Clinical Thyroidology / Original Paper

European
Thyroid Journal

Eur Thyroid J 2015;4(suppl 1):92–100
DOI: 10.1159/000381308

Received: November 3, 2014
Accepted after revision: February 24, 2015
Published online: May 28, 2015

Urine Metabolomics by ^{1}H-NMR Spectroscopy Indicates Associations between Serum 3,5-T_2 Concentrations and Intermediary Metabolism in Euthyroid Humans

Maik Pietzner[a] Georg Homuth[b] Kathrin Budde[a] Ina Lehmphul[d]
Uwe Völker[b] Henry Völzke[c] Matthias Nauck[a] Josef Köhrle[d] Nele Friedrich[a]

[a]Institute of Clinical Chemistry and Laboratory Medicine, [b]Interfaculty Institute for Genetics and Functional Genomics, and [c]Institute for Community Medicine, University Medicine Greifswald, Ernst Moritz Arndt University, Greifswald, and [d]Institut für Experimentelle Endokrinologie, Charité-Universitätsmedizin Berlin, Berlin, Germany

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

Abstract
Context: 3,5-Diiodo-L-thyronine (3,5-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-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-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}H-NMR spectroscopy. Multinomial logistic and multivariate linear regression models were used to detect associations between urine metabolites and serum 3,5-T_2 concentrations. ***Results:*** Serum 3,5-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-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-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-T_2 exhibited a unique urinary metabolic profile distinct from previously published results for the classical thyroid hormones.

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 (T_4) and 3,3′,5-triiodo-L-thyronine (T_3) – became obvious, including biological effects of nonclassical TH such as tetraiodothyroacetic acid [3, 4] and 3,5-diiodo-L-thyronine (3,5-T_2) [4], the putative

KARGER 125

E-Mail karger@karger.com
www.karger.com/etj

2235–0640/15/0045–0092$39.50/0

Maik Pietzner
Institute of Clinical Chemistry and Laboratory Medicine
University Medicine Greifswald, Ferdinand-Sauerbruch Strasse
DE–17475 Greifswald (Germany)
E-Mail maik.pietzner@uni-greifswald.de

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 β-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 (^{1}H-NMR) spectroscopy can be used [14] to collect further information on the metabolic actions of 3,5-T_2. ^{1}H-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 designed 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 ^{1}H-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 ≥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°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).

^{1}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_2O and contained sodium TSP [3-trimethylsilyl-(2,2,3,3-D4)-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 ^{1}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 ^{1}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) 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_2, 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 χ^2 test (nominal data) were used to compare men and women. Metabolite and metabolite ratios, as well as 3,5-T_2 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_2 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_2 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_2 groups as outcome were performed. After the exclusion of participants with zero values for each metabolite/ratio and 3,5-T_2 concentrations below 0.2 nM, multivariate linear models with 3,5-T_2 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_2 or TSH concentrations outside the reference range (n = 757; n = 267 with serum 3,5-T_2 <0.2 nM) was tested to analyze the influence of an altered thyroid state. A p value $<1.1 \times 10^{-3}$ (Bonferroni correction) for metabolites or $<4.6 \times 10^{-5}$ (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).

Table 1. General characteristics of the study population

Characteristics	Male (n = 351)	Female (n = 364)	p[1]
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_2, 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).
[1] The Wilcoxon rank sum test for continuous and the χ^2 test for categorical data were used for comparison.

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_2 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_2 (fig. 1, 2; table 2). In detail, the odds for sup-

