

# Urine Metabolomics by $^1\text{H-NMR}$ Spectroscopy Indicates Associations between Serum 3,5- $\text{T}_2$ Concentrations and Intermediary Metabolism in Euthyroid Humans

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## Key Words

3,5-Diiodothyronine · Trigonelline · Urine metabolome · NMR spectroscopy · Thyroid hormone

## Abstract

**Context:** 3,5-Diiodo-L-thyronine (3,5- $\text{T}_2$ ) is a thyroid hormone metabolite which exhibited versatile effects in rodent models, including the prevention of insulin resistance or hepatic steatosis typically forced by a high-fat diet. With respect to euthyroid humans, we recently observed a putative link between serum 3,5- $\text{T}_2$  and glucose but not lipid metabolism. **Objective:** The aim of the present study was to widely screen the urine metabolome for associations with serum 3,5- $\text{T}_2$  concentrations in healthy individuals. **Study Design and Methods:** Urine metabolites of 715 euthyroid participants of the population-based Study of Health in Pomerania (SHIP-TREND) were analyzed by  $^1\text{H-NMR}$  spectroscopy. Multinomial logistic and multivariate linear regression models were used to detect associations between urine metabolites and serum 3,5- $\text{T}_2$  concentrations. **Results:** Serum 3,5- $\text{T}_2$  concentrations were positively associated with urinary levels of trigonelline, pyroglutamate, acetone and hippurate. In detail, the odds for intermediate or suppressed serum 3,5- $\text{T}_2$

concentrations doubled owing to a 1-standard deviation (SD) decrease in urine trigonelline levels, or increased by 29–50% in relation to a 1-SD decrease in urine pyroglutamate, acetone and hippurate levels. **Conclusion:** Our findings in humans confirmed the metabolic effects of circulating 3,5- $\text{T}_2$  on glucose and lipid metabolism, oxidative stress and enhanced drug metabolism as postulated before based on interventional pharmacological studies in rodents. Of note, 3,5- $\text{T}_2$  exhibited a unique urinary metabolic profile distinct from previously published results for the classical thyroid hormones.

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## Introduction

During the last decade the concepts of thyroid hormone (TH) action underwent a sustainable gain in complexity [1–3]. New mechanisms of action concerning the classical TH – L-thyroxine ( $\text{T}_4$ ) and 3,3',5-triiodo-L-thyronine ( $\text{T}_3$ ) – became obvious, including biological effects of nonclassical TH such as tetraiodothyroacetic acid [3, 4] and 3,5-diiodo-L-thyronine (3,5- $\text{T}_2$ ) [4], the putative

deiodination product of  $T_3$  in vivo.  $3,5-T_2$  exhibited remarkable metabolic effects when used as a pharmacological agent in animal studies. Administered to hypothyroid rats,  $3,5-T_2$  prevented and even reversed the severe consequences typically induced by a high-fat diet, like weight gain, insulin resistance or hepatic steatosis [5–7]. The responsible mechanisms include improvement of the blood lipid profile through the stimulation of  $\beta$ -oxidation of free fatty acids (FFA) and mitochondrial uncoupling, as well as depression of gluconeogenesis in hepatocytes [5, 8]. Therefore,  $3,5-T_2$  appears to act in a distinctively different mode compared to  $T_3$ .

Beside pharmacological studies in rodents, the knowledge about circulating  $3,5-T_2$  in humans is sparse. Early human studies in small and selected cohorts reported associations with chronic renal disease, liver cirrhosis or sepsis [9–11]. Most of these early data stem from observational analyses and detection of  $3,5-T_2$  in human serum employing polyclonal antibody-based immunoassays which resulted in a wide spectrum of reported  $3,5-T_2$  serum concentrations spanning almost two orders of magnitude [for a review see 12]. Development and application of a monoclonal antibody-based chemiluminescent immunoassay recently revealed serum  $3,5-T_2$  concentrations in the range of 0.2–0.5 nM in healthy individuals [12]. These  $3,5-T_2$  values showed no correlation to serum  $T_4$  or  $T_3$  concentrations in healthy or  $T_4$ -substituted individuals, indicating that serum  $3,5-T_2$  has a metabolic fate independent of that related to  $T_4$  and  $T_3$ . Using this assay we recently observed a putative link between serum  $3,5-T_2$  and glucose but not lipid metabolism in euthyroid humans [13].

Expanding these initial rodent experimental and human observational findings on the metabolic effects of  $3,5-T_2$ , high-throughput techniques like proton nuclear magnetic resonance ( $^1H$ -NMR) spectroscopy can be used [14] to collect further information on the metabolic actions of  $3,5-T_2$ .  $^1H$ -NMR spectroscopy attempts to give a comprehensive view of small-molecule metabolites present in various biofluids, thus providing a more sensitive tool than classical clinical markers [15]. Urine, which is readily available, as a downstream product of human metabolism can especially mirror the impact of genetic determinants, environmental factors, personal behavior, nutrition and therapeutic intervention [16]. Despite these advantages, the use of metabolomics in the investigation of TH (metabolite) action is, to the best of our knowledge, limited to intervention studies in rodents [17–21] and, with the exception of a recent study on the relation between TH status and serum metabolites [22], completely lacking in humans. Therefore, the present study was de-

signed to gain further insights into the metabolic profiles associated with circulating  $3,5-T_2$  by means of urine metabolomics in a large euthyroid study population.

