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Reduced expression of thyroid hormone receptor β in human nonalcoholic steatohepatitis

Christin Krause¹, Martina Grohs¹, Alexander T El Gammal², Stefan Wolter², Hendrik Lehnert³, Oliver Mann², Jens Mittag⁴ and Henriette Kirchner¹

¹Epigenetics & Metabolism, Medical Department I, University of Lübeck, Lübeck, Germany

²Department of General, Visceral and Thoracic Surgery, University Medical Center Hamburg-Eppendorf, Hamburg, Germany

³Medical Department I, University of Lübeck, Lübeck, Germany

⁴Molecular Endocrinology, Medical Department I, University of Lübeck, Lübeck, Germany

Correspondence should be addressed to J Mittag or H Kirchner: jens.mittag@uni-luebeck.de or henriette.kirchner@uksh.de

Abstract

Hepatic thyroid hormone signaling has an important role in the development and progression of nonalcoholic steatohepatitis (NASH). While the systemic levels of thyroid hormone might remain stable, there is evidence that the intracellular signaling machinery consisting of transporters, deiodinases and receptors could be altered in NASH. However, clinical material from human liver biopsies of individuals with NASH has not been studied to date. In a cross-sectional study, we analyzed 85 liver biopsies from patients with different stages of NASH that underwent bariatric surgery. Using qPCR, we analyzed gene expression of thyroid hormone transporters *NTCP (SLC10A1)*, *MCT8 (SLC16A2)* and *OATP1C1 (SLCO1C1)*, thyroid hormone receptor α and β (*THRA* and *THRB*) and deiodinase type I, II and III (*DIO1*, *DIO2*, *DIO3*). The expression was correlated with serum TSH, triglyceride, HbA1c and NASH score and corrected for age or gender if required. While *DIO2*, *DIO3* and *SLCO1C1* were not expressed in human liver, we observed a significant negative correlation of *THRB* and *DIO1* with age, and *SLC16A2* with gender. *THRB* expression was also negatively associated with serum triglyceride levels and HbA1c. More importantly, its expression was inversely correlated with NASH score and further declined with age. Our data provide unique insight into the mRNA expression of thyroid hormone transporters, deiodinases and receptors in the human liver. The findings allow important conclusions on the intrahepatic mechanisms governing thyroid hormone action, indicating a possible tissue resistance to the circulating hormone in NASH, which becomes more prominent in advanced age.

Key Words

- ▶ thyroid hormone receptor
- ▶ thyroid hormone transporter
- ▶ deiodinase
- ▶ liver

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Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most prevalent liver disease in Western countries, affecting for instance more than 10% of all adults in the United States. NAFLD encompasses a wide spectrum of different stages, ranging from steatosis with normal hepatic function to nonalcoholic steatohepatitis (NASH) and further to cirrhotic NASH and even hepatocellular carcinoma

(for review see (1)). Several endocrine pathways are known to contribute to the development and progression of NAFLD (2), including thyroid hormone (TH). A strong connection between systemic hypothyroidism and NAFLD has been established in humans (3, 4, 5) and rodents (6). Even in the euthyroid range, a link between NAFLD and higher free 3,3',5-triiodothyronine (fT3)

and lower free thyroxine (fT4) levels has been described (7); however, the negative correlations of NAFLD with fT3 and fT4 in these euthyroid subjects suggest that the local hepatic levels of TH might be of even greater relevance for the disease pathogenesis than the systemic TH levels. In fact, a previous study on a small cohort in patients with NAFLD has found increased expression of the TH-inactivating enzyme deiodinase type 3 (DIO3) in human liver biopsies (8), suggesting that intracellular TH action might be reduced in this condition (9).

