Phenotypical characterization of sex-dependent traits in mice models of hyperthyroidism and hypothyroidism

Inaugural-Dissertation

zur

Erlangung des Doktorgrades

Dr. rer. nat.

der Fakultät für

Biologie

an der

Universität Duisburg-Essen

vorgelegt von

Helena Rakov

aus St. Petropawlowsk

April 2017

Die der vorliegenden Arbeit zugrunde liegenden Experimente wurden am Universitätsklinikum Essen in der Klinik für Endokrinologie und Stoffwechselerkrankungen durchgeführt.

1. Gutachter: Prof. Dr. Dr. Dagmar Führer-Sakel
2. Gutachter: Prof. Dr. Elke Cario

Vorsitzender des Prüfungsausschusses: Prof. Dr. Ruth Grümmer
Tag der mündlichen Prüfung: 17.07.2017

Publications

Engels Kathrin*, Rakov Helena *, Zwanziger Denise, Moeller Lars C., Homuth Georg, Köhrle Josef, Brix Klaudia, Fuhrer Dagmar. Differences in mouse hepatic thyroid hormone transporter expression with age and hyperthyroidism. *Eur Thyroid J* 2015;4(suppl 1):81–86. DOI: 10.1159/000381020. *contributed equally

Zwanziger Denise*, Rakov Helena*, Engels Kathrin, Moeller Lars C., Fuhrer Dagmar. Sex-dependent claudin-1 expression in liver of eu- and hypothyroid mice. *Eur Thyroid J*. 2015 Sep; 4(Suppl 1): 67–73. DOI: 10.1159/000431316. *contributed equally

Engels Kathrin*, Rakov Helena*, Zwanziger Denise, Hoenes Georg Sebastian, Rehders Maren, Brix Klaudia, Koehrle Josef, Moeller Lars Christian, Fuhrer Dagmar. Efficacy of protocols for induction of chronic hyperthyroidism in male and female mice. *Endocrine*. 2016 Oct;54(1):47-54. DOI: 10.1007/s12020-016-1020-8.

Rakov Helena*, Engels Kathrin*, Hönes Georg Sebastian, Strucksberg Karl-Heinz, Moeller Lars Christian, Köhrle Josef, Zwanziger Denise, Führer Dagmar. Sex-specific phenotypes of hyperthyroidism and hypothyroidism in mice. *Biol Sex Differ*. 2016 Aug 24;7(1):36. DOI: 10.1186/s13293-016-0089-3. *contributed equally

Rakov Helena*, Engels Kathrin*, Hönes Georg Sebastian, Brix Klaudia, Köhrle Josef, Moeller Lars Christian, Zwanziger Denise, Führer Dagmar. Influence of age on sex-specific phenotypical traits of thyroid dysfunctions. Submitted to *Biology of Sex Differences*. *contributed equally

Rakov Helena, Hoenes Georg Sebastian, Möller Lars Christian, Führer Dagmar, Zwanziger Denise. Bile acid homeostasis in hypothyroid mice is exerted via water transport and tightening proteins. Submitted to *The Journal of Lipid Research*.

Hönes Georg Sebastian, Rakov Helena, Logan John, Liao Xiao-Hui, Werbenko Eugenie, Pollard Andrea S., Rijntjes Eddy, Latteyer Sören, Engels Kathrin, Strucksberg Karl-Heinz, Zwanziger Denise, Klein-Hitpass Ludger, Köhrle Josef, Bassett J H Duncan, Williams Graham R., Refetoff Samuel, Führer Dagmar, Moeller Lars C.. Non-canonical thyroid hormone signalling mediates cardiometabolic effects in vivo. Submitted to *Journal of Clinical Investigation*.

Congress contributions

Rakov H, Engels K, Zwanziger D, Renders M, Brix K, Hoenes S, Moeller LC, Führer D (2013). Molecular mechanisms of sex-dependent thyroid hormone action on target tissues. 12. Tag der Forschung der Medizinischen Fakultät, 23.11.2013, Universität Duisburg-Essen, poster presentation

Rakov H (2013). Molecular mechanisms of gender-dependent thyroid hormone action on target tissues. 29th Arbeitstagung Experimentelle Schilddrüsenforschung, 12-14.12.2013, Essen, oral presentation

Rakov H, Engels K, Zwanziger D, Renders M, Brix K, Hoenes S, Moeller LC, Führer D (2014). Molecular mechanisms of sex-dependent thyroid hormone action on target tissues. 57th Deutscher Kongress für Endokrinologie, 19-22.03.2014, Dresden, poster presentation

Rakov H, Engels K (2014). Molecular mechanisms of age- and gender-dependent thyroid hormone action in target tissues. 2nd Annual Meeting of Thyroid Trans Act, 11-12.04.2014, Essen, joint oral presentation

Rakov H (2014). Molecular mechanisms of gender-dependent thyroid hormone action on target tissues. BIOME Retreat (Genetics and Cell Biology core), 09-10.07.2014, Mülheim an der Ruhr, oral presentation

Rakov H, Engels K, Zwanziger D, Renders M, Brix K, Hoenes S, Moeller LC, Führer D (2014). Molecular mechanisms of sex-dependent thyroid hormone action on target tissues. ESE Bregenz Summer School, 03-07.08.2014, Bregenz, Swiss, poster presentation

Rakov H, Engels K, Zwanziger D, Jaeger A, Rehn S, Rehders M, Brix K, Hoenes S, Moeller LC, Führer D (2014). Molecular mechanisms of sex-dependent thyroid hormone action on target tissues. 38th Annual Meeting of the ETA, 06-10.09.2014, Santiago de Compostela, Spain, poster presentation

Rakov H, Engels K, Zwanziger D, Rehders M, Brix K, Hoenes S, Moeller LC, Führer D (2014). Molecular mechanisms of sex-dependent thyroid hormone action on target

tissues. 13. Tag der Forschung der Medizinischen Fakultät, 21.11.2014, Universität Duisburg-Essen, poster presentation

Rakov H (2014). Influence of sex on thyroid hormone action in mice. 30th Arbeitstagung Experimentelle Schilddrüsenforschung, 04-05.12.2014, Bremen, oral presentation

Rakov H, Engels K, Zwanziger D, Brix K, Koehrle J, Hoenes S, Moeller LC, Fuehrer D (2014). Gender aspects of hyperthyroidism and TH action in C57BL/6N mice. 1st Thyroid Trans Act International Conference, 05-07.12.2014, Bremen, poster presentation

Rakov H, Engels K, Zwanziger D, Jaeger A, Rehn S, Hoenes S, Moeller LC, Führer D (2015). Thyroid hormone influence on lipid metabolism in brown adipose tissue and liver of male and female mice. 14. Tag der Forschung der Medizinischen Fakultät, 20.11.2015, Universität Duisburg-Essen, poster presentation

Rakov H (2015). Impact of aging on lipid metabolism under TH dysfunction. 31st Arbeitstagung Experimentelle Schilddrüsenforschung, 10-12.12.2015, Berlin, oral presentation

Rakov H, Engels K, Zwanziger D, Hoenes S, Moeller LC, Führer D (2016). Thyroid hormone influence on lipid metabolism of male and female mice. D.A.CH Tagung, 28-31.05.2016, München, poster presentation, flash talk

Rakov H (2016). Molecular mechanisms of sex-dependent thyroid hormone action on target tissues. 4th Annual Meeting of Thyroid Trans Act, 10-11.06.2016, Essen, oral presentation

Rakov H, Engels K, Zwanziger D, Hoenes S, Moeller LC, Führer D (2016). Influence of sex hormones on thyroid dysfunction. 15. Tag der Forschung der Medizinischen Fakultät 18.11.2016, Universität Duisburg-Essen, poster presentation

Rakov H, Engels K, Golchert J, Homuth G, Völker U, Hönes GS, Moeller LC, Rijntjes E, Köhrle J, Zwanziger D, Führer D (2017). Influence of age and thyroid hormones on liver transcriptome and lipid metabolism. 60th Deutscher Kongress für Endokrinologie, 15-17.03.2017, Würzburg, oral presentation

Awards

Rakov H, Engels K, Zwanziger D, Hoenes S, Moeller LC, Führer D (2016). Thyroid hormone influence on lipid metabolism of male and female mice. D.A.CH Tagung, 28-31.05.2016, München, poster award and flash talk

Zusammenfassung

Schon lange ist eine erhöhte Prävalenz von Frauen für Schilddrüsen- (SD) Funktionsstörungen wie einer Über- oder Unterfunktion bekannt. Allerdings sind die Auswirkungen einer SD-Fehlfunktion im menschlichen Organismus kaum geschlechtsspezifisch charakterisiert. Zudem steigt die Rate an SD-Funktionsstörungen mit dem Alter an und auch hierbei ist bislang nur unzureichend bekannt, ob eine Über- oder Unterfunktion bei älteren Frauen im Vergleich zu Männern ähnliche Konsequenzen hat im Hinblick auf z.B. das kardiovaskuläre System, den Energiestoffwechsel oder das Verhalten.

Um diese klinischen Fragestellungen in einem *in vivo* Mausmodell nachzustellen, wurden zunächst Protokolle für eine chronische Überfunktion in männlichen und weiblichen Tieren etabliert. Im Anschluss erfolgte eine Charakterisierung von geschlechtsspezifischen Eigenschaften einer Über- und Unterfunktion in verschiedenen Lebensabschnitten bei jungen, erwachsenen und alten Mäusen sowie Analysen zur organspezifischen SD-Hormon (SDH) Wirkung.

Im Vergleich der jungen Tiere zeigte sich, dass das Geschlecht eine wichtige Rolle bei SDH-abhängigen Veränderungen des Körpergewichts, der Körpertemperatur, der Futter- und Wasseraufnahme, den SDH-Serumkonzentrationen und neuro-muskulären Eigenschaften spielt. Im Vergleich von erwachsenen und älteren Tieren wurde sowohl eine gleichbleibende (für Körpertemperatur, SDH-Serum-konzentrationen, Aktivität) als auch verstärkte (für Körpergewicht, Muskelfunktion) geschlechtsabhängige SDH-Wirkung beobachtet. Interessanterweise ließen sich in allen drei Altersgruppen keine Unterschiede zwischen Männchen und Weibchen in der Herzfrequenz bei einer Über- und Unterfunktion beobachten. In der Analyse der organspezifischen SDH-Wirkung zeigte sich, dass die SDH-abhängige Genexpression im Herzen die geringsten geschlechtsspezifischen Unterschiede aufweist, während im braunen Fettgewebe und noch deutlicher in der Leber ausgeprägte geschlechts- und SDH-spezifische Transkriptänderungen auftraten. In weiteren Analysen konnte eine geschlechts- und SDH-abhängige Regulation von Claudin-1 in der Leber nachgewiesen werden, welche wahrscheinlich eine Relevanz für die Gallensäure Homeostase hat und einen möglichen Pathomechanismus für geschlechtsabhängige Cholelithiasis bei SD-Funktionsstörungen darstellen könnte.

Zusammenfassend sind dies die ersten Arbeiten, welche geschlechtsspezifische Aspekte einer SD-Über- oder Unterfunktion in verschiedenen Lebensaltern im Mausmodell abbilden. Diese Ergebnisse dienen als Grundlage, um die molekularen Mechanismen auf organischer und Zellebene zu verstehen. Dies kann helfen, translational zukünftig am Menschen Komorbiditäten einhergehend mir einer SD-Fehlfunktion bei Männern und Frauen besser vorzubeugen und zu therapieren.

Summary

It is well known, that the clinical situation of thyroid dysfunctions (TDs), hyper- and hypothyroidism have an increased preponderance in women than men. In contrast, a sex-specific outcome of hyper- and hypothyroidism has not been characterized yet. Moreover, the prevalence of TDs increase with age, with unknown sex-specific consequences for the cardiovascular system, energy metabolism and behaviour.

To mimic the clinical situation, first an *in vivo* mouse model for chronic hyperthyroidism in male and female mice was established, followed by induction of chronic hyper- and hypothyroidism of both sexes in young, adult and old aged mice. This allowed a comprehensive characterization of the interplay between sex and age on phenotypical traits of TD at different life stages and enabled the analysis of organ-specific thyroid hormone (TH) effects.

Metabolic parameters of body weight change, food and water intake, TH serum concentrations, heart rate, body temperature, muscle function and activity were assessed. In young mice a distinct sex impact on TH-dependent alterations was identified for body weight, body temperature, nutrient intake, TH serum concentrations and neuromuscular features. In comparison to adult and old age groups sex-effects were persistent (for body temperature, TH serum concentrations, activity) and exaggerated (for body weight and muscle function), whereas no TH-dependent differences between male and female mice were noted for heart rate at any age. Molecular investigations showed less sex influence on TH-dependent gene expression in heart, whereas in brown adipose tissue and even more significant alterations in liver were detected. Further analysis of a sex and TH-dependent regulation of Claudin-1 in livers of mice indicated relevance in an altered biliary homeostasis and a possible pathophysiological role for TH-dependent change of sex-dependency in cholelithiasis.

To conclude, these studies allow a comprehensive analysis of sex influence on phenotypical and molecular traits of hyper- and hypothyroidism at different life stages in mice for the first time and enable the subsequent analysis of the underlying mechanisms in an organ and cell-specific manner. This will help to improve our understanding of possible sex impact on complications of TD in women and men and implicate a sex-specific prevention and treatment of patients.

Contents

Chapter 1: Background

1.1 Thyroid hormones and their function

Thyroid hormones (THs), triiodothyronine (T3) and thyroxine (T4), are produced by the thyroid gland, which is a small butterfly-shaped organ located around the trachea in mammals. The production and secretion of these two essential THs is tightly regulated by the hypothalamic-pituitary-thyroid axis (HPT axis, Zoeller *et al.*, 2007). The hypothalamus produces and releases the thyrotropin releasing hormone (TRH, Segersen *et al.*, 1987a; Segersen *et al.*, 1987b), which is transported through the pituitary-portal vasculature to the anterior pituitary gland (Fig. 1; Haisenleder *et al.*, 1992). There it induces in turn the production of thyroid stimulating hormone (TSH). TSH is transported via the blood stream to the thyroid gland and regulates positively the production of T4 and T3. THs reciprocally reach the hypothalamus or pituitary via the circulation and T3 can repress TRH and TSH secretion, thereby regulating circulating TH serum concentrations. THs secreted from the thyroid gland, comprise mostly T4 (80%) and less T3 (20%) in humans, while in rodents equal amounts of T4 and T3 are secreted (Fig. 1; Chanoine *et al.*, 1993; Maia *et al.*, 2005; van der Spek *et al.*, 2017). In the blood the vast majority of circulating THs (>99.5%) are protein-bound. 60-75% of T4 and 80% of T3 are bound to thyroxine-binding globulin (TBG), 15-30% of T4 and <5% of T3 to transthyretin (TTR) and 10% of T4 and 11% of T3 to albumin (Schussler 2000; van der Spek *et al.*, 2017). However, only unbound free THs can access peripheral organs, enabling their mode of action.

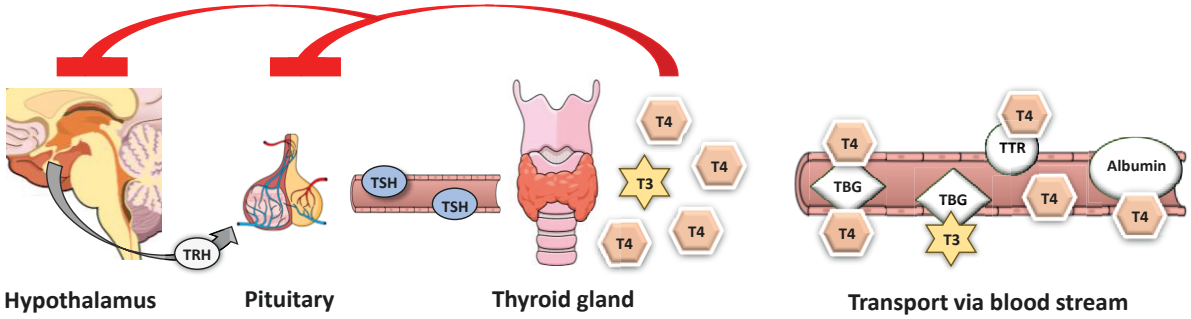

Fig. 1: Hypothalamic-pituitary-thyroid axis regulated by a negative feedback loop. Hypothalamus produces and releases the thyrotropin releasing hormone (TRH), which induces thyroid stimulating hormone (TSH) production in the pituitary gland. TSH is a positive regulator of triiodothyronine (T3) and thyroxine (T4) production in the thyroid gland. T3 represses TRH and TSH expression and secretion via a negative feedback loop in the hypothalamus and pituitary, respectively. In the blood stream THs are mainly transported

protein-bound to thyroxine-binding globulin (TBG), transthyretin (TTR) or albumin. Figures of hypothalamus, pituitary, thyroid gland and blood vessel provided by mindthegraph.com.

Uptake of T4 and T3 across the plasma membrane of cells is mediated by specific TH transporters, as amongst others by monocarboxylate transporter 8/10 (MCT8, MCT10) or organic anion-transporting polypeptide 1C1 (OATP1C1; Hennemann *et al.*, 2001; Visser *et al.*, 2008; Visser *et al.,* 2011) as a prerequisite for intracellular TH action. Within the cell T3 proceeds to the nucleus to canonically mediate repression or induction of gene expression. In the nucleus T3 binds to TH receptors (TRα or TRβ), which can already be prebound to a TH response element (TRE). TREs act as enhancer elements on the DNA and are located in promoter regions of responsive genes. To stabilize binding of the complex of T3/TR at the DNA an additional receptor, e.g. retinoid X receptor (RXR) is needed to form a heterodimer and enable induction or repression of gene transcription (Fig. 2, Yen, 2001).

TRs as ligand-dependent transcription factors exist as two isoforms, TRα and TRβ, which are differently expressed among all organs (Flamant and Gauthier, 2013). The TRα isoform is highly expressed in the heart, muscle, brain and adipose tissue, whereas TRβ is the predominant isoform in the liver (Schwartz *et al.*, 1992, Bookout *et al*., 2006).

Another alternative intracellular T3 pathway has been described in form of a cytoplasmatic complex of TR and phosphoinositide 3-kinase (Pi3K). Binding of T3 results in a rapid phosphorylation of Pi3K and activation of down-stream targets (Fig. 2; Moeller and Broecker-Preuss, 2011). In addition, extracellular TH signalling has been postulated *in vitro* to occur via T3 and T4 binding to plasma membrane receptors integrin αvβ3, thereby initiating intracellular response on e.g. gene expression by activation of Pi3K or extracellular signal-related kinases 1/2 (Fig. 2; Moeller and Broecker-Preuss, 2011; Davis *et al.*, 2015, Davis *et al.*, 2016).

In conclusion, by upregulation or repression of genes THs influence diverse metabolic functions and are highly important for the development and proper function of the brain, bones, muscles, fertility, as well as regulation of heart rate, glucose and lipid metabolism and energy homeostasis (Yen, 2001).

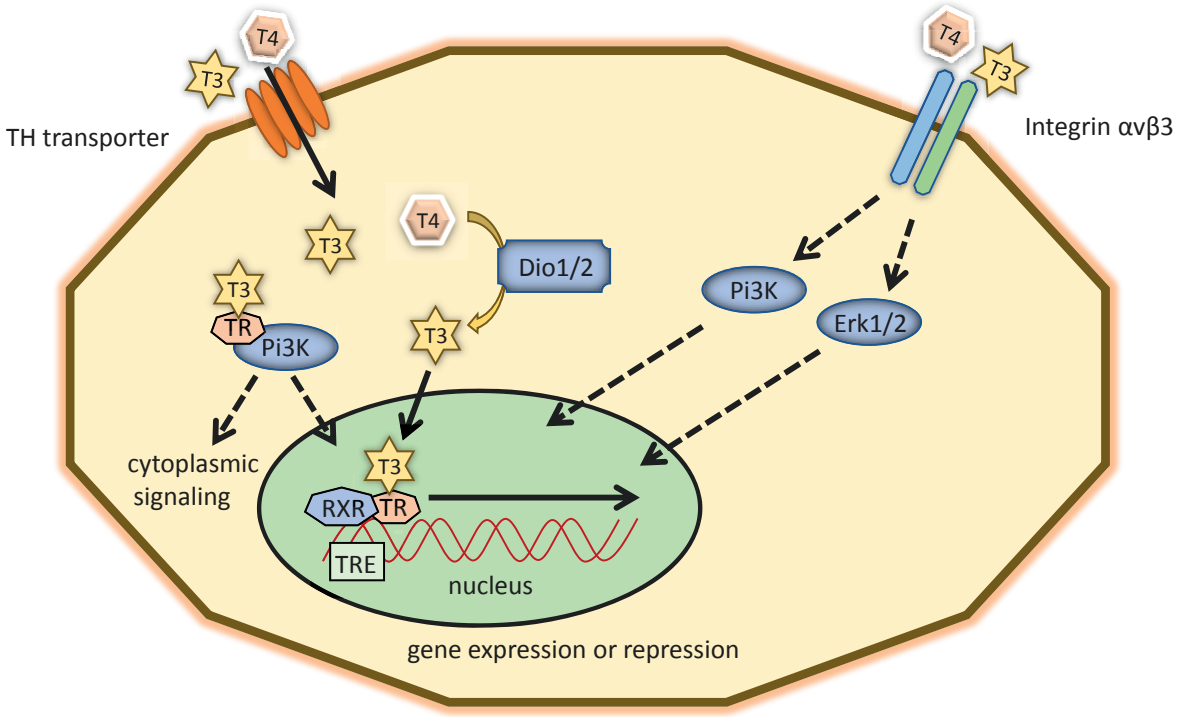

Fig. 2: Overview of possible TH action within a cell. T4 and T3 are transported across the plasma membrane via TH-specific transporters. Intracellularly, T4 is converted to T3 by deiodinase 1 or 2 (Dio1/2) and enables T3 to act by binding to either cytoplasmatic or nuclear TH receptors (TR). Canonical nuclear binding of T3 to a complex of TR, retinoid X receptor (RXR) and DNA at a TH response element (TRE) induces or represses gene transcription. Non-canonical cytoplasmatic TH action is mediated by association of T3 to a complex of TR and phosphoinositide 3-kinase (Pi3K), resulting in phosphorylation of Pi3K and rapid cytoplasmic response including activation of down-stream targets and TRE-independent regulation of gene expression (Moeller and Broecker-Preuss 2011). Moreover, extracellular binding of T3 or T4 to integrin αvβ3 activates intracellular down-stream signalling of Pi3K or extracellular signal-regulated kinases 1/2 (Erk 1/2).

Within the cell THs act exclusively via T3, thus T4 needs to be initially converted and thereby activated prior to binding to TR via specific enzymes, deiodinases. Three types of deiodinases are known (Dio1/2/3) which mediate activation or inactivation of THs. These enzymes are capable to remove specific iodine moieties by either outer-ring deiodination as by 3,3´,5,5´-T4 to 3,3´,5-T3 and 3,3´,5´-reverseT3 (rT3) to 3,3´-diiodothyronine (3,3´-T2) or inner-ring deiodination of T4 to rT3 and T3 to T2 (Fig. 3; Bianco and Kim, 2006). Moreover, deiodinases are expressed in a cell- and organ-specific pattern (Gereben *et al.*, 2008). Thus, deiodinase type 1 (Dio1) is predominantly expressed in liver, kidney, thyroid and pituitary and is responsible for generation of the major part of circulating T3 from T4. In contrast, deiodinase type 2

(Dio2) is preferably expressed in pituitary, brown adipose tissue, placenta, innate immune cells and at very low levels in skeletal muscle. Whereas deiodinase type 3 (Dio3) is highly expressed in the placenta and central nervous system (Bianco and Kim, 2006; Gereben *et al.*, 2008; van der Spek *et al.*, 2017).

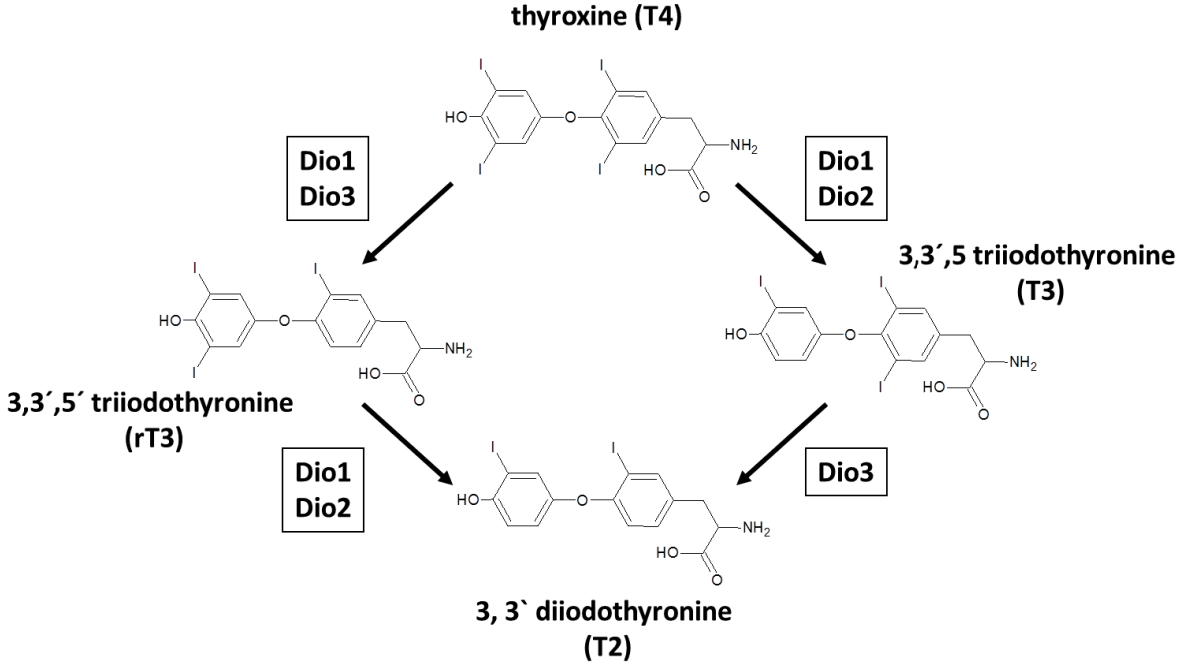

Fig. 3: Overview of TH activation and inactivation by deiodinases. T4 is converted via deiodinase 1 or 2 (Dio1/Dio2) into T3 or via deiodinase 1 or 3 (Dio1/Dio3) into reverse T3 (rT3). T3 again is inactivated via Dio3 into T2 and rT3 is further metabolised by Dio1 or Dio2 into T2 (Bianco and Kim, 2006).

1.2 Thyroid dysfunctions, causes and symptoms

Disturbed thyroid function leads to altered TH serum concentrations and thereby altered TH action within all organs, which patients recognize as symptoms of a thyroid dysfunction (TD). Two major forms of TD exist: TH deficiency named hypothyroidism and TH overproduction named hyperthyroidism. Within these two clinical relevant diseases further classifications as subclinical or overt dysfunctions are used. Subclinical hyper- or hypothyroidism are defined by altered (decreased or increased, respectively) TSH serum concentrations, but free T4 (FT4) and Free T3 (FT3) concentrations within normal ranges. Overt dysfunctions are characterized by disturbances of circulating TSH and THs. Thus, overt hypothyroidism is diagnosed by elevated TSH and decreased FT4 serum concentrations, whereas overt

hyperthyroidism is characterized by decreased TSH and elevated FT4 and/or FT3 serum concentrations (Staub *et al.*, 1992; Khandelwal and Tandon, 2012).

The major cause of hypothyroidism worldwide is still environmental iodine deficiency, however in areas of iodine sufficiency chronic autoimmune thyroiditis (Hashimoto's thyroiditis) is the most common cause for hypothyroidism. Hashimoto's thyroiditis can be associated with a thyroid enlargement (goiter) or with thyroid atrophy. Less frequent are other causes as thyroid surgery, radioiodine treatment, thyroid cancer or other non-thyroid-related head and neck malignancies, as well as secondary to pituitary and hypothalamic diseases or drug-induced hypothyroidism (Garber *et al.*, 2012).

Graves´ disease as a second known autoimmune thyroid disease (AITD) accounts for the majority of hyperthyroid patients worldwide (Bahn *et al.*, 2011). In AITDs the thyrocytes are infiltrated with sensitized T lymphocytes, which serologically result in circulating anti-thyroid antibodies. An inherited defect in immune surveillance is believed to cause AITD, by leading to an abnormal immune responsiveness and alteration of presenting antigen in the thyroid (Ban *et al.*, 2008, Menconi *et al.*, 2008). Thus, antibodies against thyroglobulin (TGAb), thyroid-peroxidase (TPOAb) and TSH receptor (TSHRAb) are known to be found in patients with AITD (Garber *et al.*, 2012). TSHRAb are most prominent thyroid autoantibodies in Graves´ disease and are able to mimic TSH signalling resulting in increased TH production (Bahn *et al.*, 2011). TGAb and TPOAb are distributed among the population and occur with a frequency of 75% in patients of AITD, but were also detected in ~10% of disease-free population. Thus, antibody concentrations should always be evaluated in context with other thyroid function parameters (Garber *et al.*, 2012).

Furthermore, hyperthyroidism frequently occurs from toxic multinodular goiter or toxic adenoma as a result of constitutively active TSH signalling. Other rare cases include acute or painless thyroiditis and drug or human chorionic gonadotrophin induced hyperthyroidism (Bahn *et al.*, 2011).

All previously mentioned causes may lead to overt hypo- or hyperthyroidism, however there is a gradual change between subclinical and overt forms in terms of clinical symptoms and morbidities (Vaidya and Pearce, 2008).

Due to the variety of functions that THs exert on many organs, alteration in TH status and or action may cause diverse and wide spread symptoms affecting the whole organism (Table 1). These include organ-specific signs, as e.g. of the skin (dryness, sweating), heart (palpitation, atrial fibrillation, bradycardia), brain and nervous system (depression, lack of concentration, anxiety, nervousness), muscles (lack of energy, weakness, aches and pains) and digestive system (weight loss, weight gain, loss of appetite, increased bowel movement).

Table 1: Symptoms and signs of hyperthyroidism and hypothyroidism (Crooks *et al.*,1959; Billewitz *et al.*, 1969; Boelaert *et al.*, 2010; Bahn *et al.*, 2011).

symptoms and signs of thyroid dysfunctions	
hypothyroidism	**hyperthyroidism**
lack of energy	irritability
puffy face	nervousness, tremor
dry hair	lack of energy, muscular weakness
dry skin	weight loss
constipation	sweating
aches and pains	osteoporosis
parasthesiae	palpitation, atrial fibrillation
cold intolerance	heat intolerance
poor memory	anxiety
lack of concentration	shortness of breath
depression	neck enlargement
heart failure	increased frequency of bowel movement
weight gain	eye symptoms
loss of appetite	heart failure
growth delay in children	impaired fertility
lowered heart rate	difficulty of falling asleep
impaired fertility	accelerated growth in children

If hyper- or hypothyroidism remains untreated, morbidities may occur and this is most likely with increasing age. Cardiac events and heart failure are among the most frequent complication of hyper- and hypothyroidism in the elderly population (Table 2, Surks *et al.*, 2004). Thus, a correlation between increased mortality and both TDs hyper- and hypothyroidism could have been shown recently in an elderly population (Grossmann *et al.*, 2016).

Table 2: The most prominent morbidities of hyperthyroid and hypothyroid patients (Surks *et al.*, 2004).

Morbidities	
hypothyroidism	**hyperthyroidism**
cardiac dysfunction	atrial fibrillation
adverse cardiac end points	adverse cardiac end points
artherosclerosis	cardiac dysfunction
elevation in total and LDL-cholesterol	systemic and neuropsychiatric symptoms
neuropsychiatric symptoms	reduced bone mineral density and fractures

1.3 Prevalence and background of sex-specificity in thyroid dysfunctions

Sex-specific characteristics of hypothyroidism

A sex-dependent distribution is well known for hypothyroidism. Large studies in different countries have shown a higher prevalence in women than men. In Europe the ratio of affected women/men with hypothyroidism varied between 1.7 to 6.8 (Table 3). In other countries the female to male ratio varied from 1.7 (in US population, Hollowell *et al.,* 2002), 2.4 (in Brazil, Camargo *et al.,* 2008), 2.0 (in Australia, O'Leary *et al.,* 2006) to 4.6 (in Japan, Konno *et al.,* 1993; Table 3).

Interestingly, little is known whether symptoms and complication of hypothyroidism differ between women and men. A recent study which focused on treatment and treatment outcome of hypothyroidism, reported a sex-dependent symptom presentation. Thus, symptom presence or absence were better predictors for overt hypothyroidism in men than women, as women reported significantly more symptoms after replacement therapy than men (Carlé *et al.*, 2015). Moreover, a tendency for overtreatment of women in comparison to men was associated with increased risk of atrial fibrillation and reduced bone mineral density in female patients (Mammen *et al.*, 2015).

Furthermore, a small study in Finland reported an association between subclinical hypothyroidism and hypertension in women, though male patients were not included in the analysis (Luboshitzky *et al.,* 2002). However, the findings of the Finish study were confirmed later in a Chinese study where subclinical hypothyroidism was shown to be a significant predictor for increased systolic blood pressure and an elevated pulse pressure in females. Serum TSH was significantly higher in the hypertensive

female group compared to the normotensive group. In contrast subclinical hypothyroidism and blood pressure did not correlated in male participants, suggesting that thyroid function could influence blood pressure to a greater extent in women compared to men (Duan *et al.,* 2009). Surprisingly, an association of hypertension with subclinical hypothyroidism could not have been shown in an Australian and a Japanese study for any gender, which indicates a need for additional studies to understand the influence of sex and hypothyroidism in regulation of blood pressure (Walsh *et al.,* 2006; Takashima *et al.,* 2007). Furthermore, a Danish study showed an association between subclinical hypothyroidism and development of cardiovascular disease with a possible role of triglycerides and C reactive protein levels in males below 50 years of age (Kvetny *et al.,* 2004). Hereby, these findings however were not confirmed so far as population studies may be influenced by ethnicity and age of participants, different experimental desings and assays applied.

Sex-dependent traits of hyperthyroidism

Hyperthyroidism as a result of a TH excess occurs more frequently in women than men. In Europe the ratio of affected women/men varies between 2.5 and 11.7 (Table 4). In other countries, the female to male ratio ranged from 1.6 (in Brazil, Camargo *et al.,* 2008) 2.4 (in US population, Hollowell *et al.,* 2002) and 8.6 (in Australia, O'Leary *et al.,* 2006; Table 4).

If this situation is also related to sex-dependent disease characteristics or outcomes has been rarely addressed. Apparently, description of symptoms of hyperthyroidism had majority of signs in common in women and men. Interestingly, some were associated with female sex, e.g. weight gain, palpitation and neck enlargement. Moreover, women were less affected by atrial fibrillation, whereas males were independently associated with higher risk of atrial fibrillation (Boelaert *et al.*, 2010). The outcome of hyperthyroidism on bones has a distinct impact on postmenopausal women as described in several studies. Thus, a significant increase in bone turnover markers and a decrease in bone mass were diagnosed in women affected by endogenous subclinical hyperthyroidism, additionally being greater in early postmenopausal patients (Tauchmanovà *et al.,* 2004). Reduced bone mineral density was found in hyperthyroid post-, but not premenopausal women or men (Uzzan *et al.*, 1996). Moreover, this may cause serious consequences as the association with an

increased risk of fractures (Bauer *et al.*, 2001; Blum *et al.*, 2015). Furthermore, overtreatment of hypothyroid women with L-thyroxine has been shown to increase fracture risk in a longitudinal study (Ko *et al.*, 2014; Bassett and Williams, 2016).

Table 3: Prevalence of hypothyroidism and gender distribution reported in studies in different countries and continents.

author	year	country	total population	hypothyroid (%)	women (%)	men (%)	ratio
Tunbridge *et al.*	1977	Great Britain	3,538	1.10	7.50	2.80	2.70
Konno *et al.*	1993	Japan	4,110	n.a.	3.13	0.68	4.60
Vanderpump *et al.*	1995	Great Britain	1,877	n.a.	4.10	0.60	6.80
Knudsen *et al.*	1999	Denmark	2,656	1.00	1.70	0.40	4.30
Bjoro *et al.*	2000	Norway	94,009	n.a.	4.80	0.90	5.40
Hollowell *et al.*	2002	US	17,353	4.60	5.80	3.40	1.70
Hoogendorn *et al.*	2006	Netherlands	6,434	4.40	5.50	3.20	1.70
O´Leary *et al.*	2006	Australia	2,115	5.64	3.64	1.80	2.00
Camargo *et al.*	2008	Brasil	678	6.54	8.40	3.43	2.40
Leese *et al.*	2008	Scotland	388,750	n.a.	5.14	0.88	5.80
Meisinger *et al.* (KORA)	2012	Germany	2,316	4.10	16.10	12.30	1.30
Meisinger *et al.* (SHIP)	2012	Germany	2,505	1.50	4.50	4.20	1.10

Table 4: Prevalence of hyperthyroidism and gender distribution reported in studies in different countries and continents.

author	year	country	total population	hyperthyroid (%)	women (%)	men (%)	ratio
Tunbridge *et al.*	1977	Great Britain	3,538	1.60	2.70	0.23	11.70
Knudsen *et al.*	1999	Denmark	2,656	1.90	2.80	1.10	2.50
Bjoro *et al.*	2000	Norway	94,009	n.a.	2.50	0.60	4.20
Hollowell *et al.*	2002	US	17,353	3.20	4.40	1.80	2.40
Hoogendorn *et al.*	2006	Netherlands	6,434	1.20	1.80	0.60	3.00
O´Leary *et al.*	2006	Australia	2,115	0.44	0.43	0.05	8.60
Camargo *et al.*	2008	Brasil	678	3.34	3.83	2.46	1.60
Leese *et al.*	2008	Scotland	388,750	0.78	1.26	0.24	5.30
Meisinger *et al.* (KORA)	2012	Germany	2,316	2.00	1.70	1.70	1.00
Meisinger *et al.* (SHIP)	2012	Germany	2,505	5.20	3.50	4.20	0.80

1.4 Is thyroid function sex-specific?

During the last 5 decades many population studies investigated the reference range of TH serum concentrations in humans, but a consistency for a sex-dependent impact could not have been defined. In some studies the TSH concentration was shown to be higher in women than men (Hollowell *et al.,* 2002; Bjoro *et al.,* 2000; Tunbridge *et al.,* 1977), while other studies reported no difference (Aghini-Lombardi *et al.,* 1999; Kapelari *et al.,* 2008; Roelfsema *et al.,* 2009) or in contrast even men having a higher TSH concentration than women (Hadlow *et al.,* 2013).

Moreover, TSH concentrations varied age-dependently in women but only if studies consisted of probands below 50 years of age (Roelfsema *et al.,* 2009; Tunbridge *et al.,* 1977). In subjects of older age the TSH concentration range increased in both gender with age consistently (Canaris *et al.,* 2000; Bjoro *et al.,* 2000) or decreased with age in women and men (Hoogendoorn *et al.,* 2006). For the FT4 and FT3 serum concentrations no significant gender-dependent difference was shown so far (Ahmed *et al.,* 2009, Dika *et al.,* 2010). However, these findings do not preclude that similar TH concentrations or similar alteration of THs in pathophysiological states will have the same effect in a male or female organism since serum thyroid state is not necessarily reflecting tissue state.

Along this line, recent findings by Porcu *et al.* in 2013 in a genome-wide study to identify common genetic variants associated with altered serum TSH and FT4 concentrations showed sex-specific differences in some loci. At the loci *phosphodiesterase type 8B* (PDE8B), *phosphodiesterase type 10A* (PDE10A) and *v-maf musculoaponeurotic fibrosarcoma oncogene homolog/LOC440389* the TSH-elevating alleles showed significantly stronger genetic impact on pituitary-thyroid function in males compared to females. Interestingly, in a sex-specific meta-analysis, alleles that are associated with higher FT4 concentrations, showed one female-specific locus and one male-specific locus which both did not appeared in the whole meta-analysis study before. The female-specific locus is located on chromosome 18q22 near gene *neuropilin and tolloid -like 1* 550 kb upstream and *F-box only protein 15* 500 kb downstream. The male-specific locus is located on chromosome 16q12.2 in intron 11 of the *lysophosphatidylcholine acyltransferase 2* gene near *calpain, small subunit 2* (CAPNS2; Porcu *et al.,* 2013). This might implicate sex-specific gene regulation in proper TH function. However, if these loci are clinically or mechanistically relevant has not been proven so far.

1.5 Interaction of sex and thyroid hormones

Basic research on the interaction of sex hormones, in particular female sex hormones and THs dates back to the 1950´s. In 1956 the effect of diethylstilbestrol (a synthetic nonsteroidal estrogen) on the binding of T4 in serum was described. Diethylstilbestrol that was administered to various patients with endocrinopathies in a dosage of 30 mg daily for periods of four to eight weeks induced increase in thyroxine-binding by TBG comparable to the observed changes during pregnancy. Moreover, this effect was not dependent of normal functioning of the thyropituitary axis (Dowling *et al.*, 1956).

In 1958 Engbring and Engstrom studied the effect of estrogen on circulating THs in humans. When administering estrogen to athyreotic patients they found the following results: Estrogen increased the thyroxine-binding capacity of serum, the concentration of serum total T4 and increased secretion of TSH at pituitary level, as well as thyroidal activity. Additionally, estrogen administration decreased the basal metabolic rate (Engbring and Engstrom, 1958). Later these findings were confirmed *in vitro* and showed that estrogen-induced serum TBG elevation may not be mediated through an increase of synthesis (Ain *et al.*, 1988). Furthermore, T4 turnover was determined during estrogenic therapy at different time spans. In all subjects, estrogen induced an increase in the binding avidity of TBG and a marked retardation of the fractional rate of peripheral turnover of T4 (Dowling *et al.*, 1960). These early pioneer studies demonstrated that estrogen and it´s derivatives are important modifiers of TH action in serum and thyroidal activity.

Conversely, THs affect reproduction ability and menstrual cycle in women, as women with TSH values outside the reference range were more likely to report shorter or longer menstrual periods than women within that range (Sowers *et al.*, 2008). Around 21.5 % of untreated thyrotoxic patients experience cycle abnormalities even though most thyrotoxic women remain ovulatory. In hypothyroid women menstrual disturbance is reported in around 23.4 % and negatively influences fertility of women (Krassas, 2000). Thus severe hypothyroidism is associated with failure of ovulation or serious problems during pregnancy with occurring abortions, stillbirths or prematurity (Krassas, 2000). The menstrual pattern is influenced by THs directly through impact on the ovaries and indirectly through impact on sex-hormone binding globulin, prolactin and gonadotropin-releasing hormone secretion and coagulation

factors (Poppe *et al.*, 2007). These findings show the interplay between sex hormones on THs and reveal TH influence on gonadal system.

1.6 Thyroid hormone action studies in mice

Distinct rodent models for studies of thyroid function have been developed over the last decades to increase the knowledge about the thyroid gland and TH action in an organism. The American Thyroid Association (ATA) summarized in 2014 a series of recommendations related to the study of TH economy and action in rodent models, as well as in cell models to improve reproducibility of experiments and tests (Bianco *et al.*, 2014). Although extensive work has been done on this topic, the majority of studies were performed in male mice only and remarkably fewer in female mice only. Thereby, sex effects within TD have not been addressed directly so far, but were reported mainly if recognized by chance or during characterization of a mouse strain.

Defining the TH status of several mouse strains, a sex-dependent difference in serum TSH concentrations in untreated mice was reported of either two-fold higher (Yeager *et al.*, 2007; Vella *et al.*, 2014) or even four-fold higher TSH concentrations in male compared to female mice (Pohlenz *et al.*, 1999). Thus, mice in contrast to humans appear to differ in their normal thyroid function between both sexes. Furthermore, functional assessment of components of TH metabolism, e.g. Dio1 showed a sexual dimorphic activity in kidney and liver of young (8 weeks) euthyroid mice. Hepatic Dio1 activity was higher in male than female mice, while renal Dio1 activity was higher in female than male mice (Riese *et al.*, 2006). Moreover, this effect was age-dependent, as sexual dimorphism for hepatic Dio1 activity vanished, but for renal Dio1 activity was preserved in adult (12 months) mice (Schomburg *et al.*, 2007).

In another crucial TH target organ, the brain, an effect of TH excess on glial morphology and function was described to be sex-dependent. Whereas T4 induced morphological change and activation of microglia and astrocytes, as well as microglial phagocytosis in male mice, no such effect was noted for female mice (Noda *et al.*, 2016).

Interestingly, in 1968 a higher uptake of T4 in the thyroid gland in female compared to male mice was found when mice were treated with desiccated thyroid or

subcutaneous injections of T4 (Schreiberova O. and Kapitola J., 1968), indicating already a sex dependent response to THs in mice. However, this topic was not followed up for a long time, until in 2014 a difference in response to T3 was noted. T3 treatment resulted in a two-fold higher increase of T3 serum concentrations in female mice, while only a 1.5-fold increase was observed in males (da Silveira *et al.*, 2014).

Other studies have found sexual dimorphism in thyroid cancer. Thus the incidence of different thyroid cancer types in mice shows a sex-dependent distribution, not dissimilar to the human situation. Thyrocyte-specific loss of phosphatase and tensin homolog resulted in the development of follicular adenomas by the age of 10 months preferably in female mice, whereas males were not affected (Yeager *et al.*, 2007). Furthermore, the laboratory mouse strain B6C3F$_1$ which is commonly used in long-term toxicity studies developed thyroid tumors in 1-10 % of the female compared with 0.8 % of male mice (Charles River Laboratories, USA) by the age of 24 months.

