Differential effects of fasting vs food restriction on liver thyroid hormone metabolism in male rats

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Abstract

A variety of illnesses that leads to profound changes in the hypothalamus–pituitary–thyroid (HPT) are axis collectively known as the nonthyroidal illness syndrome (NTIS). NTIS is characterized by decreased tri-iodothyronine (T_3) and thyroxine (T_4) and inappropriately low TSH serum concentrations, as well as altered hepatic thyroid hormone (TH) metabolism. Spontaneous caloric restriction often occurs during illness and may contribute to NTIS, but it is currently unknown to what extent. The role of diminished food intake is often studied using experimental fasting models, but partial food restriction might be a more physiologically relevant model. In this comparative study, we characterized hepatic TH metabolism in two models for caloric restriction: 36 h of complete fasting and 21 days of 50% food restriction. Both fasting and food restriction decreased serum T_4 concentration, while after 36-h fasting serum T_3 also decreased. Fasting decreased hepatic T_3 but not T_4 concentrations, while food restriction decreased both hepatic T_3 and T_4 concentrations. Fasting and food restriction both induced an upregulation of liver D3 expression and activity, D1 was not affected.
A differential effect was seen in *Mct10* mRNA expression, which was upregulated in the fasted rats but not in food-restricted rats. Other metabolic pathways of TH, such as sulfation and UDP-glucuronidation, were also differentially affected. The changes in hepatic TH concentrations were reflected by the expression of T_3-responsive genes *Fas* and *Spot14* only in the 36-h fasted rats. In conclusion, limited food intake induced marked changes in hepatic TH metabolism, which are likely to contribute to the changes observed during NTIS.

Key Words
- liver
- nutrition
- rat
- thyroid hormone metabolism

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Introduction

Profound changes occur in the hypothalamus–pituitary–thyroid (HPT) axis during illness and starvation. The non-thyroidal illness syndrome (NTIS) is characterized by decreased serum tri-iodothyronine (T_3) and thyroxine (T_4) concentrations, and inappropriately low thyroid-stimulating hormone (TSH) concentrations (Boelen *et al.* 2011). Cytokine release as part of the acute-phase response has been known to be important for the development of NTIS (Boelen *et al.* 1996). Spontaneous diminished food intake is known to be a part of a variety of illnesses,

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but has received only little attention as a potential mediator of the illness-induced changes in thyroid hormone (TH) homeostasis, despite its sometimes profound impact. For example, in our earlier studies, we found that the low serum T_3 during chronic inflammation is completely explained by the diminished food intake, indicating that this is an important factor in the pathogenesis of NTIS, especially in prolonged illness (Boelen *et al*. 2006). Whether NTIS is an adaptive or maladaptive response is still a matter of debate.

Fasting leads to a central downregulation of the HPT axis, characterized by increased type 2 deiodinase expression in hypothalamic tanycytes which leads to local increased bioavailabilty of T_3 concentration that suppresses thyrotrophin-releasing hormone (TRH) expression in hypophysiotropic neurons of the paraventricular nucleus of the hypothalamus (Fekete *et al*. 2004, 2010). In addition, the peripheral handling of THs is also markedly altered during fasting (Vagenakis *et al*. 1977, Harris *et al*. 1978).

In the liver, an important site for TH metabolism, type 1 deiodinase (D1) and type 3 deiodinase (D3) are expressed. Type 1 deiodinase is able to deiodinate both the inner and outer ring of TH, and is regarded as a TH-activating enzyme, in addition it can also contribute to the clearance of TH by degrading sulfated T_3. D3 converts T_4 to rT_3 and T_3 to 3,3′diiodothyronine (T_2) and it thus the main inactivating enzyme. Besides deiodination, sulfation by sulfotransferases and glucuronidation by UDP-glucuronosyltransferases enhance the metabolism of TH and its excretion via the bile and urine (Visser *et al*. 1990, 1998, Kester *et al*. 1999, 2003). Upstream of *Sults* and *Ugts* is the constitutive androstane receptor (CAR). CAR-target genes are the sulfotransferases *Sult1b1* and *Sult1c1* as well as the UDP-glucuronidase *Ugt1a1*, which are important for TH metabolism in the rat liver. Besides an enhanced metabolism of TH in peripheral tissues via altered deiodinases, sequestration of TH in tissues is also suggested to play a role in the decreased serum TH concentrations. In order to enter the hepatocyte, TH has to be transported over the cell membrane. This is mediated via two transporters: MCT8 and MCT10.

During fasting, the activity of hepatic type 3 deiodinase (D3) is increased (Boelen *et al*. 2012) while the activity of type 1 deiodinase (D1) is decreased (Omara *et al*. 1993, Araujo *et al*. 2008, 2009). Theoretically, these changes would result in an enhanced inactivation of TH and less conversion of T_4 to T_3. However, recent studies has indicated that the fasting-induced changes in TH serum concentrations persist in *D1*-knockout (KO) mice, and are only mildly affected in *D3*-KO mice (Galton *et al*. 2014). The precise role of sulfotransferases and UDP-glucuronidases during fasting and illness is unclear at present and seems to be species dependent, because increased hepatic expression of *Sults* and *Ugts* (Maglich *et al*. 2004, Vella *et al*. 2011) in mice, and normal or decreased expression in rats (deJong *et al*. 1992, Kester *et al*. 2003) were reported.

A recent study in a rabbit model for prolonged critical illness has revealed that the hepatic increase in *D3* and decrease in *D1* could be reverted by parenteral feeding (Mebis *et al*. 2012). By contrast, the central changes in *Trh* mRNA expression in the PVN were not affected by parenteral feeding, which is in line with earlier findings during chronic inflammation in mice, showing that, hypothalamic expression of *Dio3* and *Trh* in the hypothalamus is not primarily modulated by nutrient restriction (Boelen *et al*. 2006).

Effects of caloric restriction on TH metabolism are often studied in relative short-term fasting models such as an overnight fast or a 24–48 h fasting period. However, prolonged caloric deprivation might be a physiologically more relevant model, especially when prolonged critical illness is studied. To gain more insights into the effects of caloric restriction on TH homeostasis in the liver we compared two models. Hepatic TH metabolism was assessed in 36-h fasted rats, and in rats that received only 50% of their baseline caloric intake during 3 weeks. In both experiments, food-restricted rats were compared with rats fed *ad libitum*.

Materials and methods

Animal experiments

Male Wistar rats weighing 250–350 g and at the age of 8–12 weeks (Charles River breeding Laboratories, Sulzfeld, Germany) were housed individually in a 12 h light:12 h darkness cycle, lights were on at 0700 h. Standard laboratory chow (CRM (E) chow from Special Diet Services, Essex, UK) and water were provided *ad libitum* unless stated otherwise. All procedures were approved by the Animal Welfare Committee of the Academic Medical Center (AMC) of the University of Amsterdam.

For the short-term fasting, experiment food was removed in the evening. After 36 h (two nights, 1 day) of food deprivation, rats ($n=6$ per group) were killed at $\pm$0900 h with an overdose of pentobarbital (120 mg/kg body weight). Trunk blood samples was collected, spun down, and serum was stored at -20 °C until analysis. The right liver lobe was dissected, snap frozen in liquid

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nitrogen, and stored in −80 °C until further use. For the food restriction experiment, 24-h food intake was monitored for 4 days (baseline value). Subsequently, rats daily received 50% of their individual baseline 24-h intake for 21 days at ±1700 h. After 21 days, rats (n=6 per group) were killed as described above at ±0900 h. Both experiments had their own *ad libitum* fed control group.