These findings fit well to the emerging concept that the levels of TH in the cell or organ might differ substantially from their concentrations in the circulation due to several cellular gatekeeping mechanisms. These mechanisms control for instance the entry of the hormone into the target cell through specific TH transport proteins such as MCT8 (monocarboxylate transporter 8, SLC16A2), MCT10 (SLC16A10) or OATP1C1 (organic anion transporting polypeptide 1c1, SLCO1C1), which have distinct and different expression patterns throughout the body (10). As a second step, intracellular deiodinase enzymes catalyze the conversion of THs, thus governing the activation (DIO1 and DIO2) or inactivation (DIO1 and DIO3) of the active hormone 3,3',5'-triiodothyronine (T3) and its precursor 3,3',5,5'-tetraiodothyronine (thyroxine, T4). Finally, two different genes exist for nuclear TH receptors, *THRA* coding for TR α 1 and *THRB* for TR β , which mediate the genomic effects of the hormone and stimulate or repress gene expression in target cells. With regard to the liver, it is well established that these gatekeeping mechanisms are predominantly governed by DIO1 and TR β , while the role of the different transport proteins is less clear (11).

Targeting hepatic TH signaling has been a longterm goal in the field, evidenced by the ample number of TR β -specific compound such as GC-1, GC-24, KB141, KB2115 or MB07811 (12, 13, 14, 15). Despite their encouraging potential to induce favorable metabolic effects including lowering cholesterol levels, significant off-target effects were discovered during clinical trials (for review see (16)). Moreover, it was suggested that their liver-specific effect might be a consequence of transporter rather than TR β specificity (17). However, most recently it was shown that a low-dose TH treatment is effective to reduce hepatic lipid content in NAFLD patients (18), underlining the great potential of TH in the treatment of the disease. Unfortunately, to date, little is known regarding the different gatekeeping mechanisms for TH action in the human liver and alterations that occur during NAFLD, which could affect therapeutic strategies to target hepatic

TH signaling in this condition. To test this hypothesis, we here investigate the expression of TH transporters, deiodinases and receptors in a unique collection of human liver biopsies from patients with different stages of NASH.

Materials and methods

Study design and patients

To establish a tissue bank for metabolic disorders, liver wedge biopsies were obtained in a standardized fashion from segment III during bariatric surgery of obese subjects at University Hospital Eppendorf (UKE, Hamburg). All participants signed an informed consent. The study was approved by the local ethics committee 'Ethik Kommission der Ärztekammer Hamburg' (PV4889, 2015). As this was no direct recruitment for a study, the cohort is not matched for age and gender. Therefore, age and gender were tested as possible confounding factors and the results were corrected if necessary. NAFLD activity score was determined according to the current recommendations (19) by two expert pathologists. The patients were fasted for 6 h prior surgery, but had no dietary restrictions otherwise. Clinical parameters were measured at the time of the surgery by the Institut für Klinische Chemie und Laboratoriumsmedizin, Zentrum für Diagnostik, Universitätsklinikum Eppendorf, Hamburg, Germany according to the DIN EN ISO 15189:2014 certification. Glucose, cholesterol, HDL and triglycerides were determined using photometric assays, HbA1c was quantified using capillary electrophoresis or turbidimetric inhibition assays and folic acid and TSH were measured using luminescent oxygen channeling immunoassays (LOCI). The intra- and interassay variance for the TSH LOCI assay are typically in the range of 2.1 and 17% respectively (20). Total T3 and T4 were determined from frozen serum samples using ELISAs (EIA-1781, DRG Diagnostics, Germany for T4 and DNOV053, NovaTec Immundiagnostica GmbH, Germany).