## Material and Methods

### Study Population

SHIP-TREND is the second cohort of the Study of Health in Pomerania (SHIP), a population-based research project in West Pomerania, a rural region in north-east Germany [23]. A stratified random sample of 8,826 adults aged 20–79 years was drawn from population registries. Sample selection was facilitated by the centralization of local population registries in the Federal State of Mecklenburg-West Pomerania. The stratification variables were age, sex and city/county of residence. General baseline examinations were conducted between 2008 and 2012. Out of all the invitations, 4,420 individuals choose to participate (representing a 50.1% response rate). The study was approved by the local ethics committee and conformed to the principles of the declaration of Helsinki. For a specific SHIP-TREND subsample that encompasses 1,000 participants without self-reported diabetes who underwent an oral glucose tolerance test, a more extensive phenotyping was performed including, for example, additional laboratory measurements and metabolome analyses. This most comprehensively analyzed subsample of SHIP was chosen to ensure a maximum availability of clinically relevant information.

Of these participants, 995 were characterized by urine  $^1H$ -NMR spectra. Furthermore, we excluded subjects with one of the following conditions (overlaps existed): low urinary creatinine concentrations (<2 mM,  $n = 50$ ), missing values ( $n = 108$ ) or values more than twice the standard deviation (SD) away from mean serum  $3,5-T_2$  concentrations (>2.13 nM,  $n = 15$ ), use of thyroid medications (ATC code H03A or H03B,  $n = 98$ ) or serum thyrotropin (TSH) levels outside the reference range (0.30–3.59 mU/l,  $n = 51$ ). Ultimately, 715 subjects, aged between 21 and 81 years, were included in the present analysis.

### Measurements

Each SHIP-TREND participant underwent standardized medical examinations, blood sampling and an extensive computer-aided personal interview. Data on sociodemographic characteristics and medical histories were collected. Waist circumference (WC) was measured by an inelastic tape between the lower rib margin and the iliac crest in the horizontal plane.

Blood and urine samples (fasting  $\geq 8$  h) were collected between 6.00 a.m. and 7.00 p.m. Blood samples were drawn from the cubital vein of subjects in the supine position. Both urine and blood samples were analyzed immediately or stored at  $-80^\circ C$ . Serum TSH concentrations were measured using an immunoassay (Dimension VISTA, Siemens Healthcare Diagnostics, Eschborn, Germany) with a functional sensitivity of 0.005 mU/l. Serum  $3,5-T_2$  concentrations were measured with a recently developed monoclonal antibody-based chemiluminescence immunoassay [12]. The functional sensitivity of the assay was specified as 0.2 nM. The interassay variation was between 5.6 and 12.9%. The working range was declared as 0.2–10 nM  $3,5-T_2$ . Urine creatinine concentrations were measured using the Jaffé method (Dimension VISTA, Siemens Healthcare Diagnostics).

### <sup>1</sup>H-NMR Spectroscopic Analysis of Urinary Specimens

After thawing, urine specimens were centrifuged for 5 min at 3,000 g and the supernatant was used for spectroscopic analysis. To this purpose, 450 µl of urine were mixed with 50 µl of phosphate buffer in order to stabilize the urinary pH at 7.0 (±0.35). The phosphate buffer was prepared with D<sub>2</sub>O and contained sodium TSP [3-trimethylsilyl-(2,2,3,3-D<sub>4</sub>)-1-propionate] as the reference. Spectra were recorded at the University Medicine Greifswald, Germany, on a Bruker DRX-400 NMR spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) operating at a <sup>1</sup>H frequency of 400.13 MHz and equipped with a 4-mm selective inverse flow probe (FISEI, 120 µl active volume) with a z-gradient. Specimens were automatically delivered to the spectrometer via flow injection. The acquisition temperature was set to 300°K. A standard one-dimensional <sup>1</sup>H-NMR pulse sequence with suppression of the water peak (NOESYPREAST) was used: relaxation delay (RD) – P(90°) – 4 µs – P(90°) – mixing time (tm) – P(90°) – acquisition of the free induction decay (FID). The nonselective 90-degree hard pulse P(90°) was adjusted to 9.4 µs. The RD, the tm and the acquisition time were set to 4 s, 100 ms and 3.96 s, respectively, resulting in a total recycle time of ~8.0 s. Low-power continuous-wave irradiation on the water resonance at a field strength of ~25 Hz was applied during RD and tm for presaturation. After the application of 4 dummy scans, 64 FIDs were collected into 65,536 (64 K) complex data points using a spectral width of 20.689 parts per million (ppm). FIDs were multiplied with an exponential function corresponding to a line broadening of 0.3 Hz before Fourier transformation. Spectra were manually phase and baseline corrected and automatically referenced to the internal standard (TSP, 0.0 ppm) within TopSpin 1.3 (Bruker BioSpin).