Deiodinase measurements

For measurements of deiodinase activity, samples were homogenized on ice in ten volumes of PE buffer (0.1 M sodium phosphate, 2 mM EDTA pH 7.2) using a polytron (Kinematica, Luzern, Switzerland). 10 mM dithiothreitol (DTT) was added to the PE buffer (PED10) for measurement of D1 activity and 50 mM for D3 (PED50). Homogenates were snap frozen in aliquots and stored in −80 °C until further use. Protein concentration was measured with the Bio-Rad protein assay using BSA as the standard following the manufacturer's instructions (Bio-Rad Laboratories).

Type 1 deiodinase Liver D1 activity was measured by duplicate incubations of 75 µl of 300 times diluted homogenate (approximately between 5 and 15 µg of total protein) for 30 min at 37 °C in a final volume of 0.15 ml with 0.1 µM rT_3 with the addition of ∼1×10^5 c.p.m. [3′5′^{125}I]rT_3 in PE buffer. One sample of each group was incubated in the presence of 500 µM PTU in order to inhibit D1 activity representing a tissue blank. Reactions were stopped by the addition of 0.15 ml ice-cold ethanol. After centrifugation, 0.125 ml of the supernatant was added to 0.125 ml 0.02 M ammonium acetate (pH 4.0), and 0.1 ml of the mixture was applied to 4.6× 250 mm Symmetry C18 column connected to a waters HPLC system (model 600E pump, model 717 WISP autosampler, Waters, Etten-Leur, The Netherlands). The column was eluated with a linear gradient of acetonitrile (28–42%) for 15 min in 0.02 M ammonium acetate (pH 4.0) at a flow of 1.2 ml/min. The activity of rT_3 and T_2 in the eluate was measured online using a Radiomatic 150 TR flow scintillation analyzer (Perkin Elmer, Waltham, MA, USA). D1 activity can be calculated by subtracting the activity measured in the tissue blank from the activity measured without PTU. D1 activity was expressed as picomole 3,3′T_2 generated per minute per milligram protein, with intra-assay variation being 5.5%.

Type 3 deiodinase D3 activity was measured with the same method as D1 with the following modifications. Duplicate incubations of 75 µl of homogenate (approximately between 0.5 and 1.5 mg of total protein) were incubated for 120 min at 37 °C with the addition of 1 nM unlabeled T_3 and 2×10^5 c.p.m. [3′^{125}I]T_3 in a final volume of 0.15 ml PE buffer. One sample of each group was incubated in the presence of 500 nM unlabeled T_3 to inhibit D3 activity representing a tissue blank. The activity measured with 1 nM T_3 minus the incubation with 500 nM T_3 represent true D3 activity. D3 activity was expressed as femtomole 3,3′T_2 generated minute per milligram protein, with intra-assay variation being 5.5%.

Thyroid hormones

Serum T_3 and T_4 were measured by in-house RIAs (Wiersinga & Chopra 1982). All samples of one experiment were measured within the same assay (intra-assay variation T_3: 3.6% and T_4: 6.6%).

THs in tissue

Liver concentrations of T_3 and T_4 were measured by an LC–MS method as described previously (De Escobar *et al.* 1985, Ackermans *et al.* 2012), with some modifications. Briefly, for the determinations of T_4 and T_3 in tissues, 50 mg frozen tissue were added to a plastic 2 ml polypropylene (PP) tube containing 150 mg zirconia beads, 400 µl methanol, and 40 µl $^{13}C_6$-labeled internal standards (Ackermans *et al.* 2012). The samples were homogenized using a Magna Lyser (Roche Molecular Biochemicals) and transferred to 5 ml glass tubes. The plastic tubes were rinsed with 500 µl methanol and this was added to the homogenate. Chloroform (1.8 ml) was then added in a volume double that of the amount of methanol contained in the tissue homogenate. The samples were mixed on a Vortex for 15 s. The extraction was carried out in two steps, interspaced with centrifugations for 10 min at 1841 ***g***. In the seconds step, a mixture of 0.9 ml chloroform-methanol (2:1) was added to the pellet. The final volume of extract was about 40 times the weight of the tissue plus the volume of the internal standard, as described previously (Reyns *et al.* 2002). The chloroform–methanol extracts were transferred to glass tubes for back-extraction of the iodothyronines into an aqueous phase with 0.05% $CaCl_2$, using the following calculation: 0.05% $CaCl_2$ (ml)=final volume extracts (ml)×1/4−(tissue weight (g)×0.8)−volume internal standard (ml). The value of 0.8 represents the estimated amount of water per gram tissue in milliliter per gram.

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Table 1 Primer sequences used for qPCR

Cyclophilin	Forward: 5′-GAGACTTCACCAGGGG-3′ Reverse: 5′-CTGTCTGTCTTGGTGCTCTCC-3′
Tbp	Forward: 5′-TTCGTGCCAGAAATGCTGAA-3′ Reverse: 5′-TGCACACCATTTTCCCAGAAC-3′
Dio1	Forward: 5′-GAAGTGCAACGTCTGGGATT-3′ Reverse: 5′-CTGCCG AAGTTCAACACCA-3′
Dio3	Forward: 5′-AGCGCAGCAAGAGTACTTCAG-3′ Reverse: 5′-CCATCGTGTCCAGAA CCAG-3′
Car	Forward: 5′-AGCAAGGCCCAGAGACGCCA-3′ Reverse: 5′-CCGGAGGCCTGAACTGCACAAA-3′
Sult1b1	Forward: 5′-TCCTCGCTGGAAATGTGGCCT-3′ Reverse: 5′-TGCCCTTCCCTCTTTTCCCACCA-3′
Sult1c1	Forward: 5′-CCACCCCTCAACTCAGGTCTGGA-3′ Reverse: 5′-AGGGTACCAGGGTCAGGCAGC-3′
Ugt1a1	Forward: 5′-TGGTGTGCCGGAGCTCATGTTCG-3′ Reverse: 5′-AGACAGCAGCATACTGGAGTCCC-3′
Spot14	Forward: 5′-ACGGGGCAGGTCCTGTAGGT-3′ Reverse: 5′-AGCCGCCTTTGCATCCACCTG-3′
TRa1	Forward: 5′-CATCTTTGAACTGGGCAAGT-3′ Reverse: 5′-CTGAGGCTTTAGACTTCCTGATC-3′
TRa2	Forward: 5′-CATCTTTGAACTGGGCAAGT-3′ Reverse: 5′-GACCCTGAACAACATGCATT-3′
TRb1	Forward: 5′-TGGGCGAGCTCTATATTCCA-3′ Reverse: 5′-ACAGGTGATGCAGCGATAGT-3′
Fas	Forward: 5′-CTTGGGTGCCGATTACAACC-3′ Reverse: 5′-GCCCTCCCGTACACTCACTC-3′
Pepck	Forward: 5′-TGCCCTCTCCCCTTAAAAAAG-3′ Reverse: 5′-CGCTTCCGAAGGAGATGATCT-3′

Followed by a second extraction with pure upper phase (chloroform:methanol:0.05% $CaCl_2$ 3:49:48).