RNA isolation and gene expression measurement by qPCR

Whole-cell RNA was extracted from approximately 25 mg of snap frozen liver using the MiRNeasy mini kit (QIAGEN) as indicated by the manufacturer and quantified spectrometrically. Two micrograms of RNA were reverse transcribed into cDNA using the SuperScript VILO cDNA synthesis kit (Invitrogen). Gene expression was measured by qPCR using FastStart Universal SYBR Green (Roche) and 0.25 μ M of each primer

(sequences are available on request). The efficiency of all qPCR reactions was similar and above 95%. Absence of genomic DNA was confirmed in a reaction without reverse transcriptase. Relative gene expression was calculated with the $\Delta\Delta C_t$ method. Thirty-one potential housekeeping genes were tested with TaqMan Array Human Controls Plate (Applied Biosystems). According to the NormFinder algorithm (21), *CASC3* was the best housekeeping gene and therefore all expression data were normalized to *CASC3* expression. ΔC_t values were normalized to a 0–1 scale by $1 - (x_i - \min(x)) / (\max(x) - \min(x))$ for the visualization of gene expression, indicating 0 as lowest measured expression and 1 as highest measured expression.

Statistics

Correlations between gene expression (using ΔC_t values) and the possible confounding factors were analyzed by Pearson's correlation for continuous parameters (age) and Spearman correlation as well as Student's *t*-test for ordinal parameters (gender), according to the *Handbook of Biological Statistics* (22). As *DIO1* and *THRB* were affected by age and *SLC16A2* by gender, all subsequent *P* values and effect sizes for these genes were corrected for age or gender respectively using a linear regression model. The respective figures contain the *P* values calculated by Pearson's correlation for continuous parameters (TSH, serum triglycerides, HbA1c, blood glucose) or Spearman correlation for ordinal parameters (NASH score), as well as the age/gender corrected *P* values derived from the linear regression model. All statistic calculations were performed by MATLAB, version R2016a (The MathWorks).

Results

As TH signaling has been connected to NASH development and progression, we hypothesized that the local control of TH levels might be pathologically altered in livers of patients with different stages of NASH. We therefore tested the expression of TH transporters, deiodinases and receptors in a unique collection of human liver biopsies (Table 1). Our data showed strong expression of both TH receptors, *SLC16A2*, *SLC10A1* and *DIO1*, whereas the mRNA levels of *SLCO1C1*, *DIO2* and *DIO3* were more than 10-fold lower, suggesting negligible biological relevance (Fig. 1A). As the cohort was controlled for BMI, but not for age and gender, we tested these two factors as possible confounders, revealing that the hepatic expression of *SLC16A2* was significantly higher in females than in

males (Fig. 1A, correlation $P=0.0317$, *t*-test $P=0.0264$), and *DIO1* and *THRB* mRNA levels declined significantly with age (Fig. 1B, $P=0.0241$ for *DIO1* and $P=0.0392$ for *THRB*). Remarkably, none of the genes were significantly correlated with serum levels of thyroid-stimulating hormone (TSH) or serum T3 or T4 concentrations (Table 2); however, almost all patients were in the euthyroid range. Interestingly, one patient was severely hypothyroid, but had inconspicuous *DIO1* mRNA expression (Fig. 1C and Supplementary Fig. 1A, see section on supplementary data given at the end of this article).

We then tested whether the expression was correlated to systemic markers of lipid metabolism, revealing that *SLC16A2*, *DIO1* and *THRB* were negatively correlated with serum triglyceride levels, while *SLC10A1* and *THRA* were not affected (Fig. 2A and Supplementary Fig. 1B). However, when the respective confounding factors were used for correction, only the expression of *THRB* remained significantly correlated ($P_a=0.0175$). No correlation to LDL or total cholesterol was observed (data not shown). With regard to markers of glucose metabolism, hepatic *DIO1* and *THRB* mRNA expression were negatively associated with HbA1c (Fig. 2B and Supplementary Fig. 1C), even after correction for age ($P_a=0.0434$ for *DIO1* and $P_a=0.0268$ for *THRB*). No association for any of the genes was observed with blood glucose concentrations (data not shown), except for *THRB*, which however was not significant after correction for age (Supplementary Fig. 1C).