Although not in relation to a sex difference, TH action in mice has been studied for a long time and the prominent influence of TH on bones, brain and behaviour, heart function and energy homeostasis are well described (Schneider *et al.*, 2001; Dillmann, 2002; Shin and Osborne, 2003; Schneider *et al.*, 2006; Coppola *et al.*, 2007; Trajkovic *et al.*, 2007; Pierpaoli and Lesnikov, 2011; Lin *et al.*, 2012; Hübner *et al.*, 2014; da Silveira *et al.*, 2014; Zhang *et al.*, 2015; Bassett and Williams, 2016).
TH influences adult bone homeostasis in male mice as hyperthyroidism was found to be associated with reduced trabecular and cortical bone density, cortical thickness and high bone turnover. In contrast increased trabecular bone density and low bone turnover has been noted for hypothyroidism (Bassett and Williams, 2016).
Cardiac outcome of TH excess was associated with increased heart rate and heart to body weight ratio (Dillmann, 2002; Bachman *et al.*, 2004; Suarez *et al.*, 2010). Furthermore, functional left ventricular analysis showed a declined ejection fraction, wall thickening, systolic index and fractional shortening of hyperthyroid mice, but was partly reversible after TH cessation in young, but not in adult females (Hübner *et al.*, 2014). In contrast, propylthiouracil induced hypothyroidism in mice reduced heart rate and weight, lowered the ejection fraction and capillary density, suggesting cardiac microvascular impairment and increased sensitivity to angiogenic growth factors. Finally, one year of treatment even induced heart failure (Chen *et al.*, 2012).

Brain is known to be TH responsive, as TH excess and deprivation resulted in alterations of gene expression (Hernandez *et al.*, 2012). Furthermore, proper TH function is a prerequisite for development of pyramidal cells of the neocortex and Purkinje cells of the cerebellum, which is impaired under hypothyroidism (Bernal *et al.*, 2003). Moreover, hypothyroidism results in a major impact on the striatum of adult mice, which were described to result in motor behaviour modifications (Vallortigara *et al.*, 2008). A clear influence of TH status could be shown in a behavioural study of hypothyroid, euthyroid and hypothyroid mice supplemented with TH. Graded difference of anxiety, with TH serum concentrations was noted as adult onset of hypothyroidism in male mice induced mild anxiety which was reversible by TH-supplementation and euthyroid mice were inbetween both treatment groups (Buras *et al.*, 2014).

THs are essential for metabolic processes, thus TH excess results in hypermetabolic syndrome with increased resting and total energy expenditure, food intake (Klieverik *et al.*, 2009), reduced cholesterol levels (Lin *et al.*, 2012), increased lipolysis, and gluconeogenesis (Mullur *et al.*, 2014), decreased hepatic glycogen stores and increased body temperature (Yao *et al.*, 2014; Alvarez-Crespo *et al.*, 2016). TH deficiency in turn is accompanied by hypometabolism characterized by reduced resting energy expenditure, increased cholesterol levels, reduced lipolysis, and increased hepatic glycogen stores (Ueta *et al.*, 2011; Yao *et al.*, 2014).

Currently *in vivo* TH action is based on findings in mainly male organisms and is a bias to clinical situation of far more women being affected by TD than men. Thus, a prerequisite is to dissect the contribution of sex on TH action in a healthy or diseased organism by evaluation of male and female animals. Moreover, for a translation of experimental data it is mandatory to understand whether and to what extend sex influences TH action. Furthermore, since patients are affected at different life stages, with increased prevalence of hyper- and hypothyroidism at older age, a comprehensive analysis for sex dependent TH action also needs to consider mouse organisms of different ages.

To this aim, the focus of this work is based on the following questions:

1. What is an appropriate protocol for chronic hyperthyroidism in males and females?

2. Do males and females differ in signs and symptoms of hypo- and hyperthyroidism?

3. Is this sex difference age-dependent and thereby possibly sex hormone related?

4. Is there an organ difference in sex-specificity of hypo- and hyperthyroidism?

Chapter 2: Efficacy of protocols for induction of chronic hyperthyroidism in male and female mice

Engels Kathrin[1a], Rakov Helena[1a], Zwanziger Denise[1], Hoenes Georg Sebastian[1], Rehders Maren[2], Brix Klaudia[2], Koehrle Josef[3], Moeller Lars Christian[1], Fuhrer Dagmar[1]*

[1]Department of Endocrinology and Metabolism, University Hospital Essen, University Duisburg-Essen, Essen, Germany

[2]Department of Life Sciences and Chemistry, Jacobs University Bremen, Bremen, Germany

[3]Institut für Experimentelle Endokrinologie, Charité-Universitätsmedizin Berlin, Berlin, Germany

[a]These authors contributed equally to this work.

* corresponding author

Endocrine. 2016 Oct;54(1):47-54. Epub 2016 Jul 29; doi: 10.1007/s12020-016-1020-8.

I have conducted the study design, performed and analyzed study 2 “T4 oral treatment” (study 2 in Fig. 1, Fig.3, Fig. 4b, Fig. 6). All other experiments of T4 ip treatment were performed and analyzed together with Kathrin Engels (study 1 in Fig. 1, Fig. 2, Fig. 4a, Fig. 5, Supplemental tables 1-4) including writing of the manuscript.

2.1 Abstract

Purpose: Protocols for induction of hyperthyroidism in mice are highly variable and mostly involve short-term thyroid hormone (TH) treatment. In addition, little is known about a possible influence of sex on experimental TH manipulation. Here we analyzed the efficacy of intraperitoneal (ip) vs. oral levothyroxine administration to induce chronic hyperthyroidism in male and female mice and asked which thyroxine dosing intervals are required to achieve stable organ thyrotoxicosis.

Methods: Thyroxine was administered ip or orally over a period of 6/7 weeks. Assessment included monitoring of body weight, TH serum concentrations, serial quantitative TH target gene expression analysis in liver and heart.

Results: Our results show that both intraperitoneal and oral thyroxine treatment are reliable methods for induction of chronic hyperthyroidism in mice. Thereby thyroxine injection intervals should not exceed 48h and oral levothyroxine should be administered continuously during experiments and up to sacrifice, to ensure a hyperthyroid organ state. Furthermore, we found a sex-dependent variation in T4-induced TH serum state, with significantly higher T4 concentrations in female mice, while expression of investigated classical TH responsive genes in liver and heart did not vary with animal's sex.

Conclusions: In summary, our study shows that common approaches for rendering rodents thyrotoxic can also be used for induction of chronic hyperthyroidism in male and female mice. Thereby T4 dosing intervals are critical as are read-out parameters to verify a chronic thyrotoxic organ state.

2.2 Introduction

Hyperthyroidism is a pathological state characterized by excessive thyroid hormone (TH) action in the body and is associated with significant morbidity and mortality, mainly from cardiovascular and cerebrovascular disease [1–3]. Women are more frequently affected and in the general population, 1.7-11.7% of women and 1.2-7.6% of men are suffering from subclinical or overt hyperthyroidism [4, 5]. The most common causes of hyperthyroidism include Graves' disease, thyroid autonomy, and over-dosage of levothyroxine [5].

Animals serve as useful models to study TH action in vivo. This is also reflected in the 2014 guidelines of the American Thyroid Association (ATA), which comment on experimental settings to investigate TH economy and action in rodent and cell models [6]. The spectrum of potentially involved players has become increasingly complex and comprises distinct TH and TH derivatives, transmembrane transporters and other putative membrane targets of TH, in addition to intracellular TH metabolism and classical nuclear vs. non-genomic TH action [7].

With the aim to study molecular aspects of chronic hyperthyroidism in mice, we searched the literature for established protocols. We found, besides a preference of rats vs. mice, a great variety of protocols including application of different iodothyronines and dosages, different modes of TH administration and treatment duration as well as variable tools, endpoints and markers for monitoring thyrotoxicosis in vivo. Furthermore, data on female mice are lacking so far and little is known on sex-dependent molecular TH action in mice. In this context, the Endocrine Society has recently highlighted that sex is an important variable that needs to be considered in basic science as well as clinical research [8].

With this reasoning and in light of lack of standardized protocols for induction of chronic hyperthyroidism, we tested the efficacy of long-term intraperitoneal (ip) and oral thyroxine (T4) administration in male mice. In addition, we compared effects on serum TH and tissue thyroid status in male and female mice. Furthermore, we investigated the sustainability of ip and oral T4 supply on organ TH status to determine which T4 dosing intervals ensure stable tissue hyperthyroidism during experiments and at organ sacrifice.

2.3 Materials and Methods

Animals and treatment

For study 1 two to three months old male and female C57BL/6NTac mice (Taconic Europe A/S, Denmark) were randomly assigned to ip T4 treatment (n=6 mice/sex/treatment) or control treatment (n=3 mice/sex/treatment). For study 2 four to eight months old wild-type male mice with C57BL/6 background (Zentrales Tierlabor, University Hospital Essen) were randomly assigned to oral T4 treatment (n=12) or control treatment (n=3). After 3 weeks of T4 treatment the oral group was divided into 2 subgroups and the dosage of T4 reduced. One group (n=6) received continuous T4 treatment, while a second group (n=6) was given a recovery time of one week until T4 supply continued (Fig. 1).

Animals were housed in temperature- (23 ±1°C) and light-controlled (inverse 12:12 hour light-dark cycle) conditions two weeks prior to the start of the experiments and until all experiments were finished. 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).

In the ip treatment group, animals received ip injections of 1 µg/g body weight levothyroxine (T4; Sigma-Aldrich (T2376), St. Louis, USA; stock-solution: 2 mg/mL T4 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 PBS) or PBS (control males: 150 µl PBS; control females: 100 µl PBS) every 48h. T4 solutions (stock and injection) were prepared freshly every two weeks and were kept protected from light at 4°C.

In the oral treatment group, T4 was continuously supplemented in the drinking water containing 5 µg/mL or 1 µg/mL T4 (stock-solution: 100 µg/mL T4 dissolved in 40 mM NaOH, 0.1% BSA (Albumin from bovine serum, Sigma-Aldrich (A7906), St. Louis, USA) in tap water; solution was kept frozen at -20°C and freshly thawed before dilution) in black drinking bottles (Tecniplast, Hohenpeißenberg, Germany). Control animals received no treatment. T4 supplemented water was prepared freshly three times a week. The treatment period was six weeks for ip and seven weeks for oral mice.

T4 supply (ip or oral) was stopped after six/seven weeks and all animals were placed on tap water without change in food supply. For analysis of TH tissue status and T4 ip/oral dosing intervals, animals were sacrificed at time points 24h, 48h, 72h and 144h after last ip T4 injection or oral T4 treatment (Fig. 1).

TH measurements

Blood samples were collected from the retrobulbar venous plexus every other week at 24h after T4 injection. To allow for weekly assessment of serum TH concentrations, ip and oral T4 groups were divided so that blood was drawn from three animals/sex/treatment per week. FT3, FT4 and TT4 concentrations in serum of mice were measured using commercial ELISA kits according to the manufacturer's instructions (DRG Instruments GmbH, Marburg, Germany; FT3: EIA-2385; FT4: EIA-2386; TT4: EIA-1781). Serum samples with known TH concentrations were used as standards. According to the manufacturer's instructions, the minimum detectable TH concentration is 0.5 µg/dL for TT4, 0.05 ng/dL for FT4 and 0.05 pg/mL for FT3.

Collection of TH target organs

For tissue collection, mice were perfused with heparinized saline through a needle placed in the left ventricle. Tissues were shock frozen in liquid nitrogen, and stored at -80°C until further processing.

Quantitative real-time PCR

Total RNA from liver and heart was isolated using RNeasy Kit (Qiagen, Hilden, Germany) and stored at -80°C. 2 µg of total RNA were reverse transcribed into cDNA with SuperScriptIII (Life Technologies, Darmstadt, Germany) and random hexamer primers. Quantitative real-time PCR was performed using Roche SYBR Green I master mix (Roche, Mannheim, Germany). The primer sequences are provided in Supplementary Table 2 (PCR conditions are available upon request). In compliance with the MIQE guidelines for RT-PCR [9], we used a set of 2 to 4 reference genes per tissue to assure accurate normalization and calculation (liver: Rn18s, Ppia, Rpl13a, β-Actin in ip treated and Rn18s, Ppia in oral treated group; heart: Rn18s, Polr2a, Rpl32 in ip treated and Rn18s, Rpl32 in oral treated group). Analysis and calculation of the fold-change in gene expression were done on Ct-values ≤35 using the efficiency-corrected ΔΔCt method [10].

Statistical analysis

Calculation and plotting was done with GraphPad Prism 6 software (GraphPad, San Diego, USA). Statistical analysis was performed using two-way ANOVA followed by a Tukey's multiple comparisons test or using the unpaired two-tailed t-test, as indicated. Fold expression of RT-PCR was statistically analyzed on anti-logarithmic data.

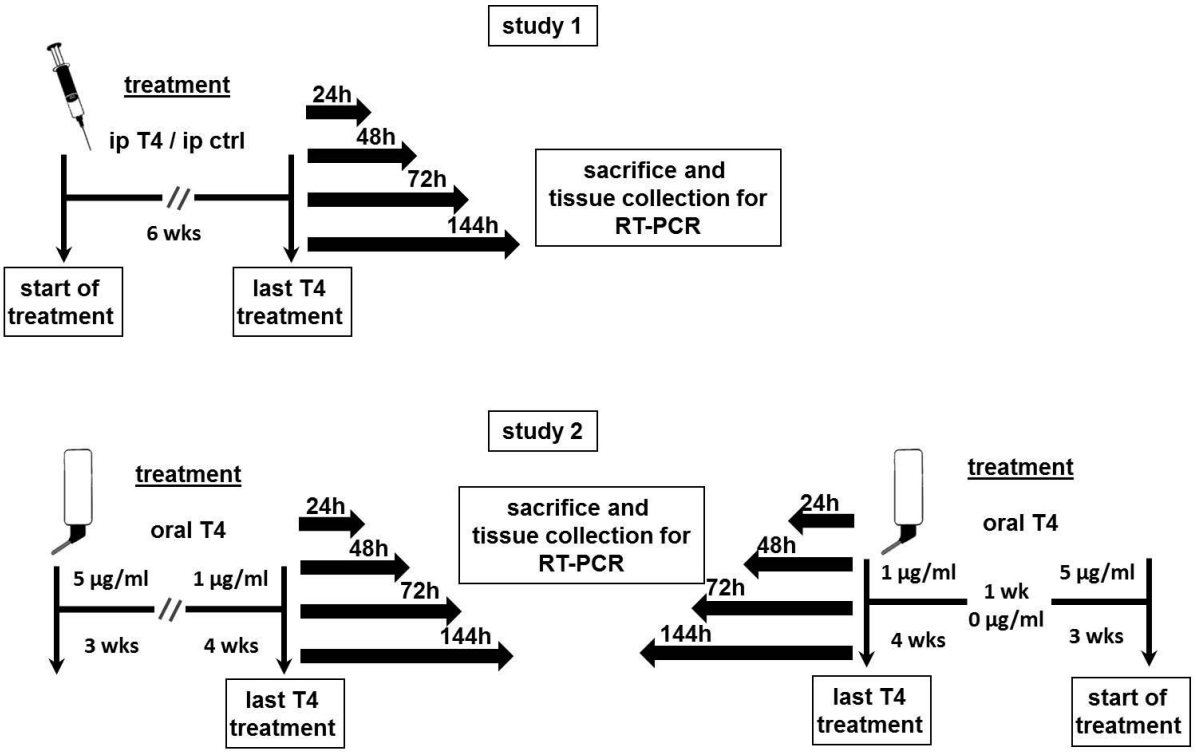

Fig. 1: Schematic illustration of the approach for comparison of ip and oral T4 supply and analysis of the sustainability of chronic T4 treatment on organ thyroid state.

Study 1: Mice received T4 intraperitoneal (ip T4, 1 µg/g BW T4 every 48h) over a period of 6 weeks (6 wks, (n=6 mice/sex). PBS treated mice served as controls (ctrl, (n=3 mice/sex). Treatment was stopped and mice were sacrificed for tissue collection at 24h, 48h, 72h and 144h after the last T4 administration to investigate TH tissue status in target organs (liver, heart).

Study 2: Male mice received oral T4 treatment (5 µg/mL T4 first 3 treatment weeks, n=12) or no treatment (n=3). After 3 weeks of T4 treatment the group was divided and one subgroup received continuous T4 treatment (1 µg/mL T4 last 4 treatment weeks, n=6), while a second subgroup was allowed a recovery time of one week until T4 supply continued (1 µg/mL T4 last 2 treatment weeks, n=6). Treatment was stopped and mice were sacrificed for tissue collection at 24h, 48h, 72h and 144h after the last T4 administration to investigate TH tissue status in target organs (liver, heart).

2.4 Results

Intraperitoneal and oral T4 treatment are suitable to induce chronic hyperthyroidism in male mice

Intraperitoneal T4 treatment (Fig. 1) resulted in marked increase in serum total thyroxine (TT4 up to 6-fold), free thyroxine (FT4 up to 3-fold) and free triiodothyronine (FT3 up to 2.5-fold) concentrations with time and was thus successful in inducing chronic serum thyrotoxicosis in the mice (Fig. 2). Intraperitoneal T4 treatment was well tolerated in all animals and as expected was accompanied by increased body weight gain compared to PBS treated controls (Fig. 4a).

In the oral T4 group, a higher T4 dosage/body weight (5 µg/mL) was used in the beginning with the rationale that the uptake would be ±65% of the oral T4 dosage in mice similarly to humans [11]. However, sequential analysis of serum TH showed that mice became severely thyrotoxic (Fig. 3) and this was also mirrored in impaired animal well-being and loss of body weight gain (Fig. 4b). Hence, at week 3 treatment was stopped for 1 week in half of the oral T4 treated mice or was continued at a much lower dosage (1 µg/mL). Both approaches resulted in complete recovery of animal well-being and comparable TT4, FT4 and FT3 serum concentrations at the end of experiment in the oral and the ip treatment group (Fig. 3). This shows that oral T4 is well absorbed in mice and that serum TH concentrations can be titrated by the oral T4 dosage (Fig. 3). No significant changes in TH serum status were observed in control animals during the entire experimental period (Fig. 2, 3).

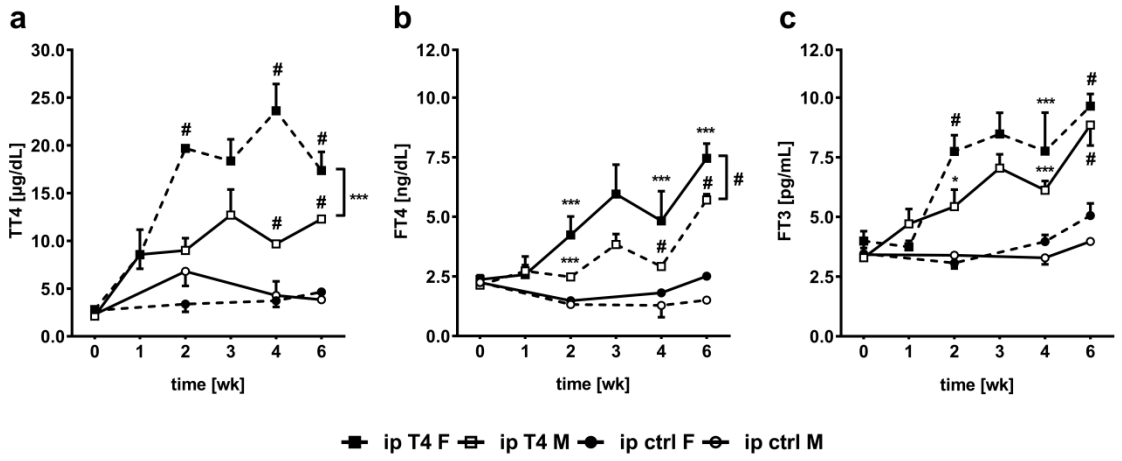

Fig. 2: Serum concentrations of thyroid hormones TT4, FT4 and FT3 in male and female mice with ip T4 treatment compared to sham treated controls. Time course of

changes in TH concentrations (a) total thyroxine (TT4), (b) free thyroxine (FT4) and (c) free triiodothyronine (FT3) determined by ELISA in sera of male (M) and female (F) mice. Mice were subjected to intraperitoneal T4 (ip T4, 1 µg/g BW ip T4 every 48h) over 6 weeks. Controls received ip PBS injections (ip ctrl every 48h) over 6 weeks. From week 2 to 3, a change in housing conditions took place (change from conventional cages to individually ventilated cages for all groups). This resulted in a temporary decrease in thyroid hormone serum concentrations in almost all animals. Data are presented as mean ±SEM, unpaired two-tailed t-test: *p<0.05, **p<0.01, ***p<0.001, #p<0.0001 (n=6 mice/sex for ip T4 treatment; n=3 mice/sex as controls).

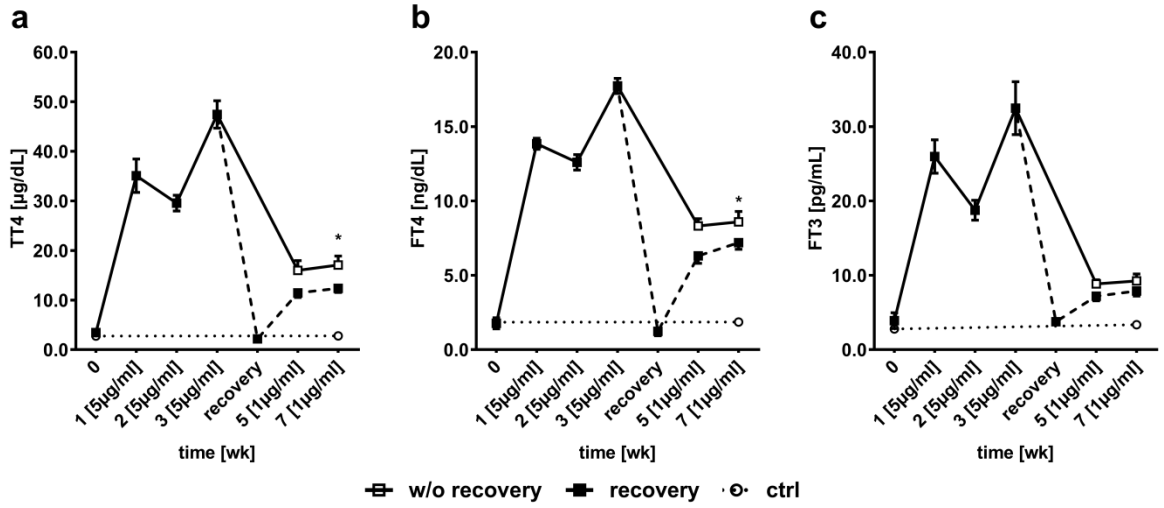

Fig. 3: Serum concentrations of thyroid hormones TT4, FT4 and FT3 in male mice with oral T4 treatment. Time course of changes in TH concentrations (a) total thyroxine (TT4), (b) free thyroxine (FT4) and (c) free triiodothyronine (FT3) determined by ELISA in sera of male mice (n=12) with or without (w/o) recovery after 3 weeks treatment. Mice were subjected to oral T4 (5 µg/mL T4) over 3 weeks and due to severe thyrotoxicosis the dosage of T4 had to be reduced (1 µg/mL T4). One group (n=6) received continuous T4 treatment (w/o recovery), while a second group (n=6) was given a recovery time of one week until T4 supply continued (recovery). Control mice (n=3) received no treatment. Data are presented as mean ±SEM; unpaired two-tailed t-test: *p<0.05, (n=6 time-point/treatment).

T4 treatment results in higher TT4/FT4 concentrations in female than male mice

As a separate aspect of our study we analysed whether sex impacts the efficacy of T4 treatment. This was only studied for the ip mouse group (study 1). Here we observed that ip T4 treatment over 6 weeks resulted in significantly higher serum TT4 (p<0.001) and FT4 (p<0.05) concentrations in female compared to male mice (Fig. 2). This was also mirrored in sex-dependent difference in weight gain. Thus in female

mice, body weight (BW) increased by 21.0% from baseline ($p<0.0001$) and in male mice by 13.7% from baseline ($p<0.0001$) compared to female and male controls (Fig. 4a, Supplementary Table 1). Importantly, while baseline body weight was higher in male compared to female mice (Supplementary Table 1), no sex-differences in serum TT4, FT4 and FT3 concentrations were found at baseline and in the control group throughout the experiment (Fig. 2). Body weight changes in control animals were in agreement with C57BL/6 wild-type mouse growth charts published by the suppliers.

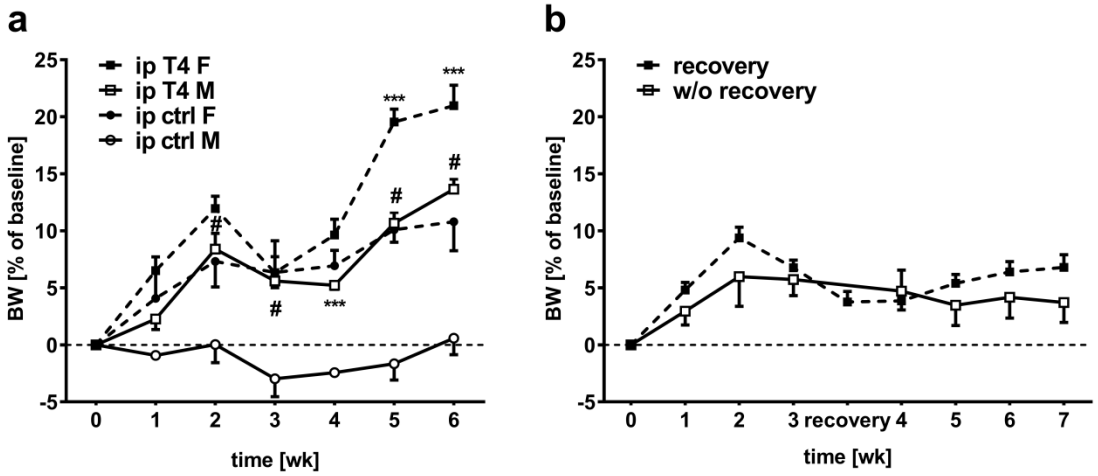

Fig. 4: Change in body weight in mice with ip and oral T4 treatment. Time course of the average body weight (BW) taken in the middle of each treatment week (wk) from (a) ip T4 treated and (b) oral T4 treated groups, as indicated, represented as the percent of baseline. Intraperitoneal T4 (ip T4, 1 µg/g BW ip T4 every 48h over 6 weeks) treatment was performed in male (M) and female (F) mice and oral T4 was supplied to male mice (oral T4, 5 µg/mL T4 in Millipore water containing 0.1% BSA continuously over first 3 weeks and 1 µg/mL over last 4 weeks). Controls received ip PBS injections (ip ctrl). In ip groups from week 2 to 3, a change in housing conditions took place in ip treated groups (change from conventional cages to individually ventilated cages for all groups). This resulted in a temporary decrease in BW in almost all animals. Data are presented as mean ±SEM, unpaired two-tailed t-test: *$p<0.05$, **$p<0.01$, ***$p<0.001$, #$p<0.0001$ (n=6 mice/sex for oral or ip T4 treatment; n=3 mice/sex as controls).

Gene expression in target tissues liver and heart confirms chronic hyperthyroid state and shows differences between ip and oral T4 administration in male mice

Changes in TH responsive genes [6, 12–16] in liver and heart of animals sacrificed at different time points after last ip or oral T4 treatment were analysed and compared to ip PBS treated controls (see Fig. 1 for experimental approach, and Supplementary Table 2 for all analyzed genes in study 1).

Changes in TH target gene expression indicating hyperthyroid state in liver and heart tissue were consistently found in ip and oral T4 treated animals at 24h after last T4 supply. Thus, in the ip group significant down-regulation of *Tbg* (0.014 fold of controls, $p<0.0001$) and up-regulation of *Dio1* (5.87 fold of controls, $p<0.0001$), *Spot14* (3.11 fold of controls, $p<0.05$) and *Me1* (2.81 fold of controls, $p<0.0001$) was found in liver tissue (Fig. 5a) and significant down-regulation of *Myh7* (0.062 fold of controls, $p<0.0001$) and up-regulation of *Hcn2* (3.771 fold of controls, $p<0.0001$) was detected in heart (Fig. 5b). Notably, no changes were found for several proposed TH target genes including *Atp2a2* and *Myh6* in heart, illustrating that these genes do not quality as markers of chronic thyrotoxicosis (Supplementary Table 3).

Comparison of liver and heart transcript levels in ip and oral T4 treated male mice showed that ip T4 treatment resulted in more pronounced effects on TH target gene expression in liver and heart compared to oral T4 treatment (Fig. 5 and 6), including up-regulation of *Spot14* and *Me1* in liver, and down-regulation of *Myh7* in heart, which were only found in ip treated mice (Fig. 5 and 6).

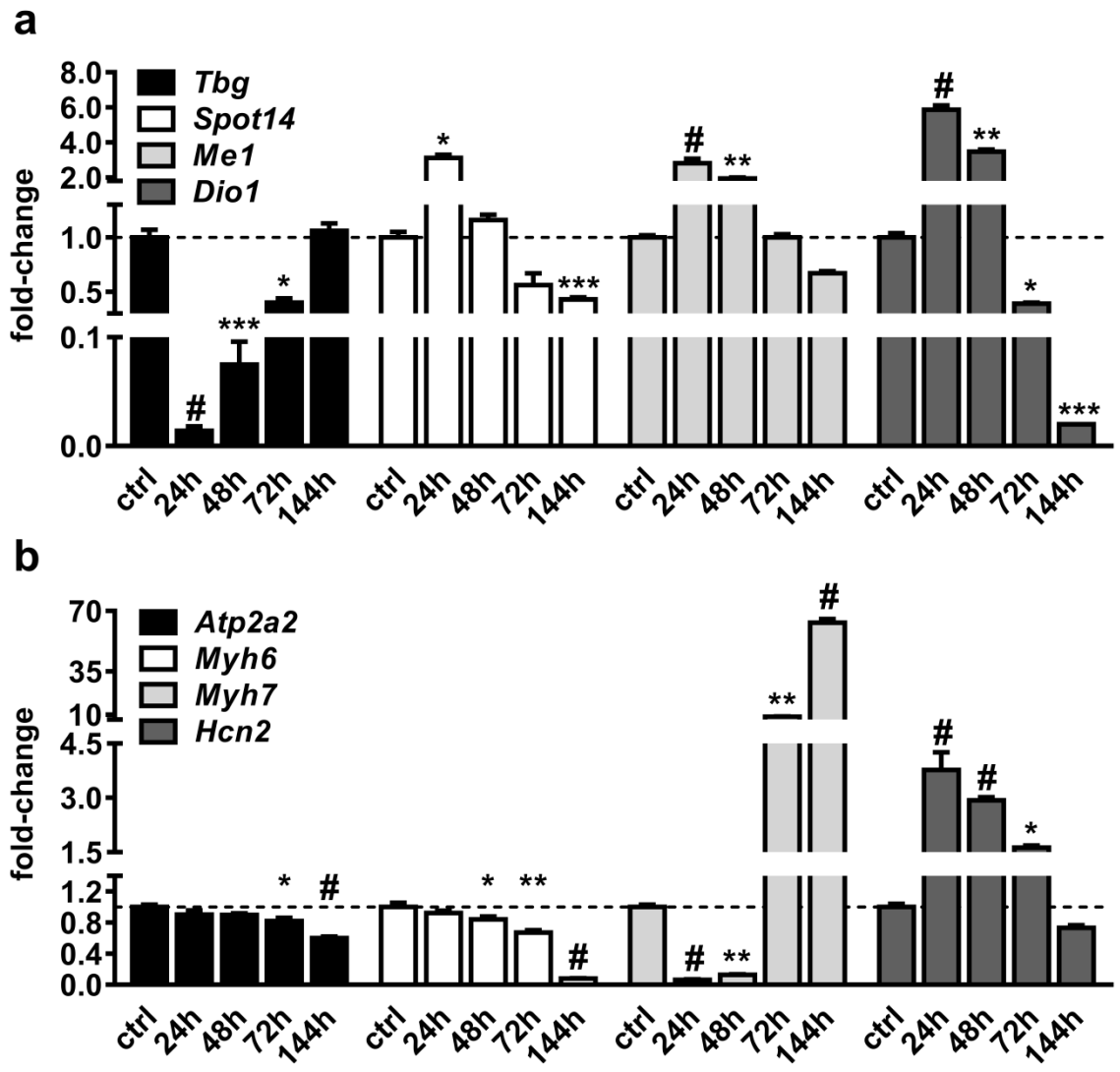

Fig. 5: Timing of T4 injection to animal sacrifice and its impact on TH responsive gene expression in (a) liver and (b) heart. (a) Expression levels of *Tbg*, *Spot14*, *Me1* and *Dio1* were measured by quantitative real-time PCR in liver tissues of mice treated for 6 weeks with intraperitoneal T4 (1 µg/g BW ip T4 every 48h) or PBS injections. Gene expression levels of mice sacrificed at 24h (n=8 mice), 48h, 72h and 144h (n=4 mice each) after the last T4 supply are shown as fold-change to PBS treated mice (ctrl, n=4-8 mice). *Rn18s*, *Ppia*, *Rpl13a* and *β-Actin* were used as reference genes. (b) Expression levels of *Atp2a2*, *Myh6*, *Myh7* and *Hcn2* were measured by quantitative real-time PCR in heart tissues of mice treated for 6 weeks with intraperitoneal T4 (1 µg/g BW ip T4 every 48h) or PBS injections. Gene expression levels in tissues of mice sacrificed at 24h (n=8 mice), 48h, 72h and 144h (n=4 mice each) after the last T4 supply are shown as fold-change to PBS treated mice (ctrl, n=4-8 mice). *Rn18s*, *Polr2a* and *Rpl32* were used as reference genes. [Data are presented

as fold-changes (mean ±SEM; efficiency-corrected ΔΔCt method, unpaired two-tailed t-test, *p<0.05, **p<0.01, ***p<0.001, #p<0.0001)].

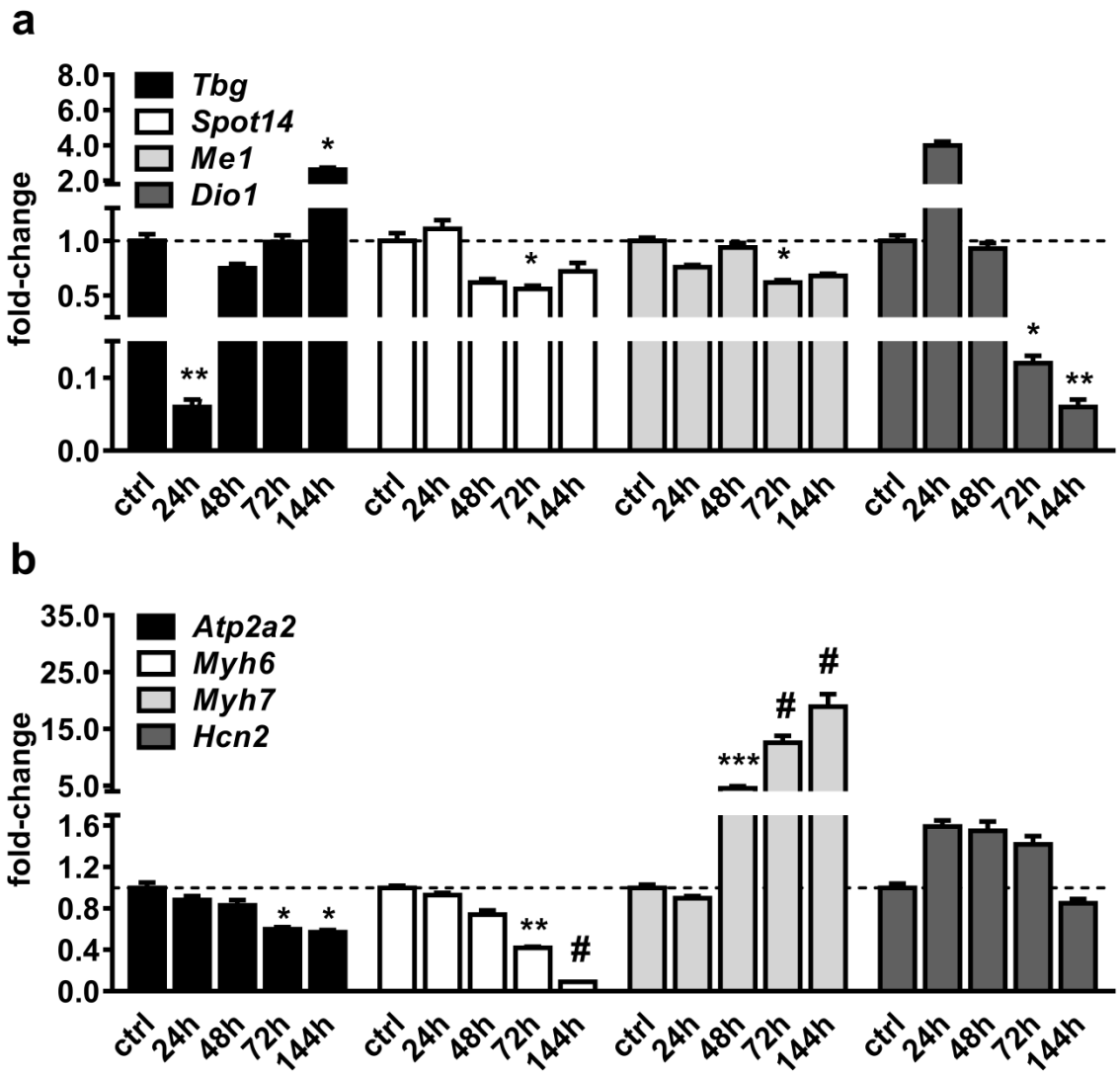

Fig.6: Timing of oral T4 administration and animal sacrifice and its impact on TH responsive gene expression in (a) liver and (b) heart. (a) Expression levels of *Tbg*, *Spot14*, *Me1* and *Dio1* were measured by quantitative real-time PCR in liver tissues of mice after continuous oral T4 treatment (5 µg/mL for the first 3 weeks and 1 µg/mL for the last 4 weeks). Gene expression levels of male mice sacrificed at 24h, 48h, 72h and 144h (n=3 mice each) after the last T4 supply are shown as fold-change to untreated controls (ctrl, n=3 mice). *Rn18s* and *Ppia* were used as reference genes. (b) Expression levels of *Atp2a2*, *Myh6*, *Myh7* and *Hcn2* were measured by quantitative real-time PCR in heart tissues of mice after continuous oral T4 supply (5 µg/mL for the first 3 weeks and 1 µg/mL for the last 4 weeks). Gene expression levels of male mice sacrificed at 24h, 48h, 72h and 144h (n=3 mice each) after the last T4 supply are shown as fold-change to untreated controls (ctrl, n=3 mice).

Rn18s and *Rpl32* were used as reference genes. [Data are presented as fold-changes (mean ±SEM; efficiency-corrected ΔΔCt method, unpaired two-tailed t-test, *p<0.05, **p<0.01, ***p<0.001, #p<0.0001)].

Sex dependent variation in T4 induced TH serum state is not reflected in target gene expression in liver and heart.

Since we found that ip T4 treatment resulted in significantly larger increases in TT4 and FT4 serum concentrations in female than male mice, we asked whether this is paralleled by sex-variation in transcript levels of identified target genes for chronic thyrotoxicosis in liver and heart. While we observed a time dependent sex-variation in *Spot14*, *Me1* and *Dio1* in liver, no sex difference was found for other investigated target genes in liver and heart of ip T4 treated male and female mice (Supplementary Table 4).

Timing of T4 administration influences organ thyroid state

To analyze the sustainability of chronic ip or oral T4 treatment on organ TH state, we performed serial mRNA expression analysis of TH responsive genes in liver and heart of mice sacrificed at 24h, 48h, 72h and 144h after cessation of ip or oral T4 supply and compared them to controls undergoing the same protocol (Fig. 1). This aspect was relevant to clarify whether after 6/7 weeks chronic treatment, stringent T4 administration during experimental assessment (e.g. behavioural testing) and for organ sacrifice e.g. before or after a weekend-break was still required.

Generally, the magnitude of T4 induced changes in target gene expression in liver and heart was larger at 24h compared to 48h and larger in ip vs. oral T4 treated mice (Fig. 5 and 6). In liver tissue, T4 induced down-regulation of *Tbg* expression and up-regulation of *Me1* was reversed reaching similar expression levels as in controls at 144h after ip T4 administration (Fig. 5). In contrast, *Dio1* expression, while up-regulated at 24 h, dropped to 0.39-fold of controls at 72h and was suppressed to 0.02-fold of controls at 144h after cessation of T4 supply (p<0.001, Fig. 5a). Furthermore, significant down-regulation of *Spot14* became apparent at 72h pertaining to 144h after last T4 supply in ip T4 treated mice (Fig. 5a)

In heart tissue, T4 induced up-regulation of *Hcn2* at 24h and 48h (Fig. 5b) normalized to control level at 144h after T4 supply (Fig. 5b). *Myh7* expression, which was down-

regulated at 24h and 48h (Fig. 5b), was increased at 72h ($p<0.01$), and even further at 144h ($p<0.0001$) after cessation of T4 supply (Fig. 5b).

Atp2a2 and *Myh6* expression in heart, while not affected by T4 treatment (Fig. 5b), was significantly down-regulated at 72h ($p<0.05$ for *Atp2a2* and $p<0.01$ for *Myh6* in ip and oral T4-treatment) and 144h after cessation of T4 supply ($p<0.0001$ for *Atp2a2* in ip and $p<0.05$ in oral treated mice; $p<0.0001$ for *Myh6*/treatment) (Fig. 5 and 6).

2.5 Discussion

In the present study we tested the efficacy of intraperitoneal vs. continuous oral T4 supply for induction of chronic hyperthyroidism. Furthermore we asked whether sex has an impact on the efficacy of chronic T4 treatment in mice.

Based on the available literature, describing ranges from 0.6 µg/g BW T4 [17] to 10 µg/g BW T4 [18] for longer-term (30 days) or short-term (8 days) treatment, respectively, we decided to administer 1 µg/g BW T4 every other day over a period of 6 weeks to induce and maintain hyperthyroidism in a mouse organism. This dose equals approximately 50 fold of daily T4 production rate, assuming that thyroidal secretion of T4 in mice is similar to that described for rats (approx. 10 ng/g BW/day) [6]. For comparison the dosage for oral T4 supply was calculated based on the weekly injected T4 amount in the ip T4 group. Here we initially assumed that the biological availability of orally ingested T4 in mice equals that described for humans, namely 65% for oral T4 intake [11]. In the literature, similar concentrations of orally supplied T4 are depicted for mice and rats, ranging from 2 µg/mL [19] to 12 µg/mL [20, 21]. T4 monotherapy was selected over T3 or T4/T3 combination therapy, since T4 has been the most widely used iodothyronine in (hyperthyroid) animal experiments, and allows for the assessment of T4 to T3 conversion in a living organism. Furthermore, levothyroxine is standard in patients requiring TH substitution.

Both ip and oral T4 treatment resulted in induction of hyperthyroidism in mice with markedly elevated serum TT4, FT4 and FT3 concentrations after 2 weeks of treatment and throughout the entire treatment period. Contrary to our expectations, oral T4 supply (5 µg/mL) was well absorbed in mice so that the dosage had to be reduced because severe thyrotoxicosis occurred. Discontinuation and/or lowering of oral T4 dosage resulted in rapid and proportional adjustment of serum TH concentrations in mice illustrating that serum TH state can be tightly manipulated by

the oral T4 dose. In summary, 1 µg/g BW T4 injection every 48h or continuous supply of 1 µg/mL T4 in drinking water led to comparable TT4, FT4 and FT3 concentrations in mice at the end of experiment and both dosages were well tolerated. On the tissue level, the magnitude of T4 induced changes in target gene expression in liver and heart was larger in ip vs. oral T4 treated mice suggesting more pronounced tissue thyrotoxicosis at time of sacrifice in the ip treated animals. For this reason we decided to compare the sustainability of tissue thyrotoxicosis in order to ensure hyperthyroid state during characterization of mice and for organ sacrifice. To this aim we performed sequential analysis of TH target gene expression in liver and heart of mice sacrificed at 24h, 48h, 72h and 144h after last ip or oral T4 treatment. We found indication for a transient hypothyroid organ state if the ip or oral T4 dosing interval exceeded 48h. A likely explanation for this observation could be a suppressed pituitary-thyroid axis in mice under chronic T4 treatment. Abrupt cessation of chronic T4 supply could hence lead to temporary secondary hypothyroidism. The strength of these effects on gene expression in target organs varied between different TH target genes in liver and heart. However, from the data it is apparent that ip T4 dosing interval in mice should not exceed 24-48h. Furthermore, we are convinced that the discontinuation of oral T4 supply prior to animal sacrifice is a likely explanation for the reduced magnitude in changes in target gene expression in oral T4 vs ip T4 treated mice since in the latter the T4 loading dose is much higher. Hence we strongly suggest that continuous T4 supply is mandatory during experimental procedure and until organ sacrifice.

Since sex could potentially impact efficacy of T4 treatment protocols for induction of hyperthyroidism, we included male and female mice in our analysis. We found that ip T4 treatment resulted in significantly higher TT4 and FT4 concentrations in female compared to male mice. We expect that this sex-difference may also hold true for oral T4 treatment, which was however not tested. Baseline TH serum status prior to start of experiments and TH status in control animals at the end of experiment did not differ for male and female mice, suggesting a sex-dependent, most likely estrogen-dependent difference in metabolism of exogenously supplied T4. Ongoing experiments in gonadectomized mice will help to clarify this issue. Of note, the sex-dependent change in TH serum status with ip T4 treatment did not proportionally transfer into TH target gene expression in liver and heart, showing that TH action in a living organism is more complex. Thus, besides biochemical assessment of serum

TH concentration, further functional and tissue specific markers need to be incorporated to assess thyroid state correctly. In this context another finding of our study was that several widely proposed TH target genes did not quality as markers of chronic organ thyrotoxicosis (Supplementary Table 3) underlining the necessity for careful selection of markers that adequately reflect tissue TH action.

In summary, intraperitoneal and oral T4 administration are reliable, safe and well-tolerated methods to induce chronic hyperthyroidism reflected by serum T4/T3 thyrotoxicosis in mice. Thereby ip T4 injection intervals should not exceed 24-48h and T4 must be continuously administered to ensure a stable hyperthyroid tissue state. Male and female mice show sex-dependent variance in hyperthyroid serum status and we propose that this may be linked to estrogen-dependent altered metabolism and/or protein binding of exogenously administered T4.

Acknowledgements

The authors are grateful to A. Jaeger and S. Rehn for their dedicated technical support.

Compliance with Ethical Standards

Ethical approval

All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. This article does not contain any studies with human participants performed by any of the authors.

Funding: This work was supported by the Deutsche Forschungsgemeinschaft (FU 356/7-1 to DF, BR 1308/11-1 to KB, KO 922/17-1 to JK, and MO 1018/2-1 to LCM) within the framework of SPP1629 “Thyroid Trans Act”.

