Thyroid
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Aging is associated with low thyroid state and organ specific sensitivity to thyroxine

Helena Rakov[1], Meri De Angelis[2], Kostja Renko[3], Georg Sebastian Hönes[1], Denise Zwanziger[1,4], Lars Christian Moeller[1], Karl-Werner Schramm[2,5], Dagmar Führer [1†]

[1]*Department of Endocrinology, Diabetes and Metabolism, University Hospital Essen, University Duisburg-Essen, Hufelandstr. 55, 45122 Essen, Germany*

[2]*Helmholtz Zentrum München - German Research Center for Environmental Health (GmbH), Molecular EXposomics, Ingolstädter Landstr. 1, 85764 Neuherberg, Germany*

[3]*Charité-Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Institut für Experimentelle Endokrinologie, Südring 10, 10117 Berlin, Germany*

[4]*Department of Endocrinology, Diabetology and Metabolism, Clinical Chemistry – Division of Laboratory Research, University Hospital Essen, University Duisburg- Essen, Hufelandstr. 55, 45122 Essen, Germany*

[5]*Technische Universität München, Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt, Department für Biowissenschaften, Weihenstephaner Steig 23, 85350 Freising, Germany*

[†]corresponding author

Full name and address of authors:

Helena Rakov, PhD

Department of Endocrinology, Metabolism and Diabetes

University Hospital Essen, University Duisburg-Essen

Hufelandstr. 55, 45122 Essen, Germany

Email: helena.rakov@uk-essen.de

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Meri De Angelis, PhD

Helmholtz Zentrum München

German Research Center for Environmental Health (GmbH), Molecular EXposomics,

Ingolstädter Landstr. 1, 85764 Neuherberg, Germany

Email: meri.deangelis@helmholtz-muenchen.de

Kostja Renko, PhD

Institut für Experimentelle Endokrinologie

Charité-Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin,

Humboldt-Universität zu Berlin, and Berlin Institute of Health

Südring 10, 10117 Berlin, Germany

Email: kostja.renko@charite.de

Georg Sebastian Hönes, PhD

Department of Endocrinology, Metabolism and Diabetes

University Hospital Essen, University Duisburg-Essen

Hufelandstr. 55, 45122 Essen, Germany

Email: sebastian.hoenes@uk-essen.de

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Denise Zwanziger, PhD

Department of Endocrinology, Metabolism and Diabetes, Division of Laboratory Research

University Hospital Essen, University Duisburg-Essen

Hufelandstr. 55, 45122 Essen, Germany

Email: denise.zwanziger@uk-essen.de

Lars Christian Moeller, MD

Department of Endocrinology, Metabolism and Diabetes

University Hospital Essen, University Duisburg-Essen

Hufelandstr. 55, 45122 Essen, Germany

Email: lars.moeller@uni-due.de

Karl-Werner Schramm, PhD, PhD

Helmholtz Zentrum München

German Research Center for Environmental Health (GmbH), Molecular EXposomics, Ingolstädter Landstr. 1, 85764 Neuherberg, Germany;

Department für Biowissenschaftliche Grundlagen, Technische Universität München, Weihenstephaner Steig 23, 85350 Freising, Germany.

Email: schramm@helmholtz-muenchen.de

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Dagmar Führer, MD PhD

Department of Endocrinology, Diabetes and Metabolism, Division of Laboratory Research, University Hospital Essen, University Duisburg-Essen

Hufelandstr.55, 45122 Essen, Germany

Email: Dagmar.Fuehrer@uk-essen.de

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Abstract:

Background: Serum thyroid state in older adults correlates with extended longevity. We hypothesized that age impacts systemic, but also organ-specific thyroid state and response to thyroxine (T_4).

Methods: Young (3 months) and old (23 months) male mice were analyzed at baseline and after acute T_4 challenge. Age effects on circulating thyroid stimulating hormone (TSH) and thyroid hormone (TH) concentrations, transcript expression in the pituitary and thyroid were compared to organ-specific responses characterized by hepatic and cardiac content of TH and TH metabolites and expression of TH-target genes, as well as hepatic deiodinase 1 activity.

Results: Circulating TH concentrations and hepatic and cardiac TH content were lower in old *vs.* young mice. After injection with T_4, conversion of T_4 to triiodothyronine was decreased in old mice while TH transport in liver and heart was not affected. Organ-specific TH response was augmented in old mice in liver but not heart, indicating age and tissue-specific sensitivity to TH. A compensatory increase of *thyroid stimulating hormone subunit beta* expression in the pituitary and increased serum TSH concentrations, but reduced expression of thyroid differentiation markers were found in old mice.

Conclusions: We suggest that a reduced activity of the aged thyroid is responsible for the systemic low TH state in old mice. Furthermore, divergent TH metabolism and tissue response in liver and heart occur after T_4 treatment in an aged organism. These rodent data are in agreement with a much narrower window for T_4 substitution in the older adults to avoid overtreatment.

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Main body

Introduction

Aging modulates thyroid hormone (TH) action and metabolism. In aged patients the prevalence of thyroid dysfunction increases, but is associated with an attenuated and oligosymptomatic clinical presentation of hyper- and hypothyroidism (1, 2). Thyroid function, defined by serum thyroid stimulating hormone (TSH), thyroxine (T_4) and triiodothyronine (T_3), has been found to change with aging towards higher TSH and lower free T_3 (fT_3) and free T_4 (fT_4) concentrations in humans (3-5). Interestingly, decreased thyroid function in older adults is suggested to contribute to an increased lifespan (6, 7). Thus, it remains under debate whether subclinical hypothyroidism, defined by TSH concentrations above a laboratory reference range, in older adults is a pathological state or whether its treatment is beneficial. Importantly, in a recent study levothyroxine treatment over 1 year did not improve symptoms in patients with biochemical subclinical hypothyroidism above 65 years of age (8).

The underlying cause of the age-dependent changes in systemic thyroid function may be caused by changes in the hypothalamic-pituitary-thyroid-axis (HPT-axis) (7) but the precise molecular mechanisms are not known. Furthermore it is still unknown whether systemic changes are also reflected in organ-specific adaptation of TH action with age.