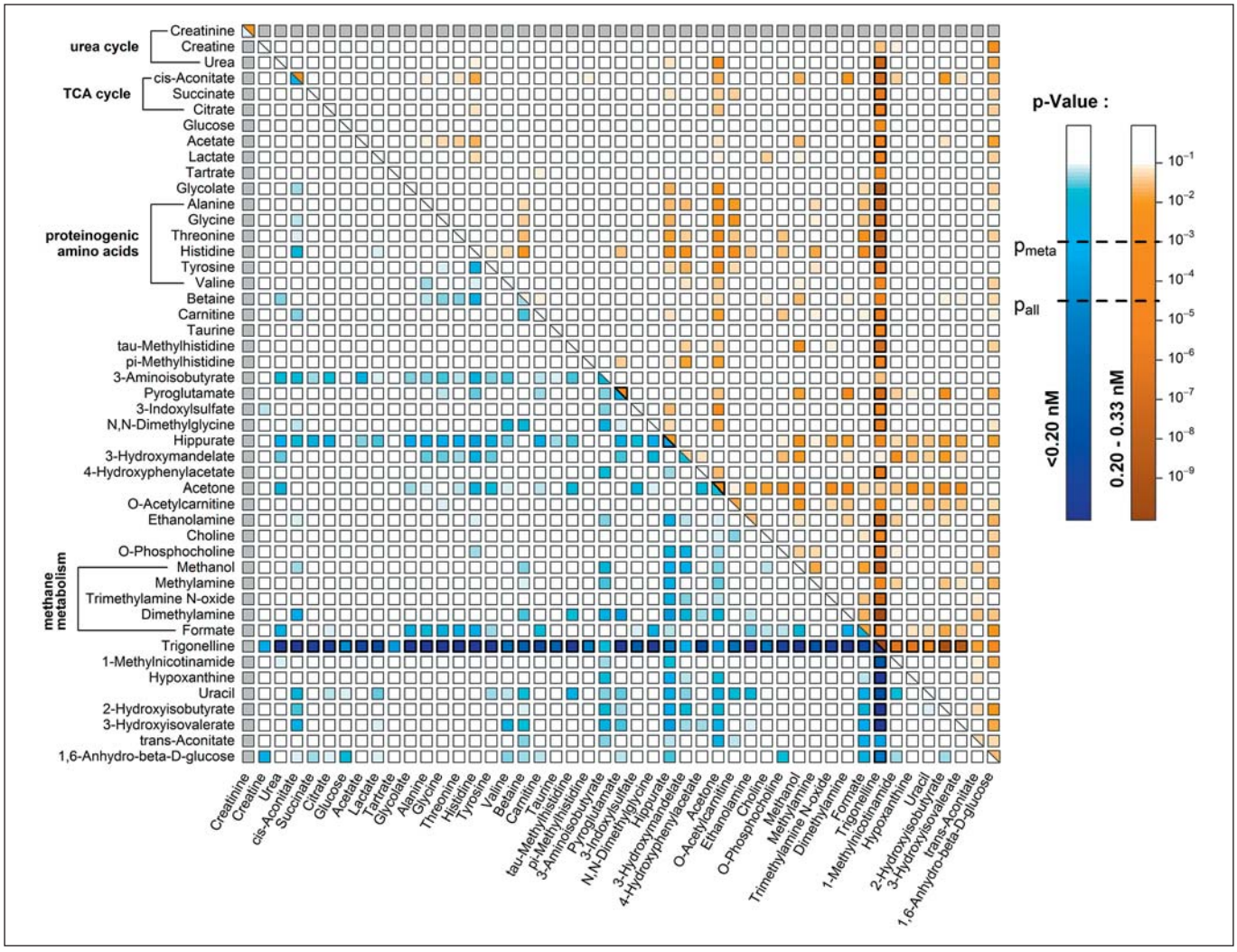

Fig. 1. Results for 3,5-T_2 from multinomial logistic regression (ref. >0.33 nM) models adjusted for age, sex and WC. p values for the suppressed (<0.20 nM) or intermediate (0.20–0.33 nM) group are colored blue and orange, respectively. Each line and column represents one metabolite; the diagonal contains 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_2 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_2 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 significantly 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_2 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.