Conflict of Interest: The authors declare that they have no conflict of interest. The authors declare no competing financial interests.

Author contributions

K.E., H.R., D.Z. and D.F. designed experiments, wrote and edited the manuscript, prepared figures; K.E., H.R., S.H., K.B. and M.R. performed experiments; all authors edited and reviewed the manuscript.

References

1. Franklyn, J.A., Sheppard, M.C., Maisonneuve, P.: Thyroid function and mortality in patients treated for hyperthyroidism. JAMA 294(1), 71–80 (2005). doi: 10.1001/jama.294.1.71

2. Klein, I., Danzi, S.: Thyroid disease and the heart. Circulation 116(15), 1725–1735 (2007). doi: 10.1161/CIRCULATIONAHA.106.678326

3. Biondi, B., Wartofsky, L.: Treatment With Thyroid Hormone. Endocrine Reviews 35(3), 433–512 (2014). doi: 10.1210/er.2013-1083

4. Meisinger, C., Ittermann, T., Wallaschofski, H., Heier, M., Below, H., Kramer, A., Doring, A., Nauck, M., Volzke, H.: Geographic variations in the frequency of thyroid disorders and thyroid peroxidase antibodies in persons without former thyroid disease within Germany. European Journal of Endocrinology 167(3), 363–371 (2012). doi: 10.1530/EJE-12-0111

5. Cooper, D.S.: Hyperthyroidism. Lancet 362(9382), 459–468 (2003). doi: 10.1016/S0140-6736(03)14073-1

6. Bianco, A.C., Anderson, G., Forrest, D., Galton, V.A., Gereben, B., Kim, B.W., Kopp, P.A., Liao, X.H., Obregon, M.J., Peeters, R.P., Refetoff, S., Sharlin, D.S., Simonides, W.S., Weiss, R.E., Williams, G.R.: American Thyroid Association Guide to investigating thyroid hormone economy and action in rodent and cell models. Thyroid 24(1), 88–168 (2014). doi: 10.1089/thy.2013.0109

7. Brix, K., Führer, D., Biebermann, H.: Molecules important for thyroid hormone synthesis and action - known facts and future perspectives. Thyroid Res 4 Suppl 1, S9 (2011). doi: 10.1186/1756-6614-4-S1-S9

8. Hammes, S.R.: Editorial: Sex matters in preclinical research. Mol. Endocrinol. 28(8), 1209–1210 (2014). doi: 10.1210/me.2014-1209

9. Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T.:

The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. Clinical Chemistry 55(4), 611–622 (2009).
doi: 10.1373/clinchem.2008.112797

10. Pfaffl, M.W.: A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29(9), e45 (2001)

11. Hasselstrom, K., Siersbaek-Nielsen, K., Lumholtz, I.B., Faber, J., Kirkegaard, C., Friis, T.: The bioavailability of thyroxine and 3,5,3'-triiodothyronine in normal subjects and in hyper- and hypothyroid patients. European Journal of Endocrinology 110(4), 483–486 (1985).
doi: 10.1530/acta.0.1100483

12. Crowe, T.C., Cowen, N.L., Loidl, N.M., Topliss, D.J., Stockigt, J.R., Barlow, J.W.: Down-regulation of thyroxine-binding globulin messenger ribonucleic acid by 3,5,3'-triiodo-thyronine in human hepatoblastoma cells. J. Clin. Endocrinol. Metab. 80(7), 2233–2237 (1995). doi: 10.1210/jcem.80.7.7608285

13. Moeller, L.C., Dumitrescu, A.M., Refetoff, S.: Cytosolic action of thyroid hormone leads to induction of hypoxia-inducible factor-1alpha and glycolytic genes. Mol. Endocrinol. 19(12), 2955–2963 (2005). doi: 10.1210/me.2004-0542

14. Moeller, L.C., Dumitrescu, A.M., Walker, R.L., Meltzer, P.S., Refetoff, S.: Thyroid hormone responsive genes in cultured human fibroblasts. J. Clin. Endocrinol. Metab. 90(2), 936–943 (2005). doi: 10.1210/jc.2004-1768

15. Flores-Morales, A., Gullberg, H., Fernandez, L., Ståhlberg, N., Lee, N.H., Vennström, B., Norstedt, G.: Patterns of liver gene expression governed by TRbeta. Mol. Endocrinol. 16(6), 1257–1268 (2002). doi: 10.1210/mend.16.6.0846

16. Feng, X., Jiang, Y., Meltzer, P., Yen, P.M.: Thyroid hormone regulation of hepatic genes in vivo detected by complementary DNA microarray. Mol. Endocrinol. 14(7), 947–955 (2000). doi: 10.1210/mend.14.7.0470

17. Klecha, A.J., Genaro, A.M., Gorelik, G., Barreiro Arcos, M.L., Silberman, D.M., Schuman, M., Garcia, S.I., Pirola, C., Cremaschi, G.A.: Integrative study of hypothalamus-pituitary-thyroid-immune system interaction: thyroid hormone-mediated modulation of lymphocyte activity through the protein kinase C signalling pathway. J. Endocrinol. 189(1), 45–55 (2006). doi: 10.1677/joe.1.06137

18. Reed, T.D., Babu, G.J., Ji, Y., Zilberman, A., Heyen, M. ver, Wuytack, F., Periasamy, M.: The expression of SR calcium transport ATPase and the

Na(+)/Ca(2+)Exchanger are antithetically regulated during mouse cardiac development and in Hypo/hyperthyroidism. J. Mol. Cell. Cardiol. 32(3), 453–464 (2000). doi: 10.1006/jmcc.1999.1095

19. Ferreira E., Silva A.E., Serakides R., Gomes A.E.S., Cassali G.D.: Model of induction of thyroid dysfunctions in adult female mice. Arquivo Brasileiro de Medicina Veterinária e Zootecnia, 1245–1249

20. Wilcoxon, J.S., Nadolski, G.J., Samarut, J., Chassande, O., Redei, E.E.: Behavioral inhibition and impaired spatial learning and memory in hypothyroid mice lacking thyroid hormone receptor α. Behavioural Brain Research 177(1), 109–116 (2007).
doi: 10.1016/j.bbr.2006.10.030

21. Messarah, M., Boumendjel, A., Chouabia, A., Klibet, F., Abdennour, C., Boulakoud, M.S., Feki, A.E.: Influence of thyroid dysfunction on liver lipid peroxidation and antioxidant status in experimental rats. Experimental and Toxicologic Pathology 62(3), 301–310 (2010).
doi: 10.1016/j.etp.2009.04.009

Supplemental Tables

Supplemental Table 1. Mean body weight (BW) in g of ip groups at start of experiments (BW start) and after six weeks of T4 or control treatment (BW final).

Male and female mice were subjected to intraperitoneal T4 (**ip T4**, 1 µg/g BW ip T4 every 48h over 6 weeks). Controls received ip PBS injections (**ip ctrl**) over 6 weeks. Data are presented as mean ±SEM.

treatment	ip T4		ip ctrl	
sex	**male (n=6)**	**female (n=6)**	**male (n=3)**	**female (n=3)**
BW start	26.8 ± 0.6	19.3 ± 0.2	26.7 ± 0.2	21.8 ± 1.4
BW final	30.5 ± 0.7	23.4 ± 0.5	26.9 ± 0.2	24.1 ± 1.1
BW gain	3.7 ± 0.9	4.1 ± 0.5	0.2 ± 0.3	2.3 ± 1.8

Supplemental Table 2. Oligonucleotides used for amplification of house-keeping genes and putative TH responsive genes in RT-PCR.

gene	Accession	forward primer	reverse primer
Rn18s	NR_003278.3	CGGCTACCACATCCAAGGAA	GCTGGAATTACCGCGGCT
Ppia	NM_008907.7	CTTGGGCCGCGTCTCCTTCG	GCGTGTAAAGTCACCACCCTG
Rpl13a	NM_009438.5	GGGCAGGTTCTGGTATTGGA	GGGGTTGGTATTCAATCCGCT
β-Actin	NM_007393.3	CTGTCGAGTCGCGTCCA	TCATCCATGGCGAACTGGTG
Polr2a	NM_001291068.	CTTTGAGGAAACGGTGGATGTC	TCCCTTCATCGGGTCACTCT
Rpl32	NM_172086.2	CACCAGTGAGACCGATATGTGAA	TGTTGTCAATGCCTCTGGGTTT
Tbg	NM_177920.5	TGGGCATGTGCTATCATCTTCA	GAGTGGCATTTTGTTGGGGC
Spot14	NM_009381.2	GAGGTGACGCGGAAATACCA	TGTCCAGGTCTCGGGTTGAT
Me1	NM_001198933.	TAAGGGTCGTGCATCTCTCAC	TGCAGCAACTCCTATGAGGG
Dio1	NM_007860.3	GGGCAGGATCTGCTACAAGG	CGTGTCTAGGTGGAGTGCAA
Klf9	NM_010638.4	GAAACACGCCTCCGAAAAGAG	AGCGCGAGAACTTTTTAAGGC
Hif1α	NM_010431.2	GTGACCATGAGGAAATGAGAGAA	CACGTTGCTGACTTGATGTTCA
Fasn	NM_007988.3	TGCCCGAGTCAGAGAACCTA	TAGAGCCCAGCCTACATCT
Bcl3	NM_033601.3	CTGAACCTGCCTACTCACCC	AGTATTCGGTAGACAGCGGC
Slc27a	NM_011978.2	GTCACTTTTACCACGCCAGC	GCCGTGATTCCACTCGACAT
Slc2a1	NM_011400.3	TCTTCGAGAAGGCAGGTGTG	CGCTCTACAACAAACAGCGAC
St6gal	NM_145933.4	CCATCCGCCTAGTGAACTCTC	TCTGGCTTCTGATACCACTGC
Igfbp1	NM_008341.4	TGCCAAACTGCAACAAGAATGG	CCAGGGATTTTCTTTCCACTCC
Adrb2	NM_007420.2	ACTTCTGGTGCGAGTTCTGG	GCTCTGGTACTTGAAGGGCG
Pfkl	NM_008826.4	ACCTCTCATGGAGTGTGTGC	GGTGGGCAAGGAGCTTGTAA
Rcan2	NM_207649.1	GAAGCCTATCAGCGATGCCA	CTGTCACACACATGAACCAC
Uqcrh	NM_025641.3	CTACTCTGGTTGCGCTTGTTG	CACTGTTGTTAGGGGGTCCA
Mlxipl	NM_021455.4	GGACAAGATCCGGCTGAACA	CAGGTTTCCGGTGCTCATCT
Atp2a2	NM_001110140.	AACTACCTGGAACAACCCGC	TCATGCAGAGGGCTGGTAGA
Myh6	NM_010856.4	CAGACAGAGATTTCTCCAACCCA	GCCTCTAGGCGTTCCTTCTC
Myh7	NM_080728.2	CACGTTTGAGAATCCAAGGCTC	CTCCTTCTCAGACTTCCGCA
Hcn2	NM_008226.2	CCAGTCCCTGGATTCGTCAC	TCACAATCTCCTCACGCAGT

House-keeping genes: ***Rn18s*****:** 18S ribosomal RNA; ***Ppia***: peptidylprolyl isomerase A; ***Rpl13a***: ribosomal protein L13A; **β-*Actin***: beta-actin; ***Polr2a***: polymerase (RNA) II (DNA directed) polypeptide A; ***Rpl32***: ribosomal protein L32; **putative TH target genes in liver**: ***Tbg****:* thyroxine binding globulin; ***Dio1****:* deiodinase 1; ***Spot14****:* thyroid hormone-responsive Spot14 homolog; ***Me1***: malic enzyme 1; ***Klf9***: Kruppel-like factor 9; ***Hif1α***: hypoxia-inducible factor 1 alpha; ***Fasn***: fatty acid synthase; ***Bcl3***: B cell leukemia/lymphoma 3; ***Slc27a2***: solute carrier family 27 (fatty acid transporter) member 2; ***Slc2a1****:* solute carrier family 2 (facilitated glucose transporter) member 1 (GLUT-1); ***St6gal1***: beta galactoside alpha 2,6 sialyltransferase 1; ***Igfbp1****:* insulin-like growth factor binding protein 1; ***Adrb2***: adrenergic receptor; ***Pfkl***: beta 2, phosphofructokinase, liver, B-type; ***Rcan2****:* regulator of calcineurin 2; ***Uqcrh***: ubiquinol-cytochrome c reductase hinge protein; ***Mlxipl***: MLX interacting protein-like;

putative TH target genes in heart: ***Atp2a2****:* sarcoplasmic/endoplasmic reticulum Ca^{2+} - ATPase; ***Myh6****:* myosin, heavy polypeptide 6; ***Myh7****:* myosin, heavy polypeptide 7; ***Hcn2***: hyperpolarization-activated, cyclic nucleotide-gated K^+ 2.

Supplemental Table 3. Changes in mRNA expression of putative TH responsive genes in liver and heart tissues of animals receiving ip T4 treatment compared to controls.

Animals were sacrificed at 48h after last ip T4 or control treatment.

organ	gene	fold-change ip T4/ ip control	Expected regulation in hyperthyroidism
liver	*Tbg*	0.07 ± 0.02	↓
	Spot14	1.16 ± 0.05	↑
	Me1	1.94 ± 0.06	↑
	Dio1	3.48 ± 0.12	↑
	Klf9	1.51 ± 0.10	↑
	Hif1α	1.30 ± 0.06	↑
	Fasn	0.70 ± 0.05	↑
	Bcl3	0.96 ± 0.09	↑
	Slc27a2	1.34 ± 0.08	↑
	Slc2a1	1.33 ± 0.13	↑
	St6gal1	0.87 ± 0.06	↓
	Igfbp1	3.62 ± 0.53	↑
	Adrb2	3.24 ± 1.22	↑
	Pfkl	0.95 ± 0.10	↑
	Rcan2	1.56 ± 0.17	↑
	Uqcrh	0.94 ± 0.05	↑
	Mlxipl	0.60 ± 0.04	↑
heart	*Atp2a2*	0.90 ± 0.02	↑
	Myh6	0.84 ± 0.04	↑
	Myh7	0.13 ± 0.01	↓
	Hcn2	2.93 ± 0.09	↑

Supplemental Table 4. Changes in mRNA expression of putative TH responsive genes in liver and heart tissues of male or female mice with ip T4 treatment (1 µg/g BW T4 every 48h over 6 weeks) compared to male or female controls (ip PBS injections every 48h over 6 weeks). Animals were sacrificed at 24h or 48h after last ip T4 treatment, as indicated.

organ	gene	fold-change ip T4 / ip control			
		male (n=4) 24h ip T4	female (n=4) 24h ip T4	male (n=2) 48h ip T4	female (n=2) 48h ip T4
liver	*Tbg*	0.01 ± 0.003	0.02 ± 0.004	0.08 ± 0.01	0.06 ± 0.03
	Spot14	1.83 ± 0.08	5.29 ± 0.25	1.53 ± 0.09	0.90 ± 0.03
	Me1	1.75 ± 0.07	4.50 ± 0.69	2.57 ± 0.13	1.51 ± 0.07
	Dio1	6.02 ± 0.16	5.73 ± 0.25	5.31 ± 0.18	2.81 ± 0.09
heart	*Atp2a2*	0.85 ± 0.02	0.95 ± 0.05	0.92 ± 0.02	0.89 ± 0.03
	Myh6	0.83 ± 0.02	1.02 ± 0.03	0.94 ± 0.05	0.78 ± 0.05
	Myh7	0.08 ± 0.01	0.05 ± 0.005	0.06 ± 0.002	0.27 ± 0.01
	Hcn2	4.42 ± 0.54	3.22 ± 0.41	3.42 ± 0.09	2.56 ± 0.11

Chapter 3: Sex-specific phenotypes of hyperthyroidism and hypothyroidism in mice

Helena Rakov[1*], Kathrin Engels[1*], Georg Sebastian Hönes[1], Karl-Heinz Strucksberg[2], Lars Christian Moeller[1], Josef Köhrle[3], Denise Zwanziger[1], Dagmar Führer[1#]

[1] *Department of Endocrinology and Metabolism, University Hospital Essen, University Duisburg- Essen, Essen, Germany*

[2]*Department of Endocrinology and Metabolism, Clinical Chemistry – Division of Laboratory Research, University Hospital Essen, University Duisburg- Essen, Essen, Germany*

[3]*Charité-Universitätsmedizin Berlin, Institute of Experimental Endocrinology, Berlin, Germany*

*HR and KE contributed equally to this work.

[#]corresponding author

Biol Sex Differ. 2016 Aug 24;7(1):36. doi: 10.1186/s13293-016-0089-3

I have written the manuscript and performed the following experiments: measurements of TH and lipid serum concentrations (Fig. 5), open field testing (Fig. 4 b,c) and analysis of all data with regard to sex-difference. Study design, treatment of mice and all other experiments (Fig. 2, 3, 4 a, d-f, 6) were performed together with Kathrin Engels.

3.1 Abstract

Background: Thyroid dysfunction is more common in the female population, however the impact of sex on disease characteristics has rarely been addressed. Using a murine model, we asked whether sex has an influence on phenotypes, thyroid hormone status and thyroid hormone tissue response in hyper- and hypothyroidism.

Methods: Hypo- and hyperthyroidism were induced in 5 months old female and male wildtype C57BL/6N mice, by LoI/MMI/ClO_4^- or T_4 ip treatment over 7 weeks, control animals underwent sham treatment (N=8 animals/sex/treatment). Animals were investigated for impact of sex on body weight, food and water intake, body temperature, heart rate, behaviour (locomotor activity, motor coordination and strength), liver function, serum thyroid hormone status and cellular TH effects on gene expression in brown adipose tissue, heart and liver.

Results: Male and female mice showed significant differences in behavioural, functional, metabolic, biochemical and molecular traits of hyper- and hypothyroidism. Hyperthyroidism resulted in increased locomotor activity in female mice, but decreased muscle strength and motor coordination preferably in male animals. Hypothyroidism led to increased water intake in male but not female mice and significantly higher serum cholesterol in male mice. Natural sex-differences in body temperature, body weight gain, food and water intake were preserved under hyperthyroid conditions. In contrast, natural sex-differences in heart rate disappeared with TH excess and deprivation. The variations of hyper- or hypothyroid traits of male and female mice were not explained by classical T_3/T_4 serum state. TH serum concentrations were significantly increased in female mice under hyperthyroidism, but no sex-differences were found under eu- or hypothyroid conditions. Interestingly, analysis of expression of TH target genes and TH transporters revealed little sex-dependency in heart, while sex-differences in target genes were present in liver and brown adipose tissue in line with altered functional and metabolic traits of hyper- and hypothyroidism.

Conclusions: These data demonstrate that the phenotypes of hypo- and hyperthyroidism differ between male and female mice and indicate that sex is an important modifier of phenotypic manifestations.

Keywords: thyroid hormone transport, thyroid hormone action, hyperthyroidism, hypothyroidism, sex-difference, sex steroid hormone, mice

3.2 Background

Thyroid dysfunction, i.e. hyper- or hypothyroidism, occurs with 2-9 fold higher prevalence in women [1], yet besides fertility aspects and bone metabolism, a possible impact of gender on disease characteristics has not been well studied, neither in the clinical setting nor in epidemiological cohorts.

Experimental approaches using murine animal models to study thyroid hormone (TH) action so far mostly include mice of only one sex or, occasionally, do not even specify the mouse sex. A momentum calling for increased awareness of sex-impact on manifestation, prognosis and treatment of diseases was published in Nature in 2014 and was afterwards re-emphasized by the Endocrine Society [2, 3]. Furthermore, the “Guide to investigating thyroid hormone economy and action in rodent and cell models analysis of TH action“ published by the American Thyroid Association in 2013 advised to study male and female rodents separately as the response to TH may be sexually dimorphic [4].

In view of the clinical situation where both women and men are affected by hyper- and hypothyroidism and consequences of disease may be gender-specific, we decided to employ a murine animal model, in which TH function status can be easily manipulated, for a comprehensive characterization of sex-impact on TH action. Hence male and female mice were studied for changes in behavioural, functional, metabolic and biochemical markers in addition to analysis of cellular TH effects on gene expression in selected target organs brown adipose tissue (BAT), heart and liver under conditions of TH excess and deprivation.

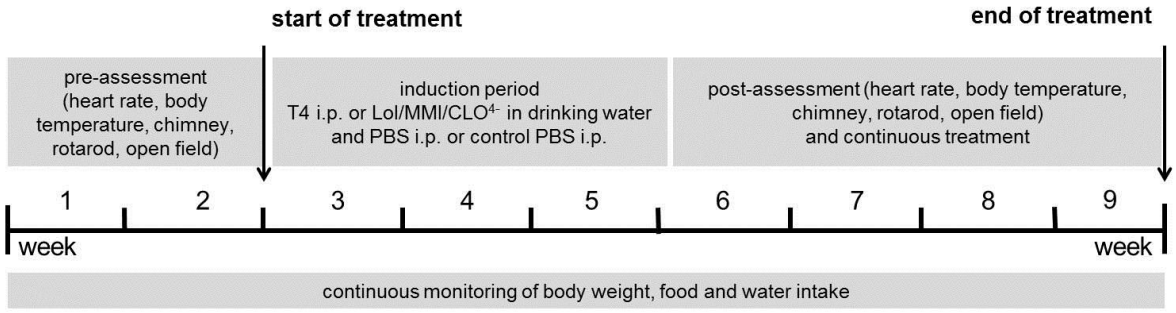

Fig. 1: Study design for phenotypic characterization of hyper- and hypothyroidism in female and male mice. Two weeks were used to study the euthyroid control state of male and female mice (run-in period), followed by 3 weeks induction period without experiments other than monitoring body weight, food and water intake. After the induction period, male

and female mice were analysed under hyper- and hypothyroid conditions over a period of 4 weeks and compared to sham treated controls.

3.3 Materials and Methods

Animals and study design

Male and female C57BL/6NTac (N=8/sex/treatment; Taconic Europe A/S, Denmark) mice aged 5 months were 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). All efforts were made to minimize suffering.

The nine-week experimental period was divided into three parts (Fig. 1), consisting of a 2-week run-in period prior to manipulation of thyroid status to phenotypically characterize each individual mouse (pre-assessment), a 3-week treatment period to induce hyper- or hypothyroidism, and a 4-week assessment period to repeat the phenotypic characterization of each individual animal under chronic TH manipulation or euthyroid control treatment.

Chronic hyperthyroidism and hypothyroidism were induced as previously described [5, 6]. Briefly, for hyperthyroidism i.p. injections of 1 µg/g body weight T_4 (Sigma-Aldrich (T2376), USA) were performed every 48h. For induction of chronic hypothyroidism, animals were fed a low-iodine diet (LoI; TD.95007, Harlan Laboratories, USA) and received drinking water supplemented with 0.02% methimazole (MMI, Sigma-Aldrich (301507), USA), 0.5% sodium perchlorate (ClO_4^-) (Sigma-Aldrich (310514), USA) and 0.3% saccharine as sweetener (Sigma-Aldrich (240931), USA) (LoI/MMI/ClO_4^-). In addition, hypothyroid animals received i.p. injections of PBS every 48 hours. Control animals were fed a control diet and received i.p. injections of PBS every 48 hours.

Blood sample collection, serum TH and cholesterol measurements

Final blood samples were stored 30 min on ice, centrifuged and free triiodothyronine (fT_3), free thyroxine (fT_4) and total T4 (TT_4) concentrations in serum of mice were measured using commercial ELISA kits according to the manufacturer's instructions (DRG Instruments GmbH, Marburg, Germany). Detection limits were 0.5 µg/dL, 0.05 ng/dL and 0.05 pg/mL for TT_4, fT_4 and fT_3, respectively. Serum TSH was measured with a sensitive, heterologous, disequilibrium double-antibody precipitation

radioimmunoassay with a detection limit of 10mU/L [7] (kindly performed by the laboratory of Prof. Refetoff at the University of Chicago, Chicago, USA). Total cholesterol concentrations in serum were detected using an enzymatic cholesterol quantitation kit (Sigma-Aldrich (MAK043), USA) according to the manufacturer's instructions. Total triglyceride concentrations in serum were detected using an enzymatic serum triglyceride determination kit (Sigma-Aldrich (TR0100), USA) according to the manufacturer´s instructions.

Monitoring of body weight, food and water intake

Body weight was measured two to three times a week by placing mice on a scale. Food consumption was determined once a week by measuring the weight of remaining food pellets in the metal cage top. Water intake was controlled by weighting of water bottles (for all to the nearest 0.1g) weekly or twice per week in control and TH manipulated groups. Male mice were caged individually, while female mice were kept in groups of three to four animals. Thereby food and water intake of female mice was calculated by dividing the measured intake by the number of animals in each cage.

For each mouse, average daily food and water intake was calculated, and adjustments for body weight were derived by dividing average intake by the average body weight, and multiplying the result by 40 g.

Measurement of body temperature

Body temperature of mice was assessed four times using a cream-covered rectal probe (RET-3 rectal probe for mice, Kent Scientific Corporation, USA) connected to a thermocouple thermometer (Acorn Temp JKT Thermocouple Meter, Kent Scientific Corporation, USA). Mice were placed on the top of the cage and the rectal probe was carefully inserted 2 cm into the rectum until a steady temperature was measured, which took approximately 8 to 10 sec.

Measurement of heart rate

Non-invasive restrained ECG recording was performed using an in-house protocol [8]. Conscious mice were placed on a platform with their paws on silver electrodes and were restrained by a half-tunnel. Signal was derived, enhanced and digitalized (Picoscope 2204, Pico Technology, United Kingdom). ECG was recorded using Picoscope 6 Software over 60-90 sec and heart rate was determined by

measurements of RR intervals over 8 sec in a stable steady state. Non-invasive restrained ECG was recorded 3 times in all animals.

Collection of organs

As previously described [5, 6] animals were euthanized 24 hours after the last T_4 treatment or continuous TH deprivation. Liver, heart and BAT were isolated and stored at -80 °C until further processing.

Isolation of RNA, cDNA synthesis and real-time PCR

RNA extraction was performed as previously described [5, 6]. Briefly, total RNA was extracted using RNeasy mini kit (Qiagen, Germany) and reversed transcribed using SuperScript III First-Strand Synthesis System for RT-PCR according to instruction manuals (Life Technologies, Germany). Exon spanning primers for amplification of TH responsive genes (suppl. table 1) were designed using PrimerBlast (NCBI) and synthesized by Eurofins (Eurofins MWG Synthesis, Germany). Quantitative real-time PCR (qRT-PCR) was performed using LightCycler® DNA Master SYBR Green I and the LightCycler®480 System (Roche, Germany). The PCR program consisted of an initial denaturation step (5 min at 95 °C) and 40 amplification cycles with 15 sec at 95 °C, 10 sec at 60 °C, 20 sec at 72 °C.

For normalization of gene expression reference genes *18S*, *Ppia* (peptidylprolyl isomerase A, cyclophilin A) and *Rpl13 a* (ribosomal protein L13a) for liver, *18S*, *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase) and *Polr2a* (polymerase RNA II) for heart and *18S*, *Ppia* and *Gapdh* for BAT were used. The following genes were studied as TH responsive: *Dio1* (deiodinase 1), *Dio2* (deiodinase 2), *Tbg* (thyroxine-binding globulin), *Me1* (malic enzyme 1), *Myh6* (myosin heavy chain 6), *Hcn4* (hyperpolarization activated cyclic nucleotide gated potassium channel 4), *Ucp1* (uncoupling protein 1) and *Pgc1α* (peroxisome proliferator-activated receptor gamma coactivator 1-alpha) [4, 9-11]. To calculate the relative quantification ratio an efficiency corrected calculation model was used [12-14].

Behavioural tests

Rotarod test

The rotarod test [15] basically consists of five 3 cm diameter cylinders, enabling five mice to be tested simultaneously. On the first testing day, mice were allowed to acclimate to the rotarod test by letting them walk 6 min on the rotating cylinder with constant acceleration from 2-20 rpm.

For each rotarod session mice were subjected to 4 trials, with a minimum resting time interval of 6 min between the trials. Rotation mode was switched to constant acceleration from 4-50 rpm within 5 min. Maximum time and speed mastered by the animal was recorded. Mice that fell off the rod or attained full speed were placed back to their home cages. Every animal was subjected to two rotarod sessions (with a suspension period of 7 days) each before and after induction of thyroid dysfunction (4 sessions with 16 trials in total). Sessions 2 and 4 were used for statistical analysis.

Chimney test

The chimney test is constituted of a plastic tube (length 30 cm, diameter 3 cm). Mice were placed inside the tube and allowed to reach the other end. Then the tube was turned into a vertical position with mice head upside down. The test consisted of determining the time taken by mice to climb up to 25 cm of height. Mice were given 90 sec of time to pass the test [16, 17].

Open Field

Open field consisted of a closed square area made of Plexiglas (50 x 50 cm). The area was divided in 4 corners, 4 walls and a center region (16 x 16 cm). Animals were tested in the dark phase of their dark/light cycle. Mice were placed in the center of the open field and allowed to move freely for 5 min. Movements were monitored and digitalized by VideoMot2 software. Software recorded entries in all areas including time, frequency, latency and distance. Occurring events of rearing, freezing, grooming and jumping were recorded manually by the investigator during the experiment. Mice were placed in their home cages after 5 min of exploring the area.

Statistical analysis

All data are shown as means ± standard deviation (SD) or standard error of the mean (SEM), as indicated. Statistical analysis using GraphPad Prism 6 Software was performed. Two-way Anova was used to compare more than two groups, followed by the Bonferroni post-hoc analysis. The effects of both thyroid dysfunctions are often opposing and inclusion of both treatments would therefore always show a significant treatment effect. To prevent false positive results statistical analysis of treatment groups were performed separately for hyper- and hypothyroid groups. Unpaired Student´s t-test to compare differences between two groups was applied. Values of $*p<0.05$, $**p<0.01$, $***p<0.001$ were considered statistically significant.

3.4 Results

Sex modifies the impact of TH on body weight, food and water consumption, body temperature and heart rate in hyper- and hypothyroid state

Male mice showed significantly increased body weight (BW) compared to female mice under euthyroid and hyperthyroid conditions (Fig. 2A, B). For euthyroid mice highest Δ BW was observed in week 9, m: <16.5% and f: <5.9% *(*$F_{(17,252)}$=9.003, p<0.0001 for time effect and $F_{(1,252)}$=67.18, p<0.0001 for sex effect, interaction: $F_{(17,252)}$=4.065, p<0.0001). Similarly, for hyperthyroid mice highest Δ BW was observed in week 9, m: <29.6% vs f: <16.5% ($F_{(17,252)}$=33.43, p<0.0001 for time effect and $F_{(1,252)}$=88.28, p<0.0001 for sex effect, interaction $F_{(17,252)}$=3.966, p<0.0001). In contrast sex-difference in body weight gain disappeared in hypothyroidism except for occasional time points in week 3, 5 and 9 (highes tΔ BW m: ~2% at week 9 vs f: ~0.7% at week 9, $F_{(17,252)}$=2.055, p=0.0093 for time effect and $F_{(1,252)}$=52.98, p<0.0001 for sex effect with no interaction $F_{(17,252)}$=1.614, p=0.0605, Fig. 2C).

Euthyroid female mice consumed more food (m: ~4.5 vs f: ~5 g/g BW*40g) and water (m: ~4.8 vs f: ~6 ml/g BW*40g) than male mice (Fig. 2D, G; food intake: $F_{(8,126)}$=61.77, p<0.0001 for time effect and $F_{(1,126)}$=121.7, p<0.0001 for sex effect, interaction: $F_{(8,126)}$=3.469, p=0.0012; water intake: $F_{(8,126)}$=31.92, p<0.0001 for time effect and $F_{(1,126)}$=385.3, p<0.0001 for sex effect, interaction: $F_{(8,126)}$=3.342, p=0.0017). T_4 administration enhanced food (m: ~5.5 vs f: ~6.5 g/g BW*40g) and water intake (m: ~6 vs f: ~7.5 ml/g BW*40g) in both sexes, again significantly more pronounced in female mice (Fig. 2E, H; food intake: $F_{(8,126)}$=11.56, p<0.0001 for time effect and $F_{(1,126)}$=78.90, p<0.0001 for sex effect, interaction: $F_{(8,126)}$=5.721, p<0.0001, water intake: $F_{(8,126)}$=7.898, p<0.0001 for time effect and $F_{(1,126)}$=90.29, p<0.0001 for sex effect, interaction: $F_{(8,126)}$=3.170, p=0.0026). Hypothyroidism abolished sex-difference in food intake (m: ~5 vs f: ~4.8 g/g BW*40g, $F_{(8,126)}$=9.004, p<0.0001 for time effect and $F_{(1,126)}$=15.25, p=0.0002 for sex effect, interaction: $F_{(8,126)}$=9.393, p<0.0001, Fig. 2F) and reversed sex-difference in water consumption with female mice showing significantly less water intake (m: ~6.8 vs f: ~4 ml/g BW*40g, $F_{(8,126)}$=13.55, p<0.0001 for time effect and $F_{(1,126)}$=90.96, p<0.0001 for sex effect, interaction: $F_{(8,126)}$=34.92, p<0.0001 , Fig. 2I).

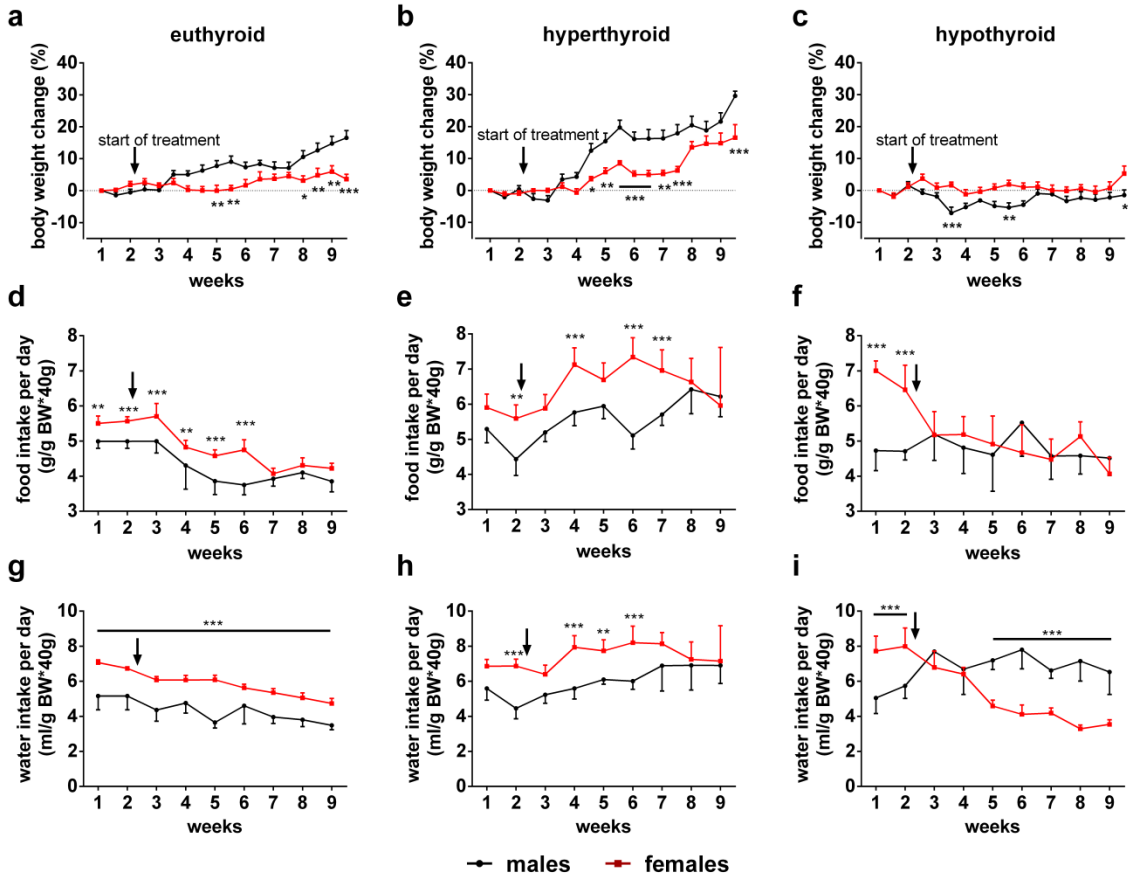

Fig. 2: Body weight change, food and water intake in euthyroid, T_4 or LoI/MMI/ClO_4^- treated mice. Time course of the average body weight (BW) of male and female mice over an experimental period of 9 weeks under (**A**) control, (**B**) T_4 and (**C**) LoI/MMI/ClO_4^- treatment. Average food intake was related to BW during experiment in (**D**) euthyroid, (**E**) hyperthyroid and (**F**) hypothyroid conditions in mice of both sexes. After run-in period mice were placed on low iodine diet for induction of hypothyroidism, or on control iodine diet by the same supplier to adapt the nutritional intake (euthyroid and hyperthyroid groups). Arrows indicate start of treatment. Time course of average water intake was monitored over the experimental period of 9 weeks under (**G**) control, (**H**) TH excess and (**I**) TH deprivation of male and female mice. Data are presented as mean ± SD, N = 8 animals/sex/treatment/time point; 2-way ANOVA followed by Bonferroni post-hoc analysis applied for time and sex effects, *p<0.05, **p<0.01, ***p<0.001 above graph represent multiple-testing results.

Body temperature, measured by a rectal probe, was higher in euthyroid female compared to male mice (m: ~37.5 vs f: ~38.2°C, p<0.05). This sex difference persisted during T_4 administration (m: ~38.1 vs f: ~38.8°C, p<0.05, $F_{(1,28)}$=21.23, p<0.0001 for sex effect, $F_{(1,28)}$=16.50, p=0.0004 for treatment effect and $F_{(1,28)}$=0.04857, p=0.8272 for interaction) and LoI/MMI/ClO_4^- treatment (m: ~37.2 vs f:

~38.2°C, p<0.01, $F_{(1,28)}$=25.77, p<0.0001 for sex effect, $F_{(1,28)}$=0.9667, p=0.3339 for treatment effect and $F_{(1,28)}$=0.7794, p=0.3848 for interaction, Fig. 3A). Interestingly, a drop in body temperature was only observed in male but not female hypothyroid mice compared to euthyroid controls.

Non-invasive ECG measurements were performed to investigate the influence of TH on heart rate (HR). Euthyroid female animals showed higher HR than male mice (m: ~704 vs f: ~738 bpm, p<0.05). Sex-difference in HR disappeared with TH excess (m: ~770 vs f: ~782 bpm, $F_{(1,28)}$=5.837, p=0.0225 for sex effect, $F_{(1,28)}$=32.65, p<0.0001 for treatment effect and $F_{(1,28)}$=1.306, p=0.2628 for interaction) or deprivation (m: ~564 vs f: ~577 bpm, $F_{(1,28)}$=5.586, p=0.0253 for sex effect, $F_{(1,28)}$=227.2, p<0.0001 for treatment effect and $F_{(1,28)}$=1.099, p=0.3034 for interaction, Fig. 3B).

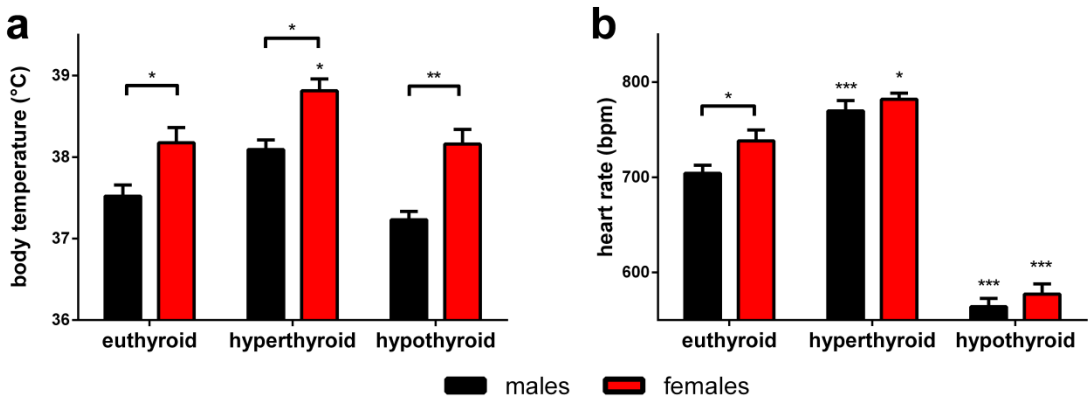

Fig. 3: Influence of sex and change of TH serum concentrations on body temperature and heart rate. (**A**) Body temperature was assessed by rectal temperature measurements and (**B**) non-invasive ECG was performed on conscious mice of both sexes under euthyroid, hyperthyroid and hypothyroid conditions. Data are presented as mean ± SD, N= 8 animals/sex/treatment; 2-way ANOVA followed by Bonferroni post-hoc analysis applied for treatment and sex effects, *p<0.05, **p<0.01, ***p<0.001 above bars represent multiple-testing results.

Male mice show pronounced impairment of muscle function and coordination while female mice exhibit increased activity under TH excess

Muscle strength, tonus and coordination of movements were examined by the chimney test. In general, female mice showed better performance in climbing up the tube than male mice (m: ~26.57 vs f: ~7.4 sec, Fig. 4A). Hyper- and hypothyroidism resulted in decrease of muscle strength and coordination in female, but even more strikingly in male mice (m: ~80.13 vs f: ~27.11 sec, p<0.001, $F_{(1,28)}$=23.94, p<0.0001

for sex effect, $F_{(1,28)}$=24.66, p<0.0001 for treatment effect and $F_{(1,28)}$=5.266, p=0.0294 for interaction (hyper) and m: ~60.71 vs. f: ~9.97 sec, p<0.05, $F_{(1,28)}$=19.23, p=0.0001 for sex effect, $F_{(1,28)}$=5.3, p=0.029 for treatment effect and $F_{(1,28)}$=3.923, p=0.0575 for interaction (hypo)). Of note, performance in the chimney test was more impaired under TH excess than TH deprivation.

To investigate changes in activity and exploration behaviour an open field test was used. Overall activity was measured by total distance travelled, while exploration was quantified by the frequency of rearing events. Control animals showed no sex difference in open field parameters. Interestingly, T_4 excess resulted in increased activity and exploratory behaviour in only female mice (m: Δ~257.9 cm vs f: Δ~1243.2 cm, p<0.001 and m: Δ~8.6 vs f: Δ~16.6 counts, p=0.05), whereas LoI/MMI/ClO_4^- treatment led to a decreased activity in male mice only (m: Δ~ 805.4 cm, vs f: Δ~227.2 cm, p<0.01, Fig. 4B, C). Sex effect under hyperthyroidism was not significant ($F_{(1,28)}$=3.164, p=0.0861 for activity, $F_{(1,28)}$=0.6493, p=0.4272 for exploratory behaviour) but treatment effect reached statistical significance ($F_{(1,28)}$=7.6, p=0.0102 for activity and $F_{(1,28)}$=10.60, p=0.0030 for exploratory behaviour). Their interaction was significant for activity ($F_{(1,28)}$=17.64, p=0.0002), but not for exploratory behaviour ($F_{(1,28)}$=1.064, p=0.3112). While under hypothyroidism no sex effect was found ($F_{(1,28)}$=0.9943, p=0.3272 for activity and $F_{(1,28)}$=0.8467, p=0.3654 for exploratory behaviour), treatment effect was significant ($F_{(1,28)}$=12.86, p=0.0013 for activity and $F_{(1,28)}$=9.231, p=0.0051 for exploratory behaviour). Furthermore we found an interaction between sex and treatment on exploratory behaviour ($F_{(1,28)}$=8.406, p=0.0072), but not on activity ($F_{(1,28)}$=4.033, p=0.0544).

In contrast to these sex-specific modulations of TH impact on behaviour, no sex differences were noted for male and female mice on the rotarod test under eu-, hyper- and hypothyroid conditions (Fig. 4D-F).

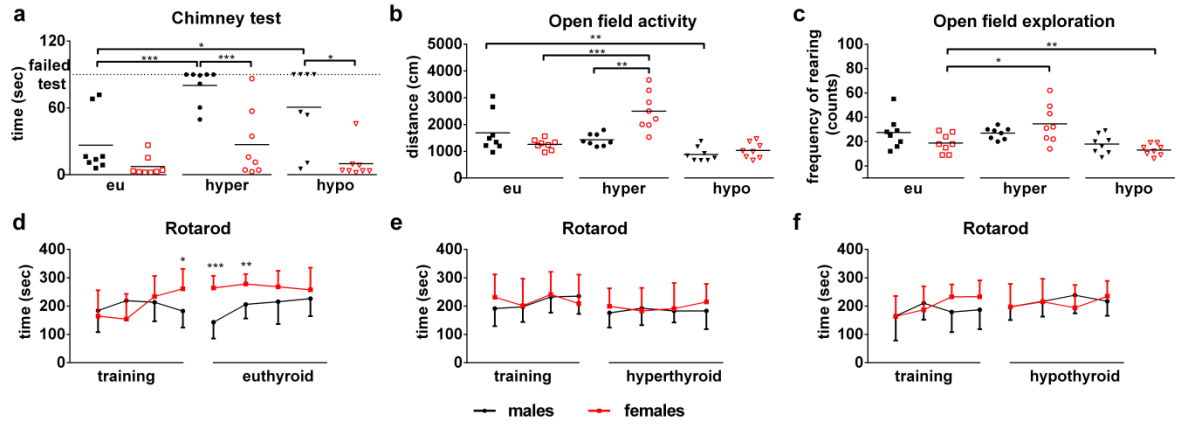

Fig. 4: Behavioural assessment of male and female mice under T_4 excess or deprivation. The chimney test was used to (**A**) examine muscle strength, tonus and coordination of movements in male and female mice under euthyroid, hyper- or hypothyroid conditions. The open field was used to investigate activity and exploration behaviour. (**B**) Total distance travelled was measured to assess activity and (**C**) frequency of rearings were determined to assess exploratory behaviour. The rotarod test was used for an overall assessment of coordination and motor function in male and female mice before start of treatment (training period) and under sham (**D**), (**E**) T_4 or (**F**) LoI/MMI/ClO_4^- treatment. Data are presented as mean ± SD, N= 8 animals/sex/treatment; 2-way ANOVA followed by Bonferroni post-hoc analysis applied for sex and treatment effects of A-C and unpaired Student´s t-test for sex-effect of D-F, *p<0.05, **p<0.01, ***p<0.001 represent multiple-testing or t-test results.