In this study we aimed to characterize the age-dependent TH state in a systemic and organ-specific manner. Systemic effects represented by circulating TSH, TH concentrations, transcript expression in the pituitary and thyroid were compared to organ-specific responses characterized by hepatic and cardiac content of TH and TH metabolites, the expression of TH-target genes, as well as hepatic deiodinase 1 activity. Furthermore, by challenging mice with a single T_4 injection, the age-dependent response was assessed in young (3 months) and old (23 months) male mice.

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Material and Methods

Animals

Male C57Bl/6J mice were purchased from Janvier Labs, St. Berthevin, France. Two age cohorts were investigated: twelve 3 months-old (young cohort) and twenty-two 23 months-old mice (aged cohort). Animals were single-housed in temperature- (23 ±1°C) and light-controlled (inverse 12:12 hour light-dark cycle) conditions. Food and water were provided *ad libitum*. All animal experiments were performed in accordance with the German regulations for Laboratory Animal Science (GVSOLAS) and the European Health Law of the Federation of Laboratory Animal Science Associations (FELASA). The protocols for animal studies were approved by the Landesamt für Natur, Umwelt und Verbraucherschutz, Nordrhein-Westfalen (LANUV-NRW), Germany.

Treatment and organ collection

Mice were randomly divided in control (n=6 for 3 months, n=8 for 23 months) or T_4 treatment (n=6 for 3 months, n=11 for 23 months) groups, twelve hours prior to sacrifice. Treated mice were injected intraperitoneally (i.p.) once with 1 µg/g body weight L-thyroxine (T_4; Sigma-Aldrich (T2376), St. Louis, USA; stock-solution: 2 mg/mL T_4 dissolved in 0.01 M NaOH, 0.1% BSA (albumin from bovine serum, Sigma-Aldrich (A7906), St. Louis, USA); injection solution: stock-solution diluted 1:10 with phosphate-buffered saline (PBS, Thermo Fisher Scientific (18912014), Waltham, USA). Control mice received an i.p. injection of 150 µl PBS. Twelve hours after treatment, mice were deeply anaesthetized by an i.p. injection of 200 µl Ketamine/Xylazine mixture (150 µl of 100 mg/ml Ketamine (Bela-pharm, Vechta, Germany) and 50 µl of 20 mg/ml Xylazine (Ceva, Düsseldorf, Germany) and final blood was obtained by heart puncture. For tissue collection, mice were perfused with heparinized saline through a needle placed in the left heart ventricle. Tissues were shock-frozen in liquid nitrogen, and stored at −80°C until further processing.

Serum thyroid hormone and TSH measurements

FT_4 and fT_3 concentrations in serum of mice were measured using commercial ELISA kits according to the manufacturer's instructions (DRG Instruments GmbH, Marburg, Germany; fT_3: EIA-2385; fT_4: EIA-2386). Serum samples with known TH concentrations were used as

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standards. According to the manufacturer's instructions, the minimum detectable TH concentration is 0.05 ng/dL for fT_4 and 0.05 pg/mL for fT_3, and the inter-assay variation was <9% for fT_4 and <11% for fT_3. TSH serum concentration was determined using the Milliplex Map mouse pituitary magnetic bead panel (EMD Millipore Corporation, Billerica, USA; MPTMAG-49K) on the Luminex 200 system (Thermo Fisher, Waltham, USA). The minimal detectable concentration is 1.9 pg/mL and the inter-assay variation <20% in this assay.

Thyroid hormone measurements in liver and heart

TH content in liver and heart tissue of mice was analyzed as previously described (9, 10) and all solvents were purchased from Promochem (Wesel, Germany). Between 10 to 50 mg of tissue were homogenized in 300 µL of pure methanol (MeOH) and sonicated using an ultrasound probe. After homogenization, 60 µL of internal standard and 600 µL of chloroform ($CHCl_3$) were added. The mixture was centrifuged at 3000 rpm for 10 min and the supernatant was then collected. This extraction procedure was repeated two more times. The $CHCl_3$-MeOH extracts were transferred into a 12 mL tube for back-extraction of the iodothyronine with 500 µL of 0.05% calcium chloride ($CaCl_2$) in water at 4 °C. The extraction procedure was repeated two more times adding 500 µL of MeOH and 500 µL of 0.05% $CaCl_2$ in water each time. The pooled aqueous phase was concentrated under nitrogen stream at 40 °C (to eliminate the excess of MeOH). Phosphoric acid was added into the combined extracts to reach a final concentration of 2%, followed by the addition of an antioxidant solution (0.2 mL; 10 mg ascorbic acid + 10 mg citricacid + 10 mg dithiothreitol in 1.5 mL MeOH). After vortexing, the mixture was loaded onto a Bond Elut Plexa PCX cartridge, which was preconditioned sequentially with, 1.5 mL of pure MeOH and 1.5 mL of water. The cartridge was first washed with 2 mL of 2% formic acid in water and then with 2 mL of MeOH: acetonitrile (1:1, v/v). Analytes were eluted into a vial with 1 mL of 5% ammonium hydroxide (28 – 30%) in MeOH: acetonitrile (1:1, v/v). The solvent was evaporated and compounds re-dissolved in 60 µL of a mixture of 20% acetonitrile in water containing 0.1% formic acid for instrumental analysis. The quantification limits were 2.5 pg injected into column for 3,5-diiodothyronine (T_2), reverse T_2 (rT_2) and T_3; 3.8 pg injected into column for reverse T_3 (rT_3) and T_4; 5 pg injected into column for

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monoiodothyronine (T_1). The inter-assay variation was determined by measuring mouse liver samples. For this experiment, three different mouse livers were chosen: (i) mouse livers in which the concentration of T_3 and T_4 was in the lower range of the experimental window, (ii) mouse livers in which the concentration of T_3 and T_4 was in the medium range of the experimental window and finally, (iii) mouse livers in which the concentration of T_3 and T_4 was in the higher range of the experimental window. Per each mouse liver, four different batches were prepared and measured at four different days within two weeks. The inter-assay variation was <15% for T_3 (low, medium and high concentration) and <20% for T_4 (low, medium, high concentration).