The Fourier-transformed and baseline-corrected NMR spectra were manually annotated by spectral pattern matching using Chenomx NMR Suite 7.0 (Chenomx Inc., Edmonton, Alta., Canada) to deduce absolute urinary concentrations of 47 metabolites; subsequently, the NMR data was reduced to these metabolites [24]. Online supplementary figure S2 (for all online suppl. materials, see [www.karger.com/doi/10.1159/000381308](http://www.karger.com/doi/10.1159/000381308)) contains example spectra as well as annotated signals for relevant metabolites in the present analysis. To account for diurnal urine dilution, metabolite concentrations were normalized by urine creatinine levels and are reported as millimoles per mole of creatinine. To reflect the relationships between metabolites, associated with circulating 3,5-T<sub>2</sub>, their ratios were calculated, resulting in 1,035 additional variables.

### Statistical Analysis

Continuous data are expressed as the median (1st; 3rd quartile), and nominal data as a percentage. For bivariate statistics the Wilcoxon rank sum test (continuous data) or  $\chi^2$  test (nominal data) were used to compare men and women. Metabolite and metabolite ratios, as well as 3,5-T<sub>2</sub> concentrations, were log-transformed to achieve a normal distribution. Furthermore, metabolite/ratio levels were scaled according to their SD to facilitate comparison between associations. Since about a third of the study population exhibited 3,5-T<sub>2</sub> concentrations below the detection limit of the used assay (0.2 nM) but distinctly higher than blank values of the standard curve, subjects were subdivided into three groups according to their 3,5-T<sub>2</sub> concentrations: <0.2 nM (n = 255) but distinct from zero, 0.2–0.33 nM (n = 230) and >0.33 nM (n = 230). Subsequently, multinomial regression models with metabolite/ratio levels as exposures and 3,5-T<sub>2</sub> groups as outcome were performed. After the ex-

**Table 1.** General characteristics of the study population

Characteristics	Male (n = 351)	Female (n = 364)	p <sup>1</sup>
Age, years	50.2 (38.6; 60.8)	48.1 (40.2; 59.5)	0.70
Smoking status, %			<0.01
Never	32.4	52.5	
Former	43.2	28.0	
Current	24.4	19.5	
Physical activity, %			0.74
>1 h/week	50.7	51.9	
<1 h/week	49.3	48.1	
WC, cm	94.0 (86.2; 102.3)	81.5 (73.7; 90.1)	<0.01
TSH, mU/l	1.11 (0.79; 1.47)	1.29 (0.91; 1.76)	<0.01
3,5-T <sub>2</sub> , nM	0.24 (0.20; 0.38)	0.25 (0.20; 0.36)	0.91
Urine creatinine, mM	12.5 (7.1; 17.0)	6.5 (4.0; 11.0)	<0.01

Data are expressed as median (25th; 75th percentile).