Finally, we tested for an association of NASH score with the respective genes. The analysis revealed that *THRB* mRNA expression was negatively associated with NASH score ($P=0.0084$, after correction for age $P_a=0.0461$), with lower expression in higher stages (Fig. 3A), while the other genes were not correlated (Supplementary Fig. 2A). The expression of *THRB* mRNA was also not correlated with APOF mRNA levels (Supplementary Fig. 2B), a molecular marker of fibrosis (23). Taken together, the data indicate that reduced *THRB* expression, which further declines with age, is connected to more progressed stages of NASH (Fig. 3B) and suggest an altered cellular responsiveness to TH during disease progression and aging (Fig. 3C).

Discussion

Our study is the first to comprehensively test the expression of genes gating local TH action in liver, including transporters, deiodinases and receptors in NAFLD patients. As liver samples cannot be obtained from healthy individuals due to ethical reasons (due to the risk

Table 1 Characteristics of the human patient cohort.

Parameter	Unit	Mean	St. Dev	Min	Max	n
Gender	m/f	24/61				85
Age	years	43.8	12.8	22	72	85
BMI	kg/m ²	52.2	10.8	32.2	84.9	85
TSH ^a	mU/L	2.5	5.0	0.1	45.7	80
T3	ng/mL	0.9	0.2	0.1	1.3	76
T4	μ g/dL	5.4	1.3	2.7	9.1	76
Triglycerides	mmol/L	212	121	57	611	85
Total cholesterol	mg/dL	188	41	109	367	85
LDL	mg/dL	101	34	12	174	79
HbA1c	%	6.5	1.9	4.3	11.8	85
Blood glucose	mg/dL	130	64	75	367	85
Diabetes ^b	Diagnosed yes/no	34/51				85
Hypertonia	Diagnosed yes/no	52/31				83
NAFLD activity	Score	2.8	2.2	0	6	73
NAS frequency	Score (number)	0 (19), 1 (5), 2 (10), 3 (8), 4 (10), 5 (12), 6 (9)				
Fibrosis	Fib-4 score	0.8	0.6	0.2	2.7	84
Fibrosis	Diagnosed yes/no	39/45				84

^a16 of which are treated with thyroxine. ^b30 of which are treated with insulin and/or metformin.

associated with a liver biopsy), our cohort is naturally limited to sick individuals, in this case exhibiting severe obesity requiring bariatric surgery. Consequently, the results cannot be compared to a healthy control group,

but were used to correlate gene expression with the severity of NAFLD, i.e. NAFLD activity score. Therefore, the results might not be representative for NAFLD patients in general, but given that obesity is the major risk factor