Quantitative real-time PCR

Total RNA from pituitaries, thyroid gland, liver and heart was isolated using the RNeasy Kit according to the manufacturer's instruction (Qiagen, Hilden, Germany) and stored at −80°C as previously described (11). RNA was reverse transcribed into cDNA with SuperScriptIII (Life Technologies, Darmstadt, Germany) and hexamer primers. Quantitative real-time PCR (qRT-PCR) was performed using Roche SYBR Green I master mix (Roche, Mannheim, Germany). Primers were designed to be intron-spanning to exclude genomic DNA signals (sequences are provided in supplementary table 1). In compliance with the MIQE guidelines for RT-PCR (12), we used a set of 2-3 reference genes per tissue to assure accurate normalization and calculation (pituitary: *18S* (18S ribosomal RNA), *Ppia* (peptidylprolyl isomerase A, cyclophilin A); thyroid gland: *18S*, *Hprt* (hypoxanthine guanine phosphoribosyl transferase); liver: *18S*, *Ppia*, *Rpl13a* (ribosomal protein L13a); heart: *18S*, *Polr2a* (polymerase RNA II), *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase)). Analysis and calculation of the fold-change in gene expression were done on Ct-values ≤ 35 using the efficiency-corrected ΔΔCt method (13).

Genes known to be involved in TH regulation (pituitary: *thyroid stimulating hormone, beta subunit* (*Tshß*), *thyrotropin releasing hormone receptor* (*Trhr*), *nuclear receptor co-repressor 2* (*Ncor2*)), TH synthesis (thyroid gland: *thyroid stimulating hormone receptor* (*Tshr*), *thyroid peroxidase* (*Tpo*), *thyroglobulin* (*Tg*), *sodium iodide symporter* (*Nis*)), TH metabolism (*deiodinases type 1, 2 and 3* (*Dio1, Dio2, Dio3*)) and organ-specific response (liver: *thyroxine binding globulin* (*Tbg*), *thyroid hormone responsive* (*Spot14*), *malic enzyme*

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(*Me1*), *B cell leukemia/lymphoma 3* (*Bcl3*); heart: *myosin heavy chain 6 and 7* (*Myh6, Myh7*), *hyperpolarization-activated, cyclic nucleotide-gated potassium channel 2 and 4* (*Hcn2, Hcn4*)) were assessed.

Hepatic deiodinase 1 activity

Liver protein samples (40 µg of total proteins; n = 6 per group) were prepared by mincing and sonification, adjusted to a defined protein concentration (5 µg/µl). Dio1 activity was assayed as previously described (14). In brief, a 50 µl reaction mixture containing 40 µg of liver microsomal proteins, 10 mM 6-n-propyl-2-thio-uracil (PTU) for controls, was mixed with 50 µl of freshly prepared substrate mix (20 µM rT_3 (Sigma-Aldrich, MO, USA), 0.2 M KPO_4 (pH 6.8), 2 mM ethylenediaminetetraacetic acid, and 80 mM dithiothreitol). The enzyme reaction lasted for 2 h at 37 °C. After centrifugation (4 °C, 15.000 ×g, 5 min), the supernatant was used for quantification of released iodide. Dowex W50-X2 resin columns were used for separation of intact rT3 and the deiodinated breakdown products from the released iodide. The iodide content was determined by the Sandell-Kolthoff reaction, using cerium solution (25 mM $(NH_4)_4Ce(SO_4)_4$ and 0.5 M H_2SO_4) and arsenite solution (25 mM $NaAsO_2$, 0.8 M NaCl, and 0.5 M H_2SO_4). The changes in absorption (OD at 415 nm) were determined at the reaction starting point and after 20 min. All protein samples were assayed in triplicate. The tubes that contained PTU were used for background subtraction. Calculation of the enzyme activity was performed by utilizing an iodide standard curve.

Histological analysis

Thyroid glands were fixed in 4% formaldehyde, embedded in paraffin and 5-µm-thick sections were used for staining. For morphological evaluation, hematoxylin and eosin (H&E) staining was performed. Staining procedures followed standard protocols. All samples were viewed on the Olympus BX51 upright microscope (Olympus, Germany) with a magnification of 10x and 20x. Quantification of follicle size was done using ImageJ (NIH) by determination of 78-245 follicles per animal (n=4 mice per group).

Statistical analysis

All data are shown as mean ± standard deviation (SD) or standard error of the mean (SEM) as indicated. Statistical analysis was performed using GraphPad Prism 6 Software. One-way

ANOVA followed Tuckey´s *post hoc* analysis or unpaired Student´s t-test were applied as indicated. Values of *p<0.05, **p<0.01, ***p<0.001, #p<0.0001 were considered statistically significant.

Results

Aging is associated with lower T_4 concentrations in serum and lower T_4 content in liver and heart of male mice

Compared to 3 months old animals, 23 months old mice displayed slightly higher serum TSH (p=0.0568, Fig. 1A) and significantly lower concentrations of serum fT_4 (young: 2.2 ng/dL and old: 0.7 ng/dL, p<0.001, Fig. 1B) and T_4 content in the liver (young: 38.5 pg/mg and old: 19.3 pg/mg, p<0.01, Fig. 1C) and heart (young: 7.2 pg/mg and old: 3.9 pg/mg, p<0.01, Fig. 1D). Furthermore, a significantly lower hepatic T_3 (young: 4.5 pg/mg and old: 2.2 pg/mg, p<0.01, Fig. 1F) content was found in old mice. Concentrations of other TH metabolites (rT_3, T_2, rT_2 and T_1) were below the detection limit in the liver and heart in all animals.

