Tissue thyroid hormone metabolism is differentially regulated during illness in mice

Anita Boelen¹, Anne H van der Spek¹, Flavia Bloise¹, Emmely M de Vries¹, Olga V Surovtseva¹, Mieke van Beeren¹, Mariette T Ackermans², Joan Kwakkel¹ and Eric Fliers¹

¹Department of Endocrinology & Metabolism, Laboratory of Endocrinology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands
²Department of Clinical Chemistry, Laboratory of Endocrinology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

Abstract
Illness induces major modifications in central and peripheral thyroid hormone (TH) metabolism, so-called nonthyroidal illness syndrome (NTIS). As a result, organ-specific changes in local TH availability occur depending on the type and severity of illness. Local TH availability is of importance for the regulation of the tissue-specific TH target genes and determined by the interplay between deiodinating enzymes, TH transport and TH receptor (TR) expression. In the present study, we evaluated changes in TH transport, deiodination and TR expression, the resulting tissue TH concentrations and the expression of TH target genes in liver and muscle in three animal models of illness. We induced (1) acute systemic inflammation by intraperitoneal injection of bacterial endotoxin (LPS), (2) chronic local inflammation by a turpentine injection in the hind limb and (3) severe pneumonia and sepsis by intranasal inoculation with Streptococcus pneumoniae. We found that all aspects of peripheral TH metabolism are differentially regulated during illness, depending on the organ studied and severity of illness. In addition, tissue TH concentrations are not equally affected by the decrease in serum TH concentrations. For example, the decrease in muscle TH concentrations is less severe than the decrease observed in liver. In addition, despite lower TH concentrations in muscle in all three models, muscle T₃ action is differentially affected. These observations help to understand the complex nature of the nonthyroidal illness syndrome.

Key Words
- nonthyroidal illness
- muscle
- liver
- thyroid hormone metabolism
- inflammation

Introduction
During illness, thyroid hormone metabolism changes profoundly. Serum thyroid hormones decrease and the classical negative feedback loop of the hypothalamic–pituitary–thyroid axis is absent. This is known as the nonthyroidal illness syndrome (NTIS). The common view is that NTIS results in an overall downregulation of metabolism via low serum T₃ concentrations to save energy. However, recent studies have shown that NTIS comprises a variety of changes in transcriptional and translational activity of genes involved in local thyroid hormone metabolism ranging from inhibition to activation. These peripheral changes vary per tissue as
well as per type and severity of illness ultimately resulting in specific changes in local thyroid hormone metabolism (Boelen et al. 2011).

Local thyroid hormone concentrations in peripheral tissues depend not only on serum thyroid hormone concentrations but also on thyroid hormone transport into the target cell and the activity of TH-converting enzymes. Among the most important, thyroid hormone transporters are MCT8 and MCT10. MCT8 transports both T₄ and T₃ and is expressed in liver, muscle, kidney and in many brain areas (Alkemade et al. 2005, Heuer et al. 2005), whereas MCT10 preferentially transports T₃ instead of T₄ and is expressed in kidney, liver and muscle (Visser et al. 2011). Once transported into the cell, thyroid hormones can be metabolised by outer or inner ring deiodination through the iodothyronine deiodinases, a selenocysteine-containing enzyme family consisting of three types: type 1 (D1), 2 (D2) and type 3 (D3) (Kohrle 2000). D1 is localised in the plasma membrane and is able to deiodinate the inner and outer ring of T₄ as well as the outer ring of rT₃. D2 is localised in the endoplasmic reticulum and deiodinates T₄ into the biologically active T₃. D2 is the main enzyme involved in the production of tissue T₃ (Burmeister et al. 1997). D3 is localised in the plasma membrane and is the major thyroid hormone-inactivating enzyme, as it catalyses inner ring deiodination of both T₄ and T₃, exclusively resulting in the production of biologically inactive rT₃ and rT₂ (Gereben et al. 2008). Currently, tissue thyroid hormone concentrations are thought to be determined by the balance between deiodinases present in the tissue rather than by serum TH concentrations alone.