Sex influences on serum thyroid function status in hyperthyroidism and liver function in hypothyroidism

Serum TT_4, fT_4, fT_3 concentrations did not differ between euthyroid male and female mice (Fig. 5A-C). TSH serum concentrations of euthyroid male and female mice were 310 ± 170 mU/l and 290 ± 30 mU/l respectively (±SEM, n=4). T_4 treatment resulted in marked sex differences in serum T_4 and T_3 status with 2.3-fold higher TT_4 and fT_4 concentrations in hyperthyroid females compared to male mice (Fig. 5A-C) and TSH concentrations below detection limit (<10 mU/l) in both sexes. LoI/MMI/ClO_4^- treatment reduced TT_4 concentrations below assay detection limit (<0.5 µg/dl) in both sexes (Fig. 5A-C) and increased TSH to 6830 ± 1070 mU/l and 7790 ± 1270 mU/l in male and female mice respectively (±SEM, n=4). Sex effects on TH serum parameters were observed for TT_4 and fT_4 under hyperthyroidism (TT_4: $F_{(1,28)}$=20.50, p=0.0001; fT_4: $F_{(1,28)}$=10.80, p=0.0027) but not for hypothyroidism (TT_4:

$F_{(1,28)}$=0.09858, fT_4: p=0.7559; $F_{(1,28)}$=0.2127, p=0.6482) and not for fT_3 (hyperthyroid: $F_{(1,28)}$=2.485, p=0.1261; hypothyroid: $F_{(1,28)}$=0.1553, p=0.6965). Treatment effects had an impact on TT_4 concentrations (hyperthyroid: $F_{(1,28)}$=95.74, p<0.0001; hypothyroid: $F_{(1,28)}$=165.8, p<0.0001) and on fT_4 and fT_3 concentrations under hyperthyroidism (fT_4: $F_{(1,28)}$=41.32, p<0.0001; fT_3: $F_{(1,28)}$=5.26, p<0.0001). No treatment impact was observed under hypothyroidism for fT_4 and fT_3 (fT_4: $F_{(1,28)}$=2.316, p=0.1393; fT_3: $F_{(1,28)}$=0.1645, p=0.6882). Interaction of TH status and sex was found for TT4 and fT4 under hyperthyroidism (TT_4: $F_{(1,28)}$=21.26, p<0.0001; fT_4: $F_{(1,28)}$=10.75, p=0.0028) but not under hypothyroidism (TT_4: $F_{(1,28)}$=0.1272, p=0.7241; fT_4: $F_{(1,28)}$=0.2350, p=0.6316) and not for fT_3 (hyperthyroid: $F_{(1,28)}$=3.658, p=0.0661; hypothyroid: $F_{(1,28)}$=0.8460, p=0.3656).

To examine the influence of sex on TH dependent liver function, liver parameters were analysed in sera collected at the end of treatment. While no changes were found in aspartate aminotransferase, creatine kinase, cholinesterase and albumin serum concentrations (data not shown) a marked sex-difference was found for total cholesterol (CHO) and triglyceride (TG) concentrations. Male mice exhibited higher CHO concentrations compared to female mice in the euthyroid state and hypothyroidism led to significantly larger increases in serum CHO concentrations in male compared to female mice (m: Δ~ 224.6 mg/dl, f: Δ~ 63.9 mg/dl). In contrast, T_4 treatment decreased total CHO concentrations in both sexes (m: Δ~106.9 mg/dl, f: Δ~ 63.1 mg/dl). Thus, sex-difference in serum CHO levels disappeared during TH excess ($F_{(1,12)}$=1.530, p=0.2397), while deprivation led to an exaggeration ($F_{(1,12)}$=35.44, p<0.0001) (Fig. 5D). The treatment effect ($F_{(1,12)}$=57.23, p<0.0001 for hyperthyroidism and $F_{(1,12)}$=54.66, p<0.0001 for hypothyroidism) was considered significant and interacted with the sex effect ($F_{(1,12)}$=3.799, p=0.0075 for hyperthyroidism and $F_{(1,12)}$=16.96, p=0.0014 for hypothyroidism). Serum TG concentrations were not different in euthyroid males and females, but increased in hyperthyroid female mice only (m: Δ~0.035 mg/ml, f: Δ~0.198 mg/ml, $F_{(1,12)}$=0.08541, p=0.7751). However, a sex-difference appeared by TH modulation and was exaggerated by LoI/MMI/ClO_4^- treatment (m: 0.288 mg/ml vs f: 0.186 mg/ml, p<0.05, $F_{(1,12)}$=15.77, p=0.0019) (Fig. 5E). The treatment effect was considered significant for hyperthyroidism ($F_{(1,12)}$=20.98, p=0.0006) and interacted with sex effect ($F_{(1,12)}$=10.23, p=0.0076), but not for hypothyroidism ($F_{(1,12)}$=3.983, p=0.0692; $F_{(1,12)}$=0.3958, p=0.5410 for interaction).

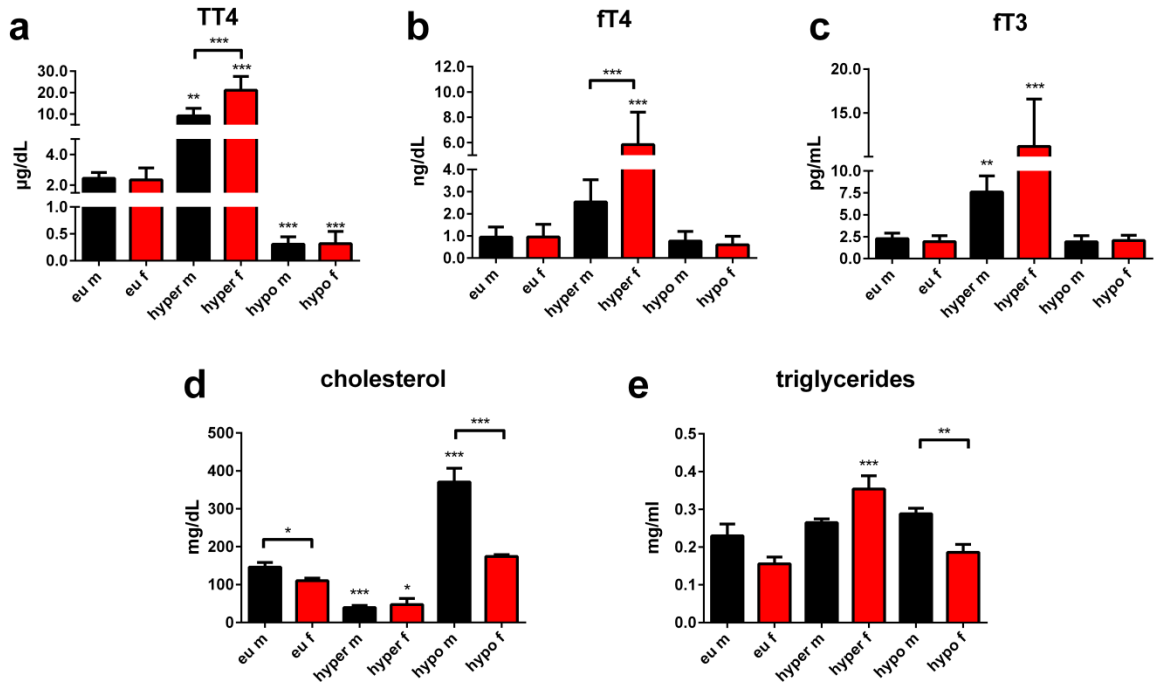

Fig. 5: Serum TH status in euthyroid controls, T_4 or LoI/MMI/ClO_4^- treated male and female mice. (**A**) Total thyroxine (TT_4), (**B**) free thyroxine (fT_4) and (**C**) free triiodothyronine (fT_3) concentrations were determined in sera by ELISA after 7 weeks of treatment. (**D**) Total cholesterol and (**E**) triglyceride serum concentrations were determined by ELISA at the end of experiment in sera of euthyroid, hyperthyroid, and hypothyroid mice of both sexes. Data are presented as mean ± SD, N= 8 animals/sex/treatment for TH concentrations, N=4/sex/treatment animals for total cholesterol concentrations; 2-way ANOVA followed by Bonferroni post-hoc analysis applied for sex and treatment effects, *p<0.05, **p<0.01, ***p<0.001 above bars represent multiple-testing results.

Evidence for a distinct impact of sex on cellular TH effects on gene expression in target organs brown adipose tissue, heart and liver

Expression of TH responsive genes and TH transporters was studied by quantitative RT-PCR in BAT, heart and liver of male and female mice under T_4 excess, TH deprivation and euthyroid condition (Fig. 6A-I). A distinct and organ specific pattern of sex-variation in gene expression was observed. In brown adipose tissue marked sex-specific alterations in *Dio2* transcript levels were detected in hyperthyroid (upregulation in male, downregulation in female mice) and for *Lat2* in hyper- and hypothyroid animals (Fig. 6A-B). Additionally, sex-dependent variation was found for expression of all investigated target genes and TH transporters in euthyroid mice

(Fig. 6C). In contrast to this data very little or no sex-impact was found on target gene or TH transporter gene expression in heart neither in euthyroid controls nor in response to T_4 or LoI/MMI/ClO_4^- treatment (Fig. 6D-F). In fact, for most investigated genes, a distinctly higher expression was found in heart tissue of male mice irrespective of thyroid function status. In line with the contribution of liver and BAT to metabolic features of thyroid dysfunction, significant sex-specific alterations for e.g. *Dio1*, *Tbg* and *Me1* as well as *Mct10* and *Lat1* expression were obvious with manipulation of thyroid status (Fig. 6G-H), while livers of male and female control mice showed little sex-variation in target gene and TH transporter expression (Fig. 6I).

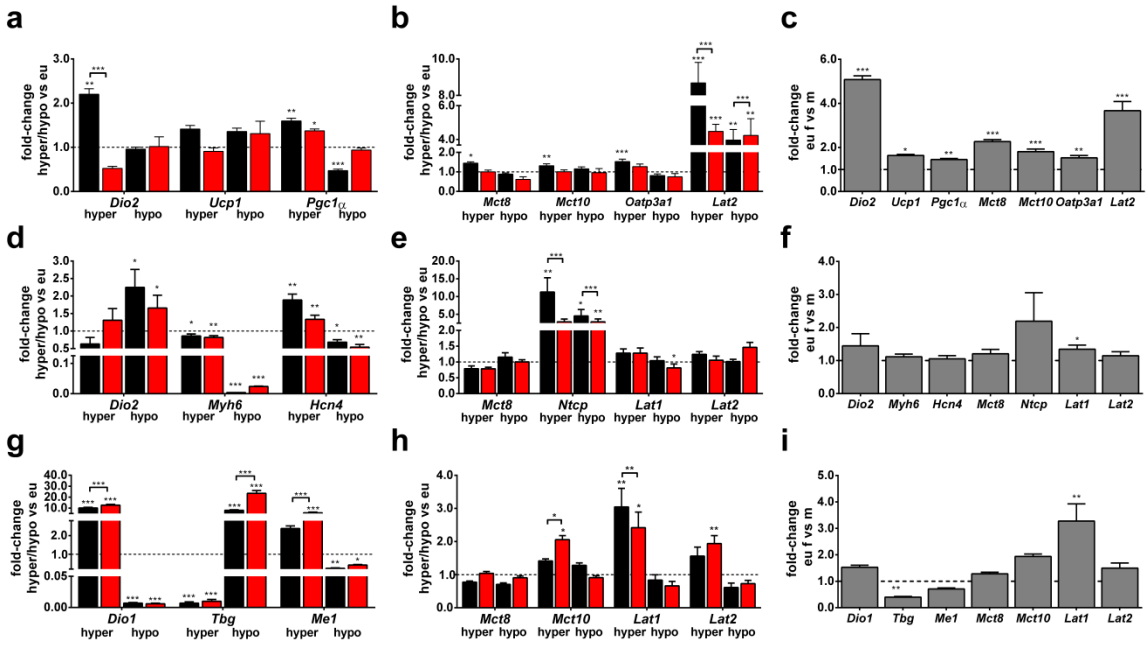

Fig. 6: TH effects in brown adipose tissue (BAT), heart and liver of male and female mice. Fold changes of representative TH responsive genes were measured by quantitative RT-PCR in (A) BAT, (D) heart and (G) liver tissue of hyperthyroid or hypothyroid mice of both sexes and normalized to euthyroid samples. For BAT *Dio2*, *Ucp1* and *PGC1α* expression for heart *Dio2*, *Myh6* and *Hcn4* expression and for liver *Dio1*, *Tbg* and *Me1* expression were quantified. Additionally, mRNA expression of TH transporter genes were analyzed in (B) BAT, (E) heart, and (H) liver. For BAT: *Mct8*, *Mct10*, *Oatp3a1*, *Lat2*, for heart: *Mct8*, *Ntcp*, *Lat1*, *Lat2* and for liver: *Mct8*, *Mct10*, *Lat1*, *Lat2*. Furthermore, euthyroid sex comparison was analysed in (C) BAT, (F) heart and (I) liver of all genes and female gene expression was normalized to male samples. Data are presented as mean ± SD, N= 5-7 animals/sex/treatment; unpaired Student´s t-test, *p<0.05, **p<0.01, ***p<0.001 represent t-test results.

3.5 Discussion

The female preponderance of thyroid dysfunction is well-known, however, whether gender has an impact on manifestation and outcome of hyper- and hypothyroidism in men and women has not been well studied and the molecular mechanisms are still unclear. Using a murine model, we found marked sex-differences in functional behaviour, metabolic and biochemical parameters in hyper- and hypothyroid mice. Sex of mice did not alter the general response of body weight and heart rate to manipulation of thyroid status, only the extent of these changes is somewhat different. In addition, a distinct sex-variation in cellular TH effects on gene expression was observed in BAT, heart and liver on the basis of TH target and TH transporter gene expression analysis.

Sex-difference for water intake reverses during TH deprivation

A clear sex-difference could be observed for water intake in euthyroid and hyperthyroid conditions as females consumed more water than male mice (Fig. 2G, H). Strikingly, this is reversed in hypothyroidism and female mice consume significantly less water than their male counterparts (Fig. 2I). This observation could indicate that female mice taste bitter substances differently due to altered taste receptor distributions. However, studies of taste receptors in mice either addressed only male mice [18] or observed no sex-differences [19]. Preferences for the primary taste qualities sweet, sour, bitter, salty and umami showed no sex-differences for C57BL/6 mice [20]. Thus, the supplemented drugs might be recognized not by usual taste receptors, but different receptor types in female and male mice. However, decreased water consumption did not affect the success of LoI/MMI/ClO_4^- treatment as TT_4 serum concentrations were below limit of detection and serum TSH was highly elevated to comparable values for both sexes. Sex-difference for water intake was reversed under euthyroid and hyperthyroid conditions as females consumed more water than male mice (Fig. 2G, H). Therefore, manipulation of TH status might also affect hormonal regulation of fluid intake, water and salt homeostasis, an observation compatible with altered water metabolism in hypothyroid patients [21, 22] and increased hypothalamic vasoactive intestinal polypeptide-immunoreactive neuron expression in hypothyroid rats [23]. For other phenotypic traits related to manipulation of TH concentration sex-specific differences persisted (e.g. body temperature in hyper- and hypothyroidism), were exaggerated (e.g. food intake in hyperthyroidism) or disappeared (e.g. BW change in hypothyroidism).

TH excess results in distinct exaggeration of behavioural traits in male vs female mice

Behavioural assessment of our mice consisted of measurements of locomotor activity, motor function and coordination tests. TH manipulation resulted in increased activity and exploratory behaviour during TH excess in female mice only, while decreased activity under TH deprivation was present in male mice only (Fig 4B, C). Previous studies with adult-onset hypothyroidism in male mice support our observation of decreased locomotor activity, with no consistent findings for anxiety [24, 25]. Impact of sex and TH excess on locomotor activity have not been addressed so far. For euthyroidism a higher locomotor activity as well as increased voluntary exercise of females compared to male mice have previously been described [26, 27] and was confirmed in our study. The chimney test investigates motor coordination and muscular strength and showed higher impairment of performance by T_4 administration than by LoI/MMI/ClO_4- treatment for both sexes (Fig. 4A). Male mice showed more pronounced affliction of chimney test performance under manipulation of TH status. Since the rotarod test evaluating motor coordination and balance in movements showed no sex or treatment differences (Fig. 4D-F) we suspect muscle function to be responsible for the observed effects in chimney test. To our knowledge, our study is the first to assess muscle strength under hyper- and hypothyroid condition for mice of both sexes. When assessing behaviour it is important to distinguish whether the differences are due to a compromised muscle function or neurological impairment. Comparison of mice of both sexes showed generally shorter contraction of isolated muscles in male mice [28]. Therefore, it has been suggested, that male muscles are generally faster and have a higher maximum power output than female muscles. On the other hand, female muscles are generally more fatigue resistant, recover faster and show less mechanical damage after exercise [28, 29]. As central nervous system and peripheral nervous system coordinate muscle function, it is not unreasonable to conclude that both neurological and primary muscular factors might contribute to observed sex-differences [30, 31]. These aspects remain to be investigated.

Sex-difference in body temperature persists throughout thyroid dysfunction and is reflected by cellular TH effects on gene expression in BAT

Persisting sex-differences in eu- and hyperthyroidism between male and female mice were found for body temperature with higher values in female mice (Fig. 3A). It has been previously described that euthyroid female mice have higher body temperature than male mice, but this is not explained by classical TH serum concentrations in euthyroid or hyperthyroid animals [26, 32]. As BAT is a TH target organ and involved in regulation of body temperature [33], gene expression analysis was performed for TH target genes and TH transporter expression. With manipulation of TH status TH-responsive sex-difference in gene expression was only found for *Dio2,* which increased in BAT of hyperthyroid male mice, but decreased in BAT of hyperthyroid female mice (Fig. 6A). However, we cannot extrapolate from transcript concentration on enzyme activity, as previous studies showed different regulation of *Dio2* under TH influence [34]. No sex-difference was found for the investigated TH-responsive genes under TH deprivation. This supports the observed persisting sex-differences in body temperature, as TH manipulation resulted in little sex-differences of TH responsive gene expression in BAT. For TH transporter genes only *Lat2* was higher expressed in BAT of hyperthyroid male compared to female mice, however *Lat2* gene expression was also increased in BAT of hypothyroid female compared to male mice (Fig. 6B). *Lat2* is known to transport not only TH (preferentially diiodothyronines), but also small neutral amino acids [35, 36]. Thus, it might reflect sex-different needs for amino acid transport in BAT during TH dysfunction.

For interpretation of cellular TH effects on gene expression in BAT it is important to remember that two mechanisms play a role in BAT activation and thermogenesis. Firstly, adjustment of body temperature in response to thermal stress which occurs due to housing temperature (23°C instead of thermoneutrality at 30°C) and therefore chronically augmented metabolism [37]. Secondly, activation of basal energy expenditure by TH results in increased thermogenesis [38, 39]. The balance between these two mechanisms has been addressed in recent studies under TH deprivation and showed that BAT thermogenic program was only down-regulated when hypothyroidism was combined with thermoneutrality [40]. In our study mice were not kept under thermoneutral condition, hence we expect an overlap between adjustment to thermal stress and activation or inactivation of basal energy expenditure by TH excess or deprivation, respectively.

Sex-difference in heart rate disappears with hyper- and hypothyroidism

Euthyroid female mice showed higher HR compared to males, but this sex-difference disappeared with a hyper- or hypothyroid state (Fig. 3B). We asked whether these changes in sex-impact would also be mirrored by cellular TH effects and hence performed gene expression analysis in heart tissue. No sex-difference in the expression of investigated TH responsive genes was found. Similarly, for TH transporters only *Ntcp* showed different expression in male and female heart tissues irrespective of TH dysfunction (Fig. 6E). Hence, this data does not explain why sex-difference in HR disappears with manipulation of TH status and it is therefore likely that the investigated genes do not reflect the total organ response. Sex-specific impact on neuronal regulation of heart rate and function might lead to these changes independent from myocardial gene expression [41].

TH dependent liver function and metabolism is sex-specific

It is well known that TH influences serum total CHO concentrations [42-45]. A correlation with serum TH status was confirmed by changes in CHO serum concentrations of our mice. A sex-difference in CHO levels was augmented during LoI/MMI/ClO_4^- treatment and disappeared under T_4 administration (Fig. 5D). Since male mice showed more pronounced changes than female mice, we suggest that CHO metabolism in livers of male mice may be more sensitive to TH manipulation compared to female mice. This observation is supported by the known sex-dependent regulation of *Cyp7a1* in transgenic mice, where hyperthyroidism decreased *Cyp7a1* in male mice, while no regulation could be found in female mice [43]. In addition, studies on changes in other functional parameters in liver tissues revealed sex-dependent regulation of tight junction proteins, such as claudin-1 and claudin-2 in cholangiocytes and hepatocytes of male and female animals under hypothyroid conditions. Further experiments including analysis of CHO synthesis pathways led to the hypothesis that claudins may be relevant for reversal of sex hormone-related susceptibility to gallstone formation in hypothyroidism [6]. On the other hand, TG serum concentration increased in hyperthyroid female mice only (Fig. 5E) and suggest a different TG metabolism response to TH in male and female mice. Gene expression profile shows more pronounced TH-dependent regulation in liver of female compared to male mice. Thus, a higher expression of TH-responsive genes *Dio1* and *Me1* was noted in hyperthyroidism in female mice liver and *Tbg* transcripts were elevated in hypothyroid female liver compared to male mice. For TH

transporters *Mct10* gene expression was increased in hyperthyroid female mice and for *Lat1* a higher expression was observed in hyperthyroid male mice (Fig. 6G, H).

Possible influence of estrogens on TT_4 and fT_4 serum concentrations during TH excess

One very obvious sex-specific alteration was the increase in TH serum concentrations in female hyperthyroid mice. In fact, 2.3-fold higher TT_4 and fT_4 serum concentrations were measured in female mice after 7 weeks of T_4 treatment (Fig. 5A-C). This is in agreement with previous findings showing a 2-fold increase in TT_3 serum concentrations in female mice under T_3 treatment, whereas male mice revealed only 50% increase in TT_3 serum concentrations [47]. In contrast, under TH deprivation no sex-differences in serum TT_4, fT_4, fT_3 and TSH concentrations were found in our mice. Sex differences in thyroid function parameters, such as TSH and classical thyroid hormones T_4 and T_3 have also been addressed in epidemiological studies, however no consistent findings were reported [48-50]. Other studies using murine models have described sex differences in thyroid function parameters at least for some strains and indicate that serum TH status needs to be considered when studying TH action [7, 51]. Thus, for every genotype TH serum concentrations should be measured in a sex-matched manner.

When studying sex-differences in animal models or humans it is likely that the most elaborated differences between male and female individuals are related to gonadal hormones. The connection between TH and estrogens has been a subject of extensive research. One major effect is an increase in thyroxine-binding globulin capacity by estrogens [52, 53]. In our mice study we observed increased TT_4 serum concentration in hyperthyroid female compared to male mice, which could be explained by higher binding capacity of thyroxine-binding globulin. However, this does not explain the sex-dependent difference in fT_4 concentrations, which were also significantly higher in female compared to male mice. Secondly, interactions between gonadal and thyroid hormones occur through the hypothalamic-pituitary-thyroid and the hypothalamic-pituitary-gonadal axis, thus hyper- and hypothyroidism can interfere with the gonadal axis resulting in infertility in particular in the female organism. Finally, there was no apparent direct linear correlation between phenotype and classical T_4/T_3 ratio in relation to observed sex-variation in phenotypical traits. Thus although higher TT_4 and fT_4 serum concentrations were present in hyperthyroid

female mice, they show exaggeration only in motor activity and exploration while other traits of hyperthyroidism are less prominent than in male animals.

Conclusions

We showed that sex is an important modifier of TH action resulting in distinct phenotypic, metabolic and biochemical traits of hyper- and hypothyroidism in male and female mice. Importantly, a direct linear link between TH serum concentrations and sex-dependent phenotype was not observed. The wide spectrum of key players in TH action, such as distinct circulating TH derivatives and metabolites, tissue-specific TH transporters, non-genomic TH effects and classical nuclear TH action illustrates the complex situation in an intact organism [54]. It seems crucial to address possible sex-difference under natural conditions, further analysis of sex-specific traits will require additional studies using e.g. gonadectomized animals.

Abbreviations

BAT: brown adipose tissue; BW: body weight; CHO: cholesterol; ClO_4-: perchlorate; ECG: electrocardiography; HR: heart rate; f: female; fT_3: free 3,3',5-triiodothyronine; fT_4: free thyroxine; LoI: low-iodine diet; m: male; MMI: methimazole; qRT-PCR: quantitative real-time PCR; T_3: 3,3',5-triiodothyronine; T_4: thyroxine; TT_4: total thyroxine; TG: triglycerides; TH: thyroid hormone; TSH: thyroid-stimulating hormone

Availability of data

The datasets analysed during the current study are available from the corresponding author on request.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was funded by DFG FU 356/7-1 to DF, MO 1018/2-1 to LCM in the framework of SPP 1629 THYROID TRANS ACT.

Authors´ contributions

HR, KE, DZ and DF conceived and designed the experiments. HR, KE and GSH performed the experiments. HR, KE and GSH analysed the data. LCM, JK, KHS, DZ and DF contributed to data interpretation and reviewed the manuscript. All authors provided valuable feedback and approved the final manuscript.

Acknowledgements

The authors are grateful to S. Rehn, A. Jaeger, J. Fleckhaus and E. Werbenko for their dedicated technical support. We are also grateful to Prof. Refetoff and Xiao Hui Liao (University of Chicago, Chicago, USA) for serum TSH measurements and Dr. Kleinbongard and Prof. Heusch (Institute for Pathophysiology, University Hospital Essen, Essen, Germany) for help with mouse ECG analysis. This work was supported by DFG FU 356/7-1 to DF, MO 1018/2-1 to LCM in the framework of SPP 1629 THYROID TRANS ACT. TH analysis was performed by Dr. Eddy Rijntjes in the team of Prof. J. Köhrle in the SPP1629 Core Facility (DFG KO 922/17-1).

References

1. Vanderpump MPJ. The epidemiology of thyroid disease. Br Med Bull. 2011;99:39-51.
2. Clayton JA, Collins FS. NIH to balance sex in cell and animals studies. Nature. 2014;509: 282–283.
3. Hammes SR. Sex Matters in Preclinical Research. Mol Endocrinol. 2014;28:1209–1210.
4. Bianco AC, Anderson G, Forrest D, Galton VA, Gereben B, Kim BW *et al.* American thyroid association guide to investigating thyroid hormone economy and action in rodent and cell models. Thyroid. 2014;24:88–168.
5. Engels K, Rakov H, Zwanziger D, Moeller LC, Homuth G, Koehrle J *et al.* Differences in mouse hepatic thyroid hormone transporter expression with age and hyperthyroidism. Eur Thyroid J. 2015; 4(Suppl 1):81-6. doi:10.1159/000381020.
6. Zwanziger D, Rakov H, Engels K, Moeller LC, Fuhrer D. Sex-dependent claudin-1 expression in liver of eu- and hypothyroid mice. Eur Thyroid J. 2015; 4(Suppl 1):67-73. doi:10.1159/000431316.
7. Pohlenz J, Maqueem A, Cua K, Weiss RE, Van Sande J, Refetoff S. Improved radioimmunoassay for measurement of mouse thyrotropin in serum: strain differences in thyrotropin concentration and thyrotroph sensitivity to thyroid hormone. Thyroid. 1999;9:1265-71.
8. Gent S, Kleinbongard P, Dammann P, Neuhäuser M, Heusch G. Heart rate reduction and longevity in mice. Basic Res Cardiol. 2015 Mar; 110(2):1-9. doi: 10.1007/s00395-014-0460-7.

9. Wulf A, Harneit A, Kröger M, Kebenko M, Wetzel MG, Weitzel JM. T3-mediated expression of PGC-1α via a far upstream located thyroid hormone response element. Mol Cell Endocrinol. 2008;287:90-95.
10. Crowe TC, Cowen NL, Loidl NM, Topliss DJ, Stockigt JR, Barlow JW. Down-regulation of thyroxine-binding globulin messenger ribonucleic acid by 3,5,3´-triiodothyronine in human hepatiblastoma cells. J Clin Endocrinol Metab. 1995;80:2233-2237.
11. Kahaly GJ, Dillmann WH. Thyroid hormone action in the heart. Endocrine Reviews. 2005;26:704-728.
12. Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper-Excel-based tool using pair-wise correlations. Biotechnol Lett. 2004;26:509–515.
13. Pfaffl MW, Horgan GW, Dempfle L. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res. 2002;30:e36.
14. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 2001;29:2002–2007.
15. Dunham NW, Miya TS. A note on a simple apparatus for detecting neurological deficit in rats and mice. J Am Pharm Ass. 1957;46:208-214.
16. Boissier JR, Tardy J, Diverres JC. Une nouvelle méthode simple pour explorer l´action “tranquillisante”: le test de la cheminée. Med Experimentalis. 1960;3:81-84.
17. Daugé V, Sebret A, Beslot F, Matsui T, Roques B. Behavioral profile of CCK2 Receptor-deficient mice. Neuropsychopharmacology. 2001;25:690-698.
18. Kim M, Kusakabe Y, Miura H, Shindo Y, Ninomiya Y, Hino A. Regional expression patterns of taste receptors and gustducin in the mouse tongue. Biochem Biophys Res Commun. 2003;312:500-506.
19. Zhang G, Zhang H, Deng S, Qin Y. Regional differences in taste bud distribution and α-gustducin expression patterns in the mouse fungiform papilla. Chem Senses. 2008;33:357-362.
20. Tordoff MG. Taste solution preferences of C57Bl/6J and 129X1/SvJ mice: Influence of age, sex, and diet. Chem Senses. 2007;32:655-671.

21. Sahún M, Villabona C, Rosel P, Navarro MA, Ramón JM, Gómez JM, Soler J. Water metabolism disturbances at different stages of primary thyroid failure. J Endocrinol. 2001 Mar;168(3):435-45.
22. Karmisholt J, Andersen S, Laurberg P. Weight loss after therapy of hypothyroidism is mainly caused by excretion of excess body water associated with myxoedema. J Clin Endocrinol Metab. 2011 Jan;96(1):E99-103. doi: 10.1210/jc.2010-1521.
23. Toni R, Mosca S, Ruggeri F, Valmori A, Orlandi G, Toni G, Lechan RM, Vezzadini P. Effect of hypothyroidism on vasoactive intestinal polypeptide-immunoreactive neurons in forebrain-neurohypophysial nuclei of the rat brain. Brain Res. 1995 Jun 5;682(1-2):101-15.
24. Vallortigara J, Alfos S, Micheau J, Higueret P, Enderlin V. T3 administration in adult hypothyroid mice modulates expression of proteins involved in striatal synaptic plasticity and improves motor behaviour. Neurobiology of Disease. 2008;31:378-385.
25. Buras A, Battle L, Landers E, Nguyen T, Vasudevan N. Thyroid hormones regulate anxiety in the male mouse. Hormones and Behavior. 2014;65:88-96.
26. Sanchez-Alavez M, Alboni S, Conti B. Sex- and age-specific differences in core body temperature of C57Bl/6 mice. Age. 2011;33:89-99.
27. De Bono JP, Adlam D, Paterson DJ, Channon KM. Novel quantitative phenotypes of exercise training in mouse models. Am J Physiol Regul Integr Comp Physiol. 2006;290:R926–R934.
28. Glenmark B, Nilsson M, Gao H, Gustafsson JA, Dahlman-Wright K, Westerblad H. Difference in skeletal muscle function in males vs. females: role of estrogens receptor-β. Am J Physiol Endocrinol Metab. 2004;287:E1125–E1131.
29. Burnes LA, Kolker SJ, Danielson JF, Walder RY, Sluka KA. Enhanced muscle fatigue occurs in male but not female ASIC3-/- mice. Am J Physiol Regul Integr Comp Physiol. 2008;294(4):R1347–R1355.
30. McCarthy MM, Konkle ATM. When is a sex difference not a sex difference? Frontiers in Neuroendocrinology. 2005;26:85–102.
31. Becker JB, Arnold AP, Berkley KJ, Blaustein JD, Eckel LA, Hampson E *et al.* Strategies and methods for research on sex differences in brain and behavior. Endocrinology. 2005;146:1650-73.

32. Gaskill BN, Gordon CJ, Pajor EA, Lucas JR, Davis JK, Garner JP. Impact of nesting material on mouse body temperature and physiology. Physiology & Behavior. 2013;110-111:87-95.
33. Obregon MJ. Adipose tissues and thyroid hormones. Frontiers in Physiology. 2014;5(479):1-12.
34. Martinez de Mena R, Scanlan TS, Obregon MJ. The T3 receptor beta1 isoform regulates UCP1 and D2 deiodinase in rat brown adipocytes. Endocrinology. 2010;151(10):5074-83.
35. Hinz KM, Meyer K, Kinne A, Schülein R, Köhrle J, Krause G. Structural insights into thyroid hormone transport mechanisms of the L-type amino acid transporter 2. Mol Endocrinol. 2015;29(6):933-42.
36. Pochini L, Scalise M, Galluccio M, Indiveri C. Membrane transporters for the special amino acid glutamine: structure/function relationships and relevance to human health. Frontiers in Chemistry. 2014;2:1–23.
37. Feldmann HM, Golozoubova V, Cannon B, Nedergaard J. UCP1 ablation induces obesity and abolishes diet-induced thermogenesis in mice exempt from thermal stress by living at thermoneutrality. Cell Metab. 2009;9:203-209.
38. Bianco AC, Maia AL, da Silva WS, Christoffolete MA. Adaptive activation of thyroid hormone and energy expenditure. Biosci Rep. 2005;25:191-208.
39. Silva JE. Thermogenic mechanisms and their hormonal regulation. Physiol Rev. 2006;86:435-464.
40. Ueta CB, Olivares EL, Bianco AC. Responsiveness to thyroid hormone and to ambient temperature underlies differences between brown adipose tissue and skeletal muscle thermogenesis in a mouse model of diet-induced obesity. Endocrinology 2011;152:3571-3581.
41. Mittag J, Lyons DJ, Sällström J, Vujovic M, Dudazy-Gralla S, Warner A *et al.* Thyroid hormone is required for hypothalamic neurons regulating cardiovascular functions.

J Clin Invest. 2013 Jan;123(1):509-16. doi: 10.1172/JCI65252.

42. Lin JZ, Martagón AJ, Hsueh WA, Baxter JD, Gustafsson JA, Webb P *et al.* Thyroid hormone receptor agonists reduce serum cholesterol independent of the LDL receptor. Endocrinology. 2012;153:6136-6144.
43. Jonas W, Lietzow J, Wohlgemuth F, Hoefig CS, Wiedmer P, Schweizer U, Köhrle J, Schürmann 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(1):389-399.

44. Zhang A, Sieglaff DH, York JP, Suh JH, Ayers SD, Winnier GE *et al.* Thyroid hormone receptor regulates most genes independently of fibroblast growth factor 21 in liver. J Endocrinol. 2015;224:289-301.
45. Shin D-J, Osborne TF. Thyroid hormone regulation and cholesterol metabolism are connected through sterol regulatory element-binding protein-2 (SREBP-2). J Biol Chem. 2003;278:34114-8.
46. Drover VAB, Angellon LB. Regulation of the human cholesterol 7α-hydroxylase gene (CYP7A1) by thyroid hormone in transgenic mice. Endocrinology. 2004;145(2):574–581.
47. Bezerra da Silveira AL, de Souza Miranda MF, Mecawi AS, Melo RL, Marassi MP, Matos da Silva AC *et al.* Sexual dimorphism in autonomic changes and in the renin-angiotensin system in the hearts of mice subjected to thyroid hormone-induced cardiac hypertrophy. Exp Physiol. 2014;99:868-880.
48. Roelfsema F, Pereira AM, Veldhuis JD, Adriaanse R, Endert E, Fliers E, Romijn JA. Thyrotropin secretion profiles are not different in men and women. J Clin Endocrinol Metab. 2009;94:3964–67.
49. Aghini-Lombardi F, Antonangeli L, Martino E, Vitti P, Maccherini D, Leoli F *et al.* The spectrum of thyroid disorders in an iodine-deficient community: the Pescopagano survey. J Clin Endocrinol Metab. 1999;84:561–566.
50. Kapelari, K, Kirchlechner C, Högler W, Schweitzer K, Virgolini I, Moncayo R. Pediatric reference intervals for thyroid hormone levels from birth to adulthood: a retrospective study. BMC Endocr Disord. 2008;8:15.
51. McLachlan Sandra M., Hamidi Sepehr, Aliesky Holly, Williams Robert W., and Rapoport Basil. Sex, genetics and the control of thyroxine and thyrotropin in mice. Thyroir. 2014; 24:1080-1087.
52. Engbring NM, Engström WW. Effects of estrogens and testosterone on circulating thyroid hormone. J Clin Endocrinol Metab. 1958;19:783-796.
53. Franklyn JA, Ramsden DB, Sheppard MC. The influence of age and sex on tests of thyroid function. Ann Clin Biochem. 1985;22:502-505.
54. Fuhrer D, Brix K, Biebermann H. Understanding the healthy thyroid state in 2015. Eur Thyroid J 2015 Sep;4(Suppl 1):1-8. doi:10.1159/000431318.

Supplemental table 1: Oligonucleotides used for amplification of house-keeping genes, TH responsive genes and TH transporters by real-time PCR.

gene	Forward primer	Reverse primer
18S	CGGCTACCACATCCAAGGAA	GCTGGAATTACCGCGGCT
Ppia	CTTGGGCCGCGTCTCCTTCG	GCGTGTAAAGTCACCACCCTGGC
RPL 13a	GGGCAGGTTCTGGTATTGGA	GGGGTTGGTATTCAATCCGCT
Gapdh	CCTCGTCCCGTAGACAAAATG	TGAAGGGGTCGTTGATGGC
ß-Actin	CTGTCGAGTCGCGTCCA	TCATCCATGGCGAACTGGTG
Polr2a	CTTTGAGGAAACGGTGGATGTC	TCCCTTCATCGGGTCACTCT
Dio1	GGGCAGGATCTGCTACAAGG	CGTGTCTAGGTGGAGTGCAA
Dio2	GTGACTGGGGAAGCAGAGTG	AGTTTAACCTGTTTGTAGGCATC
Tbg	TGGGCATGTGCTATCATCTTCA	GAGTGGCATTTTGTTGGGGC
Me1	TAAGGGTCGTGCATCTCTCAC	TGCAGCAACTCCTATGAGGG
Myh6	CAGACAGAGATTTCTCCAACCCA	GCCTCTAGGCGTTCCTTCTC
Hcn4	CAGCGTCAGAGCGGATACTT	CTTCTTGCCTATGCGGTCCA
Ucp1	GGATGGTGAACCCGACAACT	CTTGGATCTGAAGGCGGACT
PGC1α	TCTCAGTAAGGGGCTGGTTG	AGCAGCACACTCTATGTCACTC
Mct8	CTCCTTCACCAGCTCCCTAAG	ACTTCCAGCAGATACCACACC
Mct10	CGTGAGTGTCTTCACGGACA	CGATGGAGCTTACAAAAGAACTGG
Lat1	AGCGTCCCATCAAGGTGAAT	GGGCTTGTTCTTCCACCAGA
Lat2	AACAACACCGCGAAGAACCA	GGAGCCAATGATGTTCCCTACAA
Oatp3a1	CGCTACGGAAACAACTCAGC	GAGTCCGTCTGGCATTCACA
Ntcp	GGGGACATGAACCTCAGCATT	CCCTATGGCGCAAGGAATGA

Chapter 4: Influence of age on sex-specific phenotypes of thyroid dysfunction in mice

Helena Rakov[1*], Kathrin Engels[1*], Georg Sebastian Hönes[1], Klaudia Brix[2], Josef Köhrle[3], Lars Christian Moeller[1], Denise Zwanziger[1,4], Dagmar Führer[1#]

[1]*Department of Endocrinology and Metabolism, University Hospital Essen, University Duisburg- Essen, Essen, Germany*

[2]*Department of Life Sciences and Chemistry, Jacobs University Bremen, Bremen, Germany*

[3]*Charité-Universitätsmedizin Berlin, Institut für Experimentelle Endokrinologie, Berlin, Germany*

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

*HR and KE contributed equally to this work.

[#]corresponding author

Submitted to *Biology of Sex Differences* on 05.04.2017.

I have written the manuscript and performed the following experiments: measurements of TH serum concentrations (Fig. 2), open field testing (Fig. 8 C, D) and characterization of mouse cohorts for impact of sex. Study design, treatment of mice and all other experiments (Fig. 3-7 and Fig. 8 A-B) were performed together with Kathrin Engels.

4.1 Abstract

Background: Thyroid dysfunction is more common in women and increases with age. The precise role of sex hormones in pathogenesis and manifestation of thyroid diseases is still not fully understood. Using a murine model, we asked whether sex influences phenotypes of hyper- and hypothyroidism at different ages.

Methods: Hyper- and hypothyroidism were induced by i.p. T4 or MMI/ClO_4-/LoI treatment over 7 weeks in 12 and 20 months old female and male C57BL/6N mice. Control animals underwent PBS treatment (n=7-11 animals/sex/treatment). Animals were investigated for impact of sex on body weight, food and water intake, body temperature, heart rate, behaviour (locomotor activity, motor coordination and strength) and serum thyroid hormone (TH) status.

Results: Distinct sex impact was found in euthyroid and hyperthyroid mice, while phenotypic traits of hypothyroidism were similar in male and female mice, except for water intake and this was irrespective of age. T4 treatment resulted in 2-fold higher TT4, FT4 and FT3 serum concentrations in adult and old female mice. Contrary to male mice, hyperthyroid females gained body weight despite diminished food and water intake and higher activity in adult and old ages. Advanced muscle function under control conditions decreased under TH excess to a higher extent in female than male mice. Higher body temperature in female mice was confirmed in both age groups and at all TH conditions, while no sex impact was observed for heart rate in adult and old mice.

Conclusions:

By comparison of male and female young, adult and old mice with thyroid dysfunction we could identify that TH-dependent body weight change, food and water intake, muscle function and motor activity are influenced by sex hormones. An impact of sex hormones on TH serum concentrations, body temperature and heart rate was less apparent and needs further evaluation. In summary, we showed that sex differences in phenotypes were modulated by TH excess and less by TH deprivation.

Keywords: thyroid hormone, hyperthyroidism, hypothyroidism, sex-difference, thyroid dysfunction, age, mice, phenotype

4.2 Introduction

Thyroid dysfunctions (TDs) are known to occur with 2-9 fold higher prevalence in women than men [1]. However, both are affected by similar symptoms such as fatigue, cold intolerance, weight gain or bradycardia under hypothyroidism in contrast to nervousness, heat intolerance, tachycardia and weight loss, despite increased appetite under hyperthyroidism [2, 3]. Interestingly, a recent study highlighted that symptom presence or absence are better predictors for overt hypothyroidism in men than women, as women report significantly more symptoms after thyroid hormone (TH) replacement therapy than men [4]. Moreover, women are prone to be overtreated with THs in comparison to men increasing the risk of atrial fibrillation and reduced bone mineral density in female patients [5]. Yet the precise molecular mechanisms by which sex hormones or genetics may contribute to this female preponderance are still unclear.

Additionally, data on a possible sex difference in thyroid function parameters such as the thyroid-stimulating hormone and serum concentrations of classical THs thyroxine (T4) and triiodothyronine (T3) in cohort studies are inconclusive, with studies showing sex variance of TH function parameters (6-9) whilst others do not [10-12]. These contrary findings could have resulted from differences in cohort characteristics (age and gender distribution, ethnicity, iodine supply, exclusion of clinically unapparent thyroid disease) and assays employed.

In a previous study we performed a characterization of young mice with thyroid dysfunction and identified sex-specific traits of hyper- and hypothyroidism in behavioural, functional, metabolic, biochemical and molecular parameters [13].

Since the prevalence of TD increases with age we asked whether this may differentially impact male *vs.* female mice systemically. Thus, to understand the influence and interplay of sex and age in tissue and organ-specific TH action we set up a mouse study.

Male and female mice were randomized to either euthyroid or chronic hyperthyroid and hypothyroid group. First, we assessed mice of young age (5 months, [13]) and subsequently adult (12 months) and old (20 months) animals. Mice were characterized under chronic hyper- or hypothyroidism for changes in body weight

(BW), food and water intake, body temperature, heart rate, neuromuscular features (locomotor activity, muscle strength and coordination) and TH serum status (Fig.1).

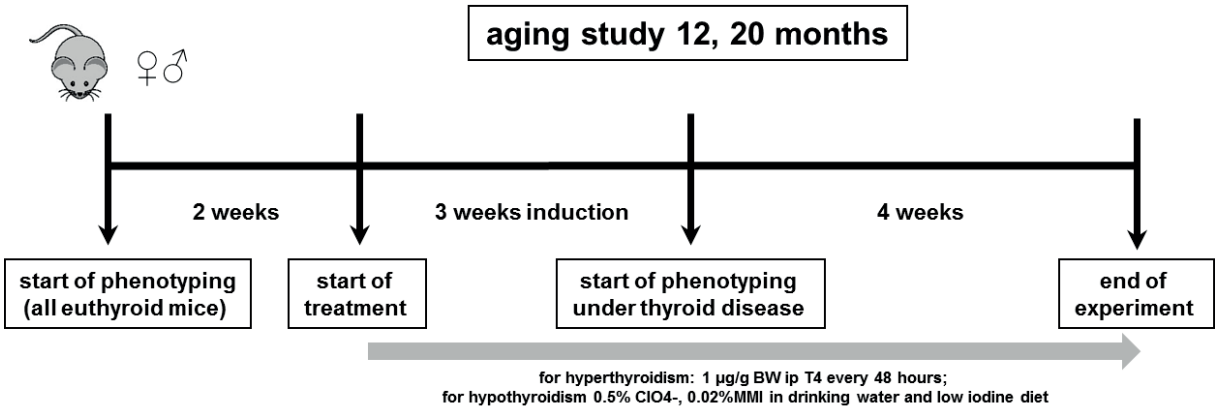

Figure 1: Study design of an approach to identify sex-differences of phenotypical traits in thyroid diseases. Aging study was performed to characterize natural sex-differences in different life stages between male and female mice in euthyroid, hyperthyroid and hypothyroid conditions [11].

