5 weeks. After 2 weeks of treatment, along with methimazole administration, rats received 0.5 ml FO orally or soybean oil, for 3 weeks. Both euthyroid and hypothyroid rats were killed by decapitation. Serum obtained from trunk blood was kept frozen at −20 °C for measurements of hormones and biochemical parameters. Carcasses were eviscerated for body composition analysis. Samples of liver tissue were frozen in liquid nitrogen and stored at −70 °C prior to extraction of total protein and RNA.

*Body composition analysis*

The procedures followed previously established protocols (Stansbie et al. 1976, Souza et al. 2010) and all reagents are analytical grade. Eviscerated carcasses were weighed, autoclaved, and homogenized in distilled water (1:1). Aliquots of homogenate were used for measurement of protein and fat content. Homogenate (3 g) was used to determine fat mass gravimetrically (Stansbie et al. 1976). Samples were hydrolyzed in a shaking water bath at 70 °C for 2 h with 30% KOH and ethanol, followed by three successive washes with petroleum ether (Vetec, Rio de Janeiro, RJ, Brazil) to remove lipids. The samples were dried at room temperature until constant weight was obtained. Protein was extracted from 1 g homogenate using 0.6 M KOH at 37 °C for 1 h, and after that samples were centrifuged at 800 × g for 10 min and supernatant was collected to measure protein concentration, using Bradford reagent (Bradford 1976).

*Biochemical parameters*

Serum cholesterol and triglycerides were measured using commercial kits (Applied BioSystems, Carlsbad, CA, USA), following recommendations of the manufacturer.

*Hormone assays*

Total serum thyroxine (T₄) and T₃ concentrations were determined using a coated tube RIA from ICN Pharmaceuticals (Costa Mesa, CA, USA), according to the manufacturer’s instructions. Minimum assay detection value was 25 ng/dl for T₃ and 1 μg/dl for T₄. Serum TSH was measured in serum samples in duplicate by a specific rat TSH RIA using reagents acquired from the National Hormone and Pituitary Program (Torrance, CA, USA), as detailed previously (Ortiga-Carvalho et al. 1997). Minimum assay detection value was 0.18 ng/ml for TSH. Within-assay variation was <5% in all hormone assays. All compared samples were measured in the same assay.

*Analysis of hepatic protein expression*

Liver samples were homogenized in lyses buffer pH 6–4 (50 mM HEPES, 1 mM MgCl₂, 10 mM EDTA, and 1% Triton X) with protease inhibitor cocktail complete (Roche). Total protein was resolved by SDS–PAGE on a 12% gel and transferred onto a polyvinylidene difluoride membrane (Westram – Whatman plc, Kent, UK). Membrane was blocked with 5% nonfat dry milk (Molico, Nestle, São Paulo, SP, Brazil) and incubated overnight at 4 °C with primary antibodies: anti-TRβ1 (THRB – Upstate Biotechnology,
Inc., Lakeplacid, NY, USA – 1:750 dilution), anti-TRα (THRA – Affinity Bioreagents, Golden, CO, USA – 1:750 dilution), anti-glutathione S-transferase α (GST; GSTA – Abcam, Cambridge, MA, USA – 1:170 000 dilution), anti-PPARα (PPARA – Abcam – 1:1000 dilution), and internal control anti-cyclophilin B (Affinity Bioreagents – 1:50 000 dilution). Membranes were then washed and incubated with peroxidase-labeled anti-rabbit IgG antibody (Amersham Biosciences, Inc. – 1:10 000 dilution) for 3 h at room temperature. All blots were then washed and incubated with a luminogen detection reagent (ECL Plus – Amersham Biosciences, Inc.) to further exposure on autoradiograph film (Kodak). The protein bands were evaluated by densitometry using the software Kodak ID3.5.