T₃ binds to thyroid hormone receptors which mediate gene transcription. Two thyroid hormone receptor genes have been described, Thra and Thrb. Through alternative splicing and alternative promoter usage many isoforms are formed, resulting in classical ligand binding nuclear receptors, non-ligand binding nuclear receptors, mitochondrial isoforms and truncated Δ-isofoms (Bassett et al. 2003). From the Thra gene the classical, ligand binding nuclear receptor TRa1 is transcribed. Two classical, ligand binding nuclear receptors isoforms are derived from the Thrb gene, TRb1 and TRb2. The TRa1 and TRb1 are important in regulating thyroid hormone target genes in peripheral tissues and are differentially expressed between organs (Brent 2000).

During illness, all aspects of thyroid hormone metabolism are changed resulting in different bioavailability and action of thyroid hormone in peripheral tissues. The aim of the present study is to present an overview of the changes in liver and muscle thyroid hormone metabolism (characterised by Slc16A2 (MCT8), Slc16A10 (MCT10), Dio1, Dio2, Dio3, Thra and Thrb expression) observed during a variety of illnesses. As changes in local thyroid hormone metabolism may lead to altered tissue TH concentrations, we also determined hepatic and muscle T₃ and T₄ tissue concentrations. Thyroid hormone action was evaluated by measuring specific T₃-responsive genes in liver and muscle. Three different NTIS models that differ in timing and severity were used: acute inflammation, chronic inflammation and bacterial sepsis. All these models represent true forms of NTIS; low serum T₃ and T₄ concentrations accompanied by a downregulation of the central HPT axis (Boelen et al. 2011).

**Materials and methods**

**Animal experiments**

We used three NTIS models to study liver and muscle thyroid hormone metabolism: acute inflammation, chronic inflammation and bacterial sepsis.

Acute inflammation was induced in female C57Bl6 mice (Harlan Sprague–Dawley, Horst, The Netherlands, 6–12 weeks of age, n=6 per group) by an intraperitoneal (i.p.) injection of 200µg LPS (Lipopolysaccharide, E. coli O127:B8; Sigma) diluted in 0.5 mL saline as described previously (Boelen et al. 2004). A separate control group was included for each time point to correct for the effect of diurnal variation. The experiment started at 09:00 h.

Chronic inflammation was induced in female C57Bl6 mice (Harlan Sprague–Dawley, Horst, The Netherlands, 6–12 weeks of age, n=6 per group) by a s.c. injection of 100 µL steam-distilled turpentine in each hindlimb as described before (Boelen et al. 2005). Control mice received 100 µL saline in each hindlimb, and the experiment started at 09:00 h.

Bacterial sepsis was induced in female C57Bl6 mice (Harlan Sprague–Dawley, Horst, The Netherlands, 6–12 weeks of age, n=6 per group) by intranasal (i.n.) inoculation of S. pneumoniae serotype 3 (American Type Culture Collection, Manassas, VA, USA) (Kwakkel et al. 2009). Briefly, S. pneumoniae were grown in Todd–Hewitt broth (Difco, Detroit, MI, USA) at 37°C, harvested at mid-logarithmic phase and washed twice in sterile saline. Bacteria were then resuspended in sterile saline.
Mice were lightly anaesthetised, and 50 µL of saline containing 5×10⁴ colony forming units was inoculated intranasally (i.n.). Control mice received 50 µL sterile saline i.n. without bacteria.

At the time points 0, 8 and 24 h after LPS (acute model, serum TH concentrations are decreased at 8 and 24 h), at 0, 24, 48 and 120 h after turpentine injection (chronic model, serum TH concentrations are decreased at these time points) and 48 h after inoculation of S. pneumonia (lethal model, end-stage illness at 48 h), six mice per group were anaesthetised with isoflurane, and blood was taken by cardiac puncture. Subsequently, mice were killed by cervical dislocation. Liver and muscle tissue were obtained and immediately stored in liquid nitrogen. All studies were approved by the local animal welfare committee.