The iodothyronines in the pooled aqueous phases were concentrated and further purified using a small Bio-Rad AG 1X2 resin column (bed volume 0.5 ml) in a Pasteur pipette, as described previously (Mallol *et al.* 1982) and eluted in a volume of 2 ml 70% acetic acid. The eluates were then evaporated to dryness and taken up in 200 µl 0.1% NH_4OH. The iodothyronines were measured on an Acquity UPLC – Xevo TQ-S tandem mass spectrometer system equipped with a Z-Spray ion source operated in positive electrospray ionization mode (Waters, Milford, MA, USA). All aspects of system operation and data acquisition were controlled using MassLynx version 4.1 software with automated data processing using the QuanLynx Application Manager (Waters).

Intra-assay was variation <9%, total-assay variation was <12% for all metabolites. LOD T_3 0.20; T_4 0.39 pg on column. LOQ T_3 1.43; T_4 1.32 pg on column. Recovery T_3 83% and T_4 77%.

RNA isolation and qPCR

RNA was isolated using the Magna Pure apparatus (Roche Molecular Biochemicals) and the Magna pure tissue III total RNA kit (Roche Molecular Biochemicals). RNA yield was determined using the Nano drop (Nanodrop, Wilmington, DW, USA) and cDNA was synthesized with equal RNA input with the First-strand cDNA synthesis kit for qPCR with oligo d(T) primers (Roche Molecular Biochemicals). As a control for genomic DNA contamination, we included a cDNA synthesis reaction without reverse transcriptase. Quantitative PCR was carried out using the Lightcycler 480 and Lightcycler480SybrGreen I Master mix (Roche Molecular Biochemicals). Quantification was carried out using the LinReg software. PCR efficiency of each sample was calculated and samples that had a deviation of

Table 2 Body weight in grams before the start of the experiment (pre) and after the fasting period (post). Mean values±S.E.M. are shown

	Body weight (g)		
	Pre	Post	%
Ad libitum	250.3±2.2	262.7±2.7	+4.9±0.3
Fasting 36 h	250.7±2.7	225.3±2.2	−10.1±0.3
Ad libitum	358.3±2.6	406.2±3.7	+13.4±1.0
Food restriction 21 days	360.2±1.4	298.5±2.1	−17.1±0.3

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more than 5% of the mean efficiency value of the assay were excluded. We tested de T_3-responsive metabolic genes fatty acid synthase (*Fas*), phosphoenolpyruvate carboxykinase (*Pepck* (*Pck1*)), and *Spot14* as a readout of metabolic status in the liver. Calculated values were normalized by the expression of housekeeping genes (*Hprt*, *Cyclophilin*, and *Tbp*), which were selected to be stable among different groups. Published primer sequences were used for *Hprt* (Boelen *et al*. 2006); *Mct8*, *Mct10* (van Beeren *et al*. 2012); other primer sequences are displayed in Table 1.

Statistical analyses

Differences between groups were evaluated using Student's *t*-tests. To correct for multiple testing, we employed the Holm's sequential Bonferonni method. All tests were performed using GraphPad prism 5 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Effects of fasting and caloric restriction on body weight

In the fasting experiment, 36 h of fasting led to a 10.1% body weight loss compared with the pre-fasting body weight. *Ad libitum* fed control animals gained 4.9% body weight during the same period. Three weeks of a 50% caloric restriction led to a 17.1% body weight loss, compared with a 13.4% body weight gain in the *ad libitum* fed control rats (Table 2).

TH concentrations in serum and liver

Serum T_3 and T_4 levels both decreased after 36 h of fasting compared with control rats (T_3: −36%, T_4: −26%), while only serum T_4 level was decreased (−19%) after food restriction for 3 weeks (Fig. 1). Serum rT_3 concentrations were not affected by fasting or food restriction (data not shown). Fasting resulted in decreased hepatic T_3 (−24%) but not T_4 concentrations, while both hepatic T_4 (−24%) and T_3 concentrations (−24%) were decreased in the food-restricted rats compared with controls (Fig. 1). Hepatic rT_3 concentrations were below the detection limit of our assay.

Hepatic deiodinase activity and expression

D3 activity and mRNA expression were significantly increased in both 36-h fasted and food-restricted rats compared with *ad libitum* fed controls. While *Dio1* mRNA expression decreased after 36 h fasting, no significant decrease was observed in D1 activity. D1 activity and expression were not affected by 3 weeks of food restriction (Fig. 2).

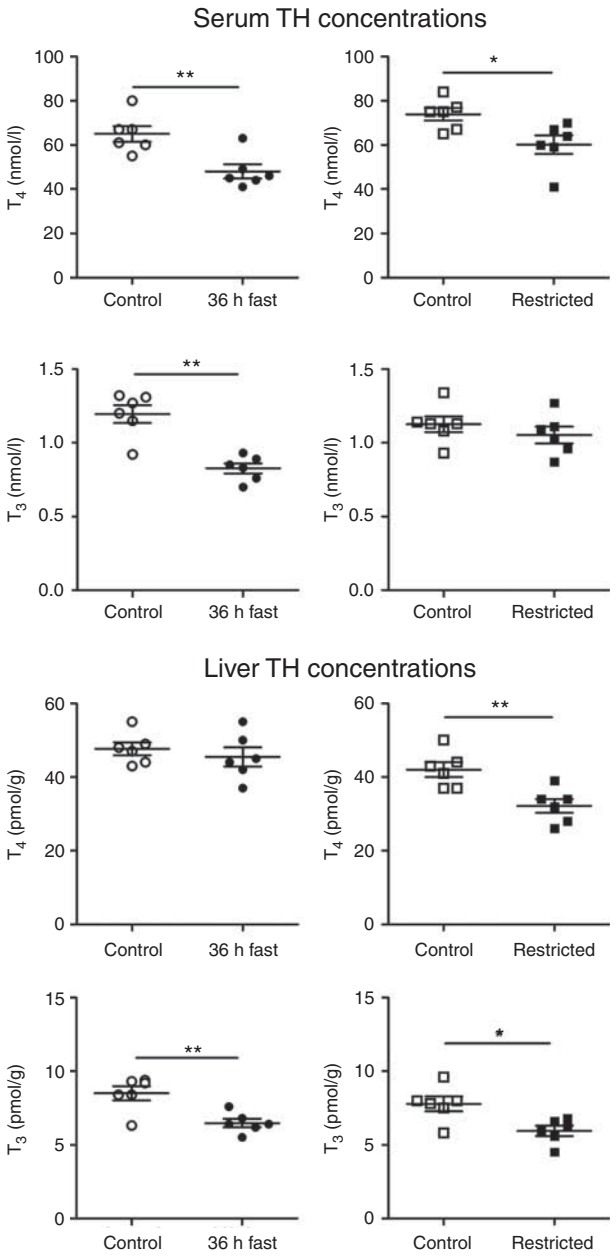

Figure 1
Serum T_4 and T_3 concentrations (upper panel) and hepatic thyroid hormone concentrations (lower panel) in the fasting (circles) and restriction (squares) experiment. Open circles and squares represent *ad libitum* fed control rats, closed circles the 36-h fasted rats, and closed squares the rats that received 50% of their baseline food intake for 21 days. Mean values ± S.E.M. are shown. Symbols indicate differences between fed vs fasted/restricted groups (*$P \leq 0.05$, **$P \leq 0.01$) as evaluated by Student's *t*-tests.