4.3 Materials and Methods

Animals

For the aging study reported herein, male and female C57BL/6NTac (n=7-11/sex/age/treatment; Taconic Europe A/S, Denmark) mice aged 12 months (adult, n=46) and 20 months (old, n=64) were used. All mice were 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). All efforts were made to minimize suffering.

The nine-week experimental period was divided into three parts, consisting of a 2-week run-in period prior to manipulation of thyroid status to phenotypically characterize each individual mouse (pre-assessment), a 3-week treatment period to induce hyper- or hypothyroidism, and a 4-week assessment period to repeat the phenotypic characterization of each individual animal under chronic TH manipulation or euthyroid control treatment (Fig. 1).

Chronic hyperthyroidism and hypothyroidism were induced as previously described [13-16]. Briefly, for hyperthyroidism i.p. injections of 1 µg/g body weight T_4 (Sigma-Aldrich (T2376), USA) were performed every 48h. For induction of chronic hypothyroidism, animals were fed a low-iodine diet (LoI; TD.95007, Harlan Laboratories, USA) and received drinking water supplemented with 0.02% methimazole (MMI, Sigma-Aldrich (301507), USA), 0.5% sodium perchlorate (ClO_4^-) (Sigma-Aldrich (310514), USA) and 0.3% saccharine as sweetener (Sigma-Aldrich (240931), USA) (LoI/MMI/ClO_4^-). In addition, hypothyroid animals received i.p. injections of PBS every 48 hours. Control animals were fed a control diet and received i.p. injections of PBS every 48 hours.

Blood sample collection and analysis of serum TH concentrations

Final blood samples were obtained by heart puncture and stored 30 min on ice before clearing by centrifugation. Free triiodothyronine (FT_3), free thyroxine (FT_4) and total T4 (TT_4) concentrations in serum of mice were measured using commercial ELISA kits according to the manufacturer's instructions (DRG Instruments GmbH, Marburg, Germany). Detection ranges were 0.5-25 µg/dL, 0.05-7.5 ng/dL and 0.05-20 pg/mL for TT_4, fT_4 and fT_3, respectively.

Monitoring of body weight, food and water intake

Body weight was measured three times a week by placing mice on a scale. Food consumption was determined once a week by measuring the weight of remaining food pellets in the metal cage top. Water intake was controlled by weighing of water bottles (each, to the nearest 0.1g) weekly (in control and hyperthyroid groups) or twice (in hypothyroid groups) per week in control and TH manipulated groups. Male mice were caged individually, while female mice were kept in groups of three to four animals. Food and water intake of female mice was calculated by dividing the measured intake by the number of animals in each cage.

For each mouse, average daily food and water intake was calculated, and adjustments for body weight were derived by dividing average intake by the average body weight, and multiplying the result by 40 g (average body weight of mice).

Measurement of body temperature

Body temperature of mice was assessed four times using a cream-covered rectal probe (RET-3 rectal probe for mice, Kent Scientific Corporation, USA) connected to a thermocouple thermometer (Acorn Temp JKT Thermocouple Meter, Kent Scientific

Corporation, USA). Mice were placed on the top of the cage and the rectal probe was carefully inserted 2 cm into the rectum until a steady temperature was measured, which took approximately 8 to 10 sec.

Measurement of heart rate

Non-invasive restrained electrocardiography (ECG) recording was performed using an in-house protocol [13]. Conscious mice were placed on a platform with their paws on silver electrodes and were restrained by a half-tunnel. Signal was derived, enhanced and digitalized (Picoscope 2204, Pico Technology, United Kingdom). ECG was recorded using Picoscope 6 Software over 60-90 sec and heart rate was determined by measurements of RR intervals over 8 sec in a stable steady state. Non-invasive restrained ECG was recorded 3 times in all animals.

Rotarod test

The rotarod test [13,17] basically consists of five 3 cm diameter cylinders, enabling five mice to be tested simultaneously. On the first testing day, mice were allowed to acclimate to the rotarod test by letting them walk 6 min on the rotating cylinder with constant acceleration from 2-20 rpm.

For each rotarod session mice were subjected to 4 trials, with a minimum resting time interval of 6 min between the trials. Rotation mode was switched to constant acceleration from 4-50 rpm within 5 min. Maximum time and speed mastered by the animal was recorded. Mice that fell off the rod or attained full speed were placed back to their home cages. Every animal was subjected to two rotarod sessions (with a suspension period of 7 days) each before and after induction of thyroid dysfunction (4 sessions with 16 trials in total). Sessions 2 and 4 were used for statistical analysis.

Chimney test

The chimney test is constituted of a plastic tube (length 30 cm, diameter 3 cm). Mice were placed inside the tube and allowed to reach the other end. Then the tube was turned into a vertical position with mice head upside down. The test consisted of determining the time taken by mice to climb up to 25 cm of height. Mice were given 90 sec of time to pass the test [13,18,19].

Open Field

The open field consisted of a closed square area made of Plexiglas (50 x 50 cm). The area was divided in 4 corners, 4 walls and a center region (16 x 16 cm). Animals were tested in the dark phase of their dark/light cycle. Mice were placed in the center

of the open field and allowed to move freely for 5 min. Movements were monitored and digitalized by VideoMot2 software. Software recorded entries in all areas including time, frequency, latency and distance. Occurring events of rearing, freezing, grooming and jumping were recorded manually by the investigator during the experiment. Mice were placed in their home cages after 5 min of exploring the arena.

Statistical analysis

All data are shown as means ± standard deviation (SD) or standard error of the mean (SEM), as indicated. Statistical analysis using GraphPad Prism 6 Software was performed. The effects of hyper- and hypothyroidism are often opposing and inclusion of both treatments would therefore always show a significant treatment effect. To prevent false positive results statistical analysis of treatment groups were performed separately for hyper- and hypothyroid groups. 2-way ANOVA, 1-way ANOVA followed Bonferroni post-hoc analysis or unpaired Student´s t-test were applied as indicated. Values of *p<0.05, **p<0.01, ***p<0.001 were considered statistically significant.

4.4 Results

Effect of T4 administration on TH serum concentrations is exaggerated in female mice irrespective of age

To examine TH serum changes with T4 excess or TH deprivation final blood samples were collected 24h after last T4 injection or continuous MMI/ClO_4-/LoI treatment.

No significant sex differences were noted in euthyroid adult and old mice for TT4, FT4 and FT3 serum concentrations (Fig. 2). Interestingly, T4 treatment resulted in a larger increase of TT4, FT4 and FT3 concentrations in sera of female compared to male mice in adult and even more in old age (p<0.001, Fig.2, Table 1). On the other hand, TH deprivation was marked by TT4 serum concentrations below assay detection limit (0.5 µg/dL) in male and female mice of both age groups (Fig. 2 A, D), whereas FT4 was not altered except for old female mice showing a reduction of 60% ($F_{(1,30)}$=5.577 for treatment effect, p=0.0249, Fig. 2 E). Moreover, MMI/ClO_4-/LoI treatment did not result in altered FT3 concentrations in male and female mice of both ages (Fig. 2 C, F).

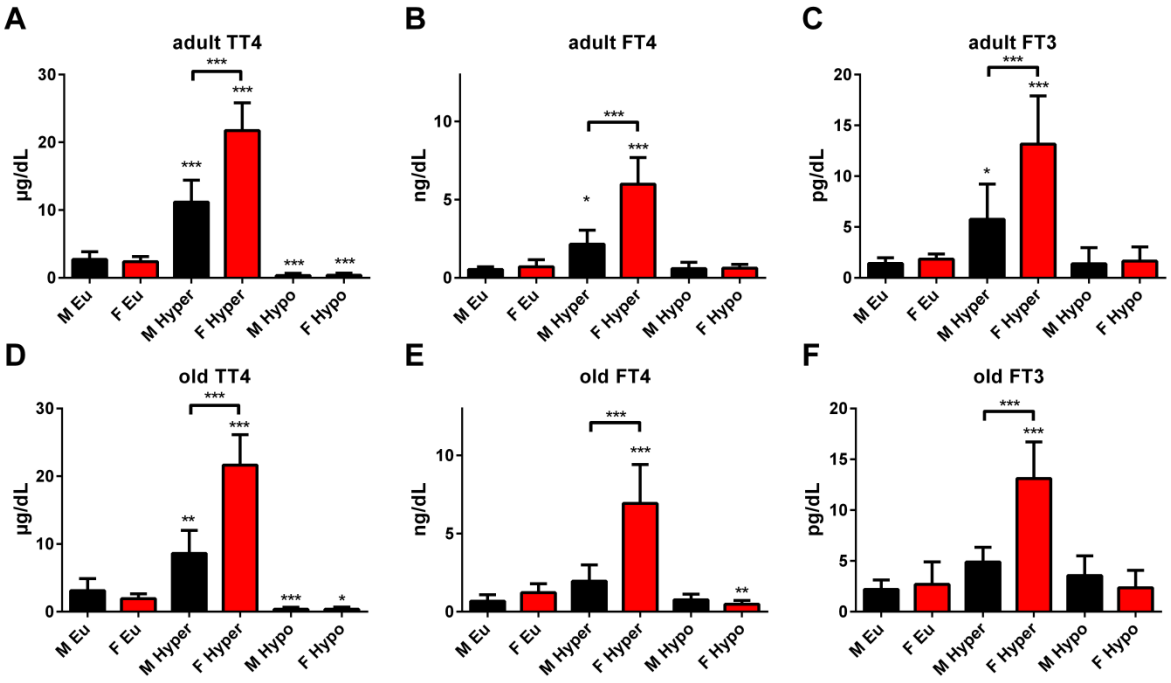

Fig. 2: Serum TH status in adult and old male and female mice under TH excess or deprivation. TT4, FT4 and FT3 serum concentrations were determined by ELISA at the end of T4 or MMI/ClO_4-/LoI treatment in sera of (**A-C**) adult and (**D-F**) old male and female mice. A distinct sex effect on T4 treatment was obvious by higher TH serum concentrations in female than male mice. Data are presented as mean ± SD, n= 7-11 animals/sex/treatment, 2-way ANOVA followed by Bonferroni post hoc analysis for each hyper- and hypothyroid conditions, respectively; *p<0.05, **p<0.01, ***p<0.001.

Table 1: Statistical analysis of TT4, FT4 and FT3 serum measurements in adult and old mice of both sexes. 2-way Anova followed by Bonferronis post hoc analysis was applied. Sex dependency was emphasized by illustration of Δ(female-male) values.

age	adult	old
Δ(female-male)	TT4=10.6 µg/dL FT4=3.8 ng/dL FT3=7.4 pg/dL	TT4=13.0 µg/dL FT4=5.0 ng/dL FT3=8.2 pg/dL
treatment effect	TT4: $F_{(1,26)}$=202.5, p<0.001 FT4: $F_{(1,26)}$=92.76, p<0.001 FT3: $F_{(1,26)}$=53.67, p<0.001	TT4: $F_{(1,26)}$=129.7, p<0.001 FT4: $F_{(1,26)}$=44.52, p<0.001 FT3: $F_{(1,26)}$=60.42, p<0.001
sex effect	TT4: $F_{(1,26)}$=27.4, p<0.001 FT4: $F_{(1,26)}$=31.48, p<0.001 FT3: $F_{(1,26)}$=13.45, p=0.0011	TT4: $F_{(1,26)}$=28.64 p<0.001 FT4: $F_{(1,26)}$=27.76 p<0.001 FT3: $F_{(1,26)}$=26.79, p<0.001
interaction	TT4: $F_{(1,26)}$=31.01, p<0.001 FT4: $F_{(1,26)}$=26.06, p<0.001 FT3: $F_{(1,26)}$=10.6, p=0.0031	TT4: $F_{(1,26)}$=41.28, p<0.001 FT4: $F_{(1,26)}$=17.83, p<0.001 FT3: $F_{(1,26)}$=20.90, p<0.001

Sex-difference in body weight change manifests with T4 treatment and aging

The impact of thyroid dysfunction on body weight (BW) of male and female mice was assessed over the entire experimental procedure and related to the individual body weight at start (Suppl. Table 1). Euthyroid male and female adult and old mice had comparable BW changes throughout the experiment (Fig. 3 A, D). In contrast, T4 treatment resulted in a marked sex-difference for BW change. While adult and old females gained weight under TH excess, male mice decreased BW at the same age (for adult: $F_{(27,371)}$=15.00 for time, $F_{(1,371)}$=180.2 for sex effect, $F_{(27,371)}$=5.726 for interaction, p<0.001; for old: $F_{(27,489)}$=8.01 for time, $F_{(1,489)}$=353.8 for sex effect, $F_{(127,489)}$=13.13 for interaction, p<0.001 Fig. 3 B, E). No sex-differences for BW changes were detected overall in adult and old hypothyroid animals (Fig. 3 C, F).

Noteworthy, to avoid different nutritional intake, all hypothyroid mouse groups were changed to low iodine diet with start of the protocol for induction of hypothyroidism, while euthyroid and hyperthyroid mice received an equivalent control diet.

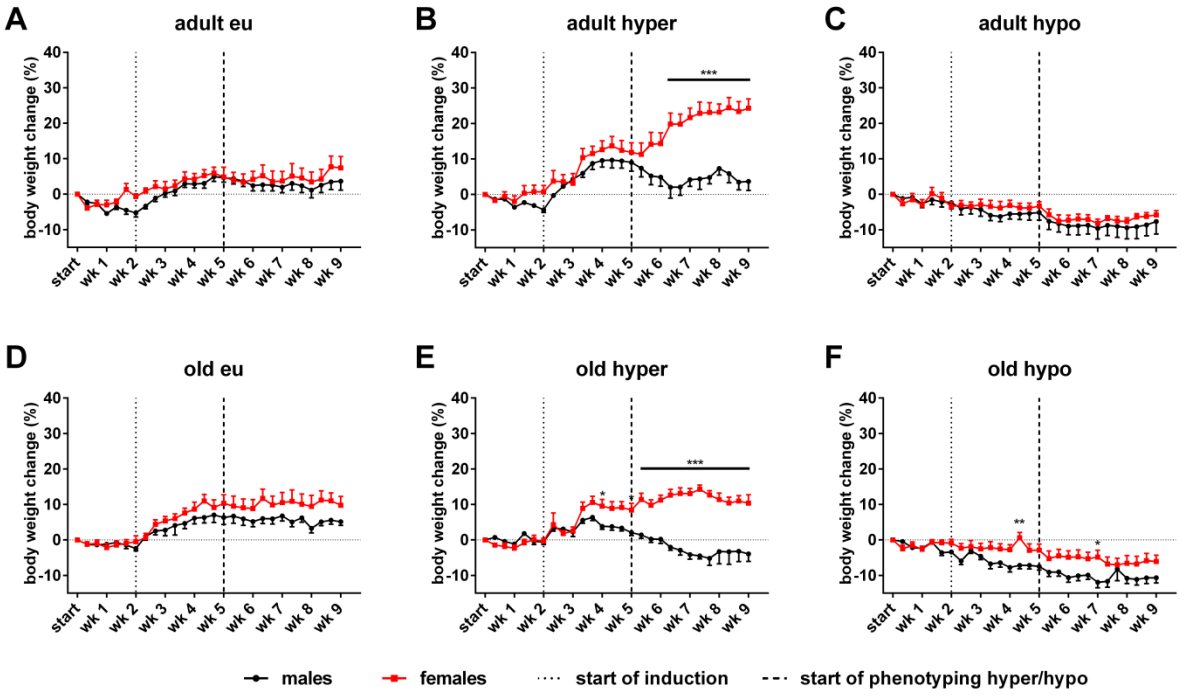

Fig. 3: Body weight change of adult and old mice of both sexes in eu-, hyper and hypothyroid conditions. BW was related to individual start weight in (**A-C**) adult and (**D-F**) old male and female mice. Sex differences in euthyroid (**A, D**), hyperthyroid (**B, E**) and hypothyroid (**C, F**) conditions were assessed. TH excess resulted in BW gain of females, while males lost BW in both ages. No sex difference was observed under TH deprivation. Data are presented as mean ± SD, n= 7-11 animals/sex/treatment, 2-way ANOVA followed by Bonferroni post hoc analysis, *p<0.05, **p<0.01, ***p<0.001.

Sex impacts food intake in euthyroid animals and is diminished by age and TH modulation

Food intake of all mice groups was measured weekly and calculated to respective BW of the individual mouse. Euthyroid control animals showed higher food intake in female compared to male mice in adult and old ages, however with larger difference in adult compared to old age (for adult: $F_{(8,108)}$=24.92 for time, $F_{(1,108)}$=430.6 for sex effect, $F_{(8,108)}$=9.204 for interaction, p<0.001; for old: $F_{(8,134)}$=15.75 for time, $F_{(1,134)}$=51.5 for sex effect, $F_{(8,134)}$=1.783 for interaction, p=0.0857; Fig. 4 A, D).

Both, T4 and MMI/ClO4-/LoI treatment diminished sex difference of food intake in adult and old ages with stronger increase of food intake in hyperthyroid males (Fig. 4 B, E) and stronger decrease in hypothyroid females, irrespective of age (Fig. 4 C, F).

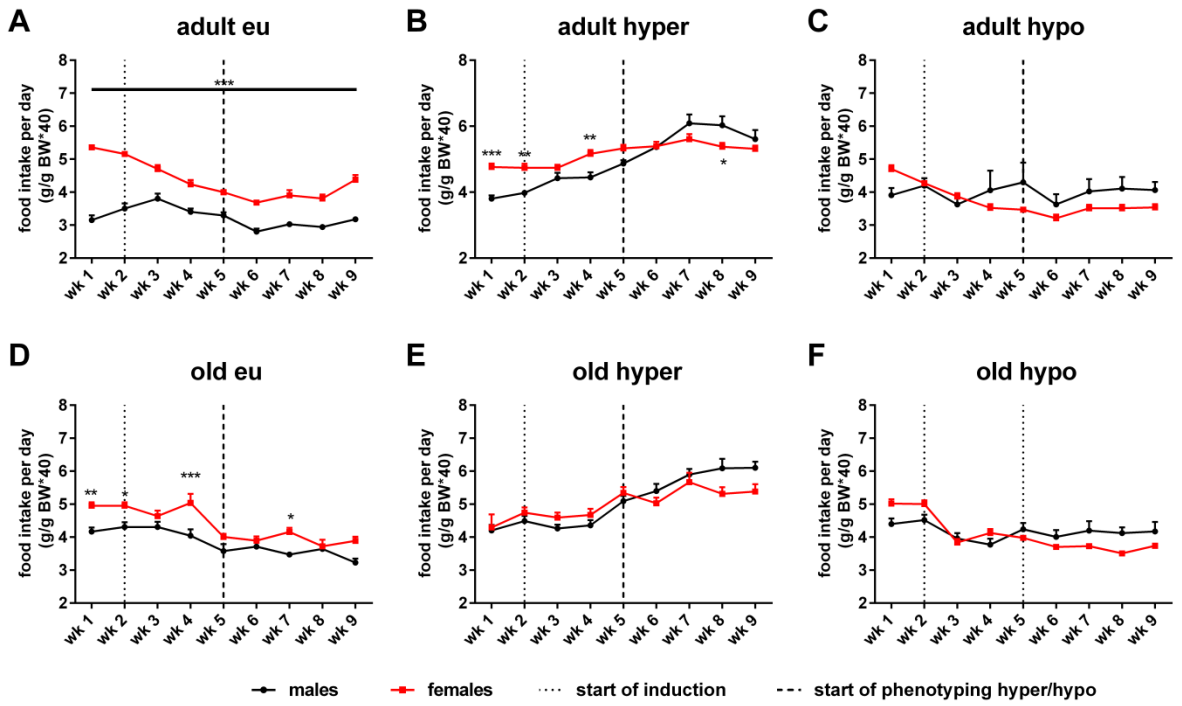

Fig. 4: Food intake behaviour during experimental procedure influenced by sex, age and TH condition. Food intake was related to BW weekly in (**A-C**) adult and (**D-F**) old male and female mice. Sex-dependency was noted for euthyroid groups, which disappeared by TH excess and deprivation. Data are presented as mean ± SD, n= 7-11 animals/sex/treatment, 2-way ANOVA followed by Bonferroni post hoc analysis, *p<0.05, **p<0.01, ***p<0.001.

Sex-difference of water intake is diminished in hyperthyroid and reversed in hypothyroid animals

Water intake of all mouse groups was measured weekly and calculated to respective BW of individual mouse. Adult and old aged female euthyroid mice had higher water intake than male mice (for adult: $F_{(8,108)}$=23.26 for time, $F_{(1,108)}$=936.2 for sex effect, $F_{(8,108)}$=5.017 for interaction, p<0.001; for old: $F_{(8,134)}$=16.38 for time, $F_{(1,134)}$=219.4 for sex effect, $F_{(8,134)}$=1.788 for interaction, p=0.0847, Fig. 5 A, D).

Sex and age-dependent differences were found for water intake under hyperthyroidism. Adult female mice did not change drinking behaviour under T4 treatment, while male mice increased water intake over time. Thus, compared to the euthyroid situation the sex-difference reversed and adult male mice consumed more water than female mice ($F_{(8,119)}$=42.04 for time, $F_{(1,119)}$=0.1882 for sex effect,

$F_{(8,119)}$=13.24 for interaction, p<0.001 Fig. 5 B). At old age, no sex-difference was observed and both male and female mice increased water intake under TH excess (Fig. 5 E).

Hypothyroidism was marked by a reversed sex-difference in drinking behaviour. While water intake in male mice did not significantly change by MMI/ClO_4-/LoI treatment, a dramatic decrease of water intake was observed in female mice, irrespective of age (for adult: $F_{(8,126)}$=15.27 for time, $F_{(1,126)}$=560.8 for sex effect, $F_{(8,126)}$=73.19 for interaction, p<0.001; for old: $F_{(8,156)}$=4.373 for time, $F_{(1,156)}$=106.0 for sex effect, $F_{(8,156)}$=9.991, p<0.001 for interaction; Fig. 5 C, F).

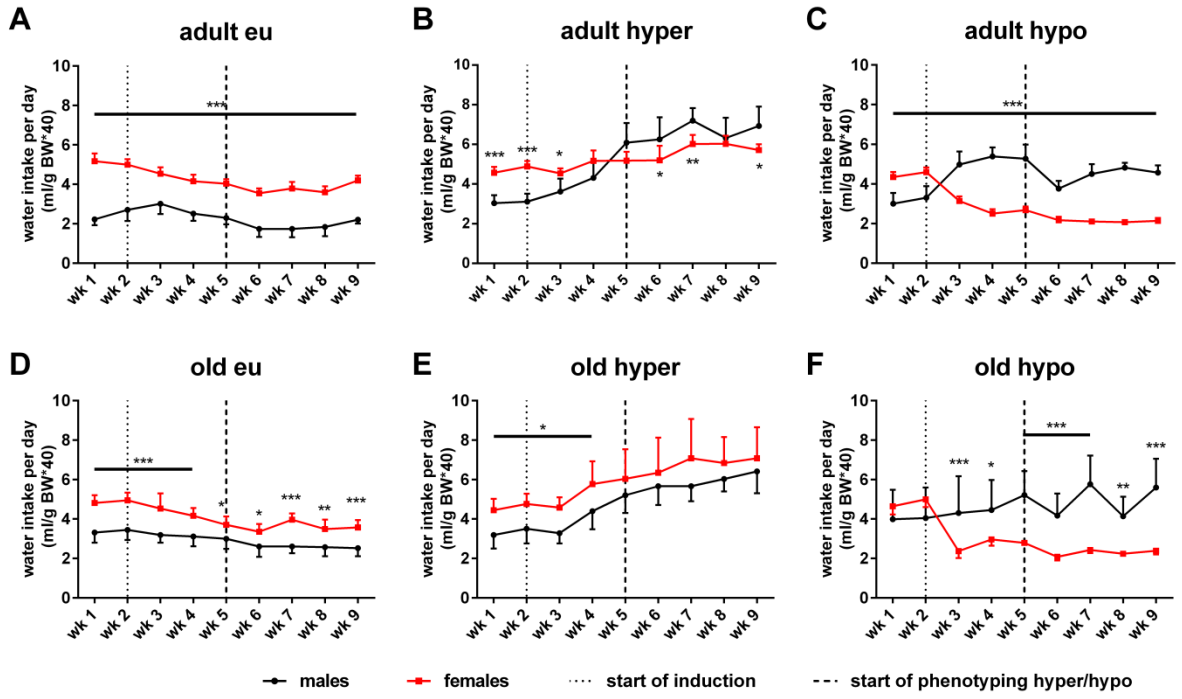

Fig. 5: Water consumption in adult and old groups of male and female mice, under control, TH excess or deprivation. Water intake was related to BW weekly in (**A-C**) adult and (**D-F**) old male and female mice under euthyroid condition, T4, or MMI/ClO_4-/LoI treatment. Data are presented as mean ± SD, n= 7-11 animals/sex/treatment, 2-way ANOVA followed by Bonferroni post hoc analysis, *p<0.05, **p<0.01, ***p<0.001.

Body temperature in contrast to heart rate, is strongly sex-dependent irrespective of TH status and age

Sex-dependency was observed throughout all measurements with a higher body temperature in female compared to male mice in adult as well as old age and at all TH conditions (Fig. 6 A, B, table 2).

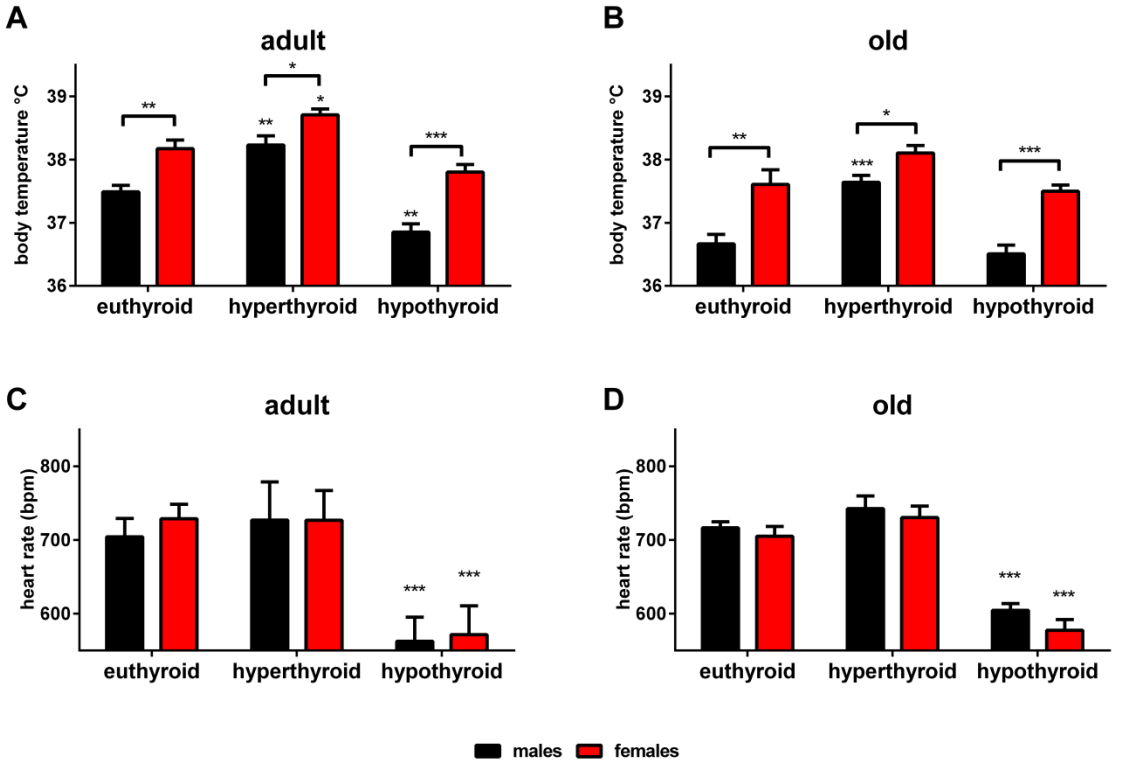

Fig. 6: Body temperature and heart rate in adult and old male and female mice with eu-, hyper- and hypothyroid state. Rectal body temperature measurements and non-invasive ECG were performed in (**A**) adult and (**B**) old male and female mice to assess body temperature and (**C, D**) to determine changes in heart rate. Under TH excess and deprivation persistent higher body temperature of female mice were noted at all TH status and ages, whereas no sex impact was noted for heart rate. Data are presented as mean ± SD, n= 7-11 animals/sex/treatment, 2-way ANOVA followed by Bonferroni post hoc analysis, *p<0.05, **p<0.01, ***p<0.001.

Table 2: Statistical analysis of body temperature measurements in adult and old mice of both sexes. 2-way ANOVA followed by Bonferronis post hoc analysis was applied for hyper- and hypothyroid conditions.

age	adult	old
Δ(female-male)	eu: 0.68°C hyper: 0.48°C hypo: 0.95°C	eu: 0.94°C hyper: 0.46°C hypo: 1.00°C
treatment effect	hyper: $F_{(1,25)}$=28.25, p<0.001 hypo: $F_{(1,26)}$=16.48, p<0.001	hyper: $F_{(1,29)}$=22.19, p<0.001 hypo: $F_{(1,31)}$=0.719, p=0.403
sex effect	hyper: $F_{(1,25)}$=23.43, p<0.001 hypo: $F_{(1,26)}$=43.08, p<0.001	hyper: $F_{(1,29)}$=20.13 p<0.001 hypo: $F_{(1,31)}$=37.18 p<0.001
interaction	hyper: $F_{(1,25)}$=0.707, p=0.408 hypo: $F_{(1,26)}$=1.152, p=0.293	hyper: $F_{(1,29)}$=2.326, p=0.138 hypo: $F_{(1,31)}$=0.0024, p=0.878

Non-invasive ECG measurements were performed to examine the influence of sex and TH on heart rate. However, no significant sex differences were found between euthyroid, hyperthyroid and hypothyroid mice at adult and old age (Fig. 6 C, D).

Sex difference on muscle strength and motor coordination is diminished by TH deprivation and TH excess in adult and old mice

To investigate the impact of TH disturbances on motor function and muscle strength in male and female mice rotarod and chimney tests were performed.

In particular, the rotarod test examines the ability to balance and walk on a rotating cylinder and thereby investigates motor coordination and function. For each mouse a training period was implemented in euthyroid state and was compared to the performance under hyper-, hypo- or again euthyroid state.

Euthyroid female mice spend more time on the rod compared to male mice at adult and old ages (for adult: $F_{(7,96)}$=1.680 for time, $F_{(1,96)}$=279.6 for sex effect, $F_{(7,96)}$=0.576 for interaction; for old: $F_{(7,120)}$=1.972 for time, $F_{(1,120)}$=80.98 for treatment effect, $F_{(7,120)}$=0.7789 for interaction; Fig. 7 A, D), showing better muscle endurance and motor coordination. T4 treatment and TH deprivation resulted in diminished sex

differences in adult and old mice with generally poorer performance of hyperthyroid adult and old mice and unchanged performance of old hypothyroid mice (Fig. 7 B-C, E-F).

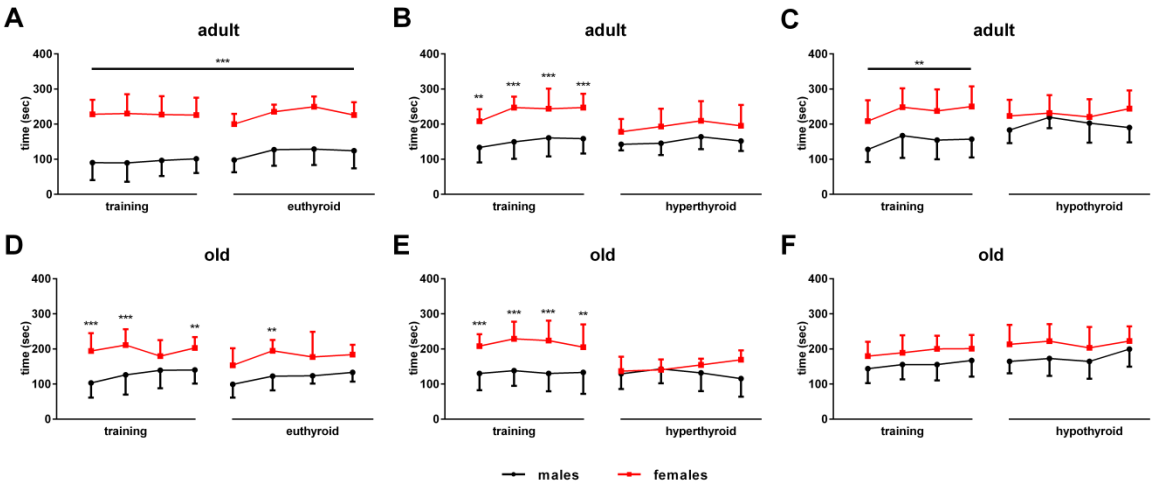

Fig. 7: Rotarod performance in all groups in both ages and sexes under control, T4 or MMI/ClO_4/LoI treatment. Time spend on the rotating cylinder was determined in (**A-C**) adult and (**D-F**) old male and female mice. Pronounced sex difference in euthyroid mice was diminished by TH excess or deprivation. Data are presented as mean ± SD, n= 7-11 animals/sex/treatment, unpaired two-tailed student´s t-test, *p<0.05, **p<0.01, ***p<0.001.

To further evaluate muscle strength, tonus and coordination of movements we used the chimney test, which measures the required time of mice to climb up in a tube. Overall better coordination of movement and muscle strength was observed in female compared to male mice (Fig. 8). Thus euthyroid control male mice failed in a high percentage to pass the test, while female mice succeeded to climb up the tube. This however was impaired under hyper- and hypothyroid conditions with more pronounced impairment under TH excess ($F_{(5,39)}$=10.13, p<0.001, Fig. 8 A). Old male mice were not able to pass the Chimney test illustrating the profound effect of age on muscle strength and coordination (Fig. 8 B).

To determine whether activity was different between male and female adult and old mice an open field test was used. Sex-differences were observed at all ages and varied with TH status. In general higher activity was found in female compared to male mice and this was persistent in eu-, hyper- and hypothyroid state irrespective of age, either as a trend or on a significant level (adult: $F_{(5,39)}$=16.42, p<0.001, Fig. 8 C and old $F_{(5,50)}$=10.34, p<0.001, Fig. 8 D).

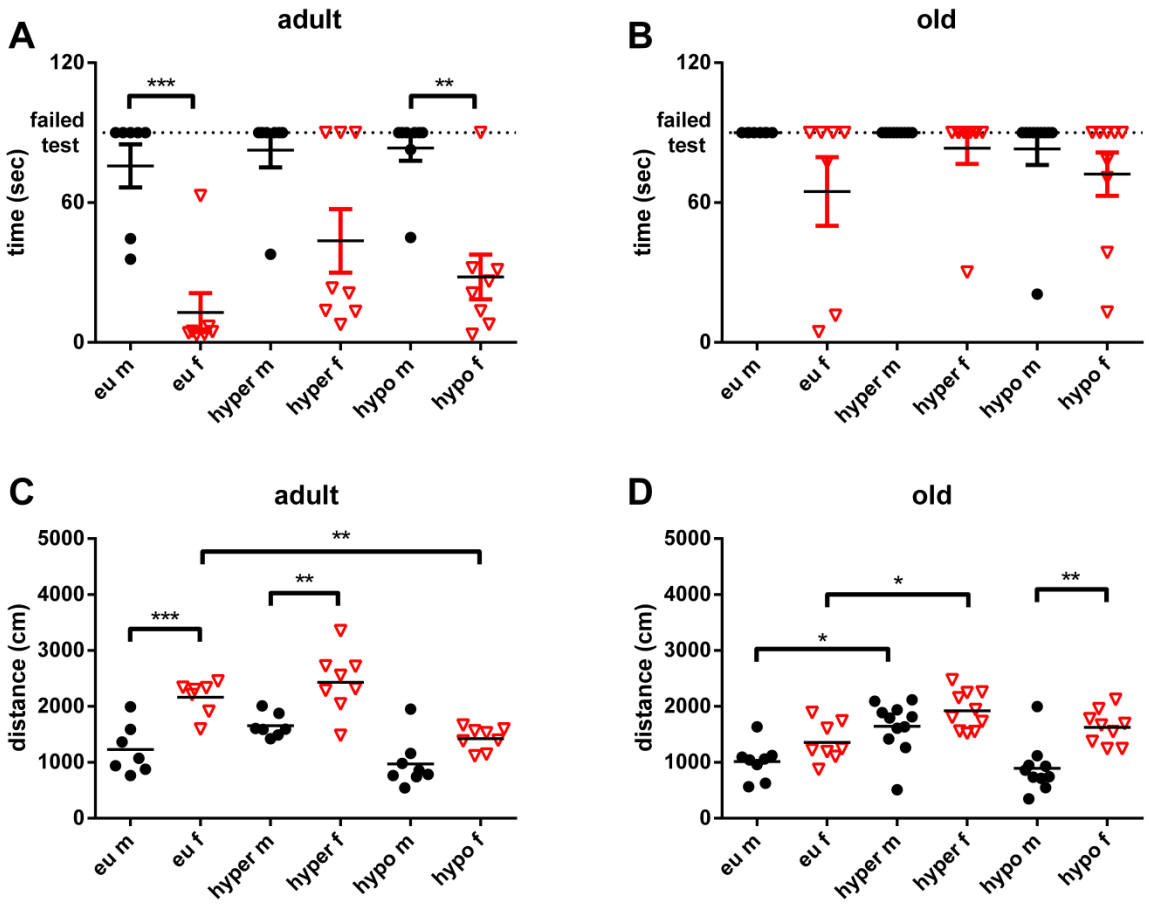

Fig. 8: Chimney test and open field activity of adult and old mice of both sexes under eu-, hypo- or hyperthyroid conditions. The Chimney test was performed in (**A**) adult and (**B**) old male and female mice to assess muscle function, while the open field test was performed to assess the activity of mice by measuring the covered distance in (**C**) adult and (**D**) old age under TH excess, deprivation or euthyroid state. Data are presented as mean ± SD, n= 7-11 animals/sex/treatment, One-way ANOVA followed by Bonferroni post hoc analysis, *p<0.05, **p<0.01, ***p<0.001.

4.5 Discussion

Thyroid dysfunction (TD) may occur at any stage of life in women and men, but vary in its prevalence and severity of symptoms. Two aspects are relevant in the clinical situation. First, TD affects far more often women than men and second, the prevalence of TD increases with age, but symptoms are diminished in elderly patients while risks for co-morbidities increase. To approach such differences under highly standardized conditions we decided to use a rodent model and investigated

mice of different ages to define specific traits of TD in male and female mice. In a first study we assessed sex-differences in young animals of 5 months [13] and subsequently continued with characterization of hyper- or hypothyroidism in adult (12 months) and old (20 months) male and female mice. Although female mice do not undergo a menopause as humans, still the hormonal activity and reproduction possibility declines with age [20, 21] and one can hypothesize that sex impact on TH action may be reduced in old compared to young female and male mice. Thus, by using our aging mice cohorts we were able to extract phenotypical traits to be likely sex hormone dependent or not. Traits of TD in young animals which were diminished or reversed at old age are suggested to be likely sex hormone dependent, in contrast to sex-differences which were consistent in all ages and might be highly controlled in terms of genetic background.

Sex difference in response of TH serum concentration to T4 treatment

An obvious difference in response to T4 treatment was observed in all age groups. Female mice of all ages had higher TT4, FT4 and FT3 serum concentrations if subjected to chronic T4 treatment ([13], Figure 2). This has been already observed by us in an earlier study [16] and by others using T3 [22], but was not expected to appear in older mice. In our aging study female mice had almost two-fold higher TT4 serum concentrations compared to male mice in young, adult and old age. In fact, this could have resulted from increased thyroxin-binding globulin (TBG) capacity by estrogen [23, 24], which has been described in human patients. However, for euthyroid mice a higher TBG concentration in sera of male in comparison to female mice was shown [25], but addressed no TH alteration. Concluding from our investigations in young mice, chronic T4 treatment strongly repressed TBG gene expression in both sexes [13]. Furthermore, TBG was reported to be the only TH responsive transport protein for TH in contrast to transthyretin (TTR) or albumin [26]. Thus TTR or albumin might caused the observed sex-dependent effects for TT4 serum concentrations under TH excess independent of TBG, but were not subjected in this study.

Interestingly, despite of sex influence on TT4 serum concentrations, larger increase of FT4 and FT3 serum concentrations were noted in females compared to male mice. Sex differences in FT3 serum concentrations in hyperthyroid adult and old mice under T4 treatment might have resulted in this chronic approach from sex-dependent

T4 to T3 conversion by deiodinase 1 (Dio1), which was previously reported to be differently active in young male and female mice. Thus, renal Dio1 was found to be more active in female than male mice, while hepatic Dio1 showed higher activity in male than female mice [27]. A subsequent study investigating an age effect on sexual dimorphism of Dio1 showed no differences in hepatic Dio1 activity at 12 months of age, but persistent sex difference in renal Dio1 activity [28]. Thus, higher renal Dio1 activity might have influenced FT3 serum values in female mice, but was not investigated in this study. Furthermore, concluding from TH serum concentrations under T4 treatment, one would expect to observe exaggerated symptoms of hyperthyroidism in female mice.

In contrast, TH deprivation induced by combined MMI/ClO_4^-/LoI treatment, resulted in all age groups in very low TT4 and unchanged FT4 and FT3 serum concentrations, except for decreased FT4 concentrations in old female mice. However, this difference was minor and might be an assay variation with little relevance on further evaluation of phenotypical traits.

Body weight changes sex-dependently under TH excess

An impact of THs on BW has been demonstrated already for young mice [16, 29, 30]. In addition, in our initial study a sex-dependent influence was noted with faster weight gain in young hyperthyroid male compared to female mice [13]. In contrast, this sex dependency under TH excess reversed with age. Adult and old hyperthyroid female mice gained more BW in comparison to male mice. Strikingly and close to the human situation, adult and old male mice even lost BW when subjected to T4 excess (Fig. 3 B, E). Thus, this suggested a more pronounced male sex hormone dependent regulation of BW change under TH excess. For young mice an increase of BW has been described during shorter induction periods, but no data for old hyperthyroid animals were available so far. To our knowledge, this was the first study to characterize BW changes in a model of chronic TH excess in mice of both sexes and at different ages.

TH deprivation resulted in stagnation of BW with no sex-differences for BW change in all age groups in line with results of TH serum concentrations ([13], Fig. 3 C, F).

Sex-differences in nutritional intake disappear with TH excess and deprivation

As differences in BW change were found especially in hyperthyroid animals, we considered nutrient intake as a possible explanation. Higher food intake was noted in euthyroid female compared to male mice in all ages, while no sex impact on this parameter was found in hypothyroid groups ([13], Fig. 4 A, C-D, F). In contrast, TH excess in young animals resulted in higher food intake in females than males [13], a sex difference that disappeared in hyperthyroid adult and old mice (Fig. 4 B, E). Thus, food intake was not in line with BW change and did not explain BW loss or gain in male and female mice across the different age groups. In fact, increased food intake was stronger correlated with less BW gain or even with BW loss (e.g. adult and old hyperthyroid males). All these observations led us to the hypothesis that at least under hyperthyroid conditions BW did not correlate with food intake and that these two traits were affected differently by THs. Moreover, regulation of food intake is a highly complex process, which can be a direct consequence of treatment or a compensatory response regulated by a central mechanism [31]. Thus, either uptake of nutrients is different or the metabolism of these nutrients could be differently regulated in male and female mice. In addition, it might be a result of several factors in which nutrient intake plays a role as food can be used for various functions e.g. energy storage in fat pads, increased development of muscles, maintaining body temperature or providing enough calories to enable high activity. Moreover, it has been shown for several mouse strains, that higher food intake was correlated with higher activity [32-34], but none of these studies evaluated different TH conditions.

As second part of nutrient metabolism we evaluated water intake, which in part reflects the activity of mice [35]. Euthyroid female mice consumed more water than male mice, irrespective of age ([13], Fig. 5 A, D). T4 treatment at the same time influenced drinking behaviour in a sex- and age-dependent manner. Sex-difference was diminished and even disappeared at the end of experiment in young mice [13]. In adult mice sex difference in water intake was even reversed with consumption in male mice, as female mice drinking behaviour did not change under chronic TH excess (Fig. 5 B). At the old age no sex difference in this parameter could be observed in hyperthyroid mice (Fig. 5 E). These findings suggest a sex hormone dependency for water intake and raise the awareness that impact of TH excess may be influenced by male and female sex hormones.

In contrast TH deprivation resulted in a vanished or even reversed sex-difference with higher water intake in young, adult and old male mice ([13], Fig. 5 C, F) and a drastic decrease of water intake in hypothyroid female mice. One reason might be different taste sensitivity for the drugs MMI or ClO_{4-} or saccharine which was added as sweetener to cover bitter taste of these drugs. As the acceptance of these drugs was low in female mice throughout all ages, it might be a genetic rather than a female sex hormone effect.

In contrast to heart rate, TH effects on body temperature with aging remain sex-dependent

Rectal body temperature was already different between young male and female mice under all TH conditions [13]. However, also adult and old female mice had higher body temperature than male mice, irrespective of treatment, suggesting a strong sex-dependent effect (Fig. 6 A, B). Non-invasive methods of rectal and surface body temperature measurements correlate very well with mice core body temperature [36]. However, a drawback of these methods is, that only certain time points can be measured and might therefore miss differences in small time frames. As reported previously in a study investigating the influence of sex hormones on body temperature via radiotelemetry [20], sex-differences were most obvious at daytime and in the proestrous and estrous period with higher values in 3 months old females than males. Ovariectomy in these mice resulted in similar body temperature pattern during proestrous and estrous as in male mice, indicating that differences in these time frames were regulated by sex hormones. Throughout all other days and nights body temperature was comparable between ovariectomized and normal female mice suggesting that sex hormones are not the only factors contributing to body temperature regulation in female mice. As no influence of THs was addressed in this study, we conclude from our measurements, that TH excess and deprivation have likely stronger influence on basal body temperature in general, rather than on distinct time points during estrous cycle.