**Tissue RNA isolation and qPCR**

Liver and muscle mRNA from S. pneumoniae-infected mice and matching controls was isolated using the Magna Pure LC mRNA tissue kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the protocol. Muscle and liver RNA from the LPS and turpentine-injected mice and matching controls was isolated using the TRIzol Reagent (Invitrogen) and Macherey Nagel kit (Macherey Nagel, Düren, DE, USA) according to manufacturer’s protocol. RNA yield was measured using the NanoDrop (NanoDrop, Wilmington, DE, USA) to be able to perform the cDNA synthesis with equal RNA input. cDNA synthesis was performed using the First Strand cDNA Synthesis Kit (AMV) for RT-PCR with oligo d(T) primers (Roche Molecular Biochemicals). Real-time PCR was performed using the Lightcycler480 (Roche Molecular Biochemicals) and SensiFAST SYBR (Bioline, London, UK). Primer pairs for mouse hypoxanthine phosphoribosyl transferase (Hprt), cyclophilin B, ubiquitin C, myogenin (Myog), Dio1, Dio2, Dio3, Thra1 and Thrb1 have been previously described (Bloise et al. 2016, Boelen et al. 2004, Kwakkel et al. 2010, Sjogren et al. 2007). In addition, we designed the following primer pairs for mouse: Slc16A2 (MCT8): forward primer 5′-GGGGGCTTGCAGGAGGCAA-3′, reverse primer 5′-TTTCCACAGTGGCGTGAGGC-3′, annealing temperature 70°C, Slc16A10 (MCT10): forward primer 5′-TGATTCCCTGTGCAGGCCGC-3′, reverse primer 5′-CCACGTCTAGGTGGCTCCGAGC-3′, annealing temperature 65°C, 3′, malic enzyme (Me1): forward primer 5′-GAAAGAGGTGTTTGCCCATGA-3′, reverse primer: 5′-AAT TGC AAC TCC TAT GAG G-3′, annealing temperature 65°C. Quantification was performed using the LinReg software (Ruijter et al. 2009). The mean of the efficiency was calculated for each assay, and samples that deviated more than 0.05 of the efficiency mean value were excluded from the analysis (0–5%). Calculated values were normalised using the geometric mean of the reference genes cyclophilin-B, ubiquitin-C and HPRT.

**Liver deiodinase activities**

Deiodinase type 1 and type 3 activity was measured as previously described (Kwakkel et al. 2010, van Zeijl et al. 2014). Tissue was homogenised on ice in PED50 buffer (0.1M sodium phosphate, 2mM EDTA pH 7.2, 50mM dithiothreitol (DTT)) using a Polytron (Kinematica, Luzern, Switzerland). Homogenates were snap-frozen and stored at −80°C until use. Protein concentration was measured with the Bio-Rad protein assay using bovine serum albumin (BSA) as the standard following the manufacturer’s instructions (Bio-Rad Laboratories). After deiodinase and tissue-specific incubations, reactions were stopped by adding ice-cold ethanol. After centrifugation and addition of 0.02M ammonium acetate (pH 4), the mixture was applied to 4.6×250mm Symmetry C18 column connected to a Waters HPLC system (Model 600E pump, Model 717 WISP autosampler, Waters, Etten-Leur, The Netherlands). The activity in the eluate was measured online using a Radiomatic 150 TR flow scintillation analyser (Perkin Elmer).

Liver D1 activity was measured in duplicate, using 75 µL 100–500 times diluted homogenate incubated for 30 min at 37°C in a final volume of 0.15 mL with 0.1 µM rT₃ and with the addition of approximately 1×10⁵ cpm (3,3′,5′-I) rT₃ in PED10 (0.1M sodium phosphate, 2mM EDTA pH 7.2, 10mM DTT). One sample of each group was incubated in the presence of 500 µM PTU to inhibit D1 activity representing a tissue blank. D1 activity was calculated by subtracting the activity measured in the tissue blank from the activity measured without PTU and expressed as pmol 3,3′ T₂ generated per minute per mg protein.

Liver D3 activity was measured in duplicate, using 75 µL undiluted homogenate incubated for 2 h at 37°C in a final volume of 0.15 mL with 1nM T₃ or 500nM T₃ with the addition of approximately 2×10⁴ cpm (3,3′,5′-I) T₃ in PE buffer. For each group, we included one sample with 500nM T₃ to saturate D3, the activity measured with the incubation with 1nM T₃ minus the incubation...
with 500nM T₃ represents true D3 activity. D3 activity was expressed as fmol generated 3,3’ T₂ per minute per mg tissue.

**Serum thyroid hormone levels**

Serum T₃ and T₄ were measured with in-house RIA’s (Wiersinga & Chopra 1982). All samples from one experiment were measured within the same assay (intra-assay variation T₃: 3.6% and T₄: 6.6%).