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Expression of transporters and TRs

We observed an upregulation of hepatic *Mct10* mRNA expression after 36 h fasting compared with *ad libitum* fed controls, but no changes after food restriction. *Mct8* mRNA expression was not affected by fasting or food restriction. Likewise, no effects of fasting or food restriction were observed on the expression of the TH receptors *TRa1*, *TRa2*, and *TRb1* (Fig. 3).

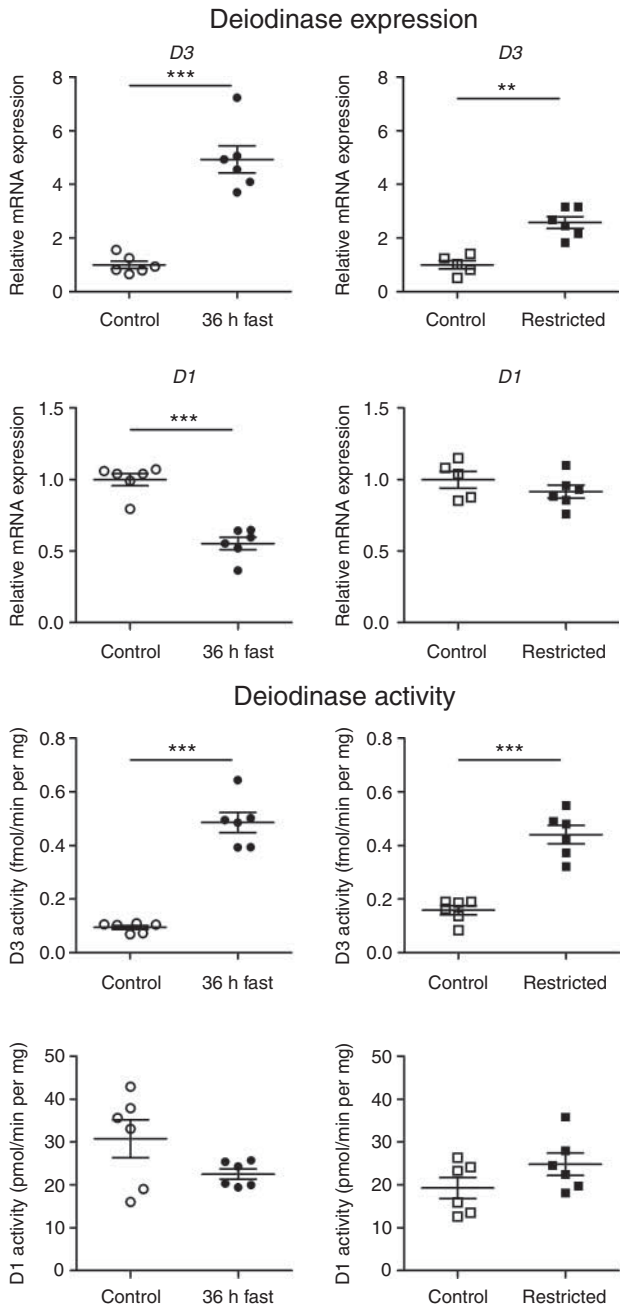

Figure 2
D1 and *D3* relative mRNA expression (upper panel) and activity (lower panel) in the fasting (circles) and restriction (squares) experiment. Open circles and squares represent *ad libitum* fed control rats, closed circles the 36-h fasted rats, and closed squares the rats that received 50% of their baseline food intake for 21 days. Mean values ± S.E.M. are shown. Symbols indicate differences between fed vs fasted/restricted groups (**$P \leq 0.01$, ***$P \leq 0.001$) as evaluated by Student's *t*-tests.

Sult and *Ugt* mRNA expression

A striking difference between the two feeding models was observed in *Car* mRNA expression. While hepatic *Car* mRNA expression increased in the 36-h fasted rats, it did not change after food restriction. This was also reflected in the upregulation of the *Car* downstream target gene *Ugt1a1*, which was significantly higher in the 36-h fasted rats, but not in the food restricted rats. Sulfotransferases *Sult1b1* and *Sult1c1* were differentially regulated. *Sult1b1* decreased after 36 h fasting but not after food restriction, while the opposite was observed for *Sult1c1* (Fig. 4).

Metabolic status in liver

Clear differences were observed in hepatic expression of the key T_3-regulated genes and metabolic enzymes *Pepck* and *Fas*. *Pepck* was increased after 36 h fasting but not after food restriction. *Fas* was decreased after 36 h fasting but not after food restriction. The TH-responsive gene *Spot14* (*Thrsp*) was downregulated after 36 h fasting, but was unaffected by food restriction (Fig. 5).

Discussion

In this study, we compared the changes in hepatic TH metabolism in two caloric restriction models, i.e., 36-h fasting or 50% caloric restriction for 3 weeks. As caloric restriction is a major part of illness, insights into the differences between these models are helpful in understanding the contribution of caloric restriction to the pathogenesis of NTIS.

Fasting for 36 h led to a significant decrease in both serum T_3 and T_4 concentrations (36 and 26% respectively), while food restriction only decreased serum T_4 concentration by 19%. This may indicate that a rigorous 36-h starvation period requires strict adaptations, including a limitation of energy expenditure, mediated by the low serum T_3 concentrations, while food restriction for a longer period may enable the organism to adapt to the new situation. Interestingly, the group of Carvalho did find significant effects on serum T_3 (−53%) and T_4 (−38%) in 25-day 40% food-restricted male rats (Araujo *et al.* 2008, 2009). As feeding schedules from their study

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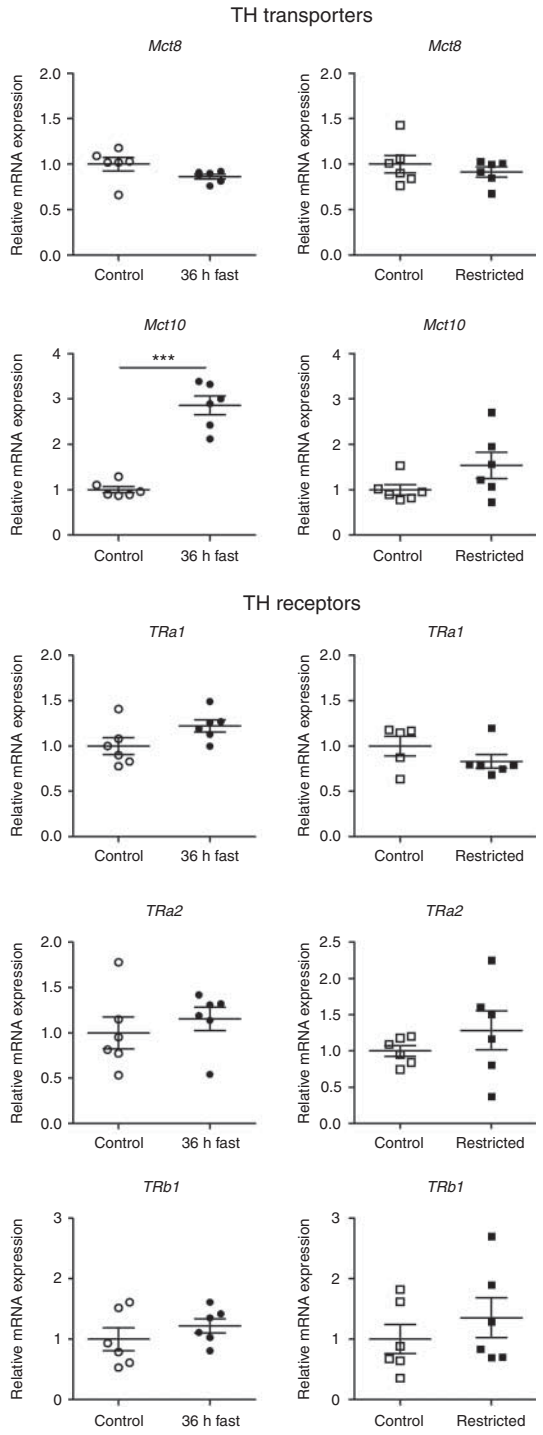