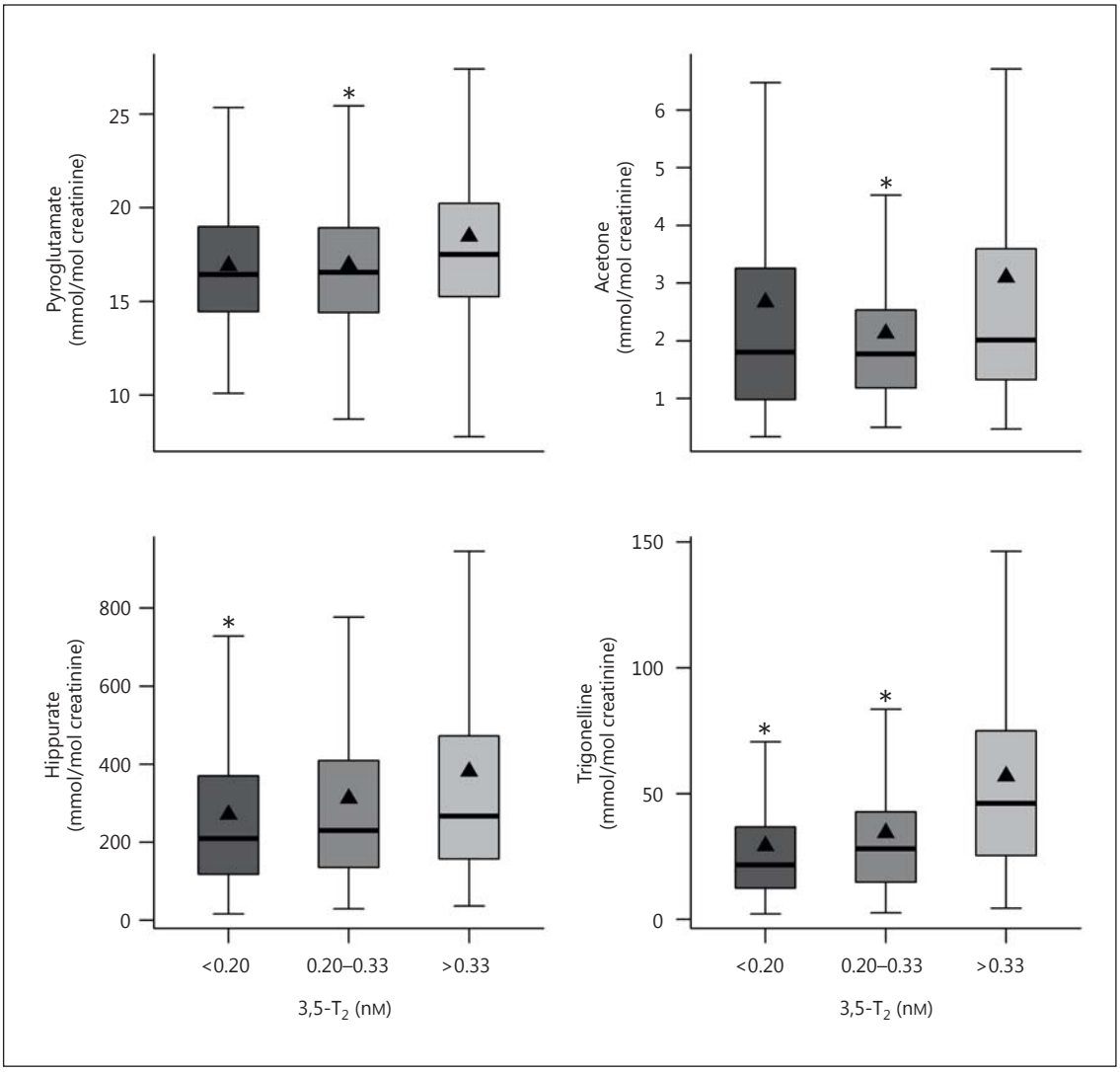

Fig. 2. Box plots of levels of urine pyroglutamate, hippurate, acetone and trigonelline by 3,5-T_2 concentration. The black triangles indicate group means. Asterisks (*) indicate a significant odds ratio compared to the highest group from the multinomial logistic regression analysis (see table 2).