Expression of TH-target genes correlates with tissue TH concentration

To assess how TH serum and tissue concentrations in young and aged mice are associated with altered TH tissue action, qRT-PCR analysis was performed in liver and heart. An age-dependent downregulation of the positively regulated TH target gene *Dio1* (p<0.001), and an increased expression for the negatively-regulated gene *Tbg* (p<0.05, Fig. 2A) was found in livers of old *vs.* young mice. Similarly, decreased TH action in the heart was suggested by downregulation of *Hcn2* (p<0.001) and upregulation of *Myh7* (p<0.001, Fig. 2B) in old mice *vs*. young mice.

The function of HPT-axis is preserved while the thyroid gland is hypoactive in old mice

Next, we addressed the functional state of the HPT-axis by measuring gene expression in pituitaries and thyroids of young and old mice. An increased expression of *Tshb* (p<0.01) was found in pituitaries of old compared to young mice (Fig. 2C) supporting the biochemical finding in serum of a systemic low TH state with a compensatory active response at the pituitary level. Transcript analysis of genes relevant for TH synthesis

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indicated a hypoactive thyroid organ with decreased *Tg* gene expression of ($p<0.001$, Fig. 2D), and a trend towards lower *Nis* (ns) and *Tpo* (ns) expression in old *vs*. young mice.

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Age alters thyroid follicle shape and function

To verify the hypothesis of a hypofunctioning thyroid gland, H&E stained formalin-fixed thyroid gland sections of 3 months and 23 months old mice were assessed (Fig. 3 A). Quantification of the follicle size displayed an age-dependent increase in size (Fig. 3 B). Furthermore, heterogeneity in follicle size with mostly flattened epithelium was noted in thyroid glands of old mice.

Conversion of T_4 into T_3 is less efficient in aged mice

Whether age has an impact on TH metabolism, and to investigate how it may affect systemic and organ specific TH response, we exposed young and aged mice to a T_4 challenge. A single injection of 1 µg T_4/g body weight (Table 1) resulted in distinct changes in serum TH concentrations and tissue content after 12 hours in young and old mice. Suppression of TSH was comparable in young and old mice (Fig. 4A), and an age-independent increase was observed for serum fT_4 concentrations and hepatic T_4 content, whereas cardiac T_4 content was higher in old mice (young: 139 pg/mg and old: 207 pg/mg, $p<0.05$; Fig. 4B-D). Interestingly, and irrespective of the T_4 increase, the conversion of T_4 into T_3 was less efficient in old mice (Fig. 4E-G). This resulted in smaller incremental increases in T_3 serum concentrations and tissue content, further illustrated by higher T_4/T_3 ratios in serum, liver and heart of old compared to young mice (Suppl. Table 2). Also, after the T_4 challenge TH metabolites (rT_3, T_2, rT_2 and T_1) were below the respective detection limit in livers and hearts of all mice.

TH responsive gene expression is age- and organ-dependent after single T_4 injection

Changes in TH responsive gene expression in organs at 12 hours after T_4 injection showed an age- and organ-specific response in mice. This was observed for gene expression in the pituitary (*Dio2*, *Trhr*, and *Tshb;* Fig. 5A) and the thyroid gland (*Tg and Nis;* Fig. 5B). Surprisingly, and in contrast to the findings in the heart, TH target gene expression in the liver differed in old *vs*. young animals after acute T_4 challenge. Whereas *Tbg*, *Spot14* and

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Bcl3 expression was not altered, liver *Dio1* was significantly upregulated at 12 hours after T_4 injection in old compared to young mice ($p<0.05$; Fig. 5C). In contrast, only marginal changes in expression were noted for TH-dependent genes in the heart after T_4 injection, and only *Hcn2* expression increased in mouse heart, irrespective of age (Fig. 5D).

Hepatic deiodinase type 1 activity after T_4 injection reflects hepatic TH content

Since induction of *Dio1* expression upon T_4 treatment was not in line with the higher increment in hepatic T_3 content in young *vs*. old mice, Dio1 activity was analyzed. No age differences were found in Dio1 activity under basal conditions, however, after T4 injection Dio1 activity increased significantly in young (Fig. 6A, $p<0.01$), but not old mice, and confirmed the differences measured for hepatic T_3 content in the animals.

Discussion

In clinical practice, age-dependent changes of thyroid function are of high relevance, frequently leading to misinterpretation of thyroid function parameters and unindicated treatment. An association between age and a decline in serum fT_4 and fT_3 concentrations, accompanied by an increase of TSH in areas with iodine sufficiency has previously been reported in humans (3, 4, 15), and may be favorably associated with longevity (6). The underlying mechanisms responsible for age-dependent changes in thyroid state and their consequence for organ-specific TH supply and action are still not fully understood. Using a rodent model and the approach of a single T_4 challenge, we report for the first time an impact of age on systemic and organ-specific TH metabolism and tissue response.

Aging *per se* is associated with mild systemic hypothyroidism caused by the thyroid gland

Old mice had lower fT_4 serum concentrations than young mice, accompanied by low hepatic and cardiac T_4 content in these animals. Increased expression of *Tshb* in the pituitaries and slightly higher TSH serum concentrations in old mice indicate an adequate function of the negative feedback loop of the HPT-axis. However, expression of genes encoding for proteins involved in TH synthesis were downregulated in the thyroid gland. Furthermore, changes in thyroid morphology including variable sizes and enlargement of

follicles were noted. Recently, changes in aged mouse thyroids have been attributed to a hypofunctioning thyroid gland (16). This is in line with the gene expression data observed in this study and suggests that the sensitivity of an ageing thyroid gland to TSH is decreased. This interpretation is further supported by elevated TSH concentrations in mice of advanced age, as shown here and as previously reported by others (17). Altered TSH efficacy on the aged thyroid gland has been reported in rats (18-20), with possible differences in TSH secretion, bioactivity and Tshr availability on the thyrocytes itself. However, the data reported here suggest a compensatory response rather than a causative role of the pituitary gland in ageing.