**Tissue thyroid hormone levels**

Liver and muscle T₃ and T₄ concentrations were determined in 20–30mg frozen tissue with an LC–MS/MS method as described before (de Vries et al. 2014) but adapted for muscle tissue. Briefly, frozen skeletal muscle tissue was added to a polypropylene 2 mL tube containing 150mg zirconia beads, 50μL PBS and 20μL ¹³C₆-labelled internal standards on ice. Samples were homogenised for 60s at 3500g using a Magna Lyser (Roche Molecular Biochemicals). Subsequently, 500μL MeOH was added, samples were mixed on a vortex, homogenised again (60s, 3500g) and transferred to 5 mL glass tubes. The polypropylene tubes were rinsed with 500μL MeOH, which was added to the homogenate. Ratio of volume aqueous compounds (tissue (mg) + volume PBS (mL)+volume IS (mL)) to MeOH (total volume) is 1:10. Chloroform (2mL) was then added in twice the volume methanol. Samples were mixed on a vortex for 15s. The extraction was carried out in two steps, interspaced with centrifugations for 10min at 2000g. In the second step, a mixture of 1mL chloroform–methanol (2:1) was added to the pellet. The final volume of the extract was about 40 times the weight of the tissue plus the volume of the internal standard plus the volume of PBS, as described by Reyns and coworkers (Reyns et al. 2002). The chloroform–methanol extract was transferred to glass tubes for back-extraction of the iodothyronines into an aqueous phase with a calculated amount of 0.05% CaCl₂ (de Vries et al. 2015) followed by a second extraction with pure upper phase (chloroform:methanol:0.05% CaCl₂ 3:49:48). The iodothyronines were measured with reversed phase chromatography (Waters BEH C18 column: 130 Å, 1.7μm, 2.1mm×50mm) on an Acquity UPLC-Xevo TQ-S tandem mass spectrometer system (Waters, Milford, MA, USA). Mobile phase A was acetonitrile:H₂O:acetic acid 5:95:0.1, mobile phase B was acetonitrile:H₂O:acetic acid 95:5:0.1. A gradient was applied with initial percentage B of 10%, which was increased linearly after 2.7 min to 40% in 5.6 min. Total runtime was 12 min. Thyroid hormones and internal standards were quantified using specific MRM transitions as described previously (Ackermans et al. 2011). All aspects of system operation and data acquisition were controlled using MassLynx, version 4.1 software with automated data processing using the TargetLynx Application Manager (Waters). The additional PBS step did not affect extraction efficiency; the recoveries of ¹³C₆-labelled T₃, T₄ and rT₃ extracted from liver with and without the additional PBS step did not differ.

**Statistical analysis**

Normal distribution of the data was tested using the Shapiro–Wilks test. Statistical significance between acute/chronic inflammation and control group was evaluated using two-way ANOVA with two grouping factors (time and treatment). When not normally distributed, data were ranked before performing ANOVA. P values in the figures represent the significant effect of the treatment. To test pair-wise comparisons, ANOVA was followed by Tukey’s post hoc test. Symbols in the figures represent the pair-wise P values. P values <0.05 were considered statistically significant. All tests were performed using GraphPad Prism 6 (GraphPad Software).

**Results**

**Illness-induced alterations in serum thyroid hormone concentrations**

Serum thyroid hormone concentrations were evaluated in three NTIS animal models; acute inflammation (induced by LPS), chronic inflammation (induced by turpentine injection) and S. pneumoniae-induced bacterial sepsis. All three models differ in timing and severity; both the acute and chronic inflammation models are non-lethal, whereas bacterial sepsis is a lethal model. All three stimuli resulted in decreased serum T₃ and T₄ concentrations within the timeframe of the experiment and can therefore be viewed as NTIS. The changes induced however differ between the models; serum T₄ decreased markedly during acute inflammation and the early phase of chronic inflammation, whereas end-stage bacterial sepsis only marginally decreased serum T₃ concentrations (Fig. 1).
Illness-induced alterations in liver thyroid hormone metabolism

In the present study, we evaluated the components of local thyroid hormone metabolism such as TH transporters (MCT8 (Slc16A2) and MCT10 (Slc16A10)), deiodinases and Thrb1 (the main TR in liver) mRNA expression. Local thyroid hormone availability was determined by measuring tissue T₃ and T₄ concentrations. Thyroid hormone action was established by measuring mRNA expression of malic enzyme (Me1), a liver-specific T₃ target gene.