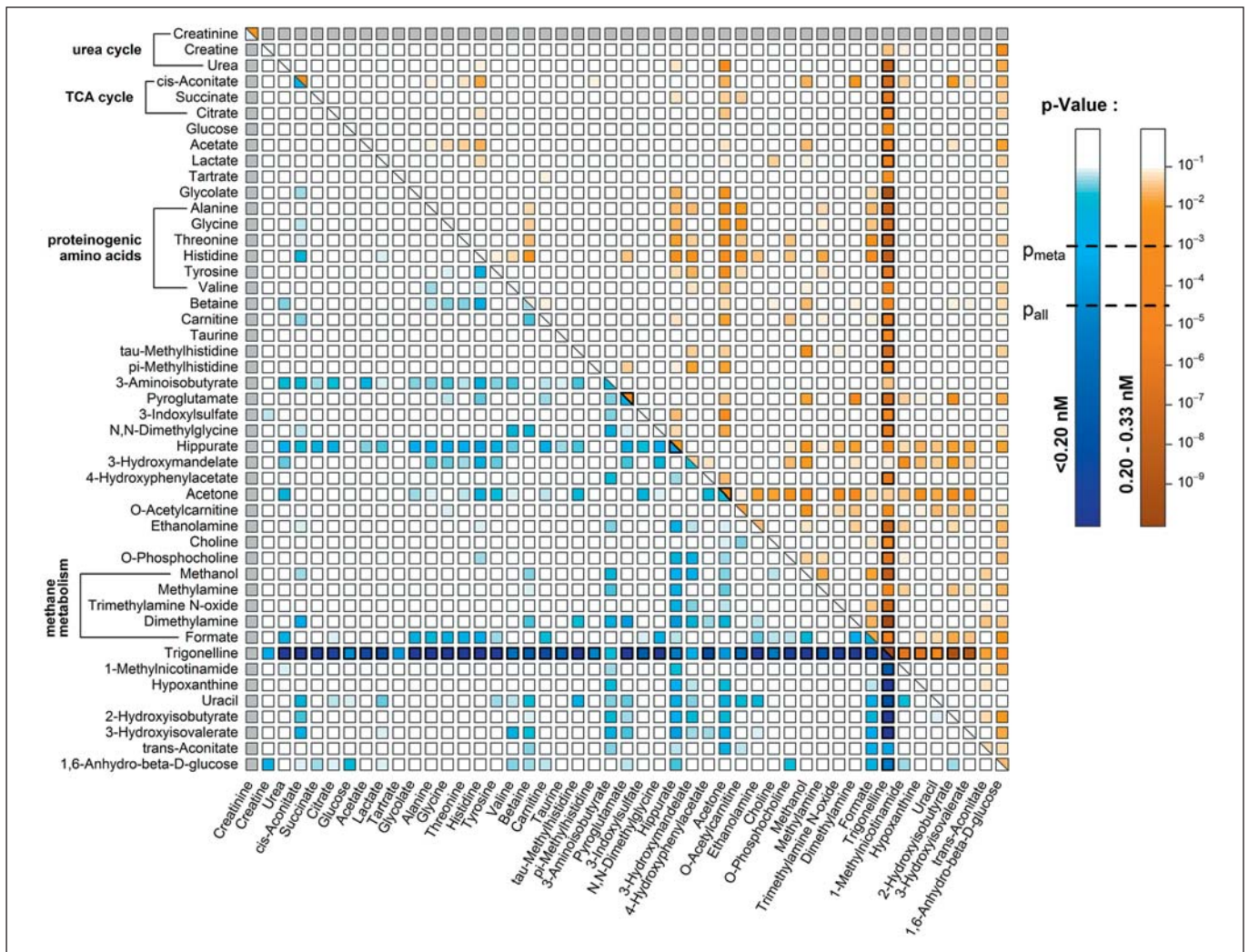
<sup>1</sup>The Wilcoxon rank sum test for continuous and the  $\chi^2$  test for categorical data were used for comparison.

clusion of participants with zero values for each metabolite/ratio and 3,5-T<sub>2</sub> concentrations below 0.2 nM, multivariate linear models with 3,5-T<sub>2</sub> concentrations as the continuous outcome were performed. All models were adjusted for age, sex and WC, as well as for TSH concentrations in sensitivity analyses. In a sensitivity analysis the influence of prior excluded subjects with high serum 3,5-T<sub>2</sub> or TSH concentrations outside the reference range (n = 757; n = 267 with serum 3,5-T<sub>2</sub> <0.2 nM) was tested to analyze the influence of an altered thyroid state. A p value <1.1 × 10<sup>-3</sup> (Bonferroni correction) for metabolites or <4.6 × 10<sup>-5</sup> (Bonferroni correction) for metabolite ratios was considered as statistically significant. Statistical analyses were performed using SAS version 9.3 (SAS statistical software, SAS Institute Inc., Cary, N.C., USA) and R 3.0.1 (R Foundation for Statistical Computing, Vienna, Austria).

### Results

The general characteristics for men and women are summarized in table 1. Women were more often never smokers and had lower values of WC, whereas serum TSH levels were higher compared to men. No sex-specific differences regarding age or 3,5-T<sub>2</sub> concentrations became obvious. Creatinine-standardized urine metabolites exhibited a sex-specific phenotype, with mostly higher values among women (online suppl. table S1).

Multinomial logistic regression models revealed several metabolites, including pyroglutamate, hippurate, acetone, formate and trigonelline, significantly associated with 3,5-T<sub>2</sub> (fig. 1, 2; table 2). In detail, the odds for sup-