Altered and organ specific T_3 supply in aged mice

Analysis of fT_3 in serum and T_3 in liver and heart showed that T_3 content in the liver was lower in old compared to young animals while only marginal age-differences were observed in serum and heart. Furthermore, repression of positively TH regulated genes (e.g. hepatic *Dio1*) and induction of negatively regulated genes (hepatic *Tbg*) was found in liver of old animals, in line with low hepatic TH content. An age-dependent change in hepatic deiodinase activities with decreased Dio1 and increased Dio3 activity was previously reported in mice above 24 months of age (17). However, in the experimental conditions used in this study Dio1 activity did not differ in the liver of young and old PBS treated animals. Surprisingly, significant changes in TH regulated genes were found in old compared to young hearts, e.g. induction of cardiac *Myh7* and repression of *Hcn2* expression. These findings suggest an organ-specific regulation of T_3 supply during ageing, which may be of relevance for the function of different tissues. Thus, these observations could also explain why TH excess is distinctly detrimental in older patients resulting in increased cardiac morbidity and mortality (1, 21, 22).

Altered response to T_4 challenge in aged mice The debate whether to treat hypothyroidism in older adults is still ongoing (8, 23, 24). In addition, recent studies have emphasized the risk of potential adverse effects of overtreatment in older adults, who may be prone to an increased likelihood of T_4-induced side effects and even increased mortality (25). Therefore, we asked whether acute organ response to T_4 might differ between young

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and old mice. Several important observations were made: first, T_4 serum concentrations correlate directly with organ T_4 concentrations in young and old mice, strongly suggesting that the specific transport of T_4 into liver and heart is not influenced by age. Secondly, conversion of T_4 to T_3 is decreased in old mice with lower systemic concentrations and tissue T_3 content compared to young mice. Consequently, the ratios of T_4/T_3 were higher in serum, liver and heart of old compared to young mice. Interestingly, the hepatic Dio1 activity measurements strongly indicate a role for Dio1 as a cause of this finding, as an increase after T_4 injection was only noted in livers of young, but not old mice, illustrating the age-dependent difference of hepatic TH content. Third, since T_3 increment is lower in serum and tissues after T_4 injection in old compared to young mice, we also expected a decreased tissue response. This was confirmed for the pituitary, thyroid and to some extent for the liver. Interestingly, in liver tissue the induction of the TH responsive gene *Dio1*, in contrast to activity, was significantly higher in old mice, possibly due to the impact of mild hypothyroidism on gene expression. After T_4 injection, expression levels of TH responsive genes were equal in the hearts of old and young mice, while in the liver, pituitary and thyroid gland fewer genes showed expression changes in old compared to young mice. This implies that ageing may differentially impact TH response in various organs (26). These findings are in agreement with the notion that an aging organism is in a "low TH state". Furthermore, and importantly, old compared to young mice show a different organ-specific response to an acute TH challenge (Fig. 6 b, c).

A shortcoming of our study is that we only determined the age effect at two time points (basal and 12 hours after acute T_4 challenge), and that we cannot extrapolate on long-term effects on the organism. However, in previous studies we have observed age-dependent differences on phenotypic traits of chronic hyper- and hypothyroidism in mice and think that they are of physiological relevance (26, 27). A strength of our study is the characterization of very old mice of 23 months of age in a direct comparison to 3 months old mice. Furthermore, we comprehensively analyzed systemic and peripheral effects of TH concentrations in untreated and T_4 treated mice and expanded the analysis by gene expression analyses hepatic TH metabolism by Dio1.

In summary, the divergent systemic and organ-specific TH states and responses to T_4 in old *vs.* young mice support the clinical notion that TH substitution, while considered adequate at the serum level, may not be equally beneficial for all tissues, particularly in an aging organism. Furthermore, age may impact local TH content and TH metabolism. Thus, our findings are in agreement with a narrower window for TH substitution in older adults and particularly careful clinical and biochemical monitoring of T_4 substituted patients in this age group.

Acknowledgments

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Abbreviations: fT_3, free T_3; fT_4, free T_4; ns, not significant; T_1, monoiodothyronine; T_2, 3,5-diiodothyronine; T_3, triiodothyronine; T_4, thyroxine; TH, thyroid hormone; TSH, thyroid stimulating hormone; rT_3, reverse T_3; rT_2, reverse T_2

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Corresponding author:

Dagmar Führer, MD PhD

Department of Endocrinology, Diabetes and Metabolism, Division of Laboratory Research, University Hospital Essen, University Duisburg-Essen

Hufelandstr.55

45122 Essen, Germany

Phone: +49 201 723 6401

Fax: +49201 723 5972

Email: Dagmar.Fuehrer@uk-essen.de

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Table 1 Body weight of young and old male mice at time of sacrifice mean ±SD

Age (months)	PBS-treated	Thyroxine-treated
3	**24.2±0.7 g (n=6)**	**25.3±0.8 g (n=6)**
23	**29.9±1.7 g (n=8)**	**32.4±1.5 g (n=11)**

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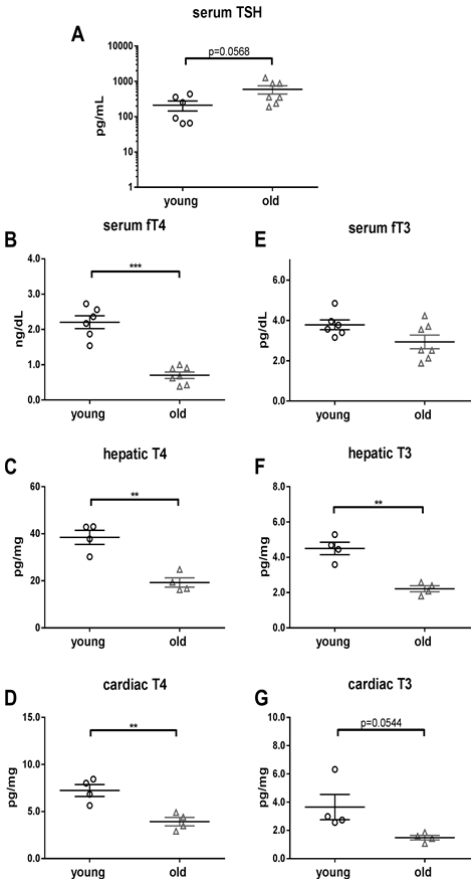

Figure 1: TSH and TH concentrations in sera, livers and hearts of young and old mice. TSH (**A**) and free T_4 concentration was measured in sera (**B**), and content of total T_4 in livers (**C**) and hearts (**D**) of PBS treated mice. The biologically active TH T_3 was analyzed as free T_3 in sera (**E**) and total T_3 in livers (**F**) and hearts (**G**) of the same mice. Lower T_4 concentrations in serum, liver, heart, and hepatic T_3 content were noted in aged mice. Values are represented as mean±SD, n=4-7, unpaired student´s t-test, **p<0.01, ***p<0.001.