Table 2. Significant associations between urinary metabolites and serum 3,5-T_2 concentrations

Metabolites per SD decrease	n	SD	OR (95% CI)[1] (ref. >0.33 nM)				n	SD	β (95% CI)[2]	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)[a]	<0.01	428	0.26	−0.059 (−0.102; −0.016)	<0.01
Hippurate	715	0.84	1.44 (1.19; 1.76)[a]	<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)[a]	<0.01	363	0.69	−0.057 (−0.104; −0.010)	0.02
Trigonelline	665	0.88	2.46 (1.96; 3.08)[a]	<0.01	2.11 (1.68; 2.65)[a]	<0.01	434	0.87	−0.152 (−0.194; −0.112)[a]	<0.01

All metabolites were in relation to creatinine. ref. = Reference tertile. [a] $p < 0.05/47 = 1.1 \times 10^{-3}$.
[1] Multinomial logistic regression models adjusted for age, sex and WC. [2] Multivariate linear regression models adjusted for age, sex and WC.

Eur Thyroid J 2015;4(suppl 1):92–100
DOI: 10.1159/000381308

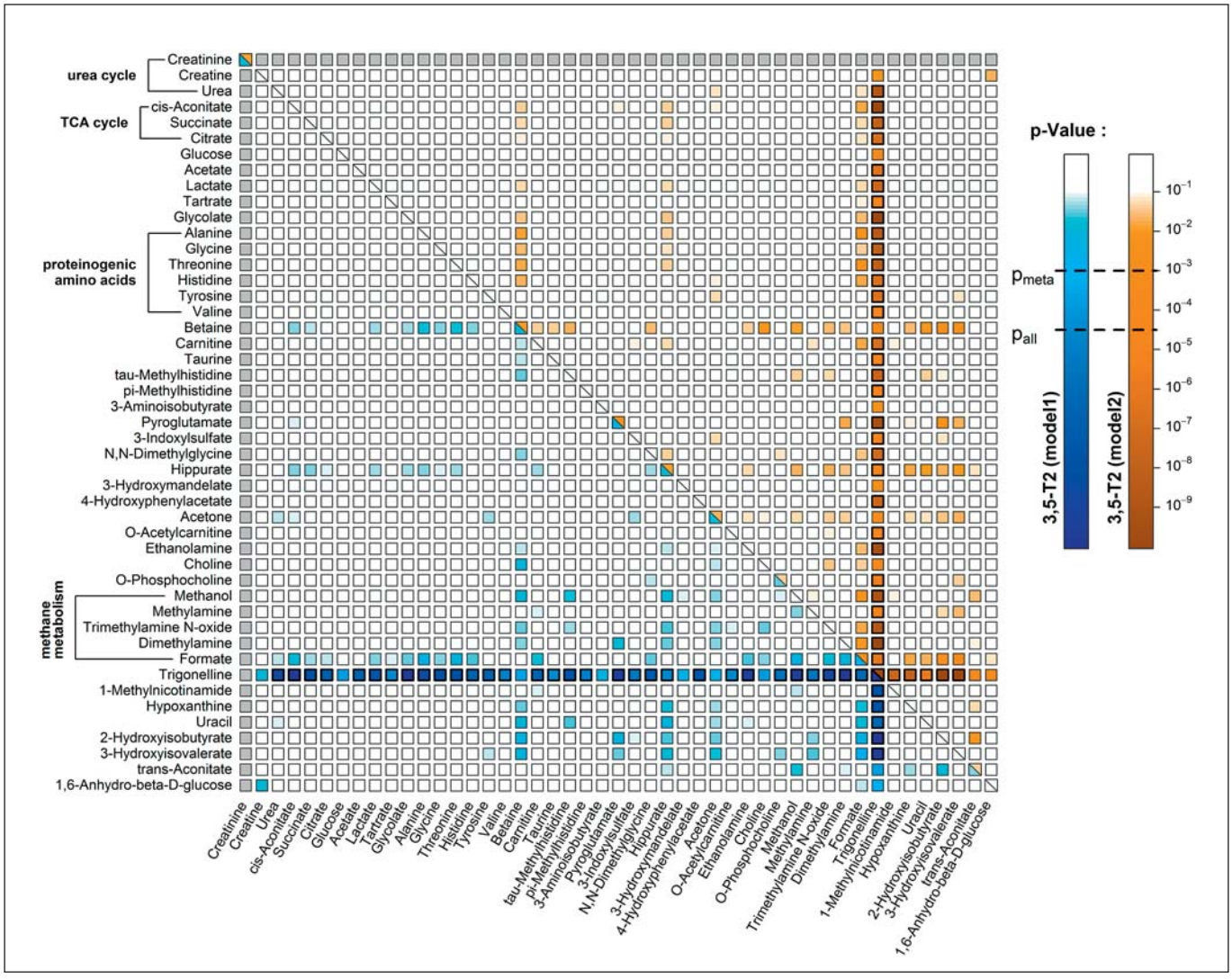

Fig. 3. Results for 3,5-T_2 colored according to p values form multivariate linear regression models adjusted for age, sex and WC (model 1; blue) and further for serum TSH levels (model 2; orange). Each line and column represents one metabolite; the diagonal contains results for single metabolites, all other boxes represent the respective ratio; light blue frames indicate significant metabolites: $p_{meta} = 0.05/47 = 1.1 \times 10^{-3}$; 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 ^{1}H-NMR spectroscopy for associations towards serum 3,5-T_2 concentrations. As discussed below, our results regarding trigonelline, pyroglutamate or acetone support and extend previously observed associations between 3,5-T_2 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_2 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_2 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_2, revealing protective effects on impaired kidney function and structure, which represents a severe sequelae of type 2 diabetes mellitus. Taken together, 3,5-T_2 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_2 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_3 production by administration of fenugreek seed extracts in rodents are of interest. These trigonelline-rich extracts inhibit T_3 production from T_4 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_2 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 γ-glutamyl cycle, which at least in astrocytes [39] was assumed to be stimulated by TH. The γ-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_2 and urine pyroglutamate levels might point towards a link between 3,5-T_2 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 β-oxidation of FFAs [41]. The positive association between 3,5-T_2 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_2. 