LPS administration resulted in a marked decrease of liver Slc16A2 (PANOVA <0.001) and Slc16A10 mRNA expression (PANOVA <0.001) within 24 h. Liver Dio1 and Dio3 mRNA expression (both PANOVA <0.001) was also markedly decreased within 24 h (data not shown). Liver D1 (PANOVA <0.001) and D3 activity (PANOVA <0.001), liver T₃ (PANOVA <0.001) and T₄ concentrations (PANOVA <0.001) and Thrb1 mRNA expression (PANOVA <0.001) significantly decreased within 24 h after LPS compared to those in controls. Liver Me1 mRNA expression (PANOVA <0.001), a positively regulated liver T₃ target gene, also decreased, which is in agreement with reduced T₃ action.

Turpentine administration, a model for chronic inflammation, resulted in decreased liver Slc16A2 mRNA expression (PANOVA <0.001) within 24 h, whereas Slc16A10 mRNA expression increased significantly within 48 h and returned to baseline after 120 h (PANOVA <0.01). In contrast, liver T₄ concentrations decreased markedly within 48 h (PANOVA <0.001) and returned to baseline after 120 h. Liver D1 activity (PANOVA <0.01) and Dio3 mRNA expression (data not shown) and D3 activity (both PANOVA <0.001) decreased after turpentine injection and remained low compared to those of controls. Liver Dio1 mRNA expression (data not shown, Pinteraction <0.01) decreased at 48 h but restored to control expression at 120 h. Liver T₃ concentrations were lower at the beginning of the inflammatory response but were restored to control concentrations after 120 h (PANOVA <0.001). Liver Thrb1 mRNA expression did not differ between chronic inflammation and control, whereas liver Me1 mRNA expression decreased (PANOVA <0.001) in line with the reduced liver T₃ concentrations indicating reduced T₃ action at the early phase of chronic inflammation.

Bacterial sepsis resulted in decreased liver Slc16A2 mRNA expression after 48 h, whereas Slc16A10 and Thrb1 did not change. Liver T₄ concentrations were markedly lower compared to those of controls. Surprisingly, liver D1 activity did not change despite significantly decreased Dio1 mRNA expression (data not shown), whereas liver Dio3 mRNA expression (data not shown) and D3 activity decreased. The net result was decreased hepatic T₃ concentrations and Me1 mRNA expression indicating reduced T₃ action in the liver during end-stage lethal bacterial sepsis.

In summary, acute inflammation lowered hepatic TH transport, hepatic T₃ and T₄ concentrations and TH action, whereas liver thyroid hormone metabolism was differentially affected during chronic inflammation. Lethal bacterial sepsis affected hepatic TH transport only marginally but lead to a marked reduction in liver D3 activity and hepatic TH concentrations and action (Fig. 2).

Illness-induced alteration in muscle thyroid hormone metabolism

In the present study, we evaluated muscle thyroid hormone metabolism by measuring the TH transporters MCT8 (Slc16A2) and MCT10 (Slc16A10), Dio2 and Dio3 mRNA expression, Thra1 (the main TR in muscle) mRNA expression and muscle T₃ and T₄ concentrations in three NTIS models. We also measured muscle myogenin (Myog)
mRNA expression, a T₃ target gene as a representative reflection of TH action.

Muscle Slc16A2 mRNA did not change after LPS administration, whereas Slc16A10 mRNA decreased 24 h after LPS (P_{ANOVA} <0.05). LPS induced a rapid increase in Dio2 mRNA expression (P_{interaction} <0.05) and a decrease in Dio3 mRNA expression (P_{interaction} <0.05). Muscle T₃ and T₄ concentrations were lower at 24 h after LPS administration (P_{ANOVA} <0.05), whereas Thra1 mRNA did not change. Muscle Myog mRNA expression was lower in LPS-treated mice at 24 h compared to that in control mice, which is in agreement with the lower muscle T₃ concentrations at 24 h.

In contrast to acute inflammation, turpentine administration did not result in significant changes in muscle Slc16A2 and Slc16A10 mRNA expression. Muscle Dio2 mRNA expression changed significantly (P_{interaction} <0.05) and Dio3 mRNA expression decreased markedly after 48 h (P_{ANOVA} <0.05). Muscle T₄ (P_{ANOVA} <0.001) and T₃ concentrations (P_{ANOVA} <0.01) and Thra1 mRNA expression (P_{ANOVA} <0.001) decreased during early chronic inflammation. In contrast, muscle Myog expression, a
T₃ target gene, was higher in turpentine-treated mice compared to that in controls suggesting increased T₃ action.