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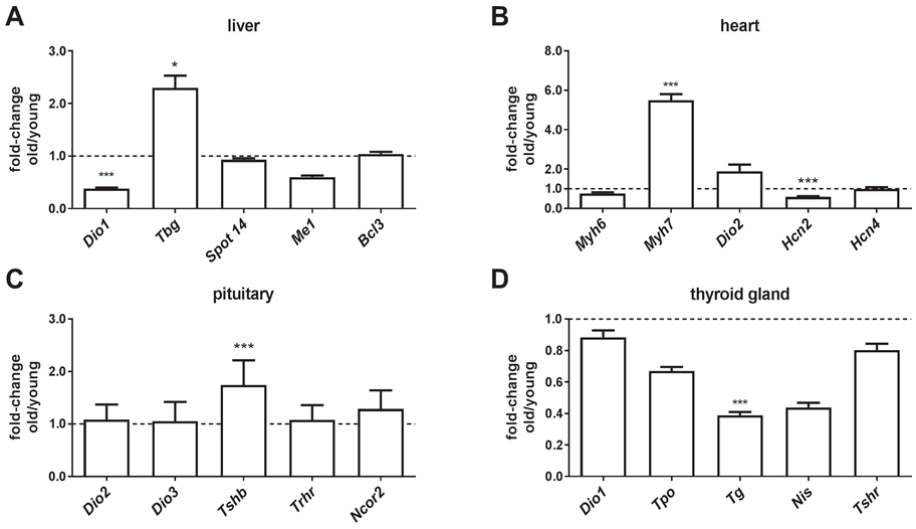

Figure 2: Age- and organ-specific expression of TH responsive genes. Transcript changes influenced by age were assessed in liver (**A**), heart (**B**), pituitary (**C**) and thyroids (**D**) of old compared to young mice. Decreased expression of positively regulated genes and increased expression of negatively regulated genes in the liver, heart and pituitary are suggestive of low TH concentrations in these tissues. Decreased expression of TH synthesis genes in the thyroid gland suggests a hypoactive organ. Dotted line highlights the fold-change in old compared to young mice set to 1 as reference. Fold-changes of old mice are represented as mean±SEM, n=6-8, unpaired student´s t-test,*p<0.05, **p<0.01, ***p<0.001.

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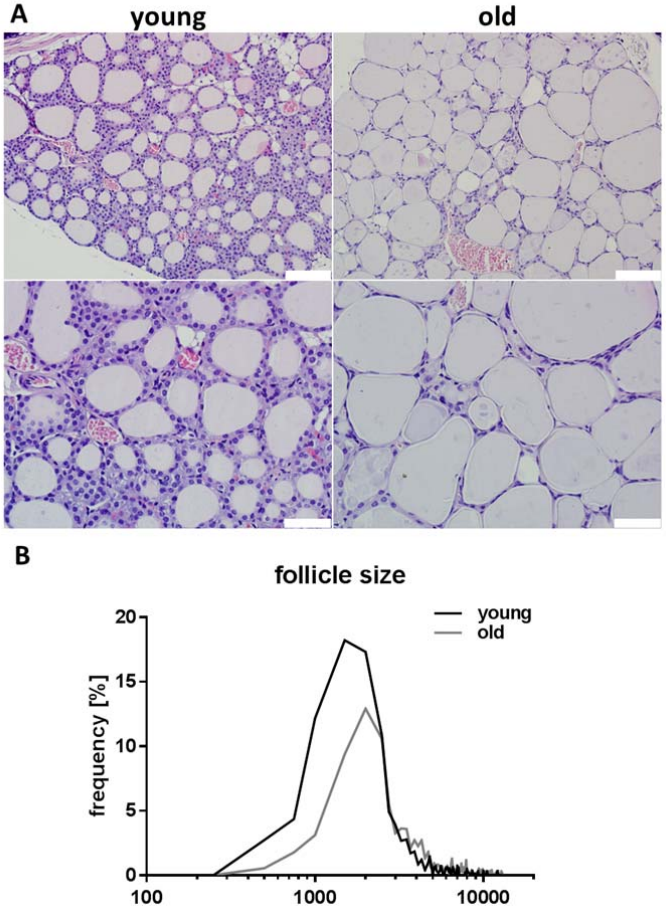

Figure 3: Morphology of thyroid glands in young and old mice. Formalin-fixed thyroid sections of 3 months and 23 months control treated mice were H&E stained (**A**). Scale bar (white block) of upper panel represents 100 µm and of lower panel 50 µm. Follicle sizes were quantified and plotted against %frequency (**B**). 78-245 follicles per thyroid gland of n=4 mice per group were counted. Representative photomicrographs taken with an Olympus BX51 upright microscope are shown.