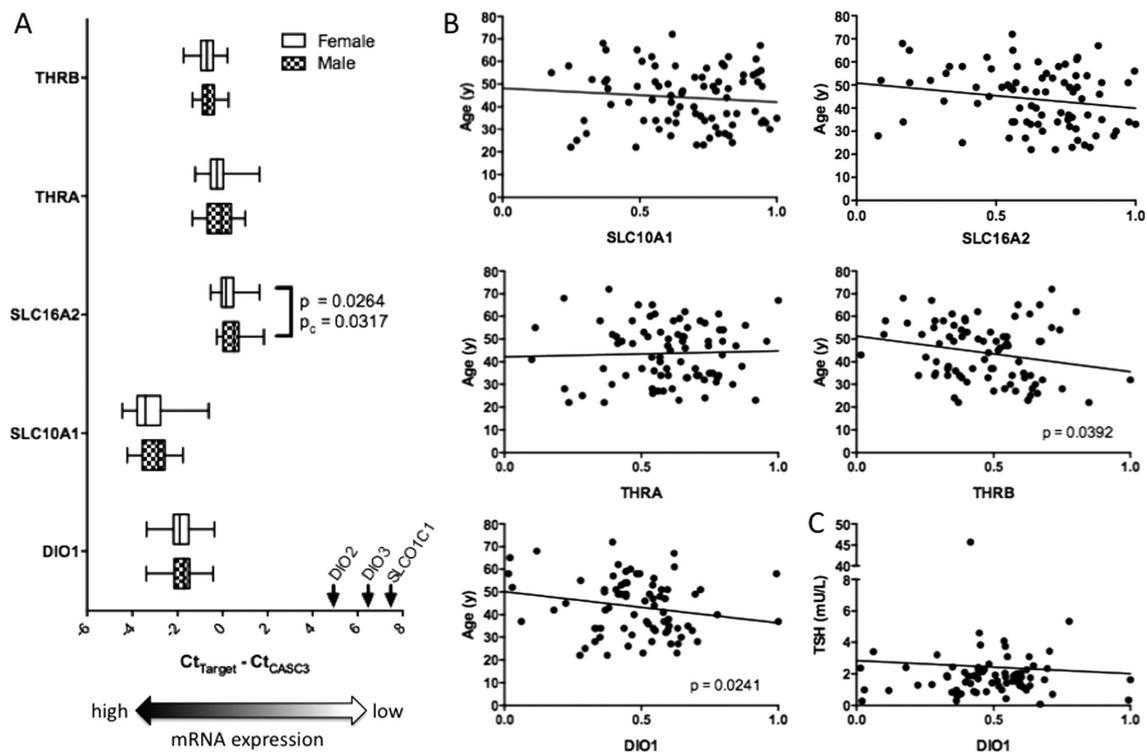


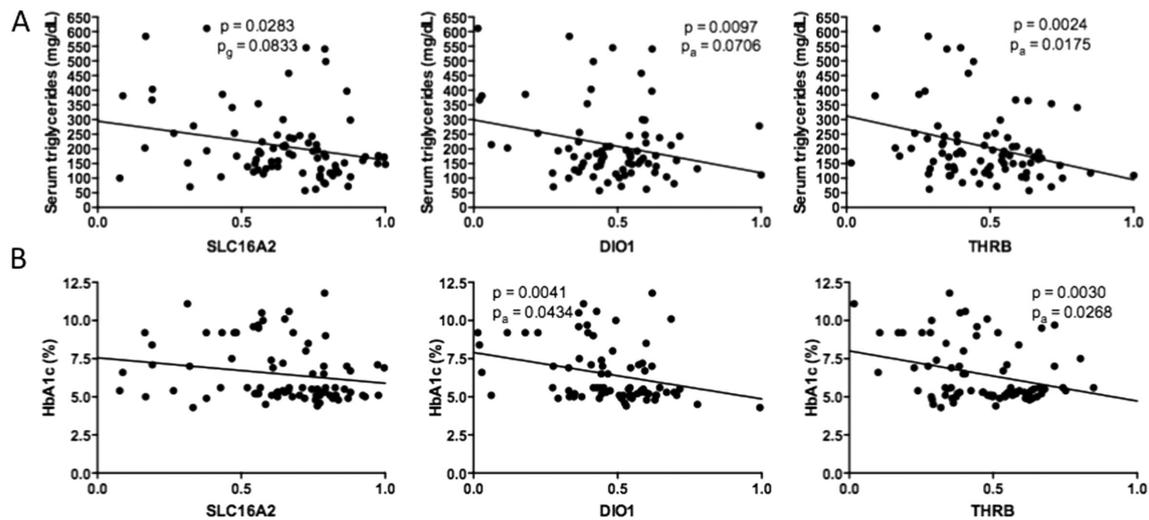
Figure 1

(A) Gene expression of thyroid hormone receptors, transporters and deiodinases in human liver biopsies of males and females, depicted as delta Ct to the housekeeping gene *CASC3* with high expression levels on the left. (B) Correlation of NCTP (*SLC10A1*), MCT8 (*SLC16A2*), thyroid hormone receptor α and β (*THR α* and *THR β*) and deiodinase type I (*DIO1*) mRNA expression with age. (C) Correlation of deiodinase type I (*DIO1*) mRNA expression with serum thyroid stimulating hormone (TSH).

Table 2 Detailed statistical analyses of the correlations in the study.