Another very important characterization was the influence of THs and sex on heart rate, as tachycardia or bradycardia are well known manifestations of TD with increased morbidity in an elderly organism.

Sex-difference for heart rate was present in young euthyroid mice but disappeared by TH excess or deprivation [13], suggesting that alteration of TH status might mask impact of sex hormones. In adult and old mice no sex impact was found for heart rate, irrespective of TH status (Fig. 6 C, D). Measurements of heart rate were performed in a non-invasive ECG, which although pre-trained to the restrainer resulted in an increased stress for all mice and could have masked small differences within the measurements.

In an aged organism TH dysfunction alters muscle function, coordination and activity more in females than males

Muscle function was assessed with two different tests. The chimney test focused on muscle strength and coordination of movement, while the rotarod test provided information on endurance and motor coordination.

In our previous study we found that TD impaired performance in young male and female mice with diminished sex difference under hyper- and hypothyroid condition while euthyroid female mice generally show better results in the rotarod test [13]. In adult and old mice the sex difference also disappeared under TH excess or deprivation (Fig. 7 A-F). Interestingly, this was caused by either a weaker performance of female mice (hyperthyroid) or by a stable performance of females but an improvement of endurance and motor coordination in males (hypothyroid).

As already described for young mice [13], also in adult mice a major sex-difference was found in the chimney test in euthyroid, hyperthyroid and hypothyroid mice. Female mice had a much higher ability to climb up the tube at all TH conditions, while male mice were incapable to pass the chimney test (Fig. 8 A). Thus, this observation suggested that female mice had a better movement coordination and muscle strength throughout the lifespan than male mice. This was supported by the assessment of chimney test in old mice, where except for one male mouse none was able to pass the test, while at least eight female mice were still able to climb up the tube (Fig. 8 B). A better muscle function in female mice has previously been described to result from a better fatigue resistance, recovery and less mechanical damage after exercise [37, 38], which is in line with our observations assessed by chimney and rotarod test in euthyroid mice. Although an advantage in female muscles was noted throughout all age stages suggesting a genetic background, TH

modulation had a stronger impact on females than males. Thus, the interplay between thyroid and sex-hormones might regulate to some extent muscle and/or neurological functions of the central and peripheral nervous system. Therefore, it is not unreasonable to conclude that both neurological and primary muscular factors might contribute to observed sex-differences [39, 40]. Noteworthy, especially the chimney test was influenced by BW and size of the mice. As females are lighter and smaller than males at all TH conditions and ages, they are less impaired by their body composition than males and are in a favourable position to climb up the tube. Thus, assessing motor function beside muscle strength and endurance, BW and size are considerable contributing factors.

Changes in activity were assessed by the open field test and were quantified by measuring the total distance travelled in 5 minutes. Significant sex-difference was noted with higher activity of female mice at different TH conditions and persisted in adult, but decreased with old age ([13], Fig. 8 C, D), which is in line with other studies confirming a higher locomotor activity and increased voluntary exercise in female mice [20, 41]. Thereby a decrease with age may indicate an influence of sex hormones on activity of female mice, irrespective of TH status.

Conclusion

Since women are more frequently affected by hyper- and hypothyroidism it is important to clarify if and how men and women may differ in manifestation and severity of symptoms of TD. To address this question, we have performed mice studies in both sexes and at different ages and found sex-specific changes in phenotypic traits by TD. Thus, by comparing experimental results of young from adult and old mice we could identify BW change, food and water intake, muscle function and activity to be likely sex hormone dependent. An impact of sex hormones on TH serum concentrations, body temperature and heart rate remains to be further investigated. Additionally, we showed that sex differences were modulated by TH excess and less so by TH deprivation.

A very important future next step is the evaluation of organ specific differences to approach molecular mechanisms which contribute to the observed phenotypical traits and this will be our focus in the following studies. In summary, traits of TD vary with

sex at least in mice, and this is sustained for several hyperthyroid signs throughout life.

Abbreviations

BW: body weight; ClO_4^-: perchlorate; ECG: electrocardiography; HR: heart rate; FT_3: free 3,3',5-triiodothyronine; FT_4: free thyroxine; LoI: low-iodine diet; MMI: methimazole; qRT-PCR: quantitative real-time PCR; T_3: 3,3',5-triiodothyronine; T_4: thyroxine; TT_4: total thyroxine; TD: thyroid dysfunction; TH: thyroid hormone; TSH: thyroid-stimulating hormone

Ethics, consent and permissions

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).

Consent publication

Not applicable.

Availability of data

The datasets analysed during the current study are available from the corresponding author on request.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was funded by DFG FU 356/7-1 to DF, MO 1018/2-1 to LCM, DFG KO 922/17-1, BR 1308/11-1 to KB and ZW 221-2/1 to DZ in the framework of SPP 1629 THYROID TRANS ACT.

Authors´ contributions

HR, KE, DZ and DF conceived and designed the experiments. HR, KE and GSH performed the experiments. HR, KE and GSH analysed the data. LCM, KB, JK, DZ and DF contributed to data interpretation and reviewed the manuscript. All authors provided valuable feedback and approved the final manuscript.

Acknowledgements

The authors are grateful to S. Rehn, A. Jaeger, J. Fleckhaus and E. Werbenko for their dedicated technical support. We are also grateful to Dr. Kleinbongard and Prof. Heusch (Institute for Pathophysiology, University Hospital Essen, Essen, Germany) for help with mouse ECG analysis. This work was supported by DFG FU 356/7-1 to DF, MO 1018/2-1 to LCM, KO 922/17-1 to JK, BR 1308/11-1 to KB, ZW 221-2/1 to DZ in the framework of SPP 1629 THYROID TRANS ACT.

References

1. Vanderpump M.P.J. The epidemiology of thyroid disease. Br Med Bull. 2011;99:39-51.
2. Ladenson P.W., Singer P.A., Ain K.B., Bagchi N., Bigos S.T., Levy E.G., Smith S.A., Daniels G.H., Cohen H.D. American Thyroid Association guidelines for detection of thyroid dysfunction. Arch Intern Med. 2000 Jun 12;160(11):1573-5.
3. Surks M.I., Ortiz E., Daniels G.H., Sawin C.T., Col N.F., Cobin R.H., Franklyn J.A., Hershman J.M., Burman K.D., Denke M.A., Gorman C., Cooper R.S., Weissman N.J.. Subclinical Thyroid Disease Scientific Review and Guidelines for Diagnosis and Management. JAMA. 2004;291(2):228-238. doi:10.1001/jama.291.2.228
4. Carlé, A., Bülow Pedersen, I., Knudsen, N., Perrild, H., Ovesen, L. and Laurberg, P. Gender differences in symptoms of hypothyroidism: a population-based DanThyr study. Clin Endocrinol, 83: 717–725. doi:10.1111/cen.12787
5. Mammen J. S., McGready J., Oxman R., Chia C. W., Ladenson P. W., and Simonsick E. M. Thyroid Hormone Therapy and Risk of Thyrotoxicosis in Community-Resident Older Adults: Findings from the Baltimore Longitudinal

Study of Aging. Thyroid. August 2015, 25(9): 979-986. doi:10.1089/thy.2015.0180

6. Tunbridge W. M., Evered D. C., Hall R., Appleton D, Brewis M, Clark F, Evans J. G., Young E., Bird T., and Smith P. A. The spectrum of thyroid disease in a community: the Whickham survey. 1977.Clin. Endocrinol. (Oxf) 7 (6). 481–93.
7. Bjoro T., Holmen J., Krüger Ø., Midthjell K., Hunstad, K., Schreiner T., Sandnes L., Brochmann H. Prevalence of thyroid disease, thyroid dysfunction and thyroid peroxidase antibodies in a large, unselected population. The Health Study of Nord-Trondelag (HUNT). 2000. Eur. J. Endocrinol. 143 (5), 639–647.
8. Hollowell J. G., Staehling N. W., Flanders W. D., Hannon W. H., Gunter E. W., Spencer C. A., Braverman L. E. Serum TSH, T(4), and thyroid antibodies in the United States population (1988 to 1994): National Health and Nutrition Examination Survey (NHANES III). 2002. J. Clin. Endocrinol. Metab. 87 (2), 489–499.
9. Hadlow N. C., Rothacker K. M., Wardrop R, Brown S. J., Lim E. M., Walsh J. P. The relationship between TSH and free T4 in a large population is complex and nonlinear and differs by age and sex. 2013. J. Clin. Endocrinol. Metab. 98 (7), 2936–2943.
10. Aghini-Lombardi F.; Antonangeli L., Martino E., Vitti P., Maccherini D., Leoli F., Rago T., Grasso L., Valeriano R., Balestrieri A., Pinchera A. The spectrum of thyroid disorders in an iodine-deficient community: the Pescopagano survey. 1999. J. Clin. Endocrinol. Metab. 84 (2), 561–566.
11. Kapelari K., Kirchlechner C., Högler W., Schweitzer K., Virgolini I., Moncayo R. Pediatric reference intervals for thyroid hormone levels from birth to adulthood: a retrospective study. 2008 BMC Endocr Disord 8: 15.
12. Roelfsema F., Pereira A. M., Veldhuis J. D., Adriaanse R., Endert E., Fliers E., and Romijn J. A. Thyrotropin secretion profiles are not different in men and women. 2009. J. Clin. Endocrinol. Metab. 94 (10): 3964–67.
13. Rakov H., Engels K., Hoenes G. S., Strucksberg K-H., Moeller L. C., Koehrle, J., Zwanziger D., Fuehrer D. Sex-specific phenotypes of hyperthyroidism and hypothyroidism in mice. Biol Sex Differ. 2016 Aug 24;7(1):36. doi: 10.1186/s13293-016-0089-3

14. Engels K, Rakov H, Zwanziger D, Moeller LC, Homuth G, Koehrle J, Brix K., Fuehrer D. Differences in mouse hepatic thyroid hormone transporter expression with age and hyperthyroidism. Eur Thyroid J. 2015; 4(Suppl 1):81-6. doi:10.1159/000381020.
15. Zwanziger D, Rakov H, Engels K, Moeller LC, Fuhrer D. Sex-dependent claudin-1 expression in liver of eu- and hypothyroid mice. Eur Thyroid J. 2015; 4(Suppl 1):67-73. doi:10.1159/000431316.
16. Engels K., Rakov H., Zwanziger D., Hoenes GS., Rehders M., Brix K., Koehrle J., Moeller L.C., Fuehrer D. Efficacy of protocols for induction of chronic hyperthyroidism in male and female mice. Endocrine (2016) 54: 47. doi:10.1007/s12020-016-1020-8.
17. Dunham N.W., Miya T.S.. A note on a simple apparatus for detecting neurological deficit in rats and mice. J Am Pharm Ass. 1957;46:208-214.
18. Boissier J.R., Tardy J., Diverres J.C. Une nouvelle méthode simple pour explorer l´action “tranquillisante”: le test de la cheminée. Med Experimentalis. 1960;3:81-84.
19. Daugé V., Sebret A., Beslot F., Matsui T., Roques B.. Behavioral profile of CCK2 Receptor-deficient mice. Neuropsychopharmacology. 2001;25:690-698.
20. Sanchez-Alavez M., Alboni S., Conti B. Sex- and age-specific differences in core body temperature of C57Bl/6 mice. Age. 2011;33:89-99.
21. Jones E.C. and Krohn P.L. The relationships between age, numbers of oocytes and fertility in virgin and multiparous mice. J Endocrinol 21, 1961 (4) 469-495
22. Bezerra da Silveira A.L., de Souza Miranda M.F., Mecawi A.S., Melo R.L., Marassi M.P., Matos da Silva A.C., Antunes-Rodrigues J., Lopes Olivares E. Sexual dimorphism in autonomic changes and in the renin-angiotensin system in the hearts of mice subjected to thyroid hormone-induced cardiac hypertrophy. Exp Physiol. 2014;99:868-880.
23. Engbring N.M., Engström W.W. Effects of estrogens and testosterone on circulating thyroid hormone. J Clin Endocrinol Metab. 1958;19:783-796.
24. Franklyn J.A., Ramsden D.B., Sheppard M.C. The influence of age and sex on tests of thyroid function. Ann Clin Biochem. 1985;22:502-505.
25. Vranckx R., Savu L., Maya M., Rouaze-Romet M., Nunez E.A. Immunological quantitation of rat and mouse thyroxine-binding globulins. Ontogenesis and

sex-dependence of the circulating levels of the thyroxine-binding globulins. Acta Endocrinol (Copenh). 1990 Dec;123(6):649-56.

26. Vranckx R., Savu L., Maya M., Nunez E.A. Characterization of a major development-regulated serum thyroxine-binding globulin in the euthyroid mouse. Biochem. J. (1990) 271, 373-379.
27. Riese C., Michaelis M., Mentrup B., Götz F., Köhrle J., Schweizer U., Schomburg L. Selenium-dependent pre- and posttranscriptional mechanisms are responsible for sexual dimorphic expression of selenoproteins in murine tissues. Endocrinology. 2006 Dec;147(12):5883-92.
28. Schomburg L., Riese C., Renko K., Schweizer U. Effect of age on sexually dimorphic selenoprotein expression in mice. Biol. Chem.,2007, Vol. 388, pp. 1035–1041, DOI 10.1515/BC.2007.128
29. Bianco A.C., Anderson G., Forrest D., Galton V.A., Gereben B., Kim B.W., Kopp P.A., Liao X.H., Obregon M.J., Peeters R.P., Refetoff S., Sharlin D.S., Simonides W.S., Weiss R.E., Williams G.R. American thyroid association guide to investigating thyroid hormone economy and action in rodent and cell models. Thyroid. 2014;24:88–168.
30. Hoefig C.S., Harder L., Oelkrug R., Meusel M., Vennström B., Brabant G., Mittag J. Thermoregulatory and Cardiovascular Consequences of a Transient Thyrotoxicosis and Recovery in Male Mice. Endocrinology, July 2016, 157(7):2957–2967, doi: 10.1210/en.2016-1095
31. Ellacott K.L.J., Morton G.J., Woods S.C., Tso P., Schwartz M.W.. Assessment of feeding behavior in laboratory mice. Cell Metab. 2010 July 4; 12(1): 10–17. doi:10.1016/j.cmet.2010.06.001.
32. .Jung A.P, Curtis T.S., Turner M.J., Lightfoot J.T. Physical Activity and Food Consumption in High- and Low-Active Inbred Mouse Strains. Med Sci Sports Exerc. 2010 October ; 42(10): 1826–1833. doi:10.1249/MSS.0b013e3181daf5e8.
33. Goodrick C.I. Effect of Voluntary Wheel Exercise on Food Intake, Water Intake, and Body Weight for C57BL/6J Mice and Mutations Which Differ in Maximal Body Weight. Physiology & Behavior 1978, Vol. 21, pp. 345—351
34. Bachmanov A.A., Reed D.R., Beauchamp G.K., Tordoff M.G. Food Intake, Water Intake, and Drinking Spout Side Preference of 28 Mouse Strains. Behav Genet. 2002 November ; 32(6): 435–443.

35. Popkin B.M., D'Anci K.E., Rosenberg I.H. Water, Hydration and Health. Nutr Rev. 2010 August ; 68(8): 439–458. doi:10.1111/j.1753-4887.2010.00304.x.
36. Newsom D.M., Bolgos G.I., Colby L., Nemzek J.A. Comparison of Body Surface Temperature Measurement and Conventional Methods for Measuring Temperature in the Mouse. CONTEMPORARY TOPICS 2004, Volume 43, No. 5, 13-18
37. Glenmark B, Nilsson M, Gao H, Gustafsson JA, Dahlman-Wright K, Westerblad H. Difference in skeletal muscle function in males vs. females: role of estrogens receptor-β. Am J Physiol Endocrinol Metab. 2004;287:E1125–E1131.
38. Burnes L.A., Kolker S.J., Danielson J.F., Walder R.Y., Sluka K.A. Enhanced muscle fatigue occurs in male but not female ASIC3-/- mice. Am J Physiol Regul Integr Comp Physiol. 2008;294(4):R1347–R1355.
39. McCarthy M.M., Konkle A.T.M. When is a sex difference not a sex difference? Frontiers in Neuroendocrinology. 2005;26:85–102.
40. Becker J.B., Arnold A.P., Berkley K.J., Blaustein J.D., Eckel L.A., Hampson E., Herman J.P, Marts S., Sadee W., Steiner M., Taylor J., Young E. Strategies and methods for research on sex differences in brain and behavior. Endocrinology. 2005;146:1650-73.
41. Konhilas J.P., Maass A.H., Luckey S.W., Stauffer B.L., Olson E.N., Leinwand L.A. Sex modifies exercise and cardiac adaptation in mice. Am J Physiol Heart Circ Physiol. 2004 December ; 287(6): H2768–H2776. doi:10.1152/ajpheart.00292.2004.

Supplemental Material

Supplemental table 1: Body weight of adult and old, male and female mice at first (start) and last (final) day of experiment.

age	sex	start	final eu	final hyper	final hypo
adult	males	40.2 ±5.0	45.8 ±3.7	40.1 ±4.1	35.6 ±2.0
	females	28.0 ±1.4	30.5 ±3.1	34.3 ±2.2	27.0 ±1.6
old	males	39.0 ±5.2	41.3 ±3.7	36.9 ±5.3	34.0 ±3.1
	females	30.3 ±3.5	32.8 ±3.2	33.7 ±4.4	28.7 ±1.7

Chapter 5: Sex-dependent claudin-1 expression in liver of eu- and hypothyroid mice

Zwanziger Denise*#, Rakov Helena*, Engels Kathrin, Moeller Lars C., Fuhrer Dagmar

University Hospital Essen, Dept. of Endocrinology and Metabolism and Division of Laboratory Research, Essen, Germany

*contributed equally

#corresponding author

Eur Thyroid J. 2015 Sep; 4(Suppl 1): 67–73. doi: 10.1159/000431316

Conflict of interest: The authors disclaim any conflict of interest.

I have performed the following experiments: treatment of mice, collection of blood and organs, measurements of TH and qRT-PCR (Fig. 1). In addition I contributed to study design, data analysis, and interpretation and reviewed the manuscript.

5.1 Abstract

Background: Clinically, a diminished bile excretion has been found in hypothyroid patients. Disorders of the biliary system are more frequent in women. However, in hypothyroidism the prevalence of gallstone diseases is higher in men. In liver the tight junction protein claudin-1 plays an important role in bile secretion by maintaining the paracellular barrier of bile canaliculi and bile duct.

Objectives: We hypothesized that hypothyroidism could lead to an altered claudin-1 expression in the liver.

Methods: We characterized claudin-1 in liver of euthyroid and hypothyroid male and female C57BL/6NTac mice. Claudin-1 mRNA expression was analyzed by real-time PCR. In addition, claudin-1 expression and localization was analyzed by Western blot and immunofluorescence.

Results: Claudin-1 is expressed in canalicular regions and the bile ducts of murine liver. Livers of female mice showed a lower claudin-1 expression than male livers. In hypothyroid mice livers, females revealed an elevated claudin-1 expression whereas diminished claudin-1 expression was found in male animals compared to the euthyroid status.

Conclusion: We demonstrate a correlation between claudin-1 expression and hypothyroidism in murine liver. Furthermore, a sex-dependent alteration of claudin-1 expression was found.

5.2 Introduction

Hypothyroidism is a pathophysiological state marked by decreased circulating thyroid hormone (TH) concentrations or impaired TH action in target tissues. In clinical practice, hypothyroidism is mostly due to afflictions of the thyroid gland per se and is defined by elevated thyroid stimulating hormone (TSH) with or without decreased free thyroxine (fT4) serum concentrations, termed overt or subclinical hypothyroidism respectively. Autoimmune thyroiditis is the most common cause of hypothyroidism in humans and a female preponderance in both autoimmune thyroid disease and hypothyroidism has long been known [1, 2]. The incidence in developing hypothyroidism in Western world is between 0.6 and 3.5 per 1.000 people [3]. In Europe women are 1.7-6.8 fold more frequently affected by hypothyroidism than men [1, 2].

The liver is an important site of TH metabolism and therefore thyroid dysfunction is associated with many hepatic alterations, such as a decrease in liver cholesterol metabolism, resulting in hypercholesterolemia in hypothyroidism [4] and diminished bile secretion from hepatocytes [5]. The reduced bilirubin and bile excretion can lead to elevated bilirubin in newborns with congenital hypothyroidism [6]. In addition, hypothyroidism increases the risk for gallbladder stones and common bile duct stones [7]. Interestingly, women seem to be more affected than men in developing common bile duct stones [7], whereas in hypothyroidism the prevalence of gallstone diseases is higher in men [8].

Tight junction (TJ) proteins like claudin-1 are involved in the regulation of the paracellular barrier integrity and paracellular permeability between epithelial and endothelial cells by TJ strand formation. Claudin-1 is involved in polarization of hepatocytes by formation of basolateral and apical plasma membrane domains [9]. In mice liver claudin-1 is expressed in the bile canalicular region of hepatocytes as well as in epithelial cells of the bile duct [10]. Biliary diseases in human can be associated with altered claudin-1 expression or mutations. For instance, in the neonatal ichthyosis-sclerosing cholangitis (NISCH) syndrome claudin-1 gene mutations may lead to increased paracellular permeability between bile duct epithelial cells [11]. Other examples are familial hypercholanemia with claudin-1 mutations [12], acute acalculous cholecystitis with decreased claudin-1 [13] and intrahepatic cholangiocarcinoma with elevated claudin-1 expression [14].

Scarce data are so far available on a potential influence of TH on claudin-1. A role of TH in TJ dynamics and maintenance in seminiferous epithelia has recently been described in rats [15]. Furthermore thyroid hormone receptor (THR) THRα1/THRß-deficient mice have been shown to exhibit defective wound healing besides impaired hair growth [16]. Since claudin-1 is known to be pivotal for an intact skin barrier [17], a role of TH receptors and or TH may be suspected. However, transfer of this speculation to other tissues or organs has to be handled carefully as TJ are in fact functionally similar but structurally quite variable between different tissues or organs and the protein composition cannot be compared necessarily. So far a direct correlation between TH, THRs and claudin-1 has not yet been studied.

In this study, we asked whether TH may impact claudin-1 expression in liver tissues and which compartments could be affected. We used male and female C57BL/6NTac mice with euthyroid and hypothyroid serum TH status to analyze claudin-1 expression and localization patterns in the liver.

5.3 Materials and Methods

Animals

Eighteen weeks old male and female (n=32) C57BL/6NTac mice (Taconic Europe, Denmark) were 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 collection of mice blood samples and livers

To establish a hypothyroid state, animals were fed a low-iodine diet (Harlan, USA) with an addition of 0.02% methimazole, 0.5% perchlorate and 3 g/l saccharin (Sigma, Germany) to drinking water for 7 weeks (n=8 animals/sex). Control groups received control diet (Harlan, USA) and regular water over the whole treatment period (n=8 animals/sex). The non-caloric sweetener saccharin is described to increase water consumption, to decrease food consumption and to alter the intestinal microbiota. However, changes in body weight in male C57BL/6 mice were not found [18].

Blood samples were collected from the retrobulbar venous plexus with a heparinated micropipette at the start and end of the experiment from each animal. Blood samples were stored on ice for 30 min for coagulation and serum was obtained by centrifugation at 4°C for 10 min at 10.000g. Serum aliquots were stored at -80°C. Total thyroxine (TT4) concentrations in serum of mice were measured using ELISA kits according to the manufacturer's instructions (DRG Instruments GmbH, Germany). Hypothyroidism in the mice was defined by decreased serum TT4 concentrations (detection limit of 0.5 µg/dL).

Mice were euthanized and perfused with heparinized saline through a needle placed in the left ventricle. Livers were isolated, frozen in liquid nitrogen, and stored at -80 °C until further processing.

RNA isolation and cDNA synthesis

For RNA extraction, tissues were homogenized in 600 µl RLT buffer (Qiagen) by 4.000 rpm and further treated with proteinase k (Qiagen) based on the manufacturers' protocol. Total RNA from tissue lysates was purified by RNeasy mini kit (Qiagen, Germany) and on-column DNase digestion using RNase-Free DNase Set (Qiagen, Germany). RNA quantity and quality were determined by NanoDrop 1000 (Thermo Scientific). 2 µg of RNA was reverse transcribed to cDNA using random hexamers and SuperScript III First-Stand Synthesis System for RT-PCR according to instruction manuals (Life Technologies, Germany).

Real-time PCR

Oligonucleotides of deiodinase 1 (*Dio1*), malic enzyme 1 (*Me1*) and *claudin-1* genes were designed using PrimerBlast (NCBI) and synthesized by eurofins (Eurofins MWG Synthesis, Germany). *Dio1* forward primer: GGGCAGGATCTGCTACAAGG, *Dio1* reverse primer: CGTGTCTAGGTGGAGTGCAA. *Me1* forward primer: CCCACAACA-GTGTCTACCCAT, *Me1* reverse primer: TCATCCAGGAAGGCGTCATA. *Claudin-1* forward primer: CAACCCGAGCCTTGATGGTA, *claudin-1* reverse primer: ACTAAT-GTCGCCAGACCTGA. Quantitative real-time PCR (RT-PCR) was performed using LightCycler® DNA Master SYBR Green I and the LightCycler®480 System (Roche, Germany). The PCR program consisted of an initial denaturation step (5 min at 95 °C) and 40 amplification cycles with 15 sec at 95 °C, 10 sec at 60 °C, 20 sec at 72 °C. For normalization of *Dio1*, *Me1* and *claudin-1* expression reference genes

18S, *Ppia* (peptidylprolyl isomerase A, cyclophilin A) and *RPL13 a* (ribosomal protein L13a) were used. Stability of housekeeping genes was determined by calculation of the coefficient of variation on the normalized relative quantities and by calculation of the geNorm M value. The geNorm value determines the most stable housekeeping genes and calculates the gene expression normalization factor based on the geometric mean of a housekeeping gene. The genomic average of the "best" three housekeeping genes (best keeper index) was calculated by repeated pair-wise correlation analysis. Target genes were correlated to the calculated index (best keeper index). Fold-changes were calculated by the relative expression software tool (REST© (provision of a subsequent statistical test to the analyzed Ct values by a pair-wise fixed reallocation randomization test, efficiency corrected ΔΔCt method, Ct<35) [19, 20].

Western blot

The following antibodies were used: anti-claudin-1 (1:250, Invitrogen, USA), anti-GAPDH (1:1.000, Cell Signalling, USA), anti-cytokeratin 19 (1:1.000, Abcam, USA), anti-ß-actin (1:2.000, Cell Signalling, USA), anti-mouse/anti-rabbit-IgG (1:2.000, Cell Signalling, USA) and anti-mouse IgG DyLight 488 conjugate (1:15.000; Life Technologies, USA). Whole protein lysates were extracted by RIPA-buffer (150mM sodium chloride, 50mM Tris pH 8.0, 1% NP-40, 0.5% sodiumdeoxycholate, 0.1% sodium dodecyl sulfate, 2mM ethylenediaminetetraacetate, 50mM sodium fluoride, protease inhibitor, Sigma, Germany). Extracted proteins were quantified by BCA protein assay (Pierce, Rockford, USA). Aliquots of proteins (40 µg) were fractionated on Any kD™ Criterion™ TGX™ SDS polyacrylamide gels and blotted by wet transfer (wet electroblotting by 10 V and 4 °C, overnight) onto PVDF membranes (BioRad, USA). Unspecific binding sites were blocked with blocking buffer (5% milk powder in phosphate buffered saline, Sigma, Germany) for 1h at room temperature. Claudin-1 GAPDH, cytokeratin 19 and ß-actin were detected by overnight incubation of the respective antibody at 4 °C in blocking buffer. GAPDH and ß-actin were used as internal protein loading controls. Both protein loading controls revealed a stable protein loading, only GAPDH is shown. Cytokeratin 19 was used to detect the amount of cholangiocytes in whole protein lysates [21]. Secondary antibodies were incubated at room temperature for 1h. Visualization was done by luminescence using the Immun-Star™ WesternC™ Kit (BioRad, USA) as well as fluorescence (VersaDoc

System, BioRad, USA). Differences in protein expression levels were quantified by densitometry using the Image Lab™ Software (BioRad, USA). Relative values of the loading controls GAPDH and cytokeratin 19 were calculated. The claudin-1 protein values were divided by the calculated relative values of either GAPDH or cytokeratin 19. The adjusted values were used to calculate the geometric mean of the loading controls and claudin-1 followed by calculation of the fold-change of claudin-1 to the respective loading controls.

Immunofluorescence

The following antibodies were used: anti-claudin-1 (1:100, Invitrogen, USA), AlexaFluor® 555 Phalloidin (1:40, Invitrogen, USA), and AlexaFluor® 488 (1:250, Invitrogen, USA) secondary antibody. Cryo liver tissues were fixed in 4% paraformaldehyde (Sigma, Germany) for 15min at room temperature and permeabilized using 0.1% Triton X100 (Sigma, Germany) in phosphate buffered saline (PBS) for 10min at room temperature. The pre-incubation in blocking solution containing 3% bovine serum albumin (BSA) and 0.3% Triton X100 in PBS was performed at room temperature for 30min. Cryo liver tissues were incubated with primary antibodies overnight at 4°C in 1%BSA/PBS. Secondary antibody AlexaFluor® 488 was incubated for 1h at room temperature in 1%BSA/PBS. The F-actin cytoskeleton was visualized by incubation of AlexaFluor® 555 Phalloidin for 20min at room temperature. The cell nuclei were stained by incubation of Hoechst 33342 (1:1.000, Invitrogen, USA) for 5min at room temperature. Cover slides were embedded by ImmuMount (Thermo Scientific, USA) and viewed on the confocal microscope Zeiss ELYRA PS.1 LSM710.

Statistical analysis

All data are shown as mean±SEM, as indicated. Statistical analysis was performed using GraphPad Prism 5 Software. For ELISA and real-time PCR data 2-way ANOVA was applied. Values of *$p \leq 0.05$, ***$p < 0.001$, $^{\#}p < 0.0001$ were considered statistically significant.

5.4 Results

To determine successful induction of hypothyroidism in C57BL/6NTac mice TT4 serum concentrations were determined by ELISA measurement (fig. 1a). Induction of hypothyroidism resulted in a significant decrease of TT4 serum concentration in male

(1.20±0.56 µg/dL vs. 2.86±0.21 µg/dL) and female mice (0.35±0.33 µg/dL vs. 3.01±0.77 µg/dL). TT4 serum concentration of female hypothyroid mice is below the detection limit of the applied assay (0.5 µg/dL) and therefore carefully to construe.

To ensure a hypothyroid state in mice livers, the mRNA levels of the two positively regulated TH responsive genes *Dio1* and *Me1* were analyzed (fig. 1b). Induction of hypothyroidism significantly decreased *Dio1* mRNA levels in male and female mice as compared to euthyroid mice (0.01±0.001 vs. 1.07±0.08 for males and 0.01±0.001 vs. 1.02±0.03 for females, respectively). Me1 mRNA levels revealed also a significant decrease in hypothyroid mice as compared to euthyroid mice of both sexes (0.24±0.02 vs. 1.07±0.06 for males and 0.42±0.03 vs. 1.02±0.06 for females, respectively).

Claudin-1 mRNA level was investigated in male and female C57BL/6NTac mice liver with normal thyroid function and under chronic hypothyroidism (fig. 1c). Claudin-1 mRNA level was not altered in hypothyroid male mice as compared to euthyroid male mice (0.86±0.06 vs. 1.12±0.09). Conversely, the induction of hypothyroidism in female mice led to a significant increase of claudin-1 mRNA level as compared to euthyroid control mice (1.98±0.26 vs. 0.97±0.15). Comparing male to female mice with normal thyroid function, a significantly lower claudin-1 mRNA level was observed in female mice (0.66±0.07 vs. 0.97±0.15).

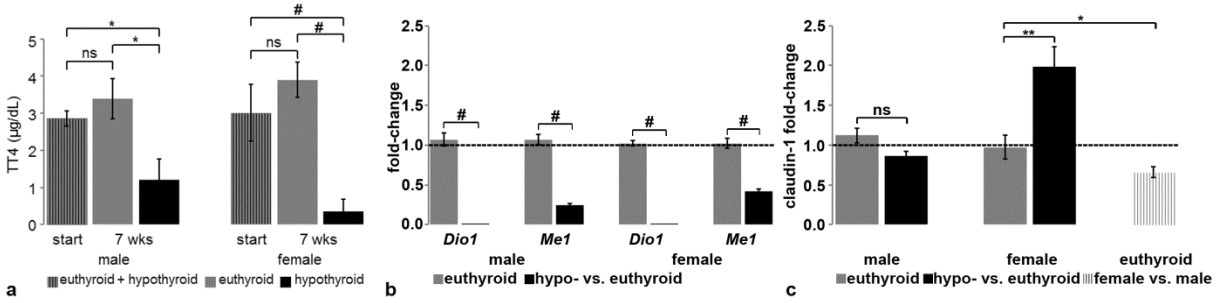

Figure 1: Serum concentration of total thyroxine (TT4) in male and female eu- and hypothyroid mice. Fold-changes of deiodinase 1, malic enzyme 1 and claudin-1 mRNA expression in euthyroid and hypothyroid male and female mice liver by quantitative real-time PCR. a TT4 serum concentration is significantly lower in male mice after induction of hypothyroidism than euthyroid male control mice. TT4 serum concentration of hypothyroid female mice is significantly lower as compared to the euthyroid female control group. TT4 was determined in mice serum by ELISA at the start and end of 7 weeks (wks) treatment. Assay detection limit of 0.5 µg/dL. Data are represented as mean±SEM, n=16 for start, n=8 for end groups, 2-way ANOVA, ns= not significant, *p<0.05, #p<0.0001. **b** Deiodinase 1 *(Dio1*) and malic enzyme 1 (*Me1*) mRNA levels are significantly decreased

in hypothyroid mice as compared to euthyroid mice for both sexes. **c** *Claudin-1* mRNA level is not altered in hypothyroid male mice as compared to euthyroid male mice. *Claudin-1* mRNA level is significantly up-regulated in female mice under hypothyroidism. *Claudin-1* mRNA level of euthyroid female mice normalized to euthyroid male mice shows a significantly lower *claudin-1* mRNA level in female animals. *18S*, *Ppia* (peptidylprolyl isomerase A, cyclophilin A) and *Rpl13 a* (ribosomal protein L13a) were used as reference genes. Data are represented as mean±SEM, n=8, efficiency corrected ΔΔCt method, 2-way ANOVA, ns= not significant, *p<0.05, **p<0.01, #p<0.0001.

To investigate, if differences in mRNA expression were also reflected on the protein level, claudin-1 expression was studied by Western blot analysis in euthyroid and hypothyroid livers of male and female mice followed by quantification (fig. 2a,b). Hypothyroid male mice showed a lower claudin-1 protein level than the euthyroid control group. In addition, mRNA results of a significantly lower claudin-1 level in euthyroid female than male mice could be confirmed on protein level. Moreover, an increased claudin-1 protein level was shown after induction of hypothyroidism in female mice. Equal amounts of cytokeratin 19 protein (protein marker of cholangiocytes [21]) in male and female mice liver with normal thyroid function and under hypothyroidism were observed.

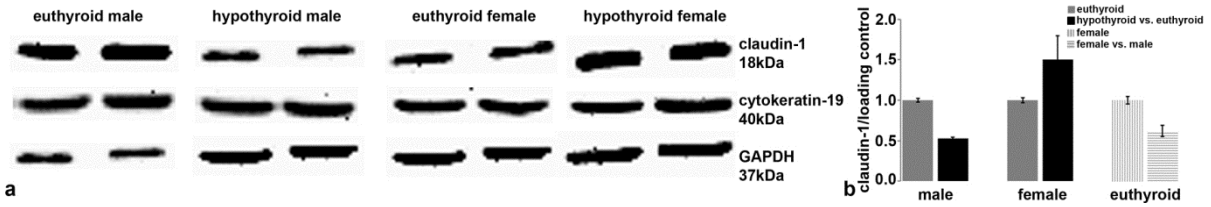

Figure 2: Claudin-1 protein expression and quantification in euthyroid and hypothyroid male and female mice liver. a/b Whole protein lysates were used. Lower claudin-1 (18 kDa) protein level in hypothyroid male mice liver than euthyroid male mice liver. Elevated claudin-1 protein level in hypothyroid female mice liver as compared to euthyroid female mice liver. Euthyroid female mice show lower claudin-1 protein amount than euthyroid male mice liver. As loading controls GAPDH (37 kDa) and cytokeratin 19 (40 kDa) as marker of cholangiocytes were used. Quantification was performed by densitometry and normalization of claudin-1 protein amount to the respective loading controls. Representative examples are shown (n=8).

To determine the localization pattern of claudin-1, immunofluorescence analysis of euthyroid and hypothyroid mice liver of both sexes was performed (fig. 3). Claudin-1 protein was found to be located in the canalicular regions of hepatocytes as well as in epithelial cells of bile ducts. In addition, a homogeneous decrease of claudin-1

protein level was observed in hypothyroid male mice liver vs. euthyroid male controls (fig 3a,b). In hypothyroid female mice liver claudin-1 level increased mainly in bile duct regions as compared to euthyroid female controls (fig. 3c,d).

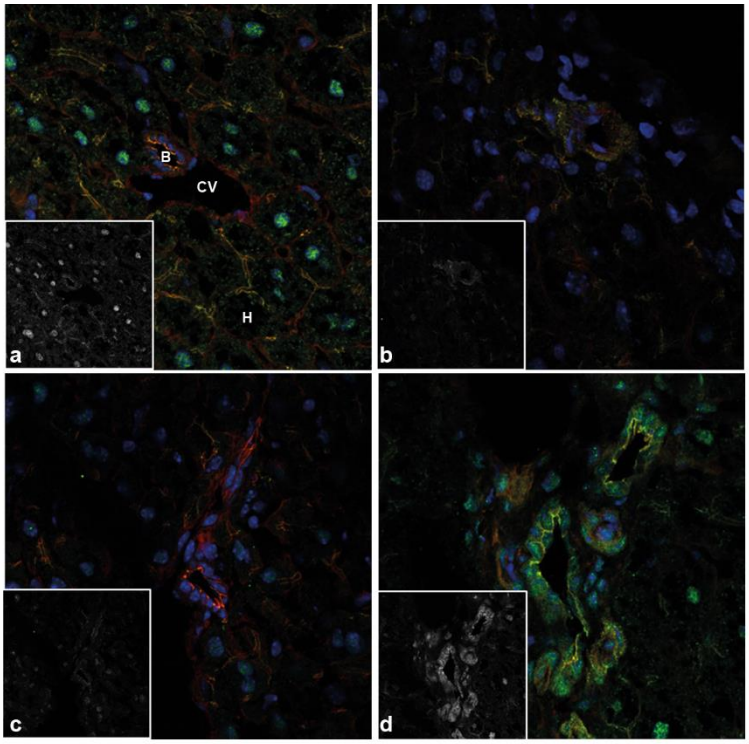

Figure 3: Immunofluorescence of claudin-1 in euthyroid and hypothyroid male and female mice liver. a Euthyroid male liver with claudin-1 (green) staining in canalicular region of liver and the bile duct. Central vein (CV), Bile duct (B) and hepatocytes (H). b Hypothyroid male liver with lower claudin-1 level than euthyroid male liver. c Euthyroid female liver with low claudin-1 level. d Hypothyroid female liver with distinct claudin-1 expression in cholangiocytes and/or epithelial cells of the bile duct. Grey scale images show only claudin-1 channel. F-actin cytoskeleton has been stained by AlexaFluor® 555 Phalloidin (red). Nuclei were stained by Hoechst 33342 (cyan). Magnification of 63x. Confocal microscope Zeiss ELYRA PS.1 LSM710. Representative examples are shown (n=4).

5.5 Discussion

Here we show that the TJ protein claudin-1 reveals sex-dependent liver expression and is distinctly altered by induction of hypothyroidism in male and female mice liver. Expression differences were found on mRNA and protein level. In euthyroid mouse liver, claudin-1 is higher expressed in males than females and seems to be localized mainly in canalicular regions of liver, cholangiocytes and epithelial cells of the bile duct, hence the biliary system. Our data contrast with a previous study, reporting elevated claudin-1 expression in female compared to male mouse livers by

immunohistochemistry [22]. However, differences could be explained by different methods used to detect claudin-1 expression and/or the investigated C57BL/6 substrain.

When comparing hypothyroid to euthyroid mice liver we found differences in claudin-1 expression between both sexes. In male mouse liver, induction of hypothyroidism decreases claudin-1 protein whereas in females an increase in claudin-1 expression could be detected. Total T4 serum concentrations of mice suggest a more hypothyroid state of females than male mice which could impact claudin-1 expression. However, if female mice are more hypothyroid than male mice it would be suspected that claudin-1 expression results in a more pronounced decrease in female than male mice liver. Furthermore, due to TH responsive gene levels in mice livers a comparable hepatic hypothyroid state of male and female mice could be speculated.

Hypothyroidism can lead to a disturbed bile transporting system in rat liver [23]. A reduced bile excretion in liver of hypothyroid mice could also be related to an altered expression of claudin-1 due to its localization within the bile duct and canalicular region. However, if claudin-1 is TH-dependently regulated by THRß, the most prevalent thyroid hormone receptor in liver [24], has to be investigated in further studies.

Hypothyroidism could indirectly affect claudin-1 expression in liver due to altered cholesterol content. In the blood-testis barrier of rabbits fed a 2% cholesterol enriched diet increased occludin and zonula occludens protein 1 (ZO-1) expression levels as well as a pronounced endosomal distribution of these TJ proteins have been observed [25]. Depletion of cell cholesterol in a human kidney cell line (Caco-2) leads to a loss of membrane localization of claudin-3, -4, -7 and occludin resulting in a diminished paracellular integrity and an increased paracellular permeability [26, 27]. However, claudin-1 distribution was not altered in Caco-2 cells under cholesterol depletion [26]. In contrast, Madin-Darby canine kidney cells (MDCK) reveal that the paracellular integrity develops more rapidly and reaches higher values of integrity the lower the cell cholesterol content is [28].

It could be speculated that the decreased claudin-1 expression in liver of hypothyroid male mice might contribute to an altered paracellular pathway of the hepatocellular

tract. In addition increased claudin-1 expression in hypothyroid female liver could result in pronounced paracellular integrity of the canalicular region and bile duct.

Very little is known about the functional consequences of altered claudin-1 expression and the regulation of claudin-1 in the bile duct epithelium. Claudin-1 deficient mice do not show biliary diseases, probably because these mice died at birth due to an impaired skin barrier [17]. It seems likely, that sex hormones are involved. Previous *in vitro* studies have shown that progesterone and estradiol are influencing TJ expression in a concentration dependent manner in human vascular endothelial and human endometrial epithelial cells [6, 29]. However, most animal studies concentrated on TJ proteins like occludin or ZO-1 and data of claudin-1 are almost missing [3]. It is thought that claudin-1 could be regulated via different signal transduction pathways like MAPkinase or PI3kinase [9], but the data are not consistent and it will be interesting to consider these pathways in the context of non-genomic TH action.

It should not disregard that a possible interaction between estradiol and TH could be involved in the sex-specific differences of claudin-1 expression as both receptors, THRs and estrogen receptors, belong to the superfamily of nuclear receptors and share similarities in TH responsive element structures [30]. It has been shown that molecular interactions between THRs and estrogen receptors are sufficient to mediate environmental effect on estrogen-controlled reproductive behavior [30]. Recently an overlapping effect of TH and estradiol on glutathione *S*-transferase-α gene expression in mice kidney has been observed [31]. However, if claudin-1 expression is also influenced in an estradiol-dependent manner can be investigated by using e.g. an ovariectomized mouse model.

In liver also other TJ proteins like occludin, claudin-2 and claudin-3 are expressed [10]. If these TJ proteins are equally affected by TH has to be determined in further studies.

However, absence or mutations of claudin-1 have been associated with distinct human biliary diseases [11-13]. It is supposed that women are more affected in developing common bile duct stones than men [32]. It could be speculated that the low claudin-1 expression in euthyroid female mice liver could prefer the formation of biliary diseases due to a less-sufficient paracellular barrier. Otherwise, there seems

to be a sex-dependent relation between hypothyroidism and gallstone diseases with a higher prevalence for men [8]. The diminished hepatic claudin-1 expression in hypothyroid male mice and an increased risk for biliary disease with low claudin-1 expression [11-13] could explain why gallstone diseases are more frequent in hypothyroid men.

The molecular mechanisms that account for the observed sex-dependent pattern of claudin-1 expression and their functional consequences have to be investigated in further studies. In summary this study shows that the TJ protein claudin-1 is altered by hypothyroidism and differs between sexes in a mice model.

Acknowledgements

Authors are grateful to S. Rehn and A. Jaeger for their dedicated technical support. This work was supported by DFG FU 356/7-1 and MO 1018/2-1.

References

1. Hoogendoorn EH, Hermus AR, de Vegt F, Ross HA, Verbeek AL, Kiemeney LA, Swinkels DW, Sweep FC, den Heijer M: Thyroid function and prevalence of anti-thyroperoxidase antibodies in a population with borderline sufficient iodine intake: influences of age and sex. Clin Chem 2006;52(1):104-111.
2. Vanderpump MP, Tunbridge WM, French JM, Appleton D, Bates D, Clark F, Grimley Evans J, Hasan DM, Rodgers H, Tunbridge F et al: The incidence of thyroid disorders in the community: a twenty-year follow-up of the Whickham Survey. Clin Endocrinol 1995;43(1):55-68.
3. Mottino AD, Hoffman T, Crocenzi FA, Sanchez Pozzi EJ, Roma MG, Vore M: Disruption of function and localization of tight junctional structures and Mrp2 in sustained estradiol-17beta-D-glucuronide-induced cholestasis. American journal of physiology Gastrointestinal Liver Physiol 2007;293(1):G391-402.
4. Hueston WJ, Pearson WS: Subclinical hypothyroidism and the risk of hypercholesterolemia. Ann Fam Med 2004;2(4):351-355.
5. Laukkarinen J, Sand J, Nordback I: The underlying mechanisms: how hypothyroidism affects the formation of common bile duct stones-a review. HPB Surg 2012;2012:102825.
6. Ye L, Martin TA, Parr C, Harrison GM, Mansel RE, Jiang WG: Biphasic effects of 17-beta-estradiol on expression of occludin and transendothelial resistance

and paracellular permeability in human vascular endothelial cells. J Cell Physiol 2003;196(2):362-369.