Figure 3
Relative mRNA expression of thyroid hormone transporters *Mct8* and *Mct10* (upper panel) and the thyroid hormone receptors *TRa1*, *TRa2*, and *TRb1* (lower panel) in the fasting (circles) and restriction (squares) experiment. Open circles and squares represent *ad libitum* fed control rats, closed circles the 36-h fasted rats, and closed squares the rats that received 50% of their baseline food intake for 21 days. Mean values ± S.E.M. are shown. Symbols indicate differences between fed vs fasted/restricted groups (***$P \leq 0.001$) as evaluated by Student's *t*-tests.

and the this study are more or less similar (21 days 50% restriction vs 25 days 40% restriction), these differences are difficult to explain. The timing of feeding and timing of killing the animal might be relevant. We gave access to the food at the beginning of the dark period and killed the animals at the beginning of the light period, but it is not known when this was done in the studies from the Carvalho group (Araujo *et al.* 2008, 2009). In addition to serum TH concentrations, we measured hepatic TH concentrations after 36-h fasting and 50% food restriction. Hepatic T_3, but not T_4 concentration, decreased after 36 h fasting while both hepatic T_3 and T_4 decreased after 3-week food restriction. This suggests a hypothyroid state of the liver after longtime diminished food intake.

In order to explain the observed changes in liver TH concentrations, we evaluated hepatic deiodinase, transporter, and TR expression in both models. As observed before, D3 mRNA expression and activity were markedly increased after 36 h fasting. A similar pattern was present after food restriction, although during food restriction the increase in mRNA expression and activity was lower compared with the fasting condition. We have observed a fasting-induced increase in liver *D3* activity before in mice after 24 and 48 h of fasting, and this was shown to be dependent on the drop in serum leptin (Boelen *et al.* 2012). As the decreased body weight in both our models is likely to represent a decrease in adiposity, and thus in leptin secretion, this may explain the increased D3 activity in both models.

Dio1 mRNA expression was only decreased after 36 h of fasting, but neither fasting nor food restriction decreased D1 activity. The 36-h fasting period is likely to be too short to observe decreases in D1 activity. Araujo *et al.* (2008, 2009) observed a decrease in D1 activity after 25 days of 40% food restriction, but this might be due to the decrease in T_3 serum concentration, which was absent in our study. Although D1 was thought to be important for the fasting-induced decrease in TH concentrations (Harris *et al.* 1978), it is more likely that D1 regulation is secondary to the serum TH concentrations, because changes in D1 occur later than the changes in serum TH concentrations (Omara *et al.* 1993), and *D1*-KO mice show no altered response to fasting (Galton *et al.* 2014).

We observed a striking increase in *Mct10* expression after 36-h fasting, but not after food restriction. MCT10 has been shown to be an effective bidirectional aromatic amino acid transporter, which has an affinity for T_3 that is even higher than MCT8 (Ramadan *et al.* 2006). Transfection of hMCT10 in cells enhances the intracellular degradation of T_3 by D3 (Friesema *et al.* 2008). Although

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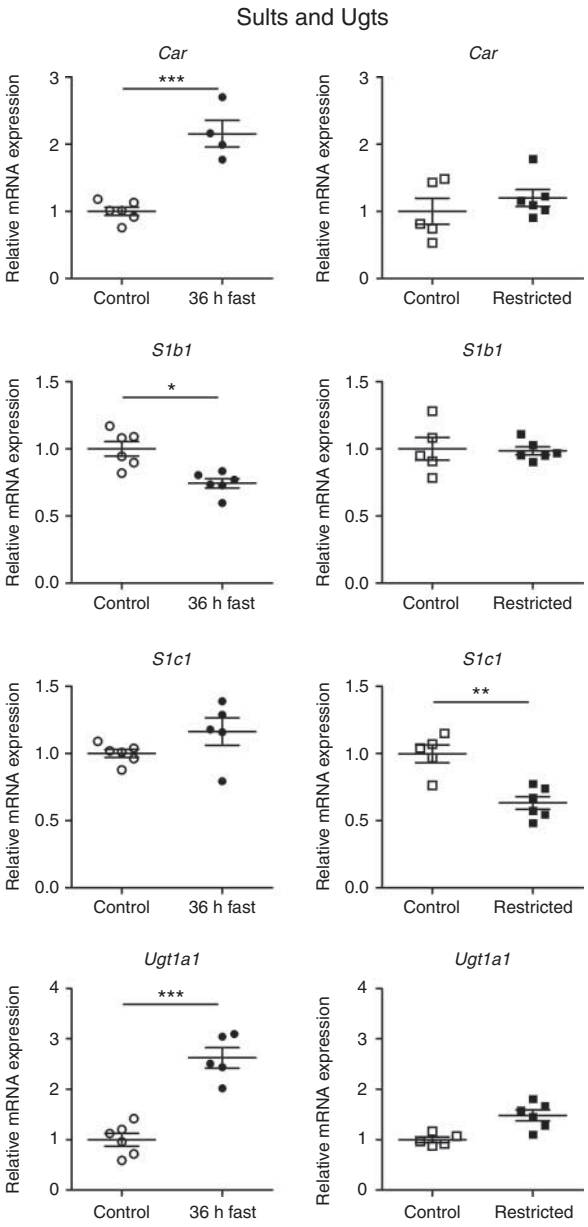

Figure 4
Relative mRNA expression of genes involved in thyroid hormone metabolism: constitutive androstane receptor (*Car*), sulfotransferase 1B1 (*S1b1*), sulfotransferase 1C1 (*Sult1c1*), and UDP-glucuronidase 1A1 (*Ugt1a1*) in the fasting (circles)and restriction (squares) experiment. Open circles and squares represent *ad libitum* fed control rats, closed circles the 36-h fasted rats, and closed squares the rats that received 50% of their baseline food intake for 21 days. Mean values±S.E.M. are shown. Symbols indicate differences between fed vs fasted/restricted groups (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$) as evaluated by Student's *t*-tests.

the higher demand for aromatic amino acids during fasting could initiate the upregulation of MCT10, it is tempting to speculate that MCT10 also has a role in the decreased serum T_3 concentrations during 36 h fasting by shuttling it into the liver, where it can be degraded by D3 to prevent a local hyperthyroid state. This concept is supported by the decreased hepatic T_3 concentrations we observed after 36 h fasting. During 3-week food restriction, liver *Mct10* expression did not change while liver *Dio3* expression increased. The combination of these changes might play a role in the decrease in hepatic T_3 in the absence of a decrease in serum T_3.