	P value Pearson	P value Spearman	P value corrected	Correlation coefficient	Effect size	Effect size corrected	n
Age							
DIO1	0.0241*			-0.2553	5.2298		78
SLC10A1	0.3516			-0.1035			83
SLC16A2	0.0827			-0.1940			81
THRA	0.7267			0.0394			81
THRB	0.0392*			-0.2282	7.9186		82
Gender							
DIO1		0.4305		0.0905			78
SLC10A1		0.0770		0.1952			83
SLC16A2		0.0317*		0.2389			81
THRA		0.4836		0.0789			81
THRB		0.7439		0.0366			82
BMI							
DIO1	0.7884			-0.0309			78
SLC10A1	0.9574			-0.0059			83
SLC16A2	0.6879			0.0453			81
THRA	0.6261			0.0549			81
THRB	0.2827			0.1200			82
TSH							
DIO1	0.7778		0.9858	-0.0334			74
SLC10A1	0.4601			0.0843			79
SLC16A2	0.3159		0.4275	0.1158			77
THRA	0.9453			-0.0080			77
THRB	0.7088		0.5356	0.0430			78
T3							
DIO1	0.8783		0.8920	0.0188			69
SLC10A1	0.9815			-0.0028			73
SLC16A2	0.5604		0.9793	-0.0697			72
THRA	0.7431			-0.0396			71
THRB	0.1590		0.2548	0.1666			73
T4							
DIO1	0.2747		0.8059	-0.1334			69
SLC10A1	0.8695			-0.0194			74
SLC16A2	0.2470		0.3490	-0.1372			73
THRA	0.4991			-0.0809			72
THRB	0.2949		0.5051	-0.1234			74
Serum triglycerides							
DIO1	0.0097		0.0706	-0.2912	59.345		78
SLC10A1	0.0611			-0.2065			83
SLC16A2	0.0283		0.0833	-0.2437	56.014		81
THRA	0.8573			0.0203			81
THRB	0.0024		0.0175*	-0.3303	110.33	81.401	82
HbA1c							
DIO1	0.0041		0.0434*	-0.3218	0.9849	0.6305	78
SLC10A1	0.9033			-0.0135			83
SLC16A2	0.0759		0.1557	-0.1983			81
THRA	0.5801			0.0624			81
THRB	0.0030		0.0268*	-0.3236	1.6621	1.1049	82
Blood glucose							
DIO1	0.0917		0.4940	-0.1923			78
SLC10A1	0.8022			-0.0279			83
SLC16A2	0.0705		0.1653	-0.2021			81
THRA	0.6621			0.0493			81
THRB	0.0114		0.0843	-0.2780	47.8810		82
NASH score							
DIO1		0.2908	0.5044	-0.1300			69
SLC10A1		0.8663		0.0202			74
SLC16A2		0.7556	0.7036	-0.0379			72
THRA		0.5448		0.0725			74
THRB		0.0084	0.0461*	-0.3106			71

As DIO1 and THRB as dependent variables were found to be significantly affected by age, all subsequent correlations of these genes were corrected for age. Likewise, SLC16A2 was significantly affected by gender; hence, the subsequent analyses for this gene were corrected for gender. *: $P < 0.05$

**Figure 2**

(A) Correlation of MCT8 (*SLC16A2*), deiodinase type I (*DIO1*) and thyroid hormone receptor β (*THRβ*) mRNA expression with serum triglyceride levels. (B) Correlation of MCT8 (*SLC16A2*), deiodinase type I (*DIO1*) and thyroid hormone receptor β (*THRβ*) mRNA expression with glycated haemoglobin A1c (HbA1c). The respective raw and corrected *P*-values for age (a) or gender (g) are given in the figures.

for NAFLD (24), the findings are still of clinical relevance. Moreover, our results are currently limited to the mRNA expression; unfortunately, however, to the best of our knowledge reliable and properly validated commercial antibodies for TH receptors, deiodinases or transporters are currently not available (25).