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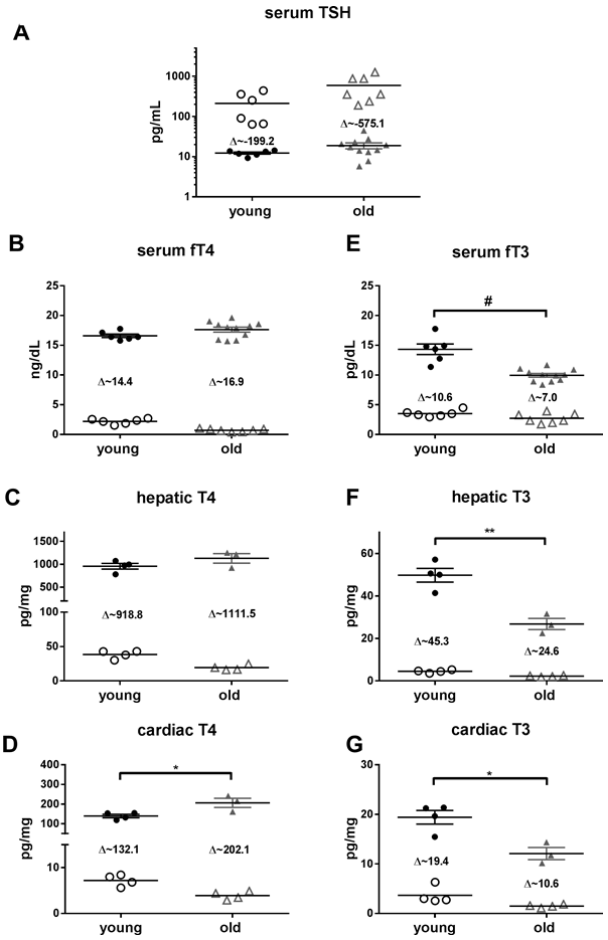

Figure 4: TSH and TH concentration changes 12 h after single T_4 injection in young and old mice. TSH (**A**) and free T_4 concentrations were measured in sera (**B**), total T_4 content in livers (**C**) and hearts (**D**) in young and old mice 12 h after T_4 injection. Similarly, free T_3 concentrations were assessed in sera (**E**), total T_3 content in livers (**F**) and hearts (**G**) of the same mice. A comparable increase of T_4 concentrations in old and young treated mice were found for serum and T_4 content in the liver. In contrast, T_3 concentrations/contents were lower in the other studied organs and the circulation. Values are represented as mean±SD, N=3-11, One-Way ANOVA with Tukey's *post hoc* comparison,*p<0.05, **p<0.01, ***p<0.001, $^{\#}$p<0.0001. Filled symbols represent concentrations of T_4 in treated mice and empty symbols of PBS injected controls. Δ represents the difference between means of T_4 treated and PBS treated mice.

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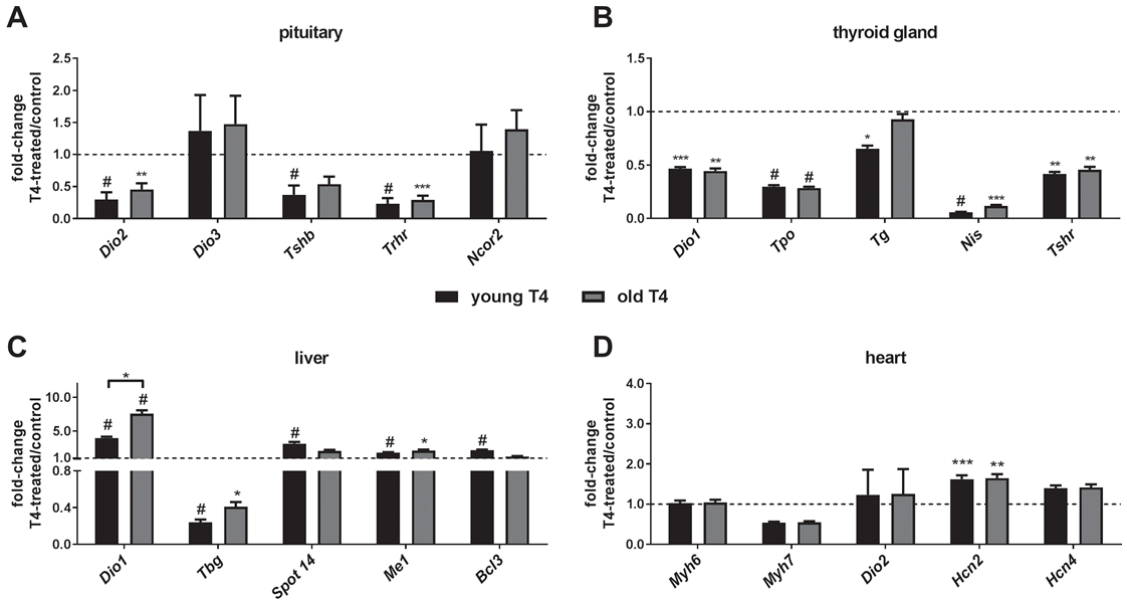

Figure 5: Effect of single T_4 injection on gene expression in young and old mice. Gene expression changes were assessed by qRT-PCR in pituitaries (**A**), thyroid glands (**B**), livers (**C**) and hearts (**D**) of young and old T_4 treated mice. The dotted line indicates the fold-change of respective age-matched control mice set to 1 as reference. Fold-changes of young and old T_4 treated mice are represented as mean±SEM, N=5-8, One-Way ANOVA with Tukey's *post hoc* comparison,*p<0.05, **p<0.01, ***p<0.001, $^{\#}$p<0.0001.

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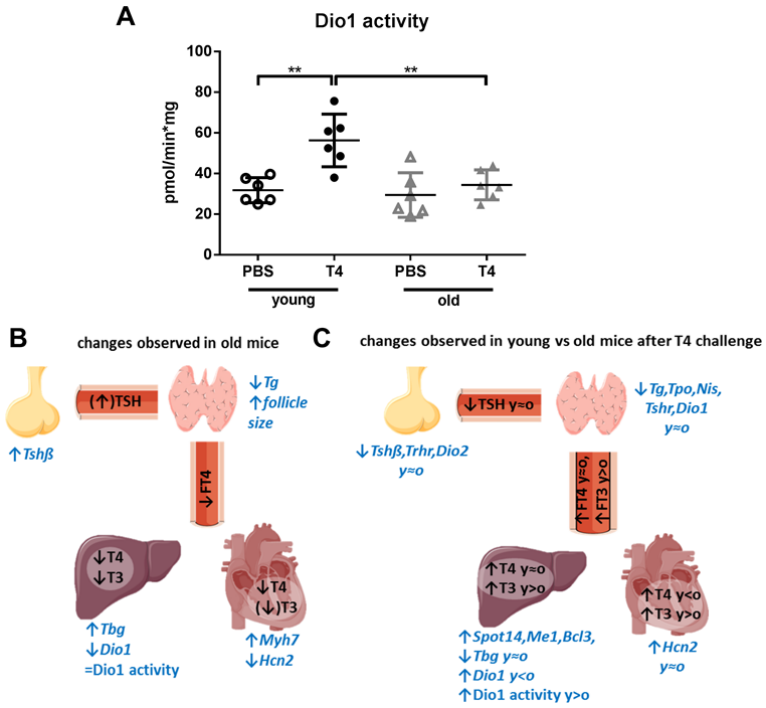