Interestingly, we found a differential regulation of *Car* mRNA expression between the two models. *Car* expression was induced after 36 h fasting, while it was unchanged after 3 weeks of 50% food restriction. The induction of *Car* was associated with an increased expression of *Ugt1a1*, which indicates a role for glucuronidation in the clearance of T_3 and T_4 from the circulation during fasting. *Car* has been shown before to be important for the fasting-induced changes in serum TH levels (Maglich *et al.* 2004). Sulfotransferase expression did not increase and *Sult1b1* was even lower after 36 h of fasting, which has been found before in rats (deJong *et al.* 1992, Kester *et al.* 2003). This indicates that increased sulfation does not play a major role in the decreased clearance of TH during fasting. In the food restriction experiment, *Sult1b1* decreased while *Ugt1a1* increased slightly without a change in *Car* expression, which points to possible involvement of other nuclear receptors such as PXR (Chai *et al.* 2013).

The key metabolic T_3-regulated enzymes Spot14, FAS, and PEPCK were differentially regulated in the two-caloric restriction models. In the fasting model, metabolic requirements clearly overrule the regulation of *Pepck*, as this gene is upregulated while *Fas* and *Spot14* are down-regulated. In the food restriction model, none of these genes were affected despite lower hepatic T_3 concentrations. Although the expression of TRs did not change, this effect might be explained by a decreased binding capacity of TRs as has been observed in a hepatoma celline upon stimulation with cytokines (Wolf *et al.* 1994).

In summary, food restriction results in a local decrease in TH concentrations in the liver, which is not always reflected in the expression of three key T_3-regulated genes. The observed alterations are different from the changes observed in experimental inflammation models where food intake was also reduced. Acute inflammation, chronic inflammation, and bacterial sepsis in rodents resulted in decreased activity of liver D1 and D3 concentrations (Boelen *et al.* 2004, 2005, 2008, 2009). However, in a rabbit model of prolonged critical illness, hepatic D3 was shown to increase and this was reversed when parenteral feeding was applied during the period of illness (Debaveye *et al.* 2005, Mebis *et al.* 2012).

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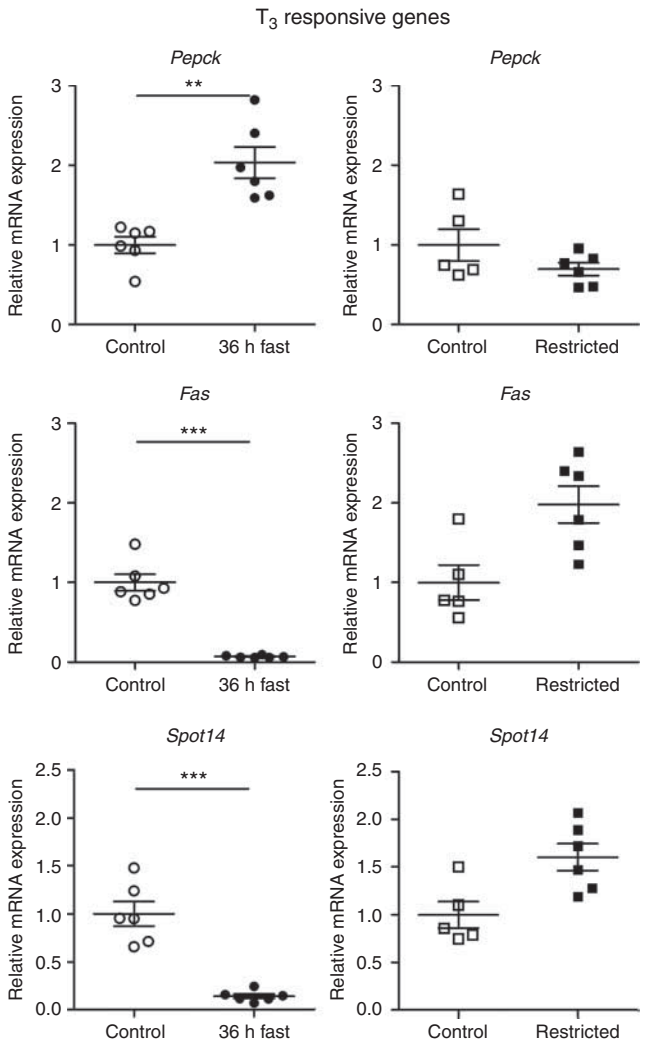

Figure 5
Relative mRNA expression of the metabolic genes phosphoenolpyruvate carboxykinase (*Pepck*), fatty acid synthase (*Fas*), and *Spot14* in the fasting (circles) and restriction (squares) experiment. Open circles and squares represent *ad libitum* fed control rats, closed circles the 36-h fasted rats, and closed squares the rats that received 50% of their baseline food intake for 21 days. Mean values ± S.E.M. are shown. Symbols indicate differences between fed vs fasted/restricted groups (**$P \leq 0.01$, ***$P \leq 0.001$) as evaluated by Student's *t*-tests.

The observed differences in D3 expression and activity may be due to differences in serum leptin concentrations. While prolonged critical illness decreases serum leptin levels, acute and chronic inflammation increase serum leptin via IL1β (Faggioni *et al.* 1998). Critically ill patients showed a minor increase in liver D3 activity, while sulfotransferase activity did not change (Peeters *et al.* 2003, 2005). A role of diminished food intake cannot be excluded in this setting. Analysis of post mortem liver tissue of prolonged critically ill patients showed reduced TH concentrations compared with acute death patients (Arem *et al.* 1993), which might be a result of decreased D1 and increased D3 activity. This would fit with our findings of reduced hepatic T_3 and T_4 concentrations after 3-week caloric restriction. Liver TH transporter expression appeared to be differentially regulated during fasting/food restriction and illness: we observed a marked increase in liver *Mct10* mRNA expression during fasting, while in critically ill patients and in critically ill rabbits, the expression of *Mct8* was increased and *Mct10* expression did not change (Mebis *et al.* 2009). In the latter studies, it was difficult to discriminate between feeding-related changes *per se* and inflammation-related changes. It is likely that the observed decrease in serum T_3 during illness depends predominantly on diminished food intake as observed before (Boelen *et al.* 2006), although it remains speculative whether liver D3 is involved.

In critically ill patients, illness-related nutrient restriction contributes to decreased TH levels. In these patients, early parenteral nutrition appeared to have a negative effect on clinical outcome compared with late parenteral nutrition (Langouche *et al.* 2013). Thus, nutrient restriction in critical illness may be a beneficial adaptation. However, it is unknown at present if the low TH concentrations in ICU patients with protracted critical illness represent a useful adaptation or rather a reflection of neuroendocrine failure. In the absence of large randomized clinical trials, there is no convincing evidence at present that treatment of these patients with TH is beneficial in terms of clinical outcome. A number of small clinical trials showed that treatment with combinations of the neuropeptides growth hormone-releasing hormone, growth hormone-releasing peptide 2, and TRH partially restores serum TH concentrations, while in addition some anabolic parameters improve in ICU patients with protracted critical illness (Van den Berghe *et al.* 1998, 1999). However, at this stage it is unknown if this approach improves inflammatory parameters and restores TH metabolism at the tissue level.

In conclusion, long-term caloric restriction may overrule the inflammatory mediated decrease in liver D3 activity that is seen in several inflammation models and likely contributes to the illness-induced changes in hepatic TH metabolism observed during prolonged illness. However, both during fasting and caloric restriction, the changes in hepatic TH metabolism are not mediating the serum decreases in TH (Kwakkel *et al.* 2008, 2010, Mebis *et al.* 2012, Galton *et al.* 2014).