Sepsis resulted in decreased muscle Slc16A2 and Slc16A10 mRNA expression. We have shown before that lethal bacterial sepsis resulted in decreased muscle Dio2 mRNA expression (Kwakkel et al. 2009), whereas Dio3 mRNA did not change. Muscle T₃ and T₄ levels and Thra1 mRNA expression were markedly lower 48 h after the onset of bacterial sepsis compared to those of control mice. However, muscle Myog mRNA expression did not differ compared to control mice suggesting unchanged TH action in muscle at end-stage bacterial sepsis.

In summary, muscle thyroid hormone metabolism is differentially affected during illness. Acute inflammation and bacterial sepsis lowered TH transport into the cell, whereas chronic inflammation did not result in altered transporter expression. Muscle TH concentrations showed a small decrease during all types of NTIS only paralleled by decreased TH action during acute illness (Fig. 3).

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Figure 3
Relative expression of Slc16A2 (MCT8), Slc16A10 (MCT10), Dio2, Dio3, Thra1 and Myog mRNA and T₃ and T₄ concentrations in the skeletal muscle of mice during acute inflammation, chronic inflammation and bacterial sepsis compared to saline-treated controls. Dio2 and Dio3 mRNA expression during bacterial sepsis has been published previously (Kwakkel et al.); Dio2 decreases during bacterial sepsis, whereas Dio3 mRNA expression does not change. White bars and open circles represent saline-injected mice, black bars and filled circles represent treated mice. Mean values ± s.e.m. are depicted. P values indicate the effect of treatment as evaluated by a two-way ANOVA. Differences between groups (n = 6 mice per group) were analyzed post hoc with Tukey’s test. *P≤0.05, **P≤0.01.
**Discussion**

It has been known for decades that illness decreases serum thyroid hormone concentrations, but the functional meaning of the observed changes in thyroid hormone concentrations, collectively known as the nonthyroidal illness syndrome (NTIS), remains incompletely understood. Although the prevailing view was that NTIS results in overall downregulation of metabolism to save energy, recent work has shown a more complex picture (Boelen et al. 2011). The present study aimed to evaluate the illness-induced alterations in liver and muscle thyroid hormone metabolism in mice using three different NTIS models that differ in timing and severity (acute inflammation, chronic inflammation and bacterial sepsis) in a systematic way. We are the first to measure liver and muscle tissue TH concentrations during NTIS in the same tissue and model at the same time point to establish the contribution of the tissue-specific changes in TH metabolism to the tissue TH concentrations.

Plasma $T_3$ and $T_4$ concentrations decreased during acute illness, chronic inflammation and lethal bacterial sepsis as observed previously (Boelen et al. 2004, 2005, 2008). The pathogenesis of the decrease in plasma TH concentrations is multifactorial; downregulation of the central part of the HPT axis, reduced production by the thyroid gland and diminished peripheral conversion in the liver (Boelen et al. 2011). Although all organs are exposed to similar circulating thyroid hormone concentrations, liver and muscle display a differential expression of deiodinating enzymes. Liver expresses D1 and D3, whereas muscle tissue expresses D2 and D3 implying a tissue specific modulation of local thyroid hormone signalling (Bianco & Kim 2006).

To become available for the deiodinating enzymes, $T_4$ must cross the plasma membrane. High-affinity transporter systems for both $T_4$ and $T_3$ have been identified, translocating the hormone across the plasma membrane. This transport is dependent on temperature, energy (ATP) and sodium and rate limiting for subsequent cellular thyroid hormone metabolism without affecting the deiodinating process or the binding of $T_3$ to the receptor (Hennemann et al. 2001).

In the liver, NTIS is known to affect TH transport at the level of the plasma membrane. Decreased liver ATP and increased concentrations of compounds that inhibit $T_4$ transport into the liver (bilirubin, non-esterified fatty acids) may contribute to reduced transport of $T_4$ into the hepatocyte (Hennemann et al. 2001). Liver TH transporter expression itself is also affected during NTIS. Interestingly, changes in liver TH transporter expression appeared to differ between the NTIS models; acute illness lowers Slc16A2 and Slc16A10 expression, whereas chronic inflammation results in decreased Slc16A2 mRNA expression and an increase in Slc16A10 mRNA. Lethal bacterial sepsis affects hepatic TH transport only marginally. However, the (sometimes small) alterations in liver Slc16A2 and Slc16A10 mRNA expression observed in our study are not determinative in affecting liver TH concentrations as the alterations observed in plasma TH concentrations are more or less identical to the decrease observed in liver TH concentrations in all three models of NTIS (Fig. 4). This is associated with a marked reduction in $T_3$ signalling represented by decreased liver Me1 mRNA expression.