Liver mGPD activity

The enzymatic activity of mGPD in the liver was measured in the mitochondrial fraction by using phenazine methosulfate (PMS – Sigma) as an electron transporter between the reduced enzyme and the iodonitrotetrazolium chloride violet (INT – Sigma). The assay was performed in the presence of 0.1 M dL-α-glycerophosphate (Sigma) diluted in KCN/potassium phosphate buffer and a solution of 0.12 mM PMS and 7.9 mM INT (Lee & Lardy 1965). Samples were analyzed in a spectrophotometer at 500 nm and the values were expressed as absorbance (O.D.)/mg mitochondrial protein.

Analysis of hepatic mRNA expression

Total liver RNA was isolated from samples using Trizol reagent (Invitrogen). Total RNA was reverse transcribed using 1 μg RNA and the Superscript III kit (Invitrogen). The mRNA of TRβ (Thrb – forward: 5’-TGCCGAGCTCTATATTTCCA-3’, reverse: 5’-ACAGTGATGCT-AGCGATAGT-3’); mGPD (Gpd2 – forward: 5’-ATTCTCCATGCTCCAGAAG-3’, reverse: 5’-ACCTCCA-TGTAATTTGGCC-3’); and type 1 deiodinase (D1; Dio1 – forward: 5’-CCTGGGTGGTGTCACG-3’, reverse: 5’-GCGGGACCGCAATGC-3’); Akamine et al. (2007, Machado et al. 2009). Products were amplified on Applied Biosystems 7500 Real-Time PCR System (Life Technologies Corp.) using SYBR Green PCR Master Mix (Applied BioSystems). Cycle parameters were 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, 58 °C for 30 s, and 70 °C for 45 s. Product purity was confirmed by agarose gel analysis. Changes in mRNA expression were calculated from the cycle threshold, after correcting for 36B4, which did not show significant variation among groups. Data are expressed as fold induction over control group.

Statistical analysis

Data are reported as mean±S.E.M. One-way ANOVA, followed by Newman–Keuls post-test, was used for comparisons between different groups (GraphPad Prism; GraphPad Software, Inc., San Diego, CA, USA). Differences were considered to be significant at P<0.05.

Results

Body weight was not significantly different between FO- and soybean oil-treated groups either in euthyroid or hypothyroid condition; however, as expected, hypothyroid rats gained less weight than euthyroid rats, regardless of the oil treatment (body weight at killing: EU SO = 393±19 g and EU FO = 353±15 g; HYPO SO = 269±7 g and HYPO FO = 276±7 g). Oral administration of FO for 3 weeks promoted beneficient effects on body composition, dependent and independent of TH status. The euthyroid animals that received FO presented a lower percentage of lipid and elevated percentage of protein content in their carcasses compared with euthyroid animals that received soybean oil (Fig. 1A and B). In hypothyroid rats, no differences were detected in lipid compartment of carcass between FO- and soybean oil-treated groups (Fig. 1A). However, the positive effect of FO treatment on protein compartment, observed in euthyroid animals, persisted in hypothyroid animals (Fig. 1B). TH status did not change lipid compartment but influenced protein compartment, promoting significant increase in hypothyroid animals (P<0.05; Fig. 1B).

Figure 1  Fish oil effects on body composition and serum lipids of euthyroid (EU) and hypothyroid (HYPO) animals. (A) Percentage of lipid content in carcass. (B) Percentage of protein content in carcass. (C) Serum cholesterol. (D) Serum triglycerides. Animals were killed after receiving oral administration of soybean oil (SO) or fish oil (FO) for 3 weeks. Data are expressed as mean±S.E.M. n=7–8 rats per group. Different letters designate significant differences between groups (P<0.05 or less).
An important hypolipidemic action of FO was observed in euthyroid animals. As depicted in Fig. 1C and D, the euthyroid FO-treated group exhibited a significant reduction of 21 and 35% in serum cholesterol and serum triglycerides, respectively, compared with euthyroid soybean oil-treated group. Hypothyroidism enhanced serum cholesterol and decreased serum triglycerides compared with euthyroid soybean oil-treated group (P<0.05). Interestingly, the hypotriglyceridemic action of FO was abolished in hypothyroidism (Fig. 1D), but the reduction in serum cholesterol was maintained (Fig. 1C), normalizing the levels to values of euthyroid soybean oil-treated group.