7. Laukkarinen J, Sand J, Autio V, Nordback I: Bile duct stone procedures are more frequent in patients with hypothyroidism. A large, registry-based, cohort study in Finland. Scand J Gastroenterol 2010;45(1):70-74.
8. Volzke H, Robinson DM, John U: Association between thyroid function and gallstone disease. WJG 2005;11(35):5530-5534.
9. Kojima T, Murata M, Yamamoto T, Lan M, Imamura M, Son S, Takano K, Yamaguchi H, Ito T, Tanaka S et al: Tight junction proteins and signal transduction pathways in hepatocytes. Histol Histopathol 2009;24(11):1463-1472.
10. Kojima T, Yamamoto T, Murata M, Chiba H, Kokai Y, Sawada N: Regulation of the blood-biliary barrier: interaction between gap and tight junctions in hepatocytes. Med Electron Microsc 2003;36(3):157-164.
11. Grosse B, Cassio D, Yousef N, Bernardo C, Jacquemin E, Gonzales E: Claudin-1 involved in neonatal ichthyosis sclerosing cholangitis syndrome regulates hepatic paracellular permeability. Hepatology 2012;55(4):1249-1259.
12. Carlton VE, Harris BZ, Puffenberger EG, Batta AK, Knisely AS, Robinson DL, Strauss KA, Shneider BL, Lim WA, Salen G et al: Complex inheritance of familial hypercholanemia with associated mutations in TJP2 and BAAT. Nat Genet 2003;34(1):91-96.
13. Laurila JJ, Karttunen T, Koivukangas V, Laurila PA, Syrjala H, Saarnio J, Soini Y, Ala-Kokko TI: Tight junction proteins in gallbladder epithelium: different expression in acute acalculous and calculous cholecystitis. J Histochem Cytochem 2007;55(6):567-573.
14. Nemeth Z, Szasz AM, Tatrai P, Nemeth J, Gyorffy H, Somoracz A, Szijarto A, Kupcsulik P, Kiss A, Schaff Z: Claudin-1, -2, -3, -4, -7, -8, and -10 protein expression in biliary tract cancers. J Histochem Cytochem 2009;57(2):113-121.
15. Gao Y, Lee WM, Cheng CY: Thyroid hormone function in the rat testis. Front Endocrinol 2014;5:188.

16. Contreras-Jurado C, Garcia-Serrano L, Martinez-Fernandez M, Ruiz-Llorente L, Paramio JM, Aranda A: Impaired hair growth and wound healing in mice lacking thyroid hormone receptors. PloS One 2014;9(9):e108137.
17. Furuse M, Hata M, Furuse K, Yoshida Y, Haratake A, Sugitani Y, Noda T, Kubo A, Tsukita S: Claudin-based tight junctions are crucial for the mammalian epidermal barrier: a lesson from claudin-1-deficient mice. J Cell Biol 2002;156(6):1099-1111.
18. Suez J, Korem T, Zeevi D, Zilberman-Schapira G, Thaiss CA, Maza O, Israeli D, Zmora N, Gilad S, Weinberger A et al: Artificial sweeteners induce glucose intolerance by altering the gut microbiota. Nature 2014;514(7521):181-186.
19. Pfaffl MW: A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001;29(9):e45.
20. Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP: Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper--Excel-based tool using pair-wise correlations. Biotechnol Lett 2004;26(6):509-515.
21. Tanimizu N, Miyajima A, Mostov KE: Liver progenitor cells develop cholangiocyte-type epithelial polarity in three-dimensional culture. Mol Biol Cell 2007;18(4):1472-1479.
22. D'Souza T, Sherman-Baust CA, Poosala S, Mullin JM, Morin PJ: Age-related changes of claudin expression in mouse liver, kidney, and pancreas. J Gerontol A Biol Sci Med Sci 2009;64(11):1146-1153.
23. Van Steenbergen W, Fevery J, De Vos R, Leyten R, Heirwegh KP, De Groote J: Thyroid hormones and the hepatic handling of bilirubin. I. Effects of hypothyroidism and hyperthyroidism on the hepatic transport of bilirubin mono- and diconjugates in the Wistar rat. Hepatology 1989;9(2):314-321.
24. Forrest D, Hanebuth E, Smeyne RJ, Everds N, Stewart CL, Wehner JM, Curran T: Recessive resistance to thyroid hormone in mice lacking thyroid hormone receptor beta: evidence for tissue-specific modulation of receptor function. EMBO J 1996;15(12):3006-3015.
25. Morgan DH, Ghribi O, Hui L, Geiger JD, Chen X: Cholesterol-enriched diet disrupts the blood-testis barrier in rabbits. Am J Physiol Endocrinol Metab 2014;307(12):E1125-1130.

26. Lambert D, O'Neill CA, Padfield PJ: Depletion of Caco-2 cell cholesterol disrupts barrier function by altering the detergent solubility and distribution of specific tight-junction proteins. Biochem J 2005;387(Pt 2):553-560.
27. Lambert D, O'Neill CA, Padfield PJ: Methyl-beta-cyclodextrin increases permeability of Caco-2 cell monolayers by displacing specific claudins from cholesterol rich domains associated with tight junctions. Cell Physiol Biochem 2007;20(5):495-506.
28. Stankewich MC, Francis SA, Vu QU, Schneeberger EE, Lynch RD: Alterations in cell cholesterol content modulate Ca(2+)-induced tight junction assembly by MDCK cells. Lipids 1996;31(8):817-828.
29. Someya M, Kojima T, Ogawa M, Ninomiya T, Nomura K, Takasawa A, Murata M, Tanaka S, Saito T, Sawada N: Regulation of tight junctions by sex hormones in normal human endometrial epithelial cells and uterus cancer cell line Sawano. Cell Tissue Res 2013;354(2):481-494.
30. Dellovade TL, Zhu YS, Krey L, Pfaff DW: Thyroid hormone and estrogen interact to regulate behavior. PNAS 1996;93(22):12581-12586.
31. Faustino LC, Almeida NA, Pereira GF, Ramos RG, Soares RM, Morales MM, Pazos-Moura CC, Ortiga-Carvalho TM: Thyroid hormone and estradiol have overlapping effects on kidney glutathione S-transferase-alpha gene expression. Am J Physiol Endocrinol Metab 2012;303(6):E787-797.
32. Laukkarinen J, Sand J, Nordback I: [Hypothyroidism is common in bile duct stone patients]. Duodecim 2010;126(19):2247-2252.

Chapter 6: Discussion

Thyroid hormones (THs) control various physiological functions, such as heart rate, bone development, lipid metabolism, body temperature (BT) and body weight (BW), as well as brain maturation and function (Yen, 2001). Thus, an untreated dysbalance of THs causes severe symptoms. Thyroid dysfunctions (TDs) resulting from an over- or underproduction of THs are known as hyper- and hypothyroidism, respectively and are frequent in the general population. Moreover, TDs show a female preponderance with women more often affected by hyper- and hypothyroidism, thyroid autoimmunity, as well as thyroid cancer (Vanderpump *et al.,* 1995; Hollowell *et al.*, 2002; Leese *et al.*, 2008).

Interestingly, little is known whether TDs have different consequences or morbidity in male or female organism.

To clarify whether TDs have sex-specific symptoms and traits the characterization of a mouse model is an important tool, in particular since it allows comprehensive analysis of behaviour, dissection of organ-specific effects and tissue-specific analysis of TH signalling. Thus, using male and female C57Bl/6 wildtype mice it is possible to mimic the clinical situation of a hyper- or hypothyroid state and to study the influence of sex on phenotypical traits of TDs.

As the onset of TDs varies in the human population a further important issue is to include mice of different ages. Thus, hyper- or hypothyroidism may have different outcomes at young, adult or old life stage. From the clinical point of view symptoms of TDs are often diminished in the elderly population although the prevalence of TD increases and are often masked by complaints about impairments related to aging (Rehman *et al.*, 2005). Furthermore, an additional advantage of using especially older female mice is the possibility to exclude influence of circulating sex hormones. Although mice do not undergo a menopause as women do (Wise, 2006), measurements of estrogen concentration in sera of old female mice showed comparable values as for ovariectomized female mice (Cai *et al.*, 2014). Thus, by using young and old female mice one can distinguish between differences, which are likely sex-hormone dependent or due to genetic background and by this approach assess the influence of circulating female sex hormones.

6.1 Induction of chronic hyper- and hypothyroidism in a mouse model

Various methods are used in the literature by different groups for an induction of hyper- and hypothyroidism in rodents (Bianco *et al.*, 2014; Reed *et al.*, 2000; Ferreira *et al.*, 2007; Wilcoxon *et al.*, 2007; Fabian *et al.*, 2015). However, TD induction protocols used in our studies had to meet certain criteria. First, well tolerated by mice in general; second, usable as long-term treatment and third, successful in induction of TD in both sexes. Prior literature research revealed more studies on rat than on mice, as well as only few studies comprising both sexes. Furthermore, to clarify the influence of sex on symptoms of TDs a treatment of several weeks is needed, an aspect which has not been addressed so far.

Methods for deprivation of TH concentrations in mice

Hypothyroidism defined as TH deficiency, can be induced by using antithyroidal drugs, which are administered via food, water or direct injection. Another more invasive procedure to induce TH deprivation is thyroidectomy, which however requires high experimental expertise and results in a considerable burden for the animals. Antithyroidal drugs are known to be well tolerated in humans, as well as in rodents (De Leo *et al.*, 2016). Most common used drugs include propylthiouracil (PTU), methimazole (MMI) and perchlorate (ClO_4-) and are in rodent studies, if applied by drinking water, combined with a sweetener as saccharine or sweet syrup. PTU can be administered via food pellets and reduces TH serum concentrations by blocking thyroid peroxidase (TPO) as well as by inhibiting Dio1 (Shiroozu *et al.*,1983; Bianco and Kim, 2006). Thus, not only the production of THs is blocked, but also the conversion of T4 to T3 in peripheral organs is reduced. MMI supplied by drinking water inhibits only TPO, and is not sufficient to decrease TH production completely (Shiroozu *et al.*, 1983). By adding ClO_4-, an inhibitor of the sodium-iodide-symporter (mediates iodide uptake in follicular cells of the thyroid gland for TH synthesis; Leung *et al.*, 2010), and in combination with a low-iodine diet a successful TH deprivation is achieved (Hönes *et al.*, unpublished). However, PTU administration is not well tolerated in long-term experiments in mice, therefore combined MMI/ClO_4- treatment in drinking water, in addition to a low-iodine diet was preferred, which achieved TT4 serum concentrations below assay detection limit after 3 weeks (Hönes *et al.*, unpublished).

Protocols to induce TH excess in mice and establishment of i.p. and oral T4 treatment

For induction of hyperthyroidism, besides species differences, different administration routes for T4 or T3, as well as a combination of both are described in the literature. However, in this work T4 was the preferred drug, as on the one hand L-thyroxine is mainly used for TH substitution in patients and on the other hand investigating the conversion of T4 to T3 was part of this study. The dosage of T4 administered via i.p. injections in the literature ranged from low (e.g. 0.6 µg/g BW for 30 days; Klecha *et al.*, 2006) to high (10 µg/g BW for 8 days; Reed *et al.*, 2000) concentrations. To prevent severe thyrotoxicosis in a long-term treatment a dosage of 1 µg/g BW T4 was used every 48 hours. In addition, the required time to achieve stable tissue thyrotoxicosis was unknown, hence induction of hyperthyroidism was conducted over a period of at least 5 weeks. Using the oral T4 supply route, ranges of 2 µg T4/mL to 12 µg T4/mL were reported (Ferreira *et al.*, 2007; Wilcoxon *et al.*, 2007; Messarah *et al.*, 2010). At the beginning of the experiment, a dosage of 5 µg T4/mL was used and enabled comparison of the efficacy of i.p. vs oral T4 supply for induction of chronic hyperthyroidism in mice.

Both i.p. and oral T4 administration resulted in elevated TH serum concentrations of TT4, FT4 and FT3 after 2-3 weeks of treatment, which remained high throughout experimental time (Chapter 2, Fig. 2, 3). Thereby, the oral dosage of 5 µg T4/mL resulted in much higher TH serum concentrations than expected and caused severe thyrotoxicosis in mice. Thus, the oral group was divided and one part received 1 µg/mL T4 continuously while in the other group, treatment of mice was stopped for 1 week to monitor if TH serum concentrations recover to normal ranges. One week was sufficient to obtain normal TH serum concentrations (Chapter 2, Fig. 3) and hence T4 treatment was continued in this group with 1 µg T4/mL. Lower oral dosage resulted in a rapid and proportional adjustment of TH serum concentrations and thus being then comparable in both methods, 1 µg/g BW T4 i.p. or 1 µg/mL T4 in drinking water.

In this context, a sex difference in TH serum state after exogenous i.p. T4 supply was found with much higher TT4 and FT4 serum concentrations in female compared to male mice (Chapter 2, Fig. 2). Since no differences were found in sham treated control male and female mice, the response to T4 administration and its metabolism is suggested to be estrogen-dependent. Previous studies by others support this

observations and reported a sex-dependent response to exogenous T3 supply with higher T3 serum concentrations in female compared to male mice (da Silveira *et al.*, 2014). However, further evaluation and testing in subsequent studies in gonadectomised mice are necessary to obtain detailed information about sex-dependent response to TH metabolism in mice sera.

Time-dependent tissue response to TH excess in mice

The next important issue of this study was to evaluate if chosen treatment timing is sufficient to maintain a hyperthyroid state over a long-term period and at the end of experiment on tissue level. Thus, liver and heart of mice sacrificed 24h, 48h, 72h and 144h after last i.p. or oral T4 administration were analysed via quantitative real-time polymerase chain reaction (qRT-PCR) for known TH-responsive gene expression. For i.p. T4 treatment a 24h or 48h dosing interval was sufficient to maintain a thyrotoxic organ state. Moreover, an extended dosing interval resulted in a transient hypothyroid organ state, most likely due to sustained suppressed HPT axis under chronic thyrotoxicosis.

Furthermore, comparison of gene expression changes between i.p. and oral T4 treated mice at the same time points showed that transcript changes in organs of oral T4 treated mice were less pronounced compared to i.p. treated animals most likely due to altered kinetics and/or dosage of altered TH exposure. Thus, an injection resulted in a rapid increase of serum TH concentrations, while oral treatment ensured continuous, but low dosages of T4. Therefore, a discontinuation of oral T4 supply should not be implemented prior to sacrifice and organ collection.

Finally, although i.p. and oral T4 administration were reliable methods to induce hyperthyroidism in mice, for subsequent studies i.p. over oral treatment was preferred due to the possibility of a better control of dosage in each individual mouse. A treatment time of 3 weeks was required to achieve stable steady state of TH serum concentrations and was therefore set as an induction period, prior to phenotypical characterization of mice.

Thus, in the following studies of young (5 months), adult (12 months) and old (20 months) male and female mice hypothyroidism was induced by 0.02% MMI, 0.5% ClO_4- and 0.3% saccharine in drinking water combined with low iodine diet, while hyperthyroidism was induced by i.p. injections of 1 µg/g BW T4 every 48h.

6.2 Distinct sex impact on TH serum concentrations under TH excess

As already described in the previous section, a difference in response to T4 treatment was noted with higher TH serum concentrations in female compared to male mice. Subsequent studies of young, adult and old mice confirmed the same sex-dependent distribution for TT4, FT4 and FT3 serum concentrations (Chapter 3, Fig. 5 a-c, Chapter 4, Fig. 2). As the degree of difference (Δ=female-male) between young, adult and old animals was similar (~2-fold), this could have not resulted from female sex-hormone influence only.

Differences in TT4 concentrations can be related to increased estrogen-dependent binding to thyroxine-binding globulin (TBG) (Engbring and Engström, 1958; Tahboub and Arafah, 2009). Thus, estrogen replacement in postmenopausal women often results in increased TT4, but decreased FT4 concentrations due to a shift of higher thyroxine binding (Abdalla *et al.*, 1984; Mazer, 2004). However, this is not transferable to this mouse studies, as high dosages of T4 were used, which might have resulted in an oversaturation of TBG and thereby higher FT4 concentrations.

Assuming a decrease of estrogen concentration in female mice with aging, additional studies are needed to evaluate further contributing factors as e.g. sex-dependent T4 uptake from peritoneum. Whether this is mediated via lymphatic or non-lymphatic absorption and if sex plays a role has not been described yet (Nagy *et al.,* 1992). Furthermore, many pathways are involved in TH metabolism as degradation and detoxification of TH excess, which can be sex-dependently altered. A single-injection of T4 is excreted to ~90% within 48h in similar parts by feces and urine in young male mice (Schneider *et al.*, 2006). Although not included in this work, one can assume a change of excretion in a long-term treatment being additionally influenced by age and sex, a hypothesis which should be assessed in future studies.

Sex-different response to T4 treatment was also noted for FT3 serum concentrations and is most likely a result of higher FT4 and TT4 concentrations in female mice, as more available T4 molecules can be converted to T3. In fact, a higher sex-dependent enzymatic Dio1 activity in female mice was described not only for young (2 months; Riese *et al.*, 2006), but also adult mice (12 months; Schomburg *et al.*, 2007) and supports this hypothesis. Thus higher renal Dio1 activity in young female compared to male mice was noted, while hepatic Dio1 was more active in males than female mice (Riese *et al.*, 2006). Aging was accompanied by persistent sex-difference in renal Dio1 activity, while no sex-difference was noted in livers of 12 months old mice

(Schomburg *et al.*, 2007). Renal Dio1 activity might contribute to the observed sex-differences in serum FT3 of hyperthyroid mice, but was not addressed in our studies so far.

TH deprivation resulted in TT4 serum concentrations below assay detection limit, in all age groups and in male and female mice confirming successful induction of hypothyroidism, independent of age and sex.

6.3 Sex-dependent influence of THs on body weight and nutrient uptake

Alterations of body weight are influenced by age, sex and TH

Patients diagnosed of TDs often complain about changes in BW. Hyperthyroidism is associated with weight loss, while hypothyroidism goes along with BW gain (Fox *et al.*, 2008). In this context one of the obvious phenotypical changes was expected for BW in our mice.

The influence of TH excess on BW of rodents has been addressed in previous studies. BW gain or no alterations of BW have been described for young mice under thyrotoxic conditions (de Luise and Harker, 1989; da Silveira *et al.,* 2014; Cordeiro *et al.*, 2013; Tancevski *et al.*, 2008), while BW loss was found only with short intra-cerebral administration of T3 (Alvarez-Crespo *et al.*, 2016) in mice. In the establishment of chronic hyperthyroidism BW gain was observed under T4 treatment in male and female mice at 10 weeks, sex-dependently more pronounced in females (Chapter 2, Fig. 4). Using young mice (5 months) BW gain could be confirmed with TH excess, but this time sex-dependently more pronounced in male mice (Chapter 3, Fig. 2 b). One very likely reason for this discrepancy is the difference in age at beginning of experiment and therefore different stages of development.

Furthermore, hyperthyroidism and aging was accompanied by a strong sex-difference in BW, observed in adult (12 months) and old (20 months) mice. T4 treated female mice gained BW to a similar amount in adult and old age as in young (5 months), while T4 treated male mice started to loose BW after 2-3 weeks of treatment in adult and directly from the start of treatment in old age (Chapter 4, Fig. 3 b, e). As this strong effect was male mice specific, BW regulation under hyperthyroid conditions could be influenced by male sex-hormones.

The ATA guidelines for investigations of thyroid hormone economy and action in rodents (Bianco *et al.*, 2014) described in 2014 a plateau in BW change to be

characteristic under systemic hypothyroidism. This was confirmed for young (5 months) male and female mice (Chapter 3, Fig. 2 c) while with increasing age adult and old mice of both sexes very slowly lost weight during experimental time (Chapter 4, Fig. 3 c, f). Thus, an aging organism might be less able to compensate TH deprivation compared to a young organism. Sex-dependency was observed only for certain time points in young and at some time points in old mice with lower BW of males compared to females. This might indicate a better tolerance of TH deficiency in female than male mice.

Sham treated control animals did not show sex-difference in BW change in adult and old age, however young male mice gained weight faster than females. This is in line with the suppliers information of juvenile male mice gaining weight over a longer time period during development compared to female mice (Taconic Biosciences, USA) and has also been reported by other studies (Samuel *et al.*, 2013). However, BW and fat content is regulated by several factors in rodents and is not dependent on THs only, as shown in a recent study. A stringent selection of high and low BW mice was used to generate over 50 generations two BW-specific lines out of the same background and strain. Interestingly, TH deprivation in both thus newly created mouse strains resulted in a decrease of BW and fat mass, but could not equalize high BW mice to low BW mice. Even after depletion of thyroid and growth hormones, BW of both mice strains were not equal, suggesting that further genetic factors contribute to BW regulation (Bünger *et al.*, 1998).

TH-dependent food and water intake is differently regulated in female and male mice

As a useful addition to changes in BW, monitoring of food and water intake was implemented to assess influences of TDs on nutrient uptake and body composition. In patients hypothyroidism can be accompanied by loss of appetite (ATA booklet Hypothyroidism, 2013) while hyperthyroidism is usually associated with increased appetite (ATA brochure Hyperthyroidism, 2014).

TH excess resulted in a sex-difference in food and water consumption in young animals, which decreased in adult and even disappeared at old age. While no difference was noted for food intake between male and female mice in all age groups under TH deprivation, water intake was reversed and young, adult and old male mice consumed more water than females. Food and water intake of mice in euthyroid

conditions was sex-dependent at all ages with higher food intake in female than male mice (Chapter 3, Fig. 2 d; Chapter 4, Fig. 4 a, d, Fig. 5 a, d).

Surprisingly, a reversed drinking behaviour of hypothyroid mice was noted in young, adult and old ages, as female mice, despite of addition of saccharine, consumed less MMI/ClO_4- water. It remains speculative whether this is related to sex-dependent preference of some substances (MMI/ClO_4-), as taste receptor distribution in mice has been shown to be sex-unspecific (Zhang *et al.*, 2008; Tordoff *et al.*, 2007). Alternatively, this could have resulted from altered hormonal regulation of fluid intake which has been described in hypothyroid patients (Sahún *et al.*, 2001; Karmisholt *et al.,* 2011), but was not addressed in mice so far. Thus, a TH influence on thirst regulation might be sex-specific in mice or rodents in general.

Both food and water intake seemed to be similarly regulated, with respect to age and sex under euthyroid and hyperthyroid conditions. Altered food intake did not explain observed differences in BW change. Moreover, increased food intake was even related to BW loss instead of gain e.g. in adult and old hyperthyroid males. In fact, this reflects the clinical situation of hyperthyroid patients of a BW loss despite of increased appetite. Thus, understanding the regulation of food intake and weight during hyperthyroidism in older male mice might help to apply the findings on human situation.

Food intake in general is known to regulate metabolic functions in mice, but is regulated itself by similar metabolic events as described in this work under TH alterations. Mice spend at least half of their energy intake for maintenance of body temperature, a critical factor which they cannot afford if food is scarce. Thus, food restriction in mice results in a fast response of metabolic changes including lowering of BT, reduction of thyroid activity, growth and reproduction and thereby reducing energy expenditure (Martinez-Sanchez *et al.*, 2014; Himms-Hagen, 1999). It has been shown in mice that thyroidal activity and serum T3 levels decrease during fasting or severe caloric restriction, but increase again fast when food becomes available (Cordeiro *et al.*, 2013; Lartey *et al.*, 2015). Other groups confirmed these findings of an increase of food intake in different types of TH excess, or decrease under TH deprivation (Cordeiro *et al.*, 2013; Kong *et al.*, 2004; Groba *et al.*, 2013). Thus, these data clearly show a highly regulated interplay between nutrient intake and thyroidal activity, which is not only different in female and male mice, but suggests an influence of sex hormones.

6.4 Sex influence on heart rate and body temperature in thyroid dysfunctions

Sex does not alter TH-dependent heart rate

Besides TH influence on body composition other functions of an organism are affected if THs are in a dysbalance. Hyperthyroidism is often attended by tachycardia and heat intolerance, while hypothyroidism goes along with bradycardia and cold intolerance in patients (Bahn *et al.*, 2011; Garber *et al.*, 2012). Thus, in all mice we determined heart rate in a non-invasive electrocardiography (ECG) system and measured BT via rectal probe to identify a possible sex-dependent influence.
Interestingly, the only sex-difference for heart rate was found for euthyroid young male and female mice. Females had a higher heart rate than male mice, a difference which disappeared by TH modulation (Chapter 3, Fig. 3 b). No sex difference was noted for adult and old mice under any TH conditions (Chapter 4, Fig. 6 c, d). Of note, in all age groups and sexes, TH deprivation resulted in a decrease of heart rate, while an increase in heart rate under TH excess was found only in young male and female mice. ECG measurements were performed in conscious mice after training to the restrainer. However, every experiment increased the stress level resulting in an increase of basal heart rate and BT. Thus, sex-differences might be masked and not distinguishable due to a high stress response. An option to exclude stress during measurements is the implantation of a telemetric probe. Using these probes long-term measurements are possible without further manipulation. But this in turn would increase the physical burden of mice in a much higher value as an operation is needed and was therefore not performed.

Body temperature is sex and TH-dependent

The aspect of a stress response to experiments and handling needs to be considered in the influence on BT as well. In fact, the results of rectal temperature measurements are considered to be consistent as they were shown to correlate well with core body temperature (Newsom *et al.*, 2004). Throughout all age groups and TH conditions female mice had a higher BT than male mice (Chapter 3, Fig. 3 a; Chapter 4, Fig. 6 a, b) suggesting less influence of circulating female sex hormones. A study assessing sex-dependency of BT by radio telemetry over several days recorded BT changes in cycling sham-treated and ovariectomized female mice, as well as in orchidectomized and sham-treated males. Two important findings were noted in this study. First, higher BT in sham-treated female mice during resting and

activity phases compared to male mice. And second, higher BT in sham-treated female mice during estrous and proestrous cycle in comparison to ovariectomized female mice. Thereby, an estrous-associated higher BT in sham-treated female mice was always correlated with higher activity in comparison to ovariectomized females. Fewer differences were noted for resting BT of these mice, suggesting an influence of circulating female sex hormones on activity, which in turn results in increase of BT. Thus, at least two factors contribute to the observed sex-differences in BT of male and female mice. First, female sex hormones are responsible for elevated BT by increasing the activity estrous-dependently and second, increased resting temperature of female mice regulated sex hormone-independently (Sanchez-Alavez *et al.*, 2011).

As sex-dependency for BT was measured in all TH conditions and ages TH modulation might have less impact on sex-dependency but more on general BT regulation, as BT measurements were performed on certain days in the active state without consideration of estrous cycle in these mice.

6.5 Activity and muscle function are sex- and TH-dependent

Fatigue, as well as hyperactivity are symptoms of hypo- and hyperthyroidism in human patients, respectively. Moreover, an influence of THs on motoric and muscle function are known for a long time (Bahn *et al.*, 2011; Garber *et al.*, 2012; Bianco *et al.*, 2014). Thus, an assessment of locomotor activity and muscle function in female and male mice under TH modulation was included. Evaluation of activity in young, adult and old mice was determined by an open field test, whereas two different muscle test were performed. To assess muscle endurance and motor coordination, mice were exposed to walk on an accelerating, rotating cylinder (Rotarod), while muscle strength and coordination was evaluated by the ability to climb up a tube in a certain time frame (Chimney test).

TH independently females show higher locomotor activity in open field than male mice

The open field test reveals an individual response to a novel environment and is known to predict a disposition to neuropsychiatric disorders as increased anxiety and depression rather than representing the normal acitivity of mice (Carreira *et al*., 2017). Although the calculation of open field activity did not always reached

significance, a higher locomotor activity of female compared to male mice was observed for eu-, hyper- and hypothyroid conditions in young animals. These sex-differences persisted in adult, but were diminished in old mice (Chapter 3, Fig. 4 b, Chaper 4, Fig. 8 c, d). Thus, female mice responded stronger to a novel environment than male mice, irrespective of age and TH condititon. Higher locomotor activity and increased voluntary exercise of female mice has already been described in euthyroid animals (Sanchez-Alavez *et al.*, 2011; Konhilas *et al.*, 2004). However, the possible influence of THs and aging on sex-dependent activity has not been addressed so far. As sex differences were noted at all ages, but diminished in old mice, this suggests a TH-independent, most likely sex hormone-dependent influence.

Muscle function is improved age-dependently in female mice

In general, muscle endurance was better in female than male mice, displayed by an improved performance of female mice in euthyroid controls in young, adult and old age. Sex difference was most pronounced in adult mice and decreased in old age. This was due to a decline in muscle function of male mice already obvious at 12 months in control animals, while no age effect was noted in females between 5 and 12 months of age. Only at 20 months, muscle function of female mice had declined, however their performance was still improved compared to 20 months old male mice. Under TH excess and deprivation, sex difference disappeared in young, adult and old mice (Chapter 3, Fig. 4 d-f, Chapter 4, Fig. 7). Hence muscle function in female mice had declined under TH excess especially in adult and old age, while male performance was already reduced per se. On the contrary, under TH deprivation female mice showed a comparable ability as euthyroid females, while male mice even improved e.g. in adult age (Chapter 4, Fig. 7 c).

These findings from the rotarod test were also in line with results of the chimney test, showing a correlation between muscle coordination and strength. Euthyroid female mice showed a better performance at all ages and needed less time to climb up the tube compared to males. While male mice showed a drastic decline in muscle strength already at 12 months of age, with a high failure rate little difference in performance was noted between 5 and 12 months old female mice. In addition, muscle strength declined with TH modulation in male and female mice, more under TH excess compared to TH deprivation, but sex effect was still persistent in young and adult age. However, for old mice the chimney test was not applicable, as only

one male and eight female mice were able to climb up the tube, and therefore neither a sex- nor a TH-effect could have been addressed properly (Chapter 4, Fig. 8 a, b).

Suggested mechanisms of TH and sex hormone influence on muscle function

Thyroid and sex hormones are likely to be involved in central mediated and direct effects on muscle coordination and function. An early study describing the influence of age, sex and TH on the energy metabolism of muscles investigated oxygen consumption of isolated soleus muscles. Mice of both sexes, different ages (5-35 weeks) and with or without T3 treatment were included. Hyperthyroidism increased oxygen consumption compared to controls; however, a sex or age effect was addressed in euthyroid mice only. Aging resulted in a reduction of oxygen consumption and thereby decreased muscle metabolic rate in male and female mice. Moreover, female compared to age-matched male mice had advanced oxygen consumption (de Luise and Harker, 1989). In addition it has been reported, that isolated male muscles contract shorter than female muscles, resulting in a higher output (Glenmark *et al.*, 2004). In contrast, female muscles are more fatigue resistant, recover faster and show less mechanical damage after exercise (Glenmark *et al.*, 2004; Burnes *et al.*, 2008). Thus, *ex vivo* investigations confirmed age and sex, as well as TH-dependent effects on muscle metabolism and function, which was observed in motoric tests in this work *in vivo*. Interestingly, deiodinase 2 (Dio2) deficient mice, which are characterized by a disrupted HPT-axis with normal T3, but elevated T4 and TSH serum concentrations (Schneider *et al.*, 2001) show an impairment in motor coordination and function in adult (6 months) but not young (3 months) male mice. However, if this was a central or direct effect is not distinguishable. At 3 and at 6 months altered T3 and T4 concentrations were noted in cerebellum and cortex of Dio2 deficient mice, while TH content of muscles was measured only at 6 months of age, showing decreased intramuscular T3 concentrations (Bárez-López *et al.*, 2014). Most likely both, central nervous system and muscle effects are responsible for disturbance of motor function in organisms with altered TH status.

6.6 Sex hormone influence on TH action in target organs

Phenotypical changes due to TH modulation in mice of different sexes and ages are caused by alterations in underlying molecular mechanisms on transcriptional and

translational level. First, gene regulations were investigated by qRT-PCR to quantify expression changes due to TH excess or deprivation in young male and female mice. As classical symptoms of TD are, amongst others, changes in heart rate, heat or cold intolerance and lipid serum profile, investigation of TH-responsive genes and transporters focused on heart, brown adipose tissue (BAT) and liver (Chapter 3, Fig. 6).

Little sex effect was noted for TH-dependent cardiac gene expression

Sex-difference observed for heart rate in young euthyroid mice disappeared with TH modulation. Thus, it was interesting, whether this was reflected in sex-dependent gene expression changes in heart tissues of these mice. However, sodium-taurocholate co-transporting polypeptide (*Ntcp)* was the only gene, which was differently regulated in male and female mice, suggesting that all other genes analysed were not representative for whole organ response (Chapter 3, Fig. 6 d-f). Moreover, heart rate might have been influenced by indirect alterations of sympathetic system, rather than resulting from a direct effect of TH excess or deprivation, but was not addressed in these studies so far.

BAT gene expression was strongly sex-dependent in euthyroid mice

Contrary to heart rate, a persistent sex-difference in BT was noted in eu-, hyper- and hypothyroid conditions. Higher expression of all investigated TH responsive and TH transporter genes were found in BAT of euthyroid female compared to male mice. TH excess resulted in a sex-different response of *Dio2* and L-type amino acid transporter 2 (*Lat2*) expression, while for hypothyroidism only *Lat2* was sex-dependently altered. These data suggest a stronger sex-dependency of BAT genes in euthyroid and less in hyper- or hypothyroid state. BAT has been shown to be crucial for maintaining BT under cold conditions as an important regulator by enabling non-shivering thermogenesis through mitochondrial uncoupling protein 1 (Ucp1) (Golozoubuva *et al.*, 2001). Moreover, BAT is highly responsive for TH, as well as cold conditions by stimulating β-adrenergic signalling centrally and directly (Silva 2006; Alvarez-Crespo *et al.*, 2016; Weiner *et al.*, 2017). Thereby, an important note is to consider the housing conditions of mice during experimental time at ~23°C, which results in a regular exposition to a cold challenge and activated BAT. In contrast thermoneutrality in mice is achieved at 30°C. Thus, to evaluate a TH effect only, one should perform

mice experiments in a thermoneutral environment. Using this approach TH excess has been suggested to cause a fever-like state, rather than an overproduction of heat (Alvarez-Crespo *et al.*, 2016). Hypothyroidism in turn inactivated BAT activity only at thermoneutrality (Ueta *et al.*, 2011). Therefore, an overlap of activating or inactivating signalling due to TH modulation and adjustment to thermal stress due to lowered housing temperatures, were expected in mice experiments of this work. In fact, these compensatory mechanisms most likely contribute to altered gene expression regulation, and one cannot easily distinguish between both effects. However, a sex effect has not been addressed on BAT activity and regulation so far, as either male or female samples were studied, but not both sexes at once (Weiner *et al.*, 2016; Alvarez-Crespo *et al.*, 2016; Sellayah and Sikder, 2014) and will be in focus in future investigations.

Hepatic gene alterations are sex and TH-dependent

Another important TH target organ is the liver which was studied in more detail to evaluate if sex has an impact on TH-dependent gene regulation. Among the investigated genes in livers of young male and female mice, sex-dependent gene expression was noted for both TH conditions hyper- and hypothyroidism. Under hyperthyroidism *Dio1*, malic enzyme (*Me1*), monocarboxylate transporter 10 (*Mct10*) and L-type amino acid transporter 1 (*Lat1*) were differently expressed, with higher transcriptional levels in female mice except for *Lat1*. Under hypothyroidism increase of *Tbg* expression was higher in female than in male mice, while no differences were noted for other TH responsive genes and transporters. Comparison of liver tissues of euthyroid control mice showed a lower basal *Tbg*, but higher *Lat1* gene expression in female compared to male mice (Chapter 3, Fig. 6 i). One could speculate that a more pronounced response of altered gene expression in female mice under hyperthyroidism could be a consequence of sex-difference in serum TH concentrations. This hypothesis cannot be rejected, but considering the gene expression profiles of hyperthyroid female and male mice in three different target organs (heart, BAT, liver) at least in young animals, a consistent gene regulation pattern in organs of females compared to male mice could not have been observed. Thus, TH serum concentrations may not entirely reflect the organ-specific TH concentrations and hence it is not possible to conclude on organ-specific response.

6.7 Focus on interplay of TH and sex in liver function and lipid metabolism

TH-influence on triglyceride and cholesterol serum concentrations is stronger in male than female mice

Another important question was, if the observed sex-dependent hepatic gene expression changes are relevant for liver function. Hence, triglyceride and cholesterol serum concentrations were determined first, as lipid metabolism is one of the main functions of the liver and is known to be altered during hyper- and hypothyroidism (Bahn *et al.*, 2011; Garber *et al.*, 2012; Bianco *et al.*, 2014). Surprisingly and in contrast to sex-dependent phenotypes under TH excess, a sex difference for both lipid parameters could not be detected under TH deprivation with lower serum triglyceride and cholesterol concentrations in female compared to male mice (Chapter 3, Fig. 5 d, e). Additionally, euthyroid control females had lower cholesterol concentrations compared to euthyroid male mice. Thus, sex-difference in cholesterol serum concentrations disappeared with TH excess and was pronounced under TH deprivation. Moreover, cholesterol metabolism of male mice is suggested to be more sensitive for TH modulation than of female mice, as magnitudes of alterations were higher in male than female mice. Changes in lipid serum composition might have not resulted from metabolic changes in the liver only, but most likely influenced additionally by TH responses from other organs as brown and white adipose tissue as well as muscles.

Previous studies have reported a sex-dependent regulation of *Cyp7a1* (cholesterol-7-αhydroxylase, promotes first step of bile acid synthesis) under TH excess, with increased expression in male than in female mice (Jonas *et al.*, 2015). Moreover, in this work other pathways were identified, which might contribute to sex-specific alteration of lipid metabolism.

Role of Claudin-1 in sex and TH-dependent alterations of hepatic function

Tight junctions are important in the formation of a paracellular barrier integrity between epithelial and endothelial cells, e.g. of the bile duct and hepatocytes. Claudin-1, one of many tight junction proteins, is expressed in the bile canalicular region of hepatocytes and is known to be involved in formation of basolateral and apical plasma membrane domains to promote polarization (Kojima *et al.*, 2003, Kojima *et al.*, 2009). A functional relevance of this protein in hepatocytes is proven by patients with altered claudin-1 expression or claudin-1 mutations, who suffer from

biliary diseases (Carlton *et al.*, 2003; Laurila *et al.*, 2007; Nemeth *et al.*, 2009; Grosse *et al.*, 2012). Moreover, a TH-dependent change in sex-dependent prevalence of biliary diseases, e.g. gallstone formation, has been described. Women are more prone to develop gallstones in euthyroid conditions than men, however the prevalence changes if patients are TH deprived since hypothyroid men are more frequently affected by gallstones than women (Volzke *et al.*, 2005; Laukkarinen *et al.*, 2010). Thus, the possible influence of sex and hypothyroidism on hepatic Claudin-1 expression was assessed in young male and female mice. *Claudin-1* was lower expressed in livers of euthyroid female compared to male mice. TH deprivation increased *Claudin-1* gene expression in livers of females, but not in male mice (Chapter 5, Fig. 1 c). Moreover, these findings were confirmed on protein level by immunoblot and fluorescence microscopy, as again livers of euthyroid male mice had higher protein expression level of *Claudin-1* than female mice, but this reversed under hypothyroidism in decreased and elevated expression level in male and female mice, respectively (Chapter 5, Fig. 2). Using fluorescence microscopy, the localization of *Claudin-1* was visualized in canalicular region of liver and the bile duct (Chapter 5, Fig. 3). Thus, a clear TH and sex-dependent alteration of *Claudin-1* was shown on transcriptional and translational level with a putative influence on paracellular pathway. Increased *Claudin-1* expression might promote paracellular integrity of the canalicular region and bile duct, while decreased expression could lead to impairment. Ongoing studies using a mouse model susceptible to gallstone formation will help to unravel the role of *Claudin-1* and other tight junction proteins, as well as enzymes of cholesterol and bile acid metabolism, in the sex-dependent prevalence especially in euthyroid and hypothyroid conditions.

6.8 Conclusion and outlook

In summary, for the first time the clinical situation of a sex- and age-dependent difference of hyper- and hypothyroidism was addressed in an *in vivo* model to enable not only the analysis of phenotypical traits but provide the ability to investigate the underlying mechanisms on a molecular level. Nutrient intake, BW, BT, heart rate and motoric and behaviour changes due to TH modulation were assessed. A complex pattern of age-dependent or independent traits under hyper- or hypothyroid conditions were found and created new hypotheses, which will be further investigated. Interesting sex-difference in TH excess on TH serum concentrations,

BW, nutrient intake and muscle function indicated an influence of sex hormones. Surprisingly little effects were noted for heart rate, while BT was persistently different in male and female mice. First molecular investigations confirmed sex- and organ-specific response on transcriptional level in heart, BAT and liver. Moreover, first hints for a clinical relevance of sex-dependency on cholesterol metabolism were found and related to sex- and TH-specific regulation of hepatic tight junction protein Claudin-1.

Future perspectives of studies on clinically relevant sex-dependent TH action

Claudin-1 regulation represents an interesting example of a different outcome of TD between women and men warranting further studies in rodents. To prove sex and TH-dependency in biliary diseases, especially as proposed for gallstone formation, a gallstone susceptible mouse strain of both sexes could be used, which in combination of a lithogenic diet results in gallstone formation (Hillebrandt *et al.*, 2003; Liu *et al.*, 2008). By additional modulation of TH status in these mice, both contributing factors sex and TH can be assessed. Moreover, the alterations in cholesterol and lipid concentrations in serum and bile can be evaluated by the underlying hepatic mechanisms and will improve the understanding of sex-dependency in gallstone formation.

Another interesting sex-dependent effect of THs is well known on bone maintenance in the adulthood. Thus, bone turnover is known to be increased in women but not men under hyperthyroidism (Bassett and Williams, 2016), which is associated with an increased risk of fractures especially in postmenopausal women (Bauer *et al.*, 2001, Blum *et al.*, 2015). Reduced bone mineral density was found in hyperthyroid post-, but not premenopausal women or men (Uzzan *et al.*, 1996). Thus, in translation to the clinical situation bone samples of mice at different ages and both sexes have been collected during experimental time and are currently under investigation. This will help to gain a deeper insight of sex and age-dependent TH action on bones.

Extending the understanding of sex effects on muscle function

Another interesting finding during phenotypical characterization of TDs in mice appeared for striking sex-difference in neuromuscular function. Female mice seemed to maintain their advanced muscle function for a much longer time than male mice. Thus, to further confirm and evaluate if this is related to improved muscle strength, additional functional tests are possible, as well as the determination of muscle size,

fibre type and the excitation-contraction process. Forelimb grip strength can be used to evaluate *in vivo* neuromuscular performance. Improved performance indicates increased muscle strength, whereas a decrease in grip strength indicate weakness and augmented fatigability (Bonetto *et al.*, 2015). In addition, the four limb-hanging test is a method to assess muscle strength of all four limbs and determines the general condition over time, but although easy to perform this method needs to be evaluated in context of other tests and consideration of BW of mice (Bonetto *et al.*, 2015). Furthermore, molecular analysis of skeletal muscle force production can be measured in isolated whole-muscle or single myofiber preparations of extensor digitorum longus (fast-switch) and soleus (slow-switch) muscles. Thus, electrical stimulation of repeated contraction and recovery phases can be used to determine mechanisms of fatigue, whereas quantification of specific force is helpful to evaluate muscle strength (Bonetto *et al.*, 2015). Taken all these experiments together a TH and sex-effect on muscle function could be studied in detail.

Organ-specific response to TH excess in male and female mice

Surprisingly, continuous sex-dependency was noted for TH excess, resulting in a stronger increase of all TH serum concentrations in female mice. Hence, it will be interesting if this distribution is also representative within organs. Measurements of TH concentrations in different tissues would further clarify, if such a sex-difference is mirrored on organ level and would help to interpret gene expression alterations. Moreover, this could reveal or discard the hypothesis of a sex and organ-specificity in TH uptake and indicate why sex-difference appears in lipid metabolism and hepatic function or why no sex-difference was noted for heart rate and cardiac gene expression.

Elimination of circulating sex hormones to unravel dependency on TH action

A very important and well established tool for studying and assessing sex-hormone influence is to apply experiments on ovariectomized and orchidectomised female and male mice, respectively. With this the influence of circulating sex hormones can be dissected. Moreover, applications of individual sex hormones as estrogen or testosterone to ovariectomized or orchidectomised mice can reveal a direct effect of these components.

This approach has been addressed in the past on euthyroid mice and a variety of topics (Riese *et al.*, 2006; Walf and Frye 2010; Sanchez-Alavez *et al.*, 2011; Faustino *et al.*, 2012). Moreover, first studies related to TH action have been performed in young euthyroid, gonadectomized male and female mice and focused on organ-specific Dio1 activity and expression. Thus, orchidectomy did not changed mRNA or activity of Dio1, whereas ovariectomy increased hepatic Dio1 activity and decreased renal *Dio1* gene expression, suggesting an influence of estrogen on Dio1 stability and efficiency (Riese *et al.*, 2006). However, a modulation of TH in combination with gonadectomy has not been performed so far and will be a focus in future studies to improve the understanding of sex-dependent TH action.

The awareness of extending the research to both sexes in animal studies has been published in 2014 by the US National Institutes of Health in Nature (Clayton and Collins, 2014) and was further encouraged in the same year by emphasizing sex as an important modifier in research studies (Hammes 2014). Thus, although an increasing amount of groups pay now more attention on the clear difference between female and male rodents, still comprehensive studies on both sexes are scarce.
Thereby, efforts of basic scientists need to be further increased to improve the applicability to human situation. Even more important, TDs as common women diseases are in need for a better understanding of female-specific differences, the leading causes and the underlying mechanisms.

6.9 References

Abdalla HI, Hart DM, Beastall GH. Reduced serum free thyroxine concentration in postmenopausal women receiving oestrogen treatment. Br Med J (Clin Res Ed). 1984 Mar 10;288(6419):754-5.

Aghini-Lombardi F., Antonangeli L., Martino E., Vitti P., Maccherini D., Leoli F, Rago T., Grasso L., Valeriano R., Balestrieri A., Pinchera A. 1999. The spectrum of thyroid disorders in an iodine-deficient community: the Pescopagano survey. J. Clin. Endocrinol. Metab. 84 (2), 561–566.