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Declaration of interest
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References

Ackermans MT, Kettelarij-Haas Y, Boelen A & Endert E 2012 Determination of thyroid hormones and their metabolites in tissue using SPE UPLC-tandem MS. *Biomedical Chromatography* **26** 485–490. (doi:10.1002/bmc.1691)

Araujo RL, de Andrade BM, da Silva ML, Marassi MP, Pereira VD, Bouskela E & Carvalho DP 2008 Low replacement doses of thyroxine during food restriction restores type 1 deiodinase activity in rats and promotes body protein loss. *Journal of Endocrinology* **198** 119–125. (doi:10.1677/JOE-08-0125)

Araujo RL, Andrade BM, da Silva ML, Ferreira AC & Carvalho DP 2009 Tissue-specific deiodinase regulation during food restriction and low replacement dose of leptin in rats. *American Journal of Physiology. Endocrinology and Metabolism* **296** E1157–E1163. (doi:10.1152/ajpendo.90869.2008)

Arem R, Wiener GJ, Kaplan SG, Kim HS, Reichlin S & Kaplan MM 1993 Reduced tissue thyroid-hormone levels in fatal illness. *Metabolism: Clinical and Experimental* **42** 1102–1108. (doi:10.1016/0026-0495(93)90266-Q)

van Beeren HC, Kwakkel J, Ackermans MT, Wiersinga WM, Fliers E & Boelen A 2012 Action of specific thyroid hormone receptor α(1) and β(1) antagonists in the central and peripheral regulation of thyroid hormone metabolism in the rat. *Thyroid* **22** 1275–1282. (doi:10.1089/thy.2012.0135)

Boelen A, Maas MA, Lowik CW, Platvoet MC & Wiersinga WM 1996 Induced illness in interleukin-6 (IL-6) knock-out mice: a causal role of IL-6 in the development of the low 3,5,3′-triiodothyronine syndrome. *Endocrinology* **137** 5250–5254. (doi:10.1210/endo.137.12.8940342)

Boelen A, Kwakkel J, Thijssen-Timmer DC, Alkemade A, Fliers E & Wiersinga WM 2004 Simultaneous changes in central and peripheral components of the hypothalamus–pituitary–thyroid axis in lipopolysaccharide-induced acute illness in mice. *Journal of Endocrinology* **182** 315–323. (doi:10.1677/joe.0.1820315)

Boelen A, Kwakkel J, Alkemade A, Renckens R, Kaptein E, Kuiper G, Wiersinga WM & Visser TJ 2005 Induction of type 3 deiodinase activity in inflammatory cells of mice with chronic local inflammation. *Endocrinology* **146** 5128–5134. (doi:10.1210/en.2005-0608)

Boelen A, Kwakkel J, Wiersinga WM & Fliers E 2006 Chronic local inflammation in mice results in decreased TRH and type 3 deiodinase mRNA expression in the hypothalamic paraventricular nucleus independently of diminished food intake. *Journal of Endocrinology* **191** 707–714. (doi:10.1677/joe.1.07056)

Boelen A, Boorsma J, Kwakkel J, Wieland CW, Renckens R, Visser TJ, Fliers E & Wiersinga WM 2008 Type 3 deiodinase is highly expressed in infiltrating neutrophilic granulocytes in response to acute bacterial infection. *Thyroid* **18** 1095–1103. (doi:10.1089/thy.2008.0090)

Boelen A, Kwakkel J, Wieland CW, St Germain DL, Fliers E & Hernandez A 2009 Impaired bacterial clearance in type 3 deiodinase-deficient mice infected with *Streptococcus pneumoniae*. *Endocrinology* **150** 1984–1990. (doi:10.1210/en.2008-1133)

Boelen A, Kwakkel J & Fliers E 2011 Beyond low plasma T_3: local thyroid hormone metabolism during inflammation and infection. *Endocrine Reviews* **32** 670–693. (doi:10.1210/er.2011-0007)

Boelen A, van Beeren M, Vos X, Surovtseva O, Belegri E, Saaltink DJ, Vreugdenhil E, Kalsbeek A, Kwakkel J & Fliers E 2012 Leptin administration restores the fasting-induced increase of hepatic type 3 deiodinase expression in mice. *Thyroid* **22** 192–199. (doi:10.1089/thy.2011.0289)

Chai X, Zeng S & Xie W 2013 Nuclear receptors PXR and CAR: implications for drug metabolism regulation, pharmacogenomics and beyond. *Expert Opinion on Drug Metabolism & Toxicology* **9** 253–266. (doi:10.1517/17425255.2013.754010)

Debaveye Y, Ellger B, Mebis L, Van Herck E, Coopmans W, Darras V & Van den Berghe G 2005 Tissue deiodinase activity during prolonged critical illness: effects of exogenous thyrotropin-releasing hormone and its combination with growth hormone releasing peptide-2. *Endocrinology* **146** 5604–5611. (doi:10.1210/en.2005-0963)

De Escobar GM, Pastor R, Obregon MJ & Delrey FE 1985 Effects of maternal hypothyroidism on the weight and thyroid-hormone content of rat embryonic-tissues, before and after onset of fetal thyroid-function. *Endocrinology* **117** 1890–1900. (doi:10.1210/endo-117-5-1890)

Faggioni R, Fantuzzi G, Fuller J, Dinarello CA, Feingold KR & Grunfeld C 1998 IL-1β mediates leptin induction during inflammation. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology* **274** R204–R208.

Fekete C, Gereben B, Doleschall M, Harney JW, Dora JM, Bianco AC, Sarkar S, Liposits Z, Rand W, Emerson C *et al.* 2004 Lipopolysaccharide induces type 2 iodothyronine deiodinase in the mediobasal hypothalamus: implications for the nonthyroidal illness syndrome. *Endocrinology* **145** 1649–1655. (doi:10.1210/en.2003-1439)

Freitas BC, Gereben B, Castillo M, Kallo I, Zeold A, Egri P, Liposits Z, Zavacki AM, Maciel RM, Jo S *et al.* 2010 Paracrine signaling by glial cell-derived triiodothyronine activates neuronal gene expression in the rodent brain and human cells. *Journal of Clinical Investigation* **120** 2206–2217. (doi:10.1172/JCI41977)

Friesema EC, Jansen J, Jachtenberg JW, Visser WE, Kester MH & Visser TJ 2008 Effective cellular uptake and efflux of thyroid hormone by human monocarboxylate transporter 10. *Molecular Endocrinology* **22** 1357–1369. (doi:10.1210/me.2007-0112)

Galton VA, Hernandez A & St Germain DL 2014 The 5′-deiodinases are not essential for the fasting-induced decrease in circulating thyroid hormone levels in male mice: possible roles for the type 3 deiodinase and tissue sequestration of hormone. *Endocrinology* **155** 3172–3181. (doi:10.1210/en.2013-1884)

Harris AR, Fang SL, Vagenakis AG & Braverman LE 1978 Effect of starvation, nutriment replacement, and hypo-thyroidism on *in vitro* hepatic T_4 to T_3 conversion in rat. *Metabolism: Clinical and Experimental* **27** 1680–1690. (doi:10.1016/0026-0495(78)90290-1)

De Jong M, Docter R, Vanderhoek HJ, Vos RA, Krenning EP & Hennemann G 1992 Transport of 3,5,3′-triiodothyronine into the perfused-rat-liver and subsequent metabolism are inhibited by fasting. *Endocrinology* **131** 463–470. (doi:10.1210/endo.131.1.1612027)