Since our previous observations (Souza et al. 2010) demonstrated that FO may alter TH action, we investigated TH signaling in these animals. Both in euthyroid and in hypothyroid animals, FO treatment did not change serum T3 (EU SO=76.77±5.11 ng/dl and EU FO=76.52±3.49 ng/dl; HYPO SO=not detectable and HYPO FO=not detectable) or TSH (EU SO=2.68±0.26 ng/ml; and EU FO=2.23±0.14 ng/ml; HYPO SO=2.01 ng/ml; and EU FO=1.98±0.13 ng/ml). However, a slightly higher serum T4 was seen in euthyroid rats that received FO (EU SO=4.67±0.22 μg/dl and EU FO=5.99±0.14 μg/dl – P<0.05; HYPO SO=2.01±0.08 μg/dl and HYPO FO=1.98±0.13 μg/dl). The protocol’s efficiency to induce hypothyroidism was observed by a significant reduction in serum T3 and T4, followed by a significant increase in serum TSH, compared with euthyroidism (P<0.05).

In the liver, the main site of the control of lipid metabolism, TH status did not change hepatic TRβ expression in the control groups, receiving soybean oil (Fig. 2A and B). FO treatment promoted an ~1.3-fold increase in TRβ1 protein content (Fig. 2A) in euthyroid rats. This increase was not observed in the hypothyroid FO-treated group which presented TRβ expression statistically similar to euthyroid and hypothyroid soybean oil-treated groups, even though a trend to lower expression was observed (Fig. 2A and B). Similar to the protein expression, the hepatic mRNA expression of TRβ was positively regulated by FO (Fig. 2B) in euthyroid condition only, presenting an increment of ~66% (P<0.01).

No significant changes were observed in hepatic TRα protein content among the experimental groups (Fig. 2C).

**Figure 2** Effect of fish oil on hepatic thyroid hormone receptor expression of euthyroid (EU) and hypothyroid (HYPO) animals. (A) Hepatic TRβ1 expression evaluated by western blotting (representative autoradiography and graphic representation of densitometric values of TRβ1 expression normalized by cyclophilin expression). (B) Hepatic mRNA TRβ expression evaluated by real-time PCR. (C) Hepatic TRz expression evaluated by western blotting (representative autoradiography and graphic representation of densitometric values of TRz expression normalized by cyclophilin expression). Values are expressed relative to EU SO group. Animals were killed after receiving oral administration of soybean oil (SO) or fish oil (FO) for 3 weeks. Data are expressed as mean±s.e.m. n=5–7 rats per group. Different letters designate significant differences between groups (P<0.05 or less).

**Figure 3** Effect of fish oil on hepatic thyroid hormone targets of euthyroid (EU) and hypothyroid (HYPO) animals. (A) Hepatic enzymatic activity of mitochondrial α-glycerophosphate dehydrogenase (mGPD) evaluated by colorimetric assay. (B) Hepatic mRNA mGPD expression evaluated by real-time PCR. (C) Hepatic GST expression evaluated by western blotting (representative autoradiography and graphic representation of densitometric values of GST expression normalized by cyclophilin expression). (D) Hepatic mRNA D1 expression evaluated by real-time PCR. Values are expressed relative to EU SO group. Animals were killed after receiving oral administration of soybean oil (SO) or fish oil (FO) for 3 weeks. Data are expressed as mean±S.E.M. n=5–7 rats per group. Different letters designate significant differences between groups (P<0.05 or less).
significantly PPAR\(\alpha\) expression in liver of euthyroid and hypothyroid animals, even though the hepatic PPAR\(\alpha\) expression in hypothyroid animals was \(~\)2-7-fold higher than that in euthyroid animals (Fig. 4).