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_2 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_1AM) treatment results in elevated serum and urine ketone bodies [21, 42]. This observation might support the hypothesis that 3,5-T_2 might be (one of) the precursor(s) of biosynthesis of 3-T_1AM [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_4 and T_3 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_2 concentrations in hypothyroidism, a decreased ratio of the putative precursor T_4 (T_3) and 3,5-T_2 could account for this observation. However, the relation between serum fT_4 as well as TSH and 3,5-T_2 concentrations in humans is not yet completely understood. Moreover, in the present study the inclusion of subjects with abnormal serum 3,5-T_2 or TSH concentrations led to weaker associations, suggesting differing roles of serum 3,5-T_2 on intermediary metabolism in different thyroid states. It is worth noting that, besides hippurate, 3,5-T_2 exhibited a unique associated urinary metabolic profile in comparison with TSH and fT_4 [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_2 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 ^{1}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_2 concentrations. Especially in epidemiological research, urine metabolomics by means of ^{1}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_2. 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 reported for rodent models of experimental hypothyroidism, whereas the majority of associations reported here constitute a new and unique association to 3,5-T_2. 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_2. 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_2 in human metabolism.

Acknowledgements

This work was funded by grants from the German Federal Ministry of Education and Research (BMBF, grants 01ZZ0403, 01ZZ0103, 01GI0883), the Ministry for Education, Research and Cultural Affairs, as well as the Ministry of Social Affairs of the Federal State of Mecklenburg-West Pomerania. This work is also part of the research project Greifswald Approach to Individualized Medicine (GANI_MED). The GANI_MED consortium is funded by the Federal Ministry of Education and Research and the Ministry of Cultural Affairs of the Federal State of Mecklenburg-West Pomerania (03IS2061A). The project was conducted within the framework of the DFG SPP 1692 'Thyroid Trans Act' (DFG WA 1328/5-1, KO 922/17-1 and VO 955/12-1) and the DFG GRK 1208-2 (TP 3 to J.K.).

Disclosure Statement

The authors declare no conflicts of interest.

References

1 Brent GA: Mechanisms of thyroid hormone action. J Clin Invest 2012;122:3035–3043.

2 Brix K, Führer D, Biebermann H: Molecules important for thyroid hormone synthesis and action – known facts and future perspectives. Thyroid Res 2011;4:S9.