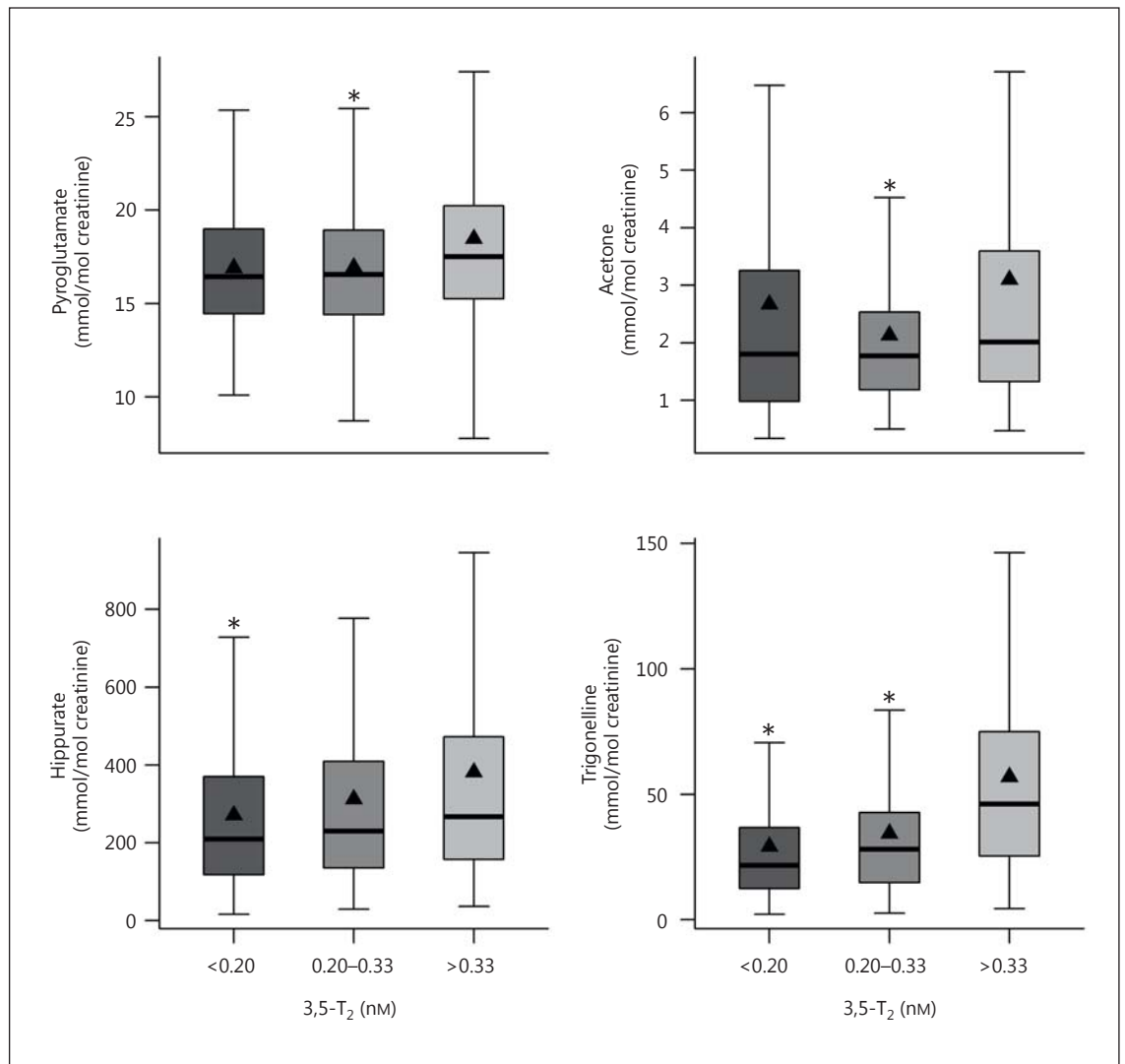
**Fig. 1.** Results for 3,5-T<sub>2</sub> from multinomial logistic regression (ref. >0.33 nM) models adjusted for age, sex and WC. p values for the suppressed (<0.20 nM) or intermediate (0.20–0.33 nM) group are colored blue and orange, respectively. Each line and column represents one metabolite; the diagonal contains results for single metabolites divided into triangles for each hormone, all other boxes

represent the respective ratio; thick-framed triangles indicate significant metabolites:  $p_{meta} = 0.05/47 = 1.1 \times 10^{-3}$ ; thick-framed squares indicate significant ratios:  $p_{all} = 0.05/1,082 = 4.6 \times 10^{-5}$  (accordingly corrected for multiple testing). Ratios with creatinine were not included in the analysis (colored gray), since all metabolites were normalized on creatinine.

pressed (<0.2 nM) or intermediate (0.2–0.33 nM) serum 3,5-T<sub>2</sub> concentrations doubled owing to a 1 SD decrease in logarithmic trigonelline levels. Furthermore, 29–50% increased odds for suppressed or intermediate serum 3,5-T<sub>2</sub> concentrations were related to a 1 SD decrease in urine pyroglutamate, acetone and hippurate levels (fig.1, 2; table 2). Multivariate linear models confirmed these findings, even if only the positive association with trigonelline reached the corrected statistical significance (fig. 3). This association was confirmed by the high number of signifi-

cantly associated metabolite ratios regarding trigonelline (fig. 3).

Adjustment for TSH levels in multinomial logistic and linear regression models did not change the above-mentioned associations (fig. 3; online suppl. fig. S1). Interestingly, the inclusion of subjects with extreme serum 3,5-T<sub>2</sub> or TSH concentrations outside the reference range led to a general loss of significance (data not shown). Regardless, the associations towards trigonelline and hippurate remained significant.