Hepatic gene expression in human liver

As local changes in TH economy are expected in livers of patients with NAFLD (9), we tested the expression of the relevant genes in our biopsy collection. As expected, we could not detect *SLCO1C1*, *DIO2* and *DIO3* transcripts. *DIO3* has been described in human fetal liver and adult livers of critically ill patients, but in line with our findings, it was reported to be absent in healthy adult human liver samples (26, 27). We identified hepatic expression of both TH receptor genes, which was not correlated to serum TSH, corroborating previous findings (28). Surprisingly, we did not find any correlation of *DIO1* with serum TSH, T4 or T3, although the gene is known as a sensitive marker of peripheral TH status in the mouse (29) and also positively correlated with T3 in critically ill patients on enzyme and transcript level (30). However, only two patients in our cohort were outside the euthyroid reference range with TSH concentrations of 0.08 mU/L and 45.7 mU/L respectively, but their *DIO1* transcript levels were not obviously altered. Interestingly, however, we observed lower *DIO1* transcripts levels correlating with elevated HbA1c as long-term marker for diabetes, which concurs

with previous animal studies showing low T3 and lower outer ring deiodination activity in type I diabetic rats (31) and mice (32). With regard to TH transporters, we observed a slightly higher expression of *SLC16A2* (MCT8) in female individuals, potentially due to the fact that it is an X-chromosomal gene (10).

Effects of aging

In the general population, decreased thyroid function is associated with longevity (33). Moreover, older adults show a prevalence for subclinical hypothyroidism, but it is currently highly controversial whether a treatment is beneficial or harmful (34). Consequently, data on the local change in TH economy on the tissue level are highly relevant to understand the underlying causes and therapeutic consequences. Several studies on animal models are available, revealing decreased liver *Slc16a2* and *Dio1* expression in old rats (35), increased *Slc10a1* but unaltered *Slc16a2* transcripts in old mice (36) and lower *DIO1* activities in several mouse models of aging (37). On the receptor level, no effect of age on *Thra* was observed, while a progressive increase in *Thrb* mRNA and total TR β protein level was found in old rats; however, most remarkably, the nuclear levels of TR β seemed to decline (35). Data on the human situation are scarce, reporting only an age-dependent decrease in *THRβ* expression in peripheral blood mononuclear cells, presumably driven by changes in promoter methylation (38). Consequently, our data on decreased hepatic *THRβ* and *DIO1* expression