Illness also differentially affects the expression and activity of liver D1 and D3; acute inflammation results in a marked decrease in both D1 and D3 activity, whereas chronic inflammation and bacterial sepsis downregulate

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**Figure 4**
Relative decreases in serum, liver and muscle $T_4$ and $T_3$ concentrations during acute inflammation, chronic inflammation and bacterial sepsis compared to saline-treated controls, which were set at 100% at each time point.
liver D3 activity. Liver D1 activity is only marginally affected. Studies in euthyroid mice have shown that D1 is not essential for TH action and should be primarily considered as a scavenger enzyme that deiodinates sulphated TH. Furthermore, as a T$_3$-responsive gene, D1 plays a role in decreasing the amount of circulating thyroid hormones during hyperthyroidism (Mullur et al. 2014). We showed in TR$\beta$ KO mice, which have almost undetectable D1 activity in the liver that the LPS-induced decrease in plasma TH concentrations is similar in KO and WT, suggesting that D1 does not play an important role in lowering plasma and liver TH concentrations during illness (Kwakkel et al. 2008).

Illness-induced reduced liver D3 activity has been observed previously in mice (Boelen et al. 2005, 2008), whereas liver D3 increases in human liver during prolonged critical illness (Peeters et al. 2003). A rabbit model for prolonged critical illness developed by van den Berghe and coworkers showed clinical, biochemical and endocrine features similar to those seen in ICU patients (Debaveye et al. 2005, Weekers et al. 2003) and liver D3 activity tends to increase in these animals (Debaveye et al. 2008). The response of D3 to inflammation/illness might be dependent on the energy status; we showed that long-term fasting results in increased liver D3 expression and activity in rodents (Boelen et al. 2012, de Vries et al. 2015), and prolonged illness is associated with diminished food intake (Langouche et al. 2013). Theoretically, the decrease in D3 might prevent/ameliorate the illness-induced decrease in liver T$_3$ concentrations. However, the relatively low D3 activity levels present in the control animals are difficult to reconcile with a robust adaptation mechanism.

In humans, most of circulating T$_3$ (80%) is produced via peripheral conversion by D1 (liver and kidney) and D2 (skeletal muscle) (Maia et al. 2005). In rodents, approximately 50% of circulating T$_3$ is produced outside the thyroid. It has been shown in rats that the amount of nuclear-bound T$_3$ derived from plasma T$_3$ or from local production differs between tissues. Local conversion of T$_4$ contributes 80% of the nuclear-bound T$_3$ in the cerebral cortex, 40% in muscle and only 5% in liver (Hennemann et al. 2001). The liver can therefore be seen as a tissue that rapidly equilibrates with plasma concentrations. Our results suggest that also during illness plasma TH concentrations are the major determinant for liver TH concentrations in mice (Fig. 4), despite the illness-induced alterations in liver Mct8, Mct10, D1 and D3. Reduced liver TH concentrations have also been described in NTIS patients (Arem et al. 1993) and critically ill rabbits (Debaveye et al. 2008).

To evaluate muscle thyroid hormone metabolism during illness, we measured TH transporters, Dio2 and Dio3 mRNA expression, Thra1 mRNA expression and muscle T$_3$ and T$_4$ concentrations in acute inflammation, chronic inflammation and bacterial sepsis. We also determined muscle myogenin (Myog) mRNA expression, a T$_3$-target gene as a representative reflection of TH action. TH concentrations were measured in muscle tissue using a previously published protocol with minor modifications (Ackermans et al. 2011). To prevent coagulation of the muscle fibres, we introduced an additional homogenisation step using small amounts of PBS before the homogenisation step with MeOH. The adapted protocol allows us to measure TH concentrations in very small amounts (20–30 mg) of muscle tissue. Muscle T$_3$ levels in control mice are around 3 pmol/g muscle tissue, slightly lower than those in liver. Muscle T$_4$ concentrations are approximately 15 pmol/g muscle tissue, more than 60% lower than those in liver T$_4$ concentrations, which is in agreement with the fact that the local conversion of T$_4$ contributes significantly to muscle T$_3$ concentrations (Gereben et al. 2008).