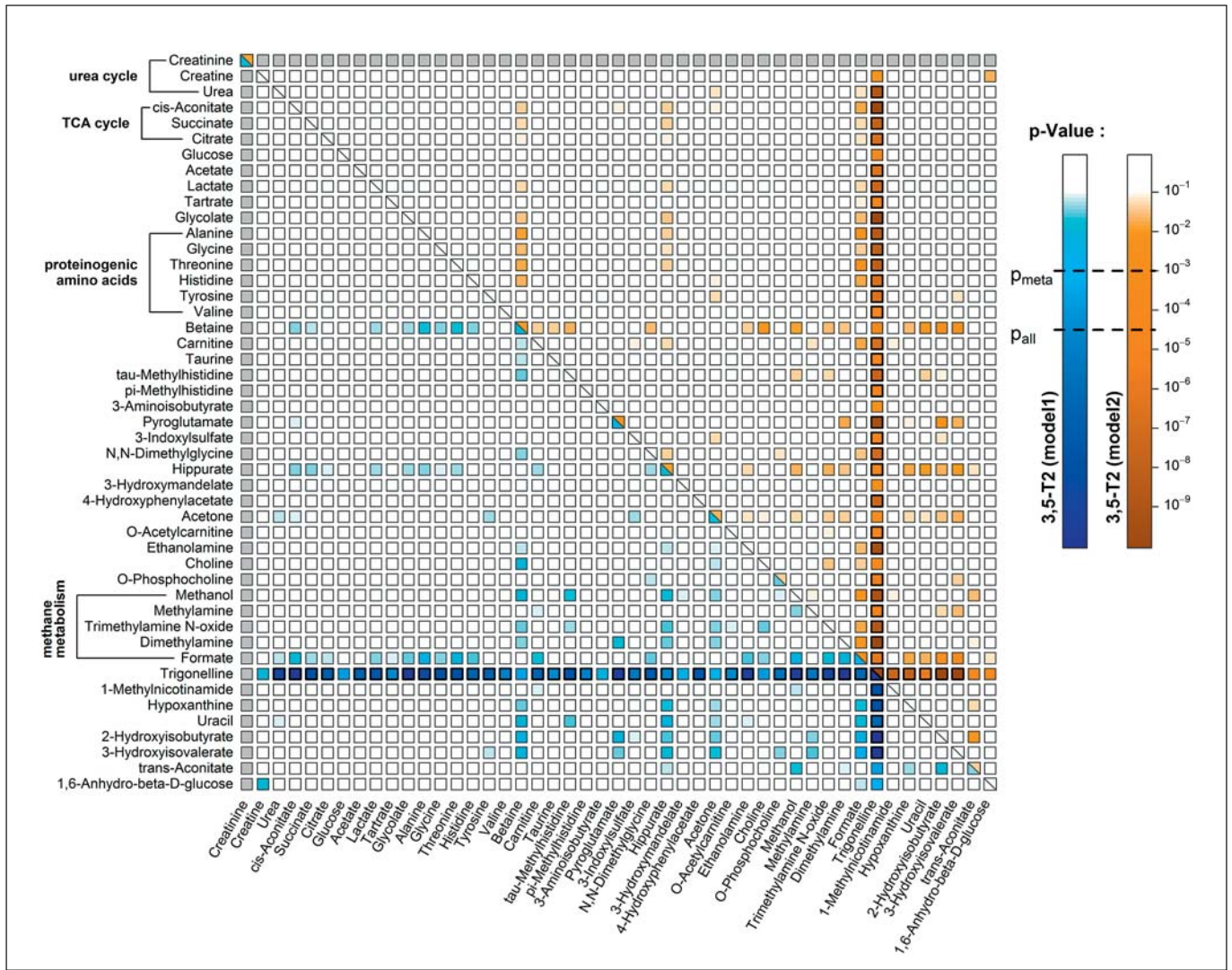
**Fig. 2.** Box plots of levels of urine pyroglutamate, hippurate, acetone and trigonelline by 3,5-T<sub>2</sub> concentration. The black triangles indicate group means. Asterisks (\*) indicate a significant odds ratio compared to the highest group from the multinomial logistic regression analysis (see table 2).

**Table 2.** Significant associations between urinary metabolites and serum 3,5-T<sub>2</sub> concentrations

Metabolites per SD decrease	n	SD	OR (95% CI) <sup>1</sup> (ref. >0.33 nM)				n	SD	β (95% CI) <sup>2</sup>	p
			<0.2 nM	p	0.2–0.33 nM	p				
Pyroglutamate	656	0.24	1.36 (1.11; 1.65)	<0.01	1.44 (1.17; 1.77) <sup>a</sup>	<0.01	428	0.26	-0.059 (-0.102; -0.016)	<0.01
Hippurate	715	0.84	1.44 (1.19; 1.76) <sup>a</sup>	<0.01	1.31 (1.07; 1.59)	<0.01	460	0.83	-0.054 (-0.098; -0.011)	0.01
Acetone	561	0.73	1.29 (1.04; 1.59)	0.02	1.50 (1.20; 1.88) <sup>a</sup>	<0.01	363	0.69	-0.057 (-0.104; -0.010)	0.02
Trigonelline	665	0.88	2.46 (1.96; 3.08) <sup>a</sup>	<0.01	2.11 (1.68; 2.65) <sup>a</sup>	<0.01	434	0.87	-0.152 (-0.194; -0.112) <sup>a</sup>	<0.01

All metabolites were in relation to creatinine. ref. = Reference tertile. <sup>a</sup> p < 0.05/47 = 1.1 × 10<sup>-3</sup>.