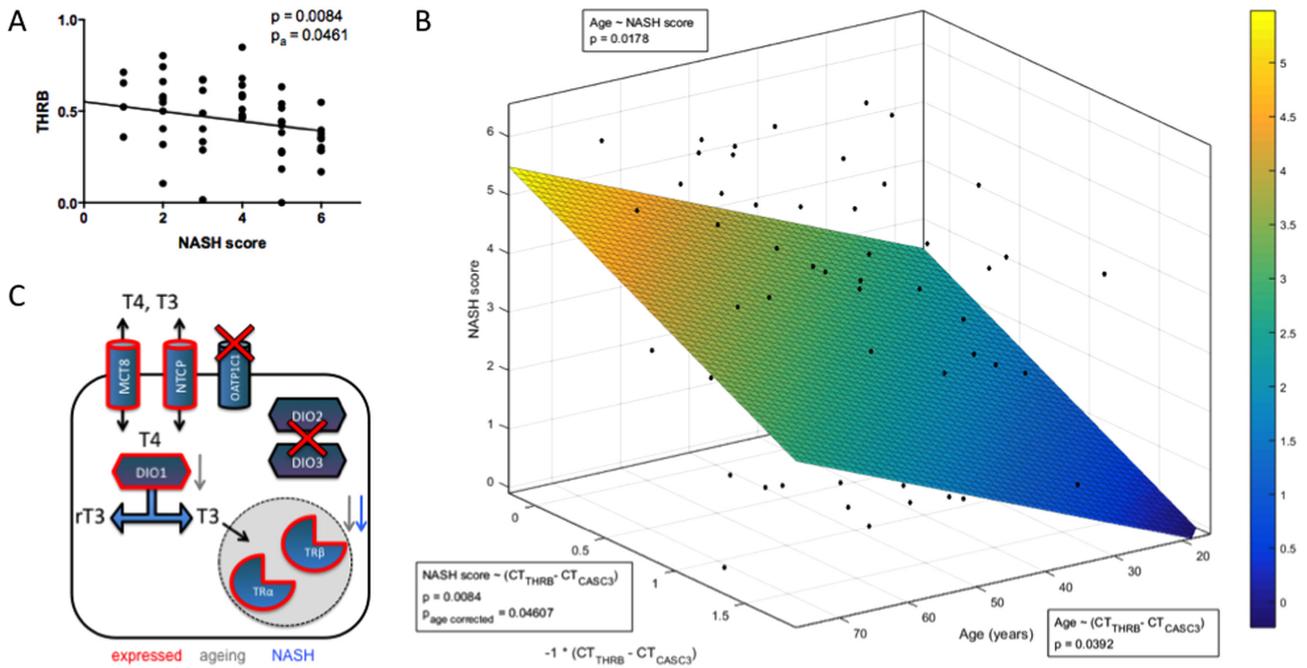


Figure 3

(A) Correlation of thyroid hormone receptor β (*THRB*) mRNA expression with NASH score. The respective raw P value and corrected for age (a) are given in the figure. (B) 3D model of the correlations between NASH score and age as well as *THRB* mRNA expression with the respective raw and corrected P values. (C) Schematic overview of the changes in liver cell thyroid hormone economy with age and NASH. DIO, deiodinase; MCT8, SLC16A2; NCTP, SLC10A1; rT3, 3,3',5'-triiodothyronine; SLCO1C1, OATP1C1; T3, 3,3',5'-triiodothyronine; T4, thyroxine; TR, thyroid hormone receptor.

are of explicit biological relevance for the understanding of TH economy in the aging liver, as they suggest that the liver might become somewhat resistant to circulating TH.

Nonalcoholic steatohepatitis

Hypothyroidism is a known risk factor for NAFLD; however, the underlying mechanisms are complex likely involving a combination of direct hepatic effects and indirect actions via adipocytes (39). The role of hepatic deiodination in this context is controversial: some suggested a higher conversion of T4 as evidenced by an elevated T3/T4 ratio in patients with NAFLD (40), while others observed increased DIO3 and reduced DIO1 in NAFLD using immunohistochemistry on a small number of liver biopsies (8). However, our data from a larger cohort now suggest that at least the mRNA levels of *DIO1* do not change over the course of the disease, while we could also not detect unusual expression of the other deiodinases.

That the expression of *THRB* decreases with higher stages of NASH is a potentially relevant finding, raising several questions for follow-up studies. Most importantly, using single-cell sequencing, it needs to be established whether the number of cells expressing *THRB* is reduced

or whether our results represent an overall reduction in *THRB* expression. This could have clinical implications for the validity of *THRB* expression as additional parameter to determine the NASH score on the molecular level. Secondly, it needs to be tested, whether the lower transcript levels are a consequence of the disorder, for example, through the suppressing effect of higher serum triglyceride levels or whether they could be causally involved in NASH development. For this, one would first need to establish whether the observed amount of reduction in *THRB* mRNA translates to reduced protein levels and cellular resistance to TH. However, it is tempting to speculate that NASH progression and declining *THRB* expression jointly initiate a vicious cycle, which could be broken by (re) activation of hepatic TH signaling. This has been tried for a long time using TR β selective compounds; unfortunately, to date, with little success due to severe side effects (16). More promising results have been obtained more recently with a liver targeted glucagon-T3 (41) or a low-dose T4 treatment in NAFLD patients (18). However, our data indicate that the efficiency of these approaches might depend on the stage of the disease, since at a certain point the cellular resistance might be irreversible due to the lack of reactivatable TR β .

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/EC-18-0499>.

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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