We observed that muscle TH metabolism is differentially affected during illness. Acute inflammation and bacterial sepsis lowered TH transport into the cell, whereas chronic inflammation did not result in altered transporter expression. Muscle Dio2 expression increases whereas Dio3 expression decreases during acute illness as observed before (Kwakkel et al. 2010). In contrast, bacterial sepsis lowers muscle Dio2 expression (Kwakkel et al. 2009). In D2-expressing cells such as muscle cells, most of the nuclear-bound T$_3$ is derived from intracellular conversion of T$_4$ into T$_3$ by D2 (Arrojo & Bianco 2011). D2 is the dominant deiodinase in muscle tissue and is thought to play an important role in local TH metabolism and muscle function. This is in agreement with the observation that the global D2KO mice display a severely hypothyroid muscle phenotype despite normal serum T$_3$ concentrations (Galton et al. 2009). Furthermore, it has been shown that tight regulation of intracellular TH levels in muscle stem cells (MSC) is essential for muscle development and function (Dentice et al. 2010). However, an elegant study by the Bianco group using mice with skeletal muscle-specific D2 knockdown showed that these mice display normal muscle fibre characteristics and only mild signs of muscle hypothyroidism, implying that thyroid hormone
signalling in muscle fibres is independent of D2 in adult muscle cells (Werneck-de-Castro et al. 2015).

Interestingly, despite variable alterations in transporter expression and deiodinating enzymes during acute, chronic inflammation and bacterial sepsis, muscle T3 and T4 concentrations were lower in all NTIS models compared to those in controls. This is partly in agreement with reduced Myog mRNA expression observed during acute inflammation. However, T3-responsive gene expression was not decreased in muscle during chronic inflammation and bacterial sepsis, which suggests that TH action in muscle is not solely dependent on muscle T3 concentrations. This is in agreement with the observation that Dio2 knockdown in myocytes does not affect T3-responsive gene expression in muscle to a large extent questioning the potential importance of myocyte-specific T3 production (Werneck-de-Castro et al. 2015).

Our experimental setting does not allow us to exclude the possibility that inflammation by itself affects Myog expression. Inflammatory signals are able to affect Myog expression (Chen et al. 2007, Zhan et al. 2007). However, an in vivo model of muscle repair demonstrated reduced Myog expression in the muscle of D2KO mice but not in WT muscle, despite both genotypes displaying inflammatory cell infiltration (and thus a pro-inflammatory environment) (Dentice et al. 2010). We therefore consider inflammation less likely to be involved in the regulation of Myog expression in our experimental setting.

We recently published the effects of bacterial sepsis and chronic inflammation on the diaphragm, the main respiratory muscle containing type I, slow twitch, fibres, and type II, fast twitch, fibres (Bloise et al. 2016). In that study, we observed that sepsis resulted in increased Dio3 expression and decreased Dio2, which was associated with reduced TH signalling as the T3-regulated genes Tnni2 and Myog were decreased. Chronic inflammation on the other hand resulted in decreased Dio3 expression in the diaphragm (Bloise et al. 2016). Thus, the changes observed in skeletal muscle in the present study are similar to the changes observed earlier in the diaphragm during illness.

Several studies postulate that the illness-induced alterations depend on the severity and type of illness. We showed that indeed differences in illness severity and timing result in differential changes in transporters, deiodinases and TR expression in muscle and liver. We are the first to measure liver and muscle TH concentrations also in addition to the genes involved in local TH metabolism. Interestingly, illness results in decreased tissue TH concentrations in addition to low serum TH concentrations, regardless of the type of inflammation. The illness-induced TH decrease is more severe in liver, however, is also present in skeletal muscle confirming the fact that almost half of the muscle TH concentrations is derived from local conversion. The (relatively small) decrease in muscle TH levels is partly associated with minor changes in T3-responsive gene expression suggesting that muscle tissue is less vulnerable to illness-induced changes in thyroid hormone metabolism by maintaining TH action at an appropriate level especially during the chronic phase of illness. Additional studies with cell-specific knockout models will be necessary to unravel the increasingly complex mechanisms involved in the pathogenesis of NTIS.

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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