Kester MH, Kaptein E, Roest TJ, van Dijk CH, Tibboel D, Meinl W, Glatt H, Coughtrie MW & Visser TJ 1999 Characterization of human iodothyronine sulfotransferases. *Journal of Clinical Endocrinology and Metabolism* **84** 1357–1364. (doi:10.1210/jcem.84.4.5590)

Kester MH, Kaptein E, Roest TJ, van Dijk CH, Tibboel D, Meinl W, Glatt H, Coughtrie MW & Visser TJ 2003 Characterization of rat iodothyronine sulfotransferases. *American Journal of Physiology. Endocrinology and Metabolism* **285** E592–E598. (doi:10.1152/ajpendo.00046.2003)

Kwakkel J, Chassande O, van Beeren HC, Wiersinga WM & Boelen A 2008 Lacking thyroid hormone receptor β gene does not influence

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alterations in peripheral thyroid hormone metabolism during acute illness. *Journal of Endocrinology* **197** 151–158. (doi:10.1677/JOE-07-0601)

Kwakkel J, Chassande O, van Beeren HC, Fliers E, Wiersinga WM & Boelen A 2010 Thyroid hormone receptor α modulates lipopolysaccharide-induced changes in peripheral thyroid hormone metabolism. *Endocrinology* **151** 1959–1969. (doi:10.1210/en.2009-1049)

Langouche L, Vander Perre S, Marques M, Boelen A, Wouters PJ, Casaer MP & Van den Berghe G 2013 Impact of early nutrient restriction during critical illness on the nonthyroidal illness syndrome and its relation with outcome: a randomized, controlled clinical study. *Journal of Clinical Endocrinology and Metabolism* **98** 1006–1013. (doi:10.1210/jc.2012-2809)

Maglich JM, Watson J, McMillen PJ, Goodwin B, Willson TM & Moore JT 2004 The nuclear receptor CAR is a regulator of thyroid hormone metabolism during caloric restriction. *Journal of Biological Chemistry* **279** 19832–19838. (doi:10.1074/jbc.M313601200)

Mallol J, Obregon MJ & Deescobar GM 1982 Analytical artifacts in radioimmunoassay of L-thyroxin in human-milk. *Clinical Chemistry* **28** 1277–1282.

Mebis L, Paletta D, Debaveye Y, Ellger B, Langouche L, D'Hoore A, Darras VM, Visser TJ & Van den Berghe G 2009 Expression of thyroid hormone transporters during critical illness. *European Journal of Endocrinology* **161** 243–250. (doi:10.1530/EJE-09-0290)

Mebis L, Eerdekens A, Guiza F, Princen L, Derde S, Vanwijngaerden YM, Vanhorebeek I, Darras VM, Van den Berghe G & Langouche L 2012 Contribution of nutritional deficit to the pathogenesis of the nonthyroidal illness syndrome in critical illness: a rabbit model study. *Endocrinology* **153** 973–984. (doi:10.1210/en.2011-1411)

Omara BA, Dittrich W, Lauterio TJ & StGermain DL 1993 Pretranslational regulation of type-I 5′-deiodinase by thyroid-hormones and in fasted and diabetic rats. *Endocrinology* **133** 1715–1723. (doi:10.1210/endo.133.4.8404614)

Peeters RP, Wouters PJ, Kaptein E, van Toor H, Visser TJ & Van den Berghe G 2003 Reduced activation and increased inactivation of thyroid hormone in tissues of critically ill patients. *Journal of Clinical Endocrinology and Metabolism* **88** 3202–3211. (doi:10.1210/jc.2002-022013)

Peeters RP, Kester MH, Wouters PJ, Kaptein E, van Toor H, Visser TJ & Van den Berghe G 2005 Increased thyroxine sulfate levels in critically ill patients as a result of a decreased hepatic type I deiodinase activity. *Journal of Clinical Endocrinology and Metabolism* **90** 6460–6465. (doi:10.1210/jc.2005-0866)

Ramadan T, Camargo SM, Summa V, Hunziker P, Chesnov S, Pos KM & Verrey F 2006 Basolateral aromatic amino acid transporter TAT1 (Slc16A10) functions as an efflux pathway. *Journal of Cellular Physiology* **206** 771–779. (doi:10.1002/jcp.20531)

Reyns GE, Janssens KA, Buyse J, Kuhn ER & Darras VM 2002 Changes in thyroid hormone levels in chicken liver during fasting and refeeding. *Comparative biochemistry and physiology. Part B, Biochemistry & Molecular Biology* **132** 239–245. (doi:10.1016/S1096-4959(01)00528-0)

Vagenakis AG, Portnay GI, Obrian JT, Rudolph M, Arky RA, Ingbar SH & Braverman LE 1977 Effect of starvation on production and metabolism of thyroxine and triiodothyronine in euthyroid obese patients. *Journal of Clinical Endocrinology and Metabolism* **45** 1305–1309. (doi:10.1210/jcem-45-6-1305)

Van den Berghe G, de Zegher F, Baxter RC, Veldhuis JD, Wouters P, Schetz M, Verwaest C, Van der Vorst E, Lauwers P, Bouillon R *et al.* 1998 Neuroendocrinology of prolonged critical illness: effects of exogenous thyrotropin-releasing hormone and its combination with growth hormone secretagogues. *Journal of Clinical Endocrinology and Metabolism* **83** 309–319.

Van den Berghe G, Wouters P, Weekers F, Mohan S, Baxter RC, Veldhuis JD, Bowers CY & Bouillon R 1999 Reactivation of pituitary hormone release and metabolic improvement by infusion of growth hormone-releasing peptide and thyrotropin-releasing hormone in patients with protracted critical illness. *Journal of Clinical Endocrinology and Metabolism* **84** 1311–1323. (doi:10.1210/jcem.83.2.4575)

Vella KR, Ramadoss P, Lam FS, Harris JC, Ye FD, Same PD, O'Neill NF, Maratos-Flier E & Hollenberg AN 2011 NPY and MC4R signaling regulate thyroid hormone levels during fasting through both central and peripheral pathways. *Cell Metabolism* **14** 780–790. (doi:10.1016/j.cmet.2011.10.009)

Visser TJ, Van Buuren JCJ, Rutgers M, Rooda SJE & Deherder WW 1990 The role of sulfation in thyroid-hormone metabolism. *Trends in Endocrinology and Metabolism* **1** 211–218. (doi:10.1016/1043-2760(90)90055-8)

Visser TJ, Kaptein E, Glatt H, Bartsch I, Hagen M & Coughtrie MW 1998 Characterization of thyroid hormone sulfotransferases. *Chemico-Biological Interactions* **109** 279–291. (doi:10.1016/S0009-2797(97)00139-7)

Wiersinga WM & Chopra IJ 1982 Radioimmunoassays of thyroxine-(T_4), 3,5,3′-triiodothyronine (T_3), 3,3′,5′-triiodothyronine (reverse T_3, Rt_3), and 3,3′-diiodothyronine (T_2). *Methods in Enzymology* **84** 272–303.

Wolf M, Hansen N & Greten H 1994 Interleukin-1-β, tumor-necrosis-factor-α and interleukin-6 decrease nuclear thyroid-hormone receptor capacity in a liver-cell line. *European Journal of Endocrinology* **131** 307–312. (doi:10.1530/eje.0.1310307)

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