3 Cheng S-Y, Leonard JL, Davis PJ: Molecular aspects of thyroid hormone actions. Endocr Rev 2010;31:139–170.

4 Moreno M, de Lange P, Lombardi A, Silvestri E, Lanni A, Goglia F: Metabolic effects of thyroid hormone derivatives. Thyroid 2008;18:239–253.

5 de Lange P, Cioffi F, Senese R, Moreno M, Lombardi A, Silvestri E, De Matteis R, Lionetti L, Mollica MP, Goglia F, Lanni A: Nonthyrotoxic prevention of diet-induced insulin resistance by 3,5-diiodo-L-thyronine in rats. Diabetes 2011;60:2730–2739.

6 Mollica MP, Lionetti L, Moreno M, Lombardi A, De Lange P, Antonelli A, Lanni A, Cavaliere G, Barletta A, Goglia F: 3,5-diiodo-L-thyronine, by modulating mitochondrial functions, reverses hepatic fat accumulation in rats fed a high-fat diet. J Hepatol 2009;51:363–370.

7 Moreno M, Silvestri E, De Matteis R, de Lange P, Lombardi A, Glinni D, Senese R, Cioffi F, Salzano AM, Scaloni A, Lanni A, Goglia F: 3,5-diiodo-L-thyronine prevents high-fat-diet-induced insulin resistance in rat skeletal muscle through metabolic and structural adaptations. FASEB J 2011;25:3312–3324.

8 Lombardi A, de Lange P, Silvestri E, Busiello RA, Lanni A, Goglia F, Moreno M: 3,5-diiodo-L-thyronine rapidly enhances mitochondrial fatty acid oxidation rate and thermogenesis in rat skeletal muscle: AMP-activated protein kinase involvement. Am J Physiol Endocrinol Metab 2009;296:E497–E502.

9 Faber J, Heaf J, Kirkegaard C, Lumholtz I, Siersbaek-Nielsen K, Kollendorf K, Friis T: Simultaneous turnover studies of thyroxine, 3,5,3′- and 3,3′,5′-triiodothyronine, 3,5-, 3,3′-, and 3′,5′-diiodothyronine, and 3′-monoiodothyronine in chronic renal failure. J Clin Endocrinol Metab 1983;56:211–217.

10 Faber J, Kirkegaard C, Thomson H, Lumholtz I, Siersbaek-Nielsen K, Friis T: The extrathyroidal conversion of 3,5,3′-triiodothyronine to 3,5-diiodothyronine in patients with liver cirrhosis. J Clin Endocrinol Metab 1983;57:428–431.

11 Pinna G, Meinhold H, Hiedra L, Thoma R, Hoell T, Graf KJ, Stoltenburg-Didinger G, Eravci M, Prengel H, Brodel O, Finke R, Baumgartner A: Elevated 3,5-diiodothyronine concentrations in the sera of patients with nonthyroidal illnesses and brain tumors. J Clin Endocrinol Metab 1997;82:1535–1542.

12 Lehmphul I, Brabant G, Wallaschofski H, Ruchala M, Strasburger CJ, Kohrle J, Wu Z: Detection of 3,5-diiodothyronine in sera of patients with altered thyroid status using a new monoclonal antibody-based chemiluminescence immunoassay. Thyroid 2014;24:1350–1360.

13 Pietzner M, Lehmphul I, Friedrich N, Schurmann C, Ittermann T, Dorr M, Nauck M, Laqua R, Volker U, Brabant G, Volzke H, Koehrle J, Homuth G, Wallaschofski H: Translating pharmacological findings from hypothyroid rodents to euthyroid humans: is there a functional role of endogenous 3,5-T_2? Thyroid 2015;25:188–197.

14 Bictash M, Ebbels TM, Chan Q, Loo RL, Yap IKS, Brown IJ, de Iorio M, Daviglus ML, Holmes E, Stamler J, Nicholson JK, Elliott P: Opening up the 'black box': metabolic phenotyping and metabolome-wide association studies in epidemiology. J Clin Epidemiol 2010;63:970–979.

15 Lindon JC, Nicholson JK, Holmes E, Everett JR: Metabonomics: metabolic processes studied by NMR spectroscopy of biofluids. Concepts Magn Reson 2000;12:289–320.