Ahmed Z., Khan M.A., ul Haq A., Attaullah S., ur Rehman J. 2009. Effect of race, gender and age on thyroid and thyroid stimulating hormone levels in North West Frontier Province, Pakistan. J FAyub Med Coll Abbottabad 21 (3), 21–24.

Ain KB, Refetoff S, Sarne DH, Murata Y. Effect of estrogen on the synthesis and secretion of thyroxine-binding globulin by a human hepatoma cell line, Hep G2. Mol Endocrinol. 1988 Apr;2(4):313-23.

Alvarez-Crespo M, Csikasz RI, Martínez-Sánchez N, Diéguez C, Cannon B, Nedergaard J, López M. Essential role of UCP1 modulating the central effects of thyroid hormones on energy balance. Mol Metab. 2016 Feb 10;5(4):271-82.

American Thyroid Association (ATA) booklet: Hypothyroidism 2013.

American Thyroid Association (ATA) brochure: Hyperthyroidism 2014.

Bachman ES, Hampton TG, Dhillon H, Amende I, Wang J, Morgan JP, Hollenberg AN. The Metabolic and Cardiovascular Effects of Hyperthyroidism Are Largely Independent of ß-Adrenergic Stimulation. Endocrinology. 2004 Jun;145(6):2767-74.

Bahn R.S, Burch H.B., Cooper D.S., Garber J.R., M. Greenlee C., Klein I., Laurberg P., McDougall I.R., Montori V.M., Rivkees S.A., Ross D.S., Sosa J.A., Stan M.N. 2011. Hyperthyroidism and Other Causes of Thyrotoxicosis: Management Guidelines of the American Thyroid Association and American Association of Clinical Endocrinologists. Thyroid Volume 21, Number 6, 2011:593-646

Ban Y, Greenberg DA, Davies TF, Jacobson E, Concepcion E, Tomer Y. Linkage analysis of thyroid antibody production: evidence for shared susceptibility to clinical autoimmune thyroid disease. J Clin Endocrinol Metab. 2008;93:3589-3596.

Bárez-López S, Bosch-García D, Gómez-Andrés D, Pulido-Valdeolivas I, Montero-Pedrazuela A, Obregon MJ, Guadaño-Ferraz A. Abnormal motor phenotype at adult stages in mice lacking type 2 deiodinase. PLoS One. 2014 Aug 1;9(8):e103857.

Bassett JH, Williams GR. Role of Thyroid Hormones in Skeletal Development and Bone Maintenance. Endocr Rev. 2016 Apr;37(2):135-87.

Bauer D.C., Ettinger B., Nevitt M.C., Stone K.L. Risk for fracture in women with low serum levels of thyroid-stimulating hormone. Ann Intern Med. 2001;134:561–568.

Bernal J, Guadaño-Ferraz A, Morte B. Perspectives in the study of thyroid hormone action on brain development and function. Thyroid. 2003 Nov;13(11):1005-12.

Bianco A.C., Anderson G., Forrest D., Galton V.A., Gereben B., Kim B.W., Kopp P.A., Liao X.H., Obregon M.J., Peeters R.P., Refetoff S., Sharlin D.S., Simonides W.S., Weiss R.E., Williams G.R. American thyroid association guide to investigating thyroid hormone economy and action in rodent and cell models. Thyroid. 2014;24:88–168.

Bianco, A.C., Kim, B.W., 2006. Deiodinases: implications of the local control of thyroid hormone action. J Clin Invest. 2006 Oct;116(10):2571-9.

Billewicz WZ, Chapman RS, Crooks J, Day ME, Cossage J, Wayne Sir E, Young A. Statistical methods applied to the diagnosis of hypothyroidism. QJ Med 1969; 38:255-66.

Bjoro T., Holmen J., Krüger Ø., Midthjell K., Hunstad K., Schreiner T., Sandnes L., Brochmann, H. 2000: Prevalence of thyroid disease, thyroid dysfunction and thyroid peroxidase antibodies in a large, unselected population. The Health Study of Nord-Trondelag (HUNT). Eur. J. Endocrinol. 143 (5), 639–647.

Blum M.R., Bauer D.C., Collet T.H., et al. Subclinical thyroid dysfunction and fracture risk: a meta-analysis. JAMA. 2015;313:2055–2065.

Boelaert K., Torlinska B, Holder R.L., Franklyn J. A. Older Subjects with Hyperthyroidism Present with a Paucity of Symptoms and Signs: A Large Cross-Sectional Study. J Clin Endocrinol Metab, June 2010, 95(6):2715–2726

Bonetto A, Andersson DC, Waning DL. Assessment of muscle mass and strength in mice. Bonekey Rep. 2015 Aug 19;4:732.

Bookout AL, Jeong Y, Downes M, Yu RT, Evans RM, Mangelsdorf DJ. Anatomical profiling of nuclear receptor expression reveals a hierarchical transcriptional network. Cell. 2006 Aug 25;126(4):789-99.

Bünger L, Wallace H, Bishop JO, Hastings IM, Hill WG. Effects of thyroid hormone deficiency on mice selected for increased and decreased body weight and fatness. Genet Res. 1998 Aug;72(1):39-53.

Buras A, Battle L, Landers E, Nguyen T, Vasudevan N. Thyroid hormones regulate anxiety in the male mouse. Horm Behav. 2014 Feb;65(2):88-96.

Burnes L.A., Kolker S.J., Danielson J.F., Walder R.Y., Sluka K.A. Enhanced muscle fatigue occurs in male but not female ASIC3-/- mice. Am J Physiol Regul Integr Comp Physiol. 2008;294(4):R1347–R1355.

Cai M, Ma YL, Qin P, Li Y, Zhang LX, Nie H, Peng Z, Dong H, Dong HL, Hou WG, Xiong LZ. The loss of estrogen efficacy against cerebral ischemia in aged postmenopausal female mice. Neurosci Lett. 2014 Jan 13;558:115-9.

Camargo R.Y.A., Tomimori E.K., Neves S.C., Rubio I.G.S., Galrão A.L., Knobel M., Medeiros-Neto G. 2008: Thyroid and the environment: exposure to excessive nutritional iodine increases the prevalence of thyroid disorders in Sao Paulo, Brazil. Eur. J. Endocrinol. 159 (3), 293–299.

Canaris G.J., Manowitz N.R., Mayor G., Ridgway E.C. 2000. The Colorado thyroid disease prevalence study. Arch. Intern. Med. 160 (4): 526–34.

Carlé, A., Bülow Pedersen, I., Knudsen, N., Perrild, H., Ovesen, L. and Laurberg, P. (2015), Gender differences in symptoms of hypothyroidism: a population-based DanThyr study. Clin Endocrinol, 83: 717–725. doi:10.1111/cen.12787

Carlton VE, Harris BZ, Puffenberger EG, Batta AK, Knisely AS, Robinson DL, Strauss KA, Shneider BL, Lim WA, Salen G et al: Complex inheritance of familial

hypercholanemia with associated mutations in TJP2 and BAAT. Nat Genet 2003;34(1):91-96.

Carreira MB, Cossio R, Britton GB. Individual and sex differences in high and low responder phenotypes. Behavioural Processes, 2017;136:20–27

Chanoine, J.P., Braverman, L.E., Farwell, A.P., Safran, M., Alex, S., Dubord, S., Leonard, J.L., 1993. The thyroid gland is a major source of circulating T3 in the rat. J Clin Invest. 1993 Jun;91(6):2709-13.

Chen J, Ortmeier SB, Savinova OV, Nareddy VB, Beyer AJ, Wang D, Gerdes AM. Thyroid hormone induces sprouting angiogenesis in adult heart of hypothyroid mice through the PDGF-Akt pathway. J Cell Mol Med. 2012 Nov;16(11):2726-35.

Clayton JA, Collins FS. NIH to balance sex in cell and animals studies. Nature. 2014;509: 282–283.

Coppola A, Liu ZW, Andrews ZB, Paradis E, Roy MC, Friedman JM, Ricquier D, Richard D, Horvath TL, Gao XB, Diano S. A Central Thermogenic-like Mechanism in Feeding Regulation: an Interplay Between Arcuate Nucleus T3 and UCP2. Cell Metab. 2007 Jan;5(1):21-33.

Cordeiro A, de Souza LL, Oliveira LS, Faustino LC, Santiago LA, Bloise FF, Ortiga-Carvalho TM, Almeida NA, Pazos-Moura CC. Thyroid hormone regulation of Sirtuin 1 expression and implications to integrated responses in fasted mice. J Endocrinol. 2013 Jan 18;216(2):181-93.

Crooks J, Murray IPC, Wayne EJ. Statistical methods applied to the clinical diagnosis of thyrotoxicosis. QJ Med 1959;28:211-34.

da Silveira A.L.B., de Souza Miranda M.F., Mecawi A.S., Melo R.L., Marassi M.P., Matos da Silva A.C., Antunes-Rodrigues J., Lopes Olivares E. Sexual dimorphism in autonomic changes and in the renin-angiotensin system in the hearts of mice subjected to thyroid hormone-induced cardiac hypertrophy. Exp Physiol. 2014;99:868-880.

Davis P.J., Goglia F, Leonard J.L.2016. Nongenomic actions of thyroid hormone. Nature Reviews Endocrinology 12, 111–121 (2016) doi:10.1038/nrendo.2015.205

Davis P.J., Sudha T., Lin H.Y., Mousa S.A. 2015. Thyroid Hormone, Hormone Analogs, and Angiogenesis. Compr Physiol. 2015 Dec 15;6(1):353-62. doi:10.1002/cphy.c150011

De Leo S, Lee S.Y., Braverman L.E., Hyperthyroidism. Lancet 2016; 388: 906–18

De Luise M, Harker M. Skeletal muscle metabolism: effect of age, obesity, thyroid and nutritional status. Horm Metab Res. 1989 Aug;21(8):410-5.

Dika H, Kasolo J., Bimenya G.S. 2010. Thyroid hormones profile in students of Makerere College of Health Sciences in Kampala Uganda. Tanzan J Health Res 12 (1), 17–22.

Dillmann W. Cellular action of thyroid hormone on the heart. Thyroid. 2002 Jun;12(6):447-52.

Dowling T., Freinkel N., Ingbar S.H. 1956. Effect of diethylstilberol on the binding of thyroxine in serum. J. Clin. Endocrinol. Metabol. 16 (11), 1491-1506

Dowling T., Freinkel N., Ingbar S.H. 1960. The effect of estrogens upon the peripheral metabolism of thyroxine. J. Clin. Invest. 39 (7), 1119-1130

Duan Y., Wang X., Peng W., Feng Y., Tang W., Wu X., Bo R., Li W., Chen J., Qin Y., Liu C., Liu C. 2009. Gender-specific associations between subclinical hypothyroidism and blood pressure in Chinese adults. Endocrine 36 (3), 438–444.

Engbring N.M., Engström W.W. 1958. Effects of estrogen and testosterone on circulating thyroid hormone. J Clin Endocrinol Metab. 19 (7), 783-796

Fabian I.D., Rosner M, Fabian I, Vishnevskia-Dai V., Zloto O., Shinderman Maman E., Cohen K., Ellis M., Lin H-Y., Hercbergs A., Davis P.J., Ashur-Fabian O. Low thyroid hormone levels improve survival in murine model for ocular melanoma. Oncotarget. 2015 May 10; 6(13): 11038–11046.

Faustino LC, Almeida NA, Pereira GF, Ramos RG, Soares RM, Morales MM, Pazos-Moura CC, Ortiga-Carvalho TM. Thyroid hormone and estradiol have overlapping effects on kidney glutathione S-transferase-α gene expression. Am J Physiol Endocrinol Metab. 2012 Sep 15;303(6):E787-97.

Ferreira E., Silva A.E., Serakides R., Gomes A.E.S., Cassali G.D.: Model of induction of thyroid dysfunctions in adult female mice. Arquivo Brasileiro de Medicina Veterinária e Zootecnia, 1245–1249 (2007).

Flamant, F. and Gauthier, K. 2013. Thyroid hormone receptors: the challenge of elucidating isotype-specific functions and cell-specific response. Biochim. Biophys. Acta 1830, 3900–3907

Fox CS, Pencina MJ, D'Agostino RB, Murabito JM, Seely EW, Pearce EN, Vasan RS. Relations of thyroid function to body weight: cross-sectional and longitudinal observations in a community-based sample. Arch Intern Med. 2008 Mar 24;168(6):587-92.

Garber J, Cobin RH, Gharib H, Hennessey JV, Klein I; Mechanick JI, Pessah-Pollack R, Singer PA, Woeber KA. Clinical practice guidelines for hypothyroidism in adults: cosponsored by American Association of Clinical Endocrinologists and the American Thyroid Association. Endocr Pract 2012; 11: 1-207.

Gereben, B., Zavacki, A.M., Ribich, S., Kim, B.W., Huang, S.A., Simonides, W.S., Zeold, A., Bianco, A.C., 2008. Cellular and molecular basis of deiodinase regulated thyroid hormone signalling. Endocr Rev. 2008 Dec;29(7):898-938.

Glenmark B, Nilsson M, Gao H, Gustafsson JA, Dahlman-Wright K, Westerblad H. Difference in skeletal muscle function in males vs. females: role of estrogens receptor-β. Am J Physiol Endocrinol Metab. 2004;287:E1125–E1131.

Golozoubova V, Hohtola E, Matthias A, Jacobsson A, Cannon B, Nedergaard J. Only UCP1 can mediate adaptive nonshivering thermogenesis in the cold. FASEB J. 2001 Sep;15(11):2048-50.

Groba C, Mayerl S, van Mullem AA, Visser TJ, Darras VM, Habenicht AJ, Heuer H. Hypothyroidism compromises hypothalamic leptin signalling in mice. Mol Endocrinol. 2013 Apr;27(4):586-97.

Grosse B, Cassio D, Yousef N, Bernardo C, Jacquemin E, Gonzales E: Claudin-1 involved in neonatal ichthyosis sclerosing cholangitis syndrome regulates hepatic paracellular permeability. Hepatology 2012;55(4):1249-1259.

Grossman A., Weiss A., Koren-Morag N., Shimon I., Beloosesky Y., Meyerovitch J.. Subclinical Thyroid Disease and Mortality in the Elderly: A Retrospective Cohort

Study. The American Journal of Medicine, Volume 129, Issue 4, April 2016, Pages 423–430

Hadlow N. C., Rothacker K.M., Wardrop R., Brown S.J., Lim E.M., Walsh J.P. 2013. The relationship between TSH and free T4 in a large population is complex and nonlinear and differs by age and sex. J. Clin. Endocrinol. Metab. 98 (7), 2936–2943.

Haisenleder D. J., Ortolano G. A., Dalkin A. C., Yasin M., Marshall J. C. Differential actions of thyrotropin (TSH)-releasing hormone pulses in the expression of prolactin and TSH subunit messenger ribonucleic acid in rat pituitary cells in vitro. Endocrinology 1992; 130: 2917–2923

Hammes SR. Sex Matters in Preclinical Research. Mol Endocrinol. 2014;28:1209–1210.

Hennemann G, Docter R, Friesema EC, de Jong M, Krenning EP, Visser TJ 2001 Plasma membrane transport of thyroid hormones and its role in thyroid hormone metabolism and bioavailability. Endocr Rev 22:451–476

Hernandez A, Morte B, Belinchón MM, Ceballos A, Bernal J. Critical role of types 2 and 3 deiodinases in the negative regulation of gene expression by T_3 in the mouse cerebral cortex. Endocrinology. 2012 Jun;153(6):2919-28.

Hillebrandt S, Matern S, Lammert F. Mouse models for genetic dissection of polygenic gastrointestinal diseases. Eur J Clin Invest 33: 155–160, 2003.

Himms-Hagen J. Physiological roles of the leptin endocrine system: differences between mice and humans. Crit Rev Clin Lab Sci. 1999 Dec;36(6):575-655.

Hollowell J.G., Staehling N.W.; Flanders W.D., Hannon W.H., Gunter E.W., Spencer C.A., Braverman L.E. 2002: Serum TSH, T(4), and thyroid antibodies in the United States population (1988 to 1994): National Health and Nutrition Examination Survey (NHANES III). J. Clin. Endocrinol. Metab. 87 (2), 489–499.

Hoogendoorn E.H., Hermus Ad.R., Vegt F.d., Ross H.A., Verbeek A.L.M. Kiemeney L.A.L.M., Swinkels D.W., Sweep F.C.G.J., Den Heijer M. 2006: Thyroid function and prevalence of anti-thyroperoxidase antibodies in a population with borderline sufficient iodine intake: influences of age and sex. Clin. Chem. 52 (1), 104–111.

Hübner NS, Merkle A, Jung B, von Elverfeldt D, Harsan LA. Analysis of left ventricular function of the mouse heart during experimentally induced hyperthyroidism and recovery. NMR Biomed. 2015 Jan;28(1):116-23.

Jonas W, Lietzow J, Wohlgemuth F, Hoefig CS, Wiedmer P, Schweizer U, Köhrle J, Schürmann A. 3,5-Diiodo-L-thyronine (3,5-T2) exerts thyromimetic effects on hypothalamus-pituitary-thyroid axis, body composition, and energy metabolism in male diet-induced obese mice. Endocrinology. 2015;156(1):389-399.

Kapelari K, Kirchlechner C., Högler W, Schweitzer K., Virgolini I., Moncayo R. 2008. Pediatric reference intervals for thyroid hormone levels from birth to adulthood: a retrospective study. BMC Endocr Disord 8: 15.

Karmisholt J, Andersen S, Laurberg P. Weight loss after therapy of hypothyroidism is mainly caused by excretion of excess body water associated with myxoedema. J Clin Endocrinol Metab. 2011 Jan;96(1):E99-103. doi: 10.1210/jc.2010-1521.

Khandelwal D., Tandon N. 2012. Overt and subclinical hypothyroidism: who to treat and how. Drugs 72 (1): 17–33.

Klecha, A.J., Genaro, A.M., Gorelik, G., Barreiro Arcos, M.L., Silberman, D.M., Schuman, M., Garcia, S.I., Pirola, C., Cremaschi, G.A.: Integrative study of hypothalamus-pituitary-thyroid-immune system interaction: thyroid hormone-mediated modulation of lymphocyte activity through the protein kinase C signalling pathway. J. Endocrinol. 189(1), 45–55 (2006). doi: 10.1677/joe.1.06137

Klieverik LP, Coomans CP, Endert E, Sauerwein HP, Havekes LM, Voshol PJ, Rensen PC, Romijn JA, Kalsbeek A, Fliers E. Thyroid hormone effects on whole-body energy homeostasis and tissue-specific fatty acid uptake in vivo. Endocrinology. 2009 Dec;150(12):5639-48.

Knudsen N., Jorgensen T., Rasmussen S., Christiansen E., Perrild H. 1999: The prevalence of thyroid dysfunction in a population with borderline iodine deficiency. Clin. Endocrinol. (Oxf) 51 (3), 361–367.

Ko Y.J., Kim J.Y., Lee J, Song H.J., Kim J.Y., Choi N.K., Park B.J. Levothyroxine dose and fracture risk according to the osteoporosis status in elderly women. J Prev Med Public Health. 2014;47:36–46.

Kojima T, Murata M, Yamamoto T, Lan M, Imamura M, Son S, Takano K, Yamaguchi H, Ito T, Tanaka S et al: Tight junction proteins and signal transduction pathways in hepatocytes. Histol Histopathol 2009;24(11):1463-1472.

Kojima T, Yamamoto T, Murata M, Chiba H, Kokai Y, Sawada N: Regulation of the blood-biliary barrier: interaction between gap and tight junctions in hepatocytes. Med Electron Microsc 2003;36(3):157-164.

Kong WM, Martin NM, Smith KL, Gardiner JV, Connoley IP, Stephens DA, Dhillo WS, Ghatei MA, Small CJ, Bloom SR. Triiodothyronine stimulates food intake via the hypothalamic ventromedial nucleus independent of changes in energy expenditure. Endocrinology. 2004 Nov;145(11):5252-8.

Konhilas JP, Maass AH, Luckey SW, Stauffer BL, Olson EN, Leinwand LA. Sex modifies exercise and cardiac adaptation in mice. Am J Physiol Heart Circ Physiol. 2004 Dec;287(6):H2768-76.

Konno N., Yuri K., Taguchi H., Miura K., Taguchi S., Hagiwara K., Murakami S. 1993: Screening for thyroid diseases in an iodine sufficient area with sensitive thyrotrophin assays, and serum thyroid autoantibody and urinary iodide determinations. Clin. Endocrinol. (Oxf) 38 (3), 273–281.

Krassas G.E. 2000. Thyroid disease and female reproduction. Fertility and Sterility 74 (6), 1063-1070.

Kvetny, J., Heldgaard, P. E., Bladbjerg, E. M. and Gram, J. 2004, Subclinical hypothyroidism is associated with a low-grade inflammation, increased triglyceride levels and predicts cardiovascular disease in males below 50 years. Clinical Endocrinology, 61: 232–238. doi:10.1111/j.1365

Lartey LJ, Werneck-de-Castro JP, O-Sullivan I, Unterman TG, Bianco AC. Coupling between Nutrient Availability and Thyroid Hormone Activation. J Biol Chem. 2015 Dec 18;290(51):30551-61.

Laukkarinen J, Sand J, Nordback I: Hypothyroidism is common in bile duct stone patients. Duodecim 2010;126(19):2247-2252.

Laurila JJ, Karttunen T, Koivukangas V, Laurila PA, Syrjala H, Saarnio J, Soini Y, Ala-Kokko TI: Tight junction proteins in gallbladder epithelium: different expression

in acute acalculous and calculous cholecystitis. J Histochem Cytochem 2007;55(6):567-573.

Leese G.P., Flynn R.V., Jung R.T., Macdonald T.M., Murphy M.J., Morris A.D. 2008. Increasing prevalence and incidence of thyroid disease in Tayside, Scotland: the Thyroid Epidemiology Audit and Research Study (TEARS). Clin. Endocrinol. (Oxf) 68 (2), 311–316.

Leung AM, Pearce EN, Braverman LE. Perchlorate, iodine and the thyroid. Best Pract Res Clin Endocrinol Metab. 2010 Feb;24(1):133-41.

Lin JZ, Martagón AJ, Hsueh WA, Baxter JD, Gustafsson JÅ, Webb P, Phillips KJ. Thyroid Hormone Receptor Agonists Reduce Serum Cholesterol Independent of the LDL Receptor. Endocrinology. 2012 Dec;153(12):6136-44.

Liu JJ, Glickman JN, Masyuk AI, Larusso NF. Cholangiocyte bile salt transporters in cholesterol gallstone-susceptible and resistant inbred mouse strains. J Gastroenterol Hepatol. 2008 Oct;23(10):1596-602.

Luboshitzky R., Aviv A., Herer P., Lavie L. 2002. Risk factors for cardiovascular disease in women with subclinical hypothyroidism. Thyroid 12 (5), 421–425.

Maia, A.L., Kim, B.W., Huang, S.A., Harney, J.W., Larsen, P.R., 2005. Type 2 iodothyronine deiodinase is the major source of plasma T3 in euthyroid humans.

Mammen J. S., McGready J., Oxman R., Chia C. W., Ladenson P. W., and Simonsick E. M. Thyroid Hormone Therapy and Risk of Thyrotoxicosis in Community-Resident Older Adults: Findings from the Baltimore Longitudinal Study of Aging. Thyroid. August 2015, 25(9): 979-986. doi:10.1089/thy.2015.0180

Martínez-Sánchez N, Alvarez CV, Fernø J, Nogueiras R, Diéguez C, López M. Hypothalamic effects of thyroid hormones on metabolism. Best Pract Res Clin Endocrinol Metab. 2014 Oct;28(5):703-12.

Mazer NA. Interaction of estrogen therapy and thyroid hormone replacement in postmenopausal women. Thyroid. 2004;14 Suppl 1:S27-34.

Meisinger C, Ittermann T, Wallaschofski H, Heier M, Below H, Kramer A, Döring A, Nauck M, Völzke H. Geographic variations in the frequency of thyroid disorders and thyroid peroxidase antibodies in persons without former thyroid disease within Germany. European Journal of Endocrinology (2012) 167 363–371

Menconi F, Monti MC, Greenberg DA, et al. Molecular amino acid signatures in the MHC class II peptide-binding pocket predispose to autoimmune thyroiditis in humans and in mice. Proc Natl Acad Sci USA. 2008;105:14034-14039.

Messarah, M., Boumendjel, A., Chouabia, A., Klibet, F., Abdennour, C., Boulakoud, M.S., Feki, A.E.: Influence of thyroid dysfunction on liver lipid peroxidation and antioxidant status in experimental rats. Experimental and Toxicologic Pathology 62(3), 301–310 (2010). doi: 10.1016/j.etp.2009.04.009

Moeller L.C. and Broecker-Preuss M. 2011. Transcriptional regulation by nonclassical action of thyroid hormone. Thyroid Research 2011: 4(Suppl 1):S6 doi: 10.1186/1756-6614-4-S1-S6

Mullur R, Liu YY, Brent GA. Thyroid hormone regulation of metabolism. Physiol Rev. 2014 Apr;94(2):355-82.

Nagy JA. Lymphatic and nonlymphatic pathways of peritoneal absorption in mice: physiology versus pathology. Blood Purif. 1992;10(3-4):148-62.

Nemeth Z, Szasz AM, Tatrai P, Nemeth J, Gyorffy H, Somoracz A, Szijarto A, Kupcsulik P, Kiss A, Schaff Z: Claudin-1, -2, -3, -4, -7, -8, and -10 protein expression in biliary tract cancers. J Histochem Cytochem 2009;57(2):113-121.

Newsom D.M., Bolgos G.I., Colby L., Nemzek J.A. Comparison of Body Surface Temperature Measurement and Conventional Methods for Measuring Temperature in the Mouse. Contemporary Topics 2004, Volume 43, No. 5, 13-18

Noda M., Mori Y, Yoshioka Y. Sex- and Age-Dependent Effects of Thyroid Hormone on Glial Morphology and Function. Opera Med Physiol 2016 Vol. 2 (2): 164-171.

O'Leary P.C., Feddema P.H., Michelangeli V.P., Leedman P.J., Chew G.T., Knuiman M. Kaye J., Walsh JP. 2006. Investigations of thyroid hormones and antibodies based on a community health survey: the Busselton thyroid study. Clin. Endocrinol. (Oxf) 64 (1), 97–104.

Pierpaoli W, Lesnikov V.A. Effects of Long-Term Intraperitoneal Injection of Thyrotropin-Releasing Hormone (TRH) on Aging- and Obesity-Related Changes in Body Weight, Lipid Metabolism, and Thyroid Functions. Current Aging Science, 2011, 4, 25-32

Pohlenz J., Maqueem A., Cua K., Weiss R.E., Van Sande J., Refetoff S. Improved Radioimmunoassay for Measurement of Mouse Thyrotropin in Serum: Strain Differences in Thyrotropin Concentration and Thyrotroph Sensitivity to Thyroid Hormone. 1999. Thyroid 9(12), 1265-1271.

Poppe K., Velkeniers B., Glinoer D. 2007.Thyroid disease and female reproduction. Clin Endocrinol 66 (3), 309–321.

Porcu E., Medici M., Pistis G., Volpato C.B., Wilson S.G., Cappola A.R. et al. 2013. A meta-analysis of thyroid-related traits reveals novel loci and gender-specific differences in the regulation of thyroid function. PLoS Genet. 9 (2), e1003266.

Reed, T.D., Babu, G.J., Ji, Y., Zilberman, A., Heyen, M. ver, Wuytack, F., Periasamy, M.: The expression of SR calcium transport ATPase and the Na(+)/Ca(2+)Exchanger are antithetically regulated during mouse cardiac development and in Hypo/hyperthyroidism. J. Mol. Cell. Cardiol. 32(3), 453–464 (2000). doi: 10.1006/jmcc.1999.1095

Rehman S.U., Cope D.W., Senseney A.D., Brzezinski W. Thyroid Disorders in Elderly Patients. South Med J. 2005;98(5):543-549.

Riese C., Michaelis M., Mentrup B., Götz F., Köhrle J., Schweizer U., Schomburg L. Selenium-dependent pre- and posttranscriptional mechanisms are responsible for sexual dimorphic expression of selenoproteins in murine tissues. Endocrinology. 2006 Dec;147(12):5883-92.

Roelfsema F., Pereira A.M., Veldhuis J.D, Adriaanse R., Endert E., Fliers E, and. Romijn J.A. 2009. Thyrotropin secretion profiles are not different in men and women. J. Clin. Endocrinol. Metab. 94 (10): 3964–67.

Sahún M, Villabona C, Rosel P, Navarro MA, Ramón JM, Gómez JM, Soler J. Water metabolism disturbances at different stages of primary thyroid failure. J Endocrinol. 2001 Mar;168(3):435-45.

Samuel P, Khan MA, Nag S, Inagami T, Hussain T. Angiotensin AT(2) receptor contributes towards gender bias in weight gain. PLoS One. 2013;8(1):e48425.

Sanchez-Alavez M., Alboni S., Conti B. Sex- and age-specific differences in core body temperature of C57Bl/6 mice. Age. 2011;33:89-99.

Schneider MJ, Fiering SN, Pallud SE, Parlow AF, St Germain DL, Galton VA. Targeted Disruption of the Type 2 Selenodeiodinase Gene (DIO2) Results in a Phenotype of Pituitary Resistance to T4. Mol Endocrinol. 2001 Dec;15(12):2137-48.

Schneider MJ, Fiering SN, Thai B, Wu SY, St Germain E, Parlow AF, St Germain DL, Galton VA. Targeted Disruption of the Type 1 Selenodeiodinase Gene (Dio1) Results in Marked Changes in Thyroid Hormone Economy in Mice. Endocrinology 2006 147(1):580–589.

Schomburg L., Riese C., Renko K., Schweizer U. Effect of age on sexually dimorphic selenoprotein expression in mice. Biol. Chem.,2007, Vol. 388, pp. 1035–1041

Schreiberova, O. and Kapitola, J.: Sex Differences in Blocking Thyroid Radioiodine Uptake in Mice. Experentia 1968 (25/1), 42.

Schussler G. C. The thyroxine-binding proteins. Thyroid 2000; 10: 141–149

Schwartz H.L., Strait K.A., Ling N.C., Oppenheimer J.H.1992. Quantitation of rat tissue thyroid hormone binding receptor isoforms by immunoprecipitation of nuclear triiodothyronine binding capacity. J. Biol. Chem. 267, 11794–11799

Segersen T. P., Hoefler H., Childers H., Wolfe H. J., Wu P., Jackson I. M.D., Lechan R. M. Localization of thyrotropin-releasing hormone prohormone messenger ribonucleic acid in rat brain by in situ hybridization. Endocrinology 1987a; 121: 98–107

Segersen T. P., Kauer J., Wolfe H. C., Mobtaker H., Wu P., Jackson I. M.D., Lechan R. M. Thyroid hormone regulates TRH biosynthesis in the paraventricular nucleus of the rat hypothalamus. Science 1987b; 238: 78–80

Sellayah D. and Sikder D. Orexin Restores Aging-Related Brown Adipose Tissue Dysfunction in Male Mice. Endocrinology, 2014, 155: 485–501

Shin DJ, Osborne TF. Thyroid Hormone Regulation and Cholesterol Metabolism Are Connected through Sterol Regulatory Element-binding Protein-2 (SREBP-2). J Biol Chem. 2003 Sep 5;278(36):34114-8.

Shiroozu A, Taurog A, Engler H, Dorris ML. Mechanism of action of thioureylene antithyroid drugs in the rat: possible inactivation of thyroid peroxidase by propylthiouracil. Endocrinology. 1983 Jul;113(1):362-70.

Silva J.E. Thermogenic Mechanisms and Their Hormonal Regulation. Physiol Rev 86: 435–464, 2006

Sowers M., Luborsky J., Perdue C., Araujo K.L.B., Goldman M.B., Harlow S.D. 2008. Blackwell Science, Ltd Thyroid stimulating hormone (TSH) concentrations and menopausal status in women at the mid-life: SWAN. Clin Endocrinol 2008 58, 340-347.

Staub J-J, Althaus B.U., Engler H.;Ryff A.S., Trabucco P., Marquardt K., Burckhardt, D., Girard J., Weintraub B.D. 1992. Spectrum of subclinical and overt hypothyroidism: effect on thyrotropin, prolactin, and thyroid reserve, and metabolic impact on peripheral target tissues. Am. J. Med. 92 (6), 631–42.

Suarez J, Scott BT, Suarez-Ramirez JA, Chavira CV, Dillmann WH. Thyroid hormone inhibits ERK phosphorylation in pressure overload-induced hypertrophied mouse hearts through a receptor-mediated mechanism. Am J Physiol Cell Physiol. 2010 Dec;299(6):C1524-9.

Surks MI, Ortiz E, Daniels GH, Sawin CT, Col NF, Cobin RH, Franklyn JA, Hershman JM, Burman KD, Denke MA, Gorman C, Cooper RS, Weissman NJ. Subclinical Thyroid Disease Scientific Review and Guidelines for Diagnosis and Management. JAMA. 2004;291(2):228-238. doi:10.1001/jama.291.2.228

Tahboub R, Arafah BM. Sex steroids and the thyroid. Best Pract Res Clin Endocrinol Metab. 2009 Dec;23(6):769-80.

Takashima N., Niwa Y., Mannami T., Tomoike H., Iwai N. 2007. Characterization of Subclinical Thyroid Dysfunction From Cardiovascular and Metabolic Viewpoints The Suita Study. Circulation Journal 71 (2), 191–195.

Tancevski I, Wehinger A, Demetz E, Eller P, Duwensee K, Huber J, Hochegger K, Schgoer W, Fievet C, Stellaard F, Rudling M, Patsch JR, Ritsch A. Reduced plasma high-density lipoprotein cholesterol in hyperthyroid mice coincides with decreased hepatic adenosine 5'-triphosphate-binding cassette transporter 1 expression. Endocrinology. 2008 Jul;149(7):3708-12.

Tauchmanovà L., Nuzzo V., Del Puente A., Fonderico F., Esposito-Del Puente A., Padulla S., Rossi A., Bifulco G., Lupoli G.; Lombardi G. 2004. Reduced bone mass detected by bone quantitative ultrasonometry and DEXA in pre- and

postmenopausal women with endogenous subclinical hyperthyroidism. Maturitas 48 (3), 299–306.

Tordoff MG. Taste solution preferences of C57Bl/6J and 129X1/SvJ mice: Influence of age, sex, and diet. Chem Senses. 2007;32:655-671.

Trajkovic M, Visser TJ, Mittag J, Horn S, Lukas J, Darras VM, Raivich G, Bauer K, Heuer H. Abnormal thyroid hormone metabolism in mice lacking the monocarboxylate transporter 8. J Clin Invest. 2007 Mar;117(3):627-35

Tunbridge W.M., Evered D.C.; Hall R., Appleton D., Brewis M., Clark F., Evans J.G., Young E., Bird T., Smith P.A. 1977. The spectrum of thyroid disease in a community: the Whickham survey. Clin. Endocrinol. (Oxf) 7 (6). 481–93.

Ueta CB, Olivares EL, Bianco AC. Responsiveness to thyroid hormone and to ambient temperature underlies differences between brown adipose tissue and skeletal muscle thermogenesis in a mouse model of diet-induced obesity. Endocrinology. 2011 Sep;152(9):3571-81

Ueta CB, Olivares EL, Bianco AC. Responsiveness to thyroid hormone and to ambient temperature underlies differences between brown adipose tissue and skeletal muscle thermogenesis in a mouse model of diet-induced obesity. Endocrinology. 2011 Sep;152(9):3571-81. doi: 10.1210/en.2011-1066

Uzzan B, Campos J, Cucherat M, Nony P, Boissel JP, Perret GY. Effects on bone mass of long term treatment with thyroid hormones: a meta-analysis. J Clin Endocrinol Metab. 1996 Dec;81(12):4278-89.

Vaidya B., Pearce S.H.S. 2008. Management of hypothyroidism in adults. BMJ 2008;337:a801, doi:10.1136/bmj.a801.

Vallortigara J, Alfos S, Micheau J, Higueret P, Enderlin V. T3 administration in adult hypothyroid mice modulates expression of proteins involved in striatal synaptic plasticity and improves motor behavior. Neurobiol Dis. 2008 Sep;31(3):378-85.

Van der Spek, A.H., Fliers E., Boelen A. The classic pathways of thyroid hormone metabolism., Molecular and Cellular Endocrinology 2017 Jan 18. pii: S0303-7207(17)30029-1. doi: 10.1016/j.mce.2017.01.025.

Vanderpump M.P.J., Tunbridge W.M., French J.M., Appleton D., Bates D., Clark F. 1995. The incidence of thyroid disorders in the community: a twenty-year follow-up of the Whickham Survey. Clin. Endocrinol. (Oxf) 43 (1), 55–68.

Vella K.R, Ramadoss P., Costa-e-Sousa R.H., Astapova I., Ye F.D., Holtz K.A., Harris J.C., Hollenberg A.N. Thyroid Hormone Signalling In Vivo Requires a Balance between Coactivators and Corepressors. 2014.Molecular and Cellular Biology 34(9), 1564–1575.

Visser E.W., Friesema E.C.H, and Visser TJ. 2011 Minireview: Thyroid Hormone Transporters: The Knowns and the Unknowns. Mol Endocrinol, January 2011, 25(1):1–14.

Visser WE, Friesema EC, Jansen J, Visser TJ. 2008 Thyroid hormone transport in and out of cells. Trends Endocrinol Metab 19:50–56.

Volzke H, Robinson DM, John U: Association between thyroid function and gallstone disease. WJG 2005;11(35):5530-5534.

Walf AA, Frye CA. Estradiol reduces anxiety- and depression-like behavior of aged female mice. Physiol Behav. 2010 Feb 9;99(2):169-74.

Walsh J.P., Bremner A.P., Bulsara M.K., O'Leary P., Leedman P.J., Feddema P., Michelangeli V. 2006. Subclinical thyroid dysfunction and blood pressure: a community-based study. Clin Endocrinol 65 (4), 486–491.

Weiner J, Hankir M, Heiker JT, Fenske W, Krause K. Thyroid hormones and browning of adipose tissue. Cell Endocrinol. 2017 Jan 12. pii: S0303-7207(17)30015-1. doi: 10.1016/j.mce.2017.01.011.

Weiner J, Kranz M, Klöting N, Kunath A, Steinhoff K, Rijntjes E, Köhrle J, Zeisig V, Hankir M, Gebhardt C, Deuther-Conrad W, Heiker JT, Kralisch S, Stumvoll M, Blüher M, Sabri O, Hesse S, Brust P, Tönjes A, Krause K. Thyroid hormone status defines brown adipose tissue activity and browning of white adipose tissues in mice. Sci Rep. 2016 Dec 12;6:38124. doi: 10.1038/srep38124.

Wilcoxon, J.S., Nadolski, G.J., Samarut, J., Chassande, O., Redei, E.E.: Behavioral inhibition and impaired spatial learning and memory in hypothyroid mice lacking thyroid hormone receptor α. Behavioural Brain Research 177(1), 109–116 (2007). doi: 10.1016/j.bbr.2006.10.030.

Wise P.M. Aging of the Female Reproductive System. Handbook of the Biology of Aging, Sixth Edition, Chapter 21, 570-590.

Yao X, Hou S, Zhang D, Xia H, Wang YC, Jiang J, Yin H, Ying H. Regulation of fatty acid composition and lipid storage by thyroid hormone in mouse liver. Cell Biosci. 2014 Jul 30;4:38.

Yeager N., Klein-Szanto A., Kimura S., Di Cristofano A. 2007. Pten Loss in the Mouse Thyroid Causes Goiter and Follicular Adenomas: Insights into Thyroid Function and Cowden Disease Pathogenesis. Cancer Research 67 (3), 959–966.

Yen P.M. 2001. Physiological and Molecular Basis of Thyroid Hormone Action. Physiological reviews Vol. 81, No. 3, July 2001: 1097-1142.

Zhang A, Sieglaff DH, York JP, Suh JH, Ayers SD, Winnier GE, Kharitonenkov A, Pin C, Zhang P, Webb P, Xia X. Thyroid hormone receptor regulates most genes independently of fibroblast growth factor 21 in liver. J Endocrinol. 2015 Mar;224(3):289-301.

Zhang G, Zhang H, Deng S, Qin Y. Regional differences in taste bud distribution and α-gustducin expression patterns in the mouse fungiform papilla. Chem Senses. 2008;33:357-362.

Zoeller T.R., Tan W.S., Tyl R.W. General Background on the Hypothalamic-Pituitary-Thyroid (HPT) Axis. 2007. Critical Reviews in Toxicology Vol. 37, Iss. 1-2.

Chapter 7: Appendix

I Abbrevations and Acronyms

AITD	autoimmune thyroid disease
ATA	American Thyroid Association
BAT	brown adipose tissue
BT	body temperature
BW	body weight
CAPNS2	calpain, small subunit 2
ClO_4-	perchlorate
CYP7A1	cholesterol-7-αhydroxylase
DIO1	deiodinase 1
DIO2	deiodinase 2
DIO3	deiodinase 3
ECG	electrocardiography
ERK1/2	extracellular signal-regulated kinases 1/2
FT3	free triiodothyronine
FT4	free thyroxine
HPT	hypothalamic-pituitary-thyroid
i.p.	intraperitoneal injection
LAT1	L-type amino acid transporter 1
LAT2	L-type amino acid transporter 2
LT4	levothyroxine
MCT10	monocarboxylate transporter 10
MCT8	monocarboxylate transporter 8
ME1	malic enzyme
MMI	methimazole
NTCP	sodium-taurocholate co-transporting polypeptide
OATP1C1	organic anion transporter protein 1 C 1
PDE8	phosphodiesterase type 8b
Pi3K	phosphoinositide 3 kinase
PTU	propylthiouracil
qRT-PCR	quantitative real-time polymerase chain reaction
rT3	3,3´,5´-reverse triiodothyronine
RXR	retinoid x receptor
T2	3,3´-diiodothyronine
T3	3,3´,5´-triiodothyronine
T4	thyroxine
TBG	thyroxine binding globulin
TD	thyroid dysfunction
TGAb	thyroglobulin antibodies
TH	thyroid hormone
TPO	thyroid peroxidase
TPOAb	thyroid peroxidase antibodies
TR	thyroid hormone receptor
TRE	thyroid hormone responsive element
TRH	thyreotropin-releasing hormone
TSH	thyroid-stimulating hormone
TSHRAb	TSH receptor antibodies
TT4	total thyroxine
TTR	transthyretin
UCP1	uncoupling protein 1

II List of Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

III List of Tables

Chapter 1

Chapter 2

Chapter 3

Chapter 4

IV Bestätigung Betreuerin

Bestätigung:

Hiermit bestätige ich die Darstellung zu den Anteilen von Frau Rakov an Konzeption, Durchführung und Abfassung jeder Publikation (Chapter 2-5) gemäß der Promotionsordnung der Fakultät für Biologie zur Erlangung des Dr. rer. nat.

Essen, den _____ _____

Prof. Dr. Dr. Dagmar Führer-Sakel

V Eidesstattliche Erklärungen

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, g der Promotionsordnung der Fakultät für Biologie zur Erlangung der Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema „***Phenotypical characterization of sex-dependent traits in mice models of hyperthyroidism and hypothyroidism***“ zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von (***Helena Rakov***) befürworte.

Essen, den_____ _____

Prof. Dr. Dr. Dagmar Führer-Sakel

Erklärung:

Hiermit erkläre ich, gem. § 7 Abs. 2, d und f der Promotionsordnung der Fakultät für Biologie zur Erlangung des Dr. rer. nat., dass ich die vorliegende Dissertation selbständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient habe und alle wörtlich oder inhaltlich übernommenen Stellen als solche gekennzeichnet habe.

Essen, den _____ _____

Helena Rakov

Erklärung:

Hiermit erkläre ich, gem. § 7 Abs. 2, e und g der Promotionsordnung der Fakultät für Biologie zur Erlangung des Dr. rer. nat., dass ich keine anderen Promotionen bzw. Promotionsversuche in der Vergangenheit durchgeführt habe, dass diese Arbeit von keiner anderen Fakultät abgelehnt worden ist, und dass ich die Dissertation nur in diesem Verfahren einreiche.

Essen, den _____ _____

Helena Rakov

VI Curriculum Vitae

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

VII Acknowledgement

First, and foremost I would like to express my deepest gratitude to my supervisor Prof. Dr. Dr. Dagmar Führer-Sakel for giving me the opportunity to work at this extremely interesting topic. Your immense knowledge, enthusiasm and motivation has been inspiring for me and I deeply appreciate your continued support, encouragement and guidance throughout my PhD time. Thank you for always taking time for me, your trust, helpful ideas and our many productive and stimulating discussions.

I would also like to thank the Thyroid Trans Act (TTA) members Prof. Dr. Josef Köhrle (Charite, Berlin), PD Dr. Lars Möller and my TTA mentor Prof. Dr. Klaudia Brix (Jacobs University, Bremen) for constructive comments and suggestions on my project, which always helped me to improve my work.

A special thank goes to Denise, for your uncountable advices, for proofreading of many manuscripts, abstracts, my PhD thesis and an always great and enjoyable time at work and off work.

Furthermore, I would like to take the opportunity to thank each team member during my PhD journey who made my lab life so much easier. Kathrin, Sebastian, Julia, Judith, Eugi, Sören, Daniela, Janina, Steffi, Andrea, Julius, Kathrin and Sabrina. You all contributed to our great team spirit, helped me when I needed a *third hand* or when *plan A* didn´t worked. I particularly thank Kathrin, for being a great team partner and spending hours and hours at the *dark side* of IG1 with me for more than a year. Everything you did was with heart and mind. Thank you, Sebastian for your always appreciated feedback on my manuscripts, my thesis and our daily discussions as you always know how to solve a problem. Thanks to Julia and Judith for our great talks and laughs, Steffi for your incredible experimental knowledge, I can´t remember you ever saying „No, I never did it before“. Thank you everyone for your support, helpful advices and a great time! Thanks to the whole ZTL team, for answering me every question about mice and mouse experiments and a good care of our animals.

I am infinitely grateful to my parents, who travelled thousands of kilometers to make it possible for me to be here and do what I love. Who always believe in me, support me and encourage me to reach my goals. Thank you to my brother and his little family

for being my sunshine and brightening my life. Lastly, I would like to thank my best friend and my second half. Thank you for being by my side for so many years and sharing my life, for enduring my moods and making me always laugh.