**Discussion**

This study highlights *in vivo* cross-talking mechanisms between the n-3 PUFA from FO and the TH signaling in lipid metabolism.

Previously, we had reported that rats fed an FO-enriched diet, from lactation to adulthood, exhibited higher hepatic TR\(\beta\)1 expression (Souza *et al.* 2010) accompanied by higher enzymatic activity of the mGPD, a well-known target of TH, involved in glycerol metabolism and in the oxidative mitochondrial process. In this study, we observed that FO given to adult animals, as a supplement by oral administration, during a shorter period of treatment (3 weeks) also provoked the enhancement of not only TR\(\beta\)1 protein content and mGPD activity but also their respective mRNAs, suggesting the involvement of a transcriptional mechanism. This consistent induction of TR\(\beta\)1 expression by FO suggests a role for TH signaling in the n-3 PUFA effects on hepatic metabolism, given the well-known hypolipidemic actions of TH, mediated by TR\(\beta\)1 isoform (Gullberg *et al.* 2002, Johansson *et al.* 2005, Hashimoto *et al.* 2006).

However, liver GST and D1 expressions, also TH targets via TR\(\beta\)1 (Sadow *et al.* 2003, Faustino *et al.* 2011), were not affected by the treatment with FO. Therefore, the data suggest that n-3 PUFA are able to not only increase TR\(\beta\)1 expression but also enhance TH signaling at specific targets. This is further reinforced by the fact that the ability of FO treatment to increase TR\(\beta\)1 and mGPD expression was abrogated in the context of hypothyroidism.

The relevance of selective upregulation of hepatic TH signaling for n-3 PUFA action became clear in hypothyroid animals. In the context of hypothyroidism, the hypocholesterolemic effect of n-3 PUFA is still observed, while the hypotriglyceridemic effect of these fatty acids was lost. Hypothyroidism was not associated with changes in liver TR\(\beta\)1 or TR\(\alpha\) expression, in disagreement with some studies (Zandieh-Doulabi *et al.* 2004), and in agreement with others (Hodin *et al.* 1990, Strait *et al.* 1990). On the other hand, hypothyroidism induced a marked increase in liver expression of PPAR\(\alpha\). Apparently, the control of serum lipids by n-3 PUFA involves different signaling pathways, a PPAR\(\alpha\)-dependent one for cholesterol and another, PPAR\(\alpha\) independent for triglycerides (Dallongeville *et al.* 2001, Sugiyama *et al.* 2008, Wakatsu *et al.* 2010). Indeed, our finding of marked increase in PPAR\(\alpha\) protein content in the liver of hypothyroid rats may justify the normalization of serum cholesterol in hypothyroid livers and in addition supports those previous reports arguing that the hypotriglyceridemic effect of n-3 PUFA is not dependent on PPAR\(\alpha\). Other nuclear proteins participate in the mediation of n-3
PUFA actions in the liver (Yoshikawa et al. 2002, Howell et al. 2009), and our study proposes TRβ as another transcriptional factor involved.

The mechanisms by which FO treatment led to increased hepatic TRβ in euthyroid condition and the reason for its absence in hypothyroidism are unclear. Since a previous study had shown that clofibrate, a PPARα-selective agonist, administered chronically to rats reduced liver TRβ expression (Bonilla et al. 2001), it is possible that higher PPARα in hypothyroid livers is counteracting the FO stimulus on TRβ expression.