<sup>1</sup> Multinomial logistic regression models adjusted for age, sex and WC. <sup>2</sup> Multivariate linear regression models adjusted for age, sex and WC.



**Fig. 3.** Results for 3,5-T<sub>2</sub> colored according to p values from multivariate linear regression models adjusted for age, sex and WC (model 1; blue) and further for serum TSH levels (model 2; orange). Each line and column represents one metabolite; the diagonal contains results for single metabolites, all other boxes represent the respective ratio; light blue frames indicate significant metabo-

lites:  $p_{meta} = 0.05/47 = 1.1 \times 10^{-3}$ ; dark blue frames indicate significant ratios:  $p_{all} = 0.05/1,082 = 4.6 \times 10^{-5}$  (accordingly corrected for multiple testing). Ratios with creatinine were not included in the analysis (colored gray), since all metabolites were normalized on creatinine.

### Discussion

The present study screened the human urine metabolite profile assessed by <sup>1</sup>H-NMR spectroscopy for associations towards serum 3,5-T<sub>2</sub> concentrations. As discussed below, our results regarding trigonelline, pyroglutamate or acetone support and extend previously observed associations between 3,5-T<sub>2</sub> and glucose or lipid metabolism as demonstrated by various animal models [4, 5, 8, 25–29]. Furthermore, we observed thyromimetic, but TSH

independent, associations regarding hippurate and pyroglutamate, as observed in recent animal studies [17, 19].

Among all associated metabolites, trigonelline exhibited the strongest association with 3,5-T<sub>2</sub> and remains significant even after adjustment for serum or urine glucose levels, possibly confounding the association. The origin of urine trigonelline could either be exogenous, as food ingredients, or endogenous as a product of niacin metabolism [30, 31]. Administration of trigonelline reduced blood glucose levels by improving insulin sensitivity after

experimental induction of type 2 diabetes mellitus [32–34]. Similar effects were reported for 3,5-T<sub>2</sub> when used as a pharmacological agent to prevent insulin resistance forced by a high-fat diet [5]. Further animal studies [27, 35, 36] confirmed the antidiabetic effect of both trigonelline and 3,5-T<sub>2</sub>, revealing protective effects on impaired kidney function and structure, which represents a severe sequelae of type 2 diabetes mellitus. Taken together, 3,5-T<sub>2</sub> and trigonelline were linked to glucose metabolism, exhibiting the potential to improve glucose homeostasis in the case of diminished insulin sensitivity. Since a decrease in 3,5-T<sub>2</sub> concentrations was associated with a decrease in urine trigonelline levels, a mutual interaction or interdependency appears possible. In this context previous reports on inhibition of T<sub>3</sub> production by administration of fenugreek seed extracts in rodents are of interest. These trigonelline-rich extracts inhibit T<sub>3</sub> production from T<sub>4</sub> concomitant to decreasing hepatic superoxide dismutase activity, but leaving lipid peroxidation and catalase activity unaltered [37], thus supporting links between TH status, glucose metabolism and trigonelline, which still need to be unraveled in detail.

Glucose metabolism was further related to 3,5-T<sub>2</sub> via stimulation of glucose-6-phosphate dehydrogenase (G6PD) [38], a key enzyme of the pentose phosphate pathway. G6PD catalyzes the conversion of D-glucose-6-phosphate to 6-phospho-D-glucono-1,5-lactone, thereby producing nicotinamide adenine dinucleotide phosphate (NADPH). NADPH in turn is necessary for the reduction of oxidized glutathione, to restore intracellular levels of reduced glutathione after response to oxidative stress. Beside this redox cycle, glutathione synthesis is assured by the  $\gamma$ -glutamyl cycle, which at least in astrocytes [39] was assumed to be stimulated by TH. The  $\gamma$ -glutamyl cycle involves the formation of pyroglutamate (5-oxoproline), which could be converted to glutamate and hence integrated in glutathione synthesis, but its conversion depends on glutathione synthase activity. Depressed glutathione synthase activity leads to a rise of pyroglutamate excretion in blood and subsequently in urine [40]. In this context, the observed positive association between 3,5-T<sub>2</sub> and urine pyroglutamate levels might point towards a link between 3,5-T<sub>2</sub> and antioxidant defense, supporting observations from a recent proteomics study [29]. Shifts in hepatic cysteine flux, altered TH homeostasis and lipid metabolism have recently been observed in a study on the hepatic profile in mice with suboptimal hepatic and systemic expression of enzymes involved in cellular redox regulation due to mild selenium deficiency [20].