16 Bouatra S, Aziat F, Mandal R, Guo AC, Wilson MR, Knox C, Bjorndahl TC, Krishnamurthy R, Saleem F, Liu P, Dame ZT, Poelzer J, Huynh J, Yallou FS, Psychogios N, Dong E, Bogumil R, Roehring C, Wishart DS: The human urine metabolome. PLoS One 2013;8:e73076.

17 Montoya GA, Strauss V, Fabian E, Kamp H, Mellert W, Walk T, Looser R, Herold M, Krennrich G, Peter E, van Ravenzwaay B: Mechanistic analysis of metabolomics patterns in rat plasma during administration of direct thyroid hormone synthesis inhibitors or compounds increasing thyroid hormone clearance. Toxicol Lett 2014;225:240–251.

18 Wu S, Gao Y, Dong X, Tan G, Li W, Lou Z, Chai Y: Serum metabonomics coupled with ingenuity pathway analysis characterizes metabolic perturbations in response to hypothyroidism induced by propylthiouracil in rats. J Pharm Biomed Anal 2013;72:109–114.

19 Wu S, Tan G, Dong X, Zhu Z, Li W, Lou Z, Chai Y: Metabolic profiling provides a system understanding of hypothyroidism in rats and its application. PLoS One 2013;8:e55599.

20 Geillinger KE, Rathmann D, Kohrle J, Fiamoncini J, Daniel H, Kipp AP: Hepatic metabolite profiles in mice with a suboptimal selenium status. J Nutr Biochem 2014;25:914–922.

21 Haviland JA, Reiland H, Butz DE, Tonelli M, Porter WP, Zucchi R, Scanlan TS, Chiellini G, Assadi-Porter FM: NMR-based metabolomics and breath studies show lipid and protein catabolism during low dose chronic T_1AM treatment. Obesity 2013;21:2538–2544.

22 Jourdan C, Linseisen J, Meisinger C, Petersen A-K, Gieger C, Rawal R, Illig T, Heier M, Peters A, Wallaschofski H, Nauck M, Kastenmüller G, Suhre K, Prehn C, Adamski J, Koenig W, Roden M, Wichmann HE, Völzke H: Associations between thyroid hormones and serum metabolite profiles in an euthyroid population. Metabolomics 2014;10:152–164.

23 Völzke H, Alte D, Schmidt CO, et al: Cohort profile: the study of health in Pomerania. Int J Epidemiol 2011;40:294–307.

24 Slupsky CM, Rankin KN, Wagner J, Fu H, Chang D, Weljie AM, Saude EJ, Lix B, Adamko DJ, Shah S, Greiner R, Sykes BD, Marrie TJ: Investigations of the effects of gender, diurnal variation, and age in human urinary metabolomic profiles. Anal Chem 2007;79:6995–7004.

25 Cavallo A, Priore P, Gnoni GV, Papa S, Zanotti F, Gnoni A: 3,5-Diiodo-L-thyronine administration to hypothyroid rats rapidly enhances fatty acid oxidation rate and bioenergetic parameters in liver cells. PLoS One 2013;8:e52328.

26 Cimmino M, Mion F, Goglia F, Minaire Y, Geloen A: Demonstration of in vivo metabolic effects of 3,5-diiodothyronine. J Endocrinol 1996;149:319–325.

27 Shang G, Gao P, Zhao Z, Chen Q, Jiang T, Zhang N, Li H: 3,5-Diiodo-L-thyronine ameliorates diabetic nephropathy in streptozotocin-induced diabetic rats. Biochim Biophys Acta 2013;1832:674–684.

28 Jonas W, Lietzow J, Wohlgemuth F, Hoefig CS, Wiedmer P, Schweizer U, Kohrle J, Schurmann A: 3,5-Diiodo-L-thyronine (3,5-T_2) exerts thyromimetic effects on hypothalamus-pituitary-thyroid axis, body composition, and energy metabolism in male diet-induced obese mice. Endocrinology 2015;156:389–399.