Figure 6: Hepatic deiodinase 1 activity and summary of the impact of age on systemic and organ-specific TH metabolism and response. Hepatic Dio1 activity was determined in young and old PBS and T4 treated mice **(A)**. Values are presented as mean±SD, N=6, One-Way ANOVA with Tukey's *post hoc* comparison, **p<0.01. Summary of age-dependent effects on TSH, TH serum and organ concentrations, hepatic Dio1 activity and transcriptional regulation of TH target genes in the pituitary, thyroid, liver and heart under basal conditions (**B**) and after a single T_4 treatment (**C**). Organ illustrations were obtained from Servier Medical Art.

Supplemental table 1: Oligonucleotides for quantitative RT-PCR. Oligonucleotides were designed using PrimerBlast (NCBI) and synthesized by Eurofins (Eurofins MWG Synthesis, Ebersberg, Germany).

Gene name	Forward primer	Reverse primer
18S	*CGGCTACCACATCCAAGGAA*	*GCTGGAATTACCGCGGCT*
Ppia	*CTTGGGCCGCGTCTCCTTCG*	*GCGTGTAAAGTCACCACCCTGGC*
Rpl13a	*GGGCAGGTTCTGGTATTGGA*	*GGGGTTGGTATTCAATCCGCT*
Hprt	*TGGGCTTACCTCACTGCTTT*	*TCATCGCTAATCACGACGCT*
Polr2a	*CTTTGAGGAAACGGTGGATGTC*	*TCCCTTCATCGGGTCACTCT*
Gapdh	*CCTCGTCCCGTAGACAAAATG*	*TGAAGGGGTCGTTGATGGC*
Dio1	*GGGCAGGATCTGCTACAAGG*	*CGTGTCTAGGTGGAGTGCAA*
Dio2	*GTGACTGGGGAAGCAGAGTG*	*AGTTTAACCTGTTTGTAGGCATC*
Dio3	*GATAGGGAAAGGGTGGGCAG*	*CTTTAGGCGCTGTTTCGAGC*
Tshß	*GGCAAGCAGCATCCTTTTGT*	*TTGCCATTGATATCCCGTGTC*
Trhr	*GCTTGGCCTCTGAGAACTAAAGA*	*TCCCAGTATGTAACTTGCCTGA*
Ncor2	*CAGCCAGCATAGAGGGACTC*	*GTAAGTAGTCCTCCTGCGCC*
Tpo	*AGCTCAAGACACTGGACAGGAAC*	*GCCAATGTCTGGCTCCAAAG*
Tg	*CGCATTTTGGACTGTGGCTC*	*TGTCACTCCATACAGCAGGC*
Nis	*GTTTCTGTGGATGTGCGTGG*	*AGCGCAGTTCTAGGTACTGGT*
Tshr	*TGGACCAACTTTGCTAGATGTGT*	*AGTCCAGGTGTCTTTTGCGA*
Tbg	*TGGGCATGTGCTATCATCTTCA*	*GAGTGGCATTTTGTTGGGGC*
Spot14	*GAGGTGACGCGGAAATACCA*	*TGTCCAGGTCTCGGGTTGAT*
Me1	*TAAGGGTCGTGCATCTCTCAC*	*TGCAGCAACTCCTATGAGGG*
Bcl3	*CTGAACCTGCCTACTCACCC*	*AGTATTCGGTAGACAGCGGC*
Myh6	*CAGACAGAGATTTCTCCAACCCA*	*GCCTCTAGGCGTTCCTTCTC*

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Myh7	*CACGTTTGAGAATCCAAGGCTC*	*CTCCTTCTCAGACTTCCGCA*
Hcn2	*CCA GTC CCT GGA TTC GTC AC*	*TCA CAA TCT CCT CAC GCA GT*
Hcn4	*CAGCGTCAGAGCGGATACTT*	*CTTCTTGCCTATGCGGTCCA*

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Supplemental table 2: T_4/T_3 ratios in serum, liver and heart of young and old mice under basal conditions and after T_4 treatment.

	serum	liver	heart
young basal	28 pM fT_4 0.06 pM fT_3 ~500:1 fT_4/fT_3 ratio	50 pmol/g T_4 7 pmol/g T_3 ~7:1 T_4/T_3 ratio	9.3 pmol/g T_4 5.6 pmol/g T_3 ~1.8:1 T_4/T_3 ratio
old basal	9 pM fT_4 0.045 pM fT_3 ~200:1 fT_4/fT_3 ratio	25 pmol/g T_4 3.4 pmol/g T_3 ~7.5:1 T_4/T_3 ratio	5 pmol/g T_4 2.3 pmol/g T_3 ~2.2:1 T_4/T_3 ratio
young T_4 treated	214 pM fT_4 0.22 pM fT_3 ~990:1 fT_4/fT_3 ratio	1232 pmol/g T_4 76 pmol/g T_3 ~16:1 T_4/T_3 ratio	179 pmol/g T_4 30 pmol/g T_3 ~6:1 T_4/T_3 ratio
old T_4 treated	227 pM fT_4 0.15 pM fT_3 ~1500:1 fT_4/fT_3 ratio	1455 pmol/g T_4 41 pmol/g T_3 ~37:1 T_4/T_3 ratio	265 pmol/g T_4 18.6 pmol/g T_3 ~15:1 T_4/T_3 ratio

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