Acetone is a ketone body derived by a spontaneous decarboxylation of acetoacetate, which in turn is derived from acetyl-CoA. Generally, acetyl-CoA serves as substrate for the tricarboxylic acid cycle by condensing with oxaloacetate. In a state of low oxaloacetate levels, acetyl-CoA is redirected to ketogenesis. This displays a switch in energy metabolism from glucose to FFA utilization. Urine ketone body levels were considered as markers for mitochondrial  $\beta$ -oxidation of FFAs [41]. The positive association between 3,5-T<sub>2</sub> concentrations and urine acetone levels observed in our study is in concordance with several animal studies [8, 25, 26] showing rapid enhanced FFA transportation and subsequent oxidation in the mitochondria of skeletal muscle and hepatocytes of hypothyroid rats treated with 3,5-T<sub>2</sub>. As the molecular target carnitine palmitoyltransferase-1 (CPT1) was identified, which constitutes the main gateway for FFAs to mitochondria [8, 25]. Of note, related enzymes, including CPT1, were reported to be altered by 3,5-T<sub>2</sub> treatment in the liver of rats fed with a high-fat diet [29]. Furthermore, enhanced ketogenesis following TH metabolite administration was even observed in two previous studies, where 3-iodothyronamine (3-T<sub>1</sub>AM) treatment results in elevated serum and urine ketone bodies [21, 42]. This observation might support the hypothesis that 3,5-T<sub>2</sub> might be (one of) the precursor(s) of biosynthesis of 3-T<sub>1</sub>AM [12, 43, 44]. Consequently, it should be considered to expand the concept of TH action on (lipid) metabolism to metabolically active TH derivatives originating from the classical TH T<sub>4</sub> and T<sub>3</sub> by further deiodination and decarboxylation in target tissues such as liver, skeletal muscle and adipose tissue.

In concordance with this hypothesis, we observed an association with urine levels of hippurate, which were shown to be elevated in the case of experimental induction of hypothyroidism in rats [19]. Since previous work [12] suggested unaltered serum 3,5-T<sub>2</sub> concentrations in hypothyroidism, a decreased ratio of the putative precursor T<sub>4</sub> (T<sub>3</sub>) and 3,5-T<sub>2</sub> could account for this observation. However, the relation between serum fT<sub>4</sub> as well as TSH and 3,5-T<sub>2</sub> concentrations in humans is not yet completely understood. Moreover, in the present study the inclusion of subjects with abnormal serum 3,5-T<sub>2</sub> or TSH concentrations led to weaker associations, suggesting differing roles of serum 3,5-T<sub>2</sub> on intermediary metabolism in different thyroid states. It is worth noting that, besides hippurate, 3,5-T<sub>2</sub> exhibited a unique associated urinary metabolic profile in comparison with TSH and fT<sub>4</sub> [19].

Our study has some strengths and some potential limitations. Metabolomics is a powerful tool for endocrine

research, since it has the capability of jointly capturing versatile influences, like genetics or health behavior, in intermediate phenotypes. These intermediate phenotypes even enable the analysis of effects of moderate endocrine actors. In contrast to the previously performed interventional studies on animals, the present study is limited by the cross-sectional and agnostic design, whereby no prediction of time courses and detection of intervention effects are possible. Moreover, the functional sensitivity of the assay used to measure serum 3,5-T<sub>2</sub> represented a strong limitation in the statistical analysis, since one third of study participants (n = 255) exhibited concentrations below this detection limit. Our findings should therefore be regarded as hypothesis generating. It is widely accepted that <sup>1</sup>H-NMR spectroscopy is hampered by lower sensitivity in comparison with chromatographic coupled mass spectrometry. Hence, we expect that further metabolites and subsequent pathways are associated with 3,5-T<sub>2</sub> concentrations. Especially in epidemiological research, urine metabolomics by means of <sup>1</sup>H-NMR spectroscopy has the advantage of robust and reproducible measurements of very large sample numbers.

In conclusion, we observed, for the first time, associations between urine metabolites and serum 3,5-T<sub>2</sub>. The detected metabolites are related to glucose (trigonelline) and lipid metabolism (acetone), as well as the response to oxidative stress or drug metabolism (pyroglutamate), and are in concordance with previously published liver proteome analyses [29]. Interestingly, some of the associations (hippurate and pyroglutamate) were recently re-

ported for rodent models of experimental hypothyroidism, whereas the majority of associations reported here constitute a new and unique association to 3,5-T<sub>2</sub>. In concordance with several animal studies, our findings emphasize the need to consider the extension of classical TH action by additional metabolically active TH metabolites, such as 3,5-T<sub>2</sub>. Further studies with complementary metabolomics tools like mass spectrometry and the conduction of other biofluids, such as serum, could provide further insight into the role of serum 3,5-T<sub>2</sub> in human metabolism.

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### Disclosure Statement

The authors declare no conflicts of interest.

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