29 Silvestri E, Cioffi F, Glinni D, Ceccarelli M, Lombardi A, de Lange P, Chambery A, Severino V, Lanni A, Goglia F, Moreno M: Pathways affected by 3,5-diiodo-L-thyronine in liver of high fat-fed rats: evidence from two-dimensional electrophoresis, blue-native page, and mass spectrometry. Mol Biosyst 2010;6:2256–2271.

30 Ludwig IA, Clifford MN, Lean ME, Ashihara H, Crozier A: Coffee: biochemistry and potential impact on health. Food Funct 2014;5:1695–1717.

31 Yuyama S, Suzuki T: The excretion of N1-methyl-2-pyridone-5-carboxylic acid and related compounds in human subjects after oral administration of nicotinic acid, trigonelline and N1-methyl-2-pyridone-5-carboxylic acid. Adv Exp Med Biol 1991;294:475–479.

32 Hamden K, Bengara A, Amri Z, Elfeki A: Experimental diabetes treated with trigonelline: effect on key enzymes related to diabetes and hypertension, β-cell and liver function. Mol Cell Biochem 2013;381:85–94.

33 van Dijk AE, Olthof MR, Meeuse JC, Seebus E, Heine RJ, van Dam RM: Acute effects of decaffeinated coffee and the major coffee components chlorogenic acid and trigonelline on glucose tolerance. Diabetes Care 2009;32:1023–1025.

34 Yoshinari O, Sato H, Igarashi K: Anti-diabetic effects of pumpkin and its components, trigonelline and nicotinic acid, on Goto-Kakizaki rats. Biosci Biotechnol Biochem 2009;73:1033–1041.

35 Ghule AE, Jadhav SS, Bodhankar SL: Trigonelline ameliorates diabetic hypertensive nephropathy by suppression of oxidative stress in kidney and reduction in renal cell apoptosis and fibrosis in streptozotocin induced neonatal diabetic (nSTZ) rats. Int Immunopharmacol 2012;14:740–748.

36 Hamden K, Mnafgui K, Amri Z, Aloulou A, Elfeki A: Inhibition of key digestive enzymes related to diabetes and hyperlipidemia and protection of liver-kidney functions by trigonelline in diabetic rats. Sci Pharm 2013;81:233–246.

37 Panda S, Tahiliani P, Kar A: Inhibition of triiodothyronine production by fenugreek seed extract in mice and rats. Pharmacol Res 1999;40:405–409.

38 Lombardi A, Beneduce L, Moreno M, Diano S, Colantuoni V, Ursini MV, Lanni A, Goglia F: 3,5-Diiodo-L-thyronine regulates glucose-6-phosphate dehydrogenase activity in the rat. Endocrinology 2000;141:1729–1734.

39 Dasgupta A, Das S, Sarkar PK: Thyroid hormone promotes glutathione synthesis in astrocytes by up regulation of glutamate cysteine ligase through differential stimulation of its catalytic and modulator subunit mrnas. Free Radic Biol Med 2007;42:617–626.

40 Shi ZZ, Habib GM, Rhead WJ, Gahl WA, He X, Sazer S, Lieberman MW: Mutations in the glutathione synthetase gene cause 5-oxoprolinuria. Nat Genet 1996;14:361–365.

41 Comstock JP, Garber AJ: Ketonuria; in Walker HK, Hall WD, Hurst JW (eds): Clinical Methods: The History, Physical, and Laboratory Examinations. Boston, Butterworth, 1990.

42 Braulke LJ, Klingenspor M, DeBarber A, Tobias SC, Grandy DK, Scanlan TS, Heldmaier G: 3-Iodothyronamine: a novel hormone controlling the balance between glucose and lipid utilisation. J Comp Physiol B 2008;178:167–177.

43 Hoefig CS, Renko K, Piehl S, Scanlan TS, Bertoldi M, Opladen T, Hoffmann GF, Klein J, Blankenstein O, Schweizer U, Kohrle J: Does the aromatic L-amino acid decarboxylase contribute to thyronamine biosynthesis? Mol Cell Endocrinol 2012;349:195–201.

44 Piehl S, Hoefig C, Scanlan T, Köhrle J: Thyronamines – past, present, and future. Endocr Rev 2011;32:64–80.