mGPD gene is a classical target of TH action and its expression is upregulated via the TRβ1 at transcriptional level (Zavacki et al. 2005). In our present study, FO treatment led to a slight increase in serum T₄, with no changes in serum T₃ or TSH, only in euthyroid status, due to an unknown mechanism. Because in our previous study animals fed a FO-enriched diet exhibited no alterations in serum TH levels, but still presented higher TRβ1 and mGPD expression (Souza et al. 2010), we believe that the induction of the enzyme, in euthyroid condition, was due to the increased availability of TRβ1 caused by FO treatment, independent of serum TH. Liver mGPD is also a target of PPARα (Patsouris et al. 2004), but hypothyroidism has been shown to attenuate the effect of a PPARα agonist on mGPD activity (Shoemaker & Yamazaki 1991, Pacot et al. 1993). In this study, we showed that even though hypothyroid livers have higher availability of PPARα, n-3 PUFA from FO were unable to induce mGPD, reinforcing the role of TRβ in the FO induction of mGPD expression.

The influence of TH on PPARα expression is unclear and controversial, since both upregulation and downregulation of the mRNA encoding PPARα in the liver have been reported (Flores-Morales et al. 2002, Weitzel et al. 2003). To our knowledge, our study is the first to show that PPARα expression is upregulated by TH insufficiency at the protein level. Other studies had pointed out the existence of variable patterns of cross-talking between TR and PPAR pathways. Not only do they coregulate specific genes but the activity or availability of one receptor can influence the other’s activity (Lu & Cheng 2010). It has been demonstrated, both in transgenic animals and in cell transfection assays, that TR, in the absence of T₃ binding, is able to reduce PPARα-mediated transcription (Buroker et al. 2007, Liu et al. 2007, Ying et al. 2007). This mechanism is particularly of interest to hypothyroid status, since the unliganded TR may blunt not only the action of PPARα (Liu et al. 2007) but also the action of other nuclear receptors in the liver, as described by others (Hashimoto et al. 2006). Therefore, even though there is a higher expression of PPARα in the liver of hypothyroid rats, the functional consequence of hypothyroidism to the transcriptional activity of PPARα remains to be elucidated. Nevertheless, our study reinforces the involvement of TR and PPAR signaling on common pathways, as demonstrated by other authors in the regulation of muscular uncoupling protein 3 (de Lange et al. 2007) and hepatic fibroblast growth factor 21 expression (Adams et al. 2010).

In euthyroid rats, n-3 PUFA from FO promoted beneficial effects on body composition, reducing lipid compartment and increasing protein compartment of the carcasses. These findings have been reported by some studies (Su & Jones 1993, Gaiva et al. 2001), but not others (Awad et al. 1990), and the underlying mechanism has not been fully elucidated. Such as in the liver, in adipose tissue, n-3 PUFA have been shown to repress lipogenic gene expression while enhancing β-oxidation, which contributes not only to reduction of fat depot but also to lower serum triglycerides (Yu et al. 2010). Our data suggest that TH are involved in the reduction of lipid compartment caused by FO, because this effect was abolished in hypothyroid condition. TH or selective TRβ1 agonist administration reduced fat content of carcass (Villicev et al. 2007), and it remains to be investigated whether similar changes to the liver TRβ1 may occur to TR expression in adipose tissue.

FO ingestion in humans has proven efficacy to reduce serum lipids (Agren et al. 1996); however, it remains to be proved that the same mechanisms proposed herein are operating in humans, since, even though the rat is a good model, there are species differences in response to metabolic demands (Menahan & Sobocinski 1983, Couture & Hulbert 1995) and in the relative content of different PUFA in membranes (Hulbert et al. 2006).

In conclusion, this study shows that the ability of n-3 PUFA from FO to induce enhancement in liver expression of TRβ and of its target mGPD was lost in hypothyroidism, along with the abrogation of n-3 PUFA effects on serum triglycerides and on body lipid content, but preservation of cholesterol-lowering effect of such fatty acids. Thus, data suggest that n-3 PUFA effects on lipid metabolism are dependent on TH signaling in specific steps and, together with the marked upregulation of PPARα in liver of hypothyroid rats, suggests an important cross-talk between fatty acids and TH pathways in liver metabolism, with potential physiological and therapeutic implications.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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