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Sex-Dependent Claudin-1 Expression in the Liver of Euthyroid and Hypothyroid Mice

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Key Words
Claudin-1 · Liver · Sex-dependent expression · Hypothyroid · Biliary disease

Abstract
Background: In the liver the tight junction protein claudin-1 plays an important role in bile secretion by maintaining the paracellular barrier of bile canaliculi and the bile duct. A diminished bile excretion has been found in hypothyroid patients, and the prevalence of gallstones is increased in hypothyroidism. This association, however, only applies for men and is in contrast to the well-established female preponderance of biliary disease in the general population. ***Objectives:*** We hypothesized that hypothyroidism could lead to altered claudin-1 expression in the liver, and that this effect may be sex specific. ***Methods:*** We characterized claudin-1 expression and localization in livers of euthyroid and hypothyroid male and female C57BL/6NTac mice by real-time PCR, Western blot and immunofluorescence. ***Results:*** Claudin-1 is expressed in canalicular regions and the bile ducts of the murine liver. Livers of female mice showed lower claudin-1 expression than male livers. In hypothyroid livers, female animals showed an elevated claudin-1 expression, whereas reduced claudin-1 expression was found in male animals compared to the euthyroid controls. ***Conclusion:*** We demonstrate a correlation between claudin-1 expression and hypothyroidism in the murine liver. Furthermore, a sex-dependent alteration of claudin-1 expression was found.

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 (fT_4) 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 of developing hypothyroidism in the Western world ranges between 0.6

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and 3.5 per 1,000 people [3]. In Europe women are 1.7- to 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 the polarization of hepatocytes by the 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 humans can be associated with altered claudin-1 expression or mutations. For instance, in the neonatal ichthyosis-sclerosing cholangitis 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, TH receptor (THR)α1-/THRβ-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 been studied.

In this study, we investigated 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 a euthyroid and hypothyroid serum TH status to analyze claudin-1 expression and localization patterns in the liver.

Materials and Methods

Animals

Eighteen-week-old male and female (n = 32) C57BL/6NTac mice (Taconic Europe, Ry, 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 (GVSO-LAS) 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 Murine Blood Samples and Livers

To establish a hypothyroid state, animals were fed a low-iodine diet (Harlan Laboratories, Madison, Wis., USA) with an addition of 0.02% methimazole, 0.5% perchlorate and 3 g/l saccharin (Sigma-Aldrich, Munich, Germany) to drinking water for 7 weeks (n = 8 animals/sex). Control groups received a control diet (Harlan Laboratories) and regular water over the whole treatment period (n = 8 animals/sex). The noncaloric 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 10,000 *g*. Serum aliquots were stored at –80°C. Total thyroxine (TT_4) concentrations in the serum of mice were measured using ELISA kits according to the manufacturer's instructions (DRG Instruments GmbH, Marburg, Germany). Hypothyroidism in the mice was defined by decreased serum TT_4 concentrations (detection limit 0.5 µg/dl).

Mice were euthanized and perfused with heparinized saline through a needle placed in the left ventricle. Livers were then 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, Hilden, Germany) by 4,000 rpm and further treated with proteinase K (Qiagen) based on the manufacturer's protocol. Total RNA from tissue lysates was purified by RNeasy mini kit (Qiagen) and on-column DNase digestion using an RNase-Free DNase Set (Qiagen). RNA quantity and quality were determined by NanoDrop 1000 (Thermo Scientific). Two micrograms of RNA was reverse transcribed to cDNA using random hexamers and the SuperScript III First-Strand Synthesis System for RT-PCR according to instruction manuals (Life Technologies, Darmstadt, 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, Ebersberg, 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 the LightCycler® DNA Master SYBR Green I and the LightCycler® 480 System (Roche, Mannheim, Germany). The PCR program consisted of an initial denaturation step (5 min at 95°C) and 40 amplification cycles with 15 s at 95°C, 10 s at 60°C and 20 s at 72°C. For the 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. The 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 pairwise 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 pairwise 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, Carlsbad, Calif., USA), anti-GAPDH (1:1,000; Cell Signaling, Danvers, Mass., USA), anti-cytokeratin 19 (1:1,000; Abcam, Cambridge, Mass., USA), anti-β-actin (1:2,000; Cell Signaling), anti-mouse/anti-rabbit-IgG (1:2,000, Cell Signaling) and anti-mouse IgG DyLight 488 conjugate (1:15,000; Life Technologies, Thermo Fisher, Waltham, Mass., USA). Whole protein lysates were extracted by RIPA-buffer (150 mM sodium chloride, 50 mM Tris pH 8.0, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 2 mM ethylenediaminetetraacetate, 50 mM sodium fluoride, protease inhibitor; Sigma-Aldrich). Extracted proteins were quantified by BCA protein assay (Pierce, Rockford, Ill., USA). The 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 (Bio-Rad, Hercules, Calif., USA). Unspecific binding sites were blocked with blocking buffer (5% milk powder in phosphate-buffered saline, PBS; Sigma-Aldrich) for 1 h at room temperature. Claudin-1, GAPDH, cytokeratin 19 and β-actin were detected by overnight incubation of the respective antibody at 4°C in blocking buffer. GAPDH and β-actin were used as internal protein loading controls. Both protein loading controls revealed a stable protein loading, although 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 1 h. Visualization was done by luminescence using the Immun-Star™ WesternC™ Kit (Bio-Rad) as well as fluorescence (VersaDoc System; Bio-Rad). Differences in protein expression levels were quantified by densitometry using the Image Lab™ Software (Bio-Rad). 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), AlexaFluor® 555 phalloidin (1:40; Invitrogen) and AlexaFluor® 488 (1:250; Invitrogen) secondary antibody. Cryo-liver tissues were fixed in 4% paraformaldehyde (Sigma-Aldrich) for 15 min at room temperature and permeabilized using 0.1% Triton X-100 (Sigma-Aldrich) in PBS for 10 min at room temperature. The preincubation in blocking solution containing 3% bovine serum albumin (BSA) and 0.3% Triton X-100 in PBS was performed at room temperature for 30 min. Cryo-liver tissues were incubated with primary antibodies overnight at 4°C in 1% BSA/PBS. Secondary antibody AlexaFluor® 488 was incubated for 1 h at room temperature in 1% BSA/PBS. The F-actin cytoskeleton was visualized by incubation of AlexaFluor® 555 phalloidin for 20 min at room temperature. The cell nuclei were stained by incubation of Hoechst 33342 (1:1,000; Invitrogen) for 5 min at room temperature. Cover slides were embedded by Immu-Mount (Thermo Fisher Scientific) and viewed on the confocal microscope Zeiss ELYRA PS.1 LSM710.

Statistical Analysis

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

Results

To determine the successful induction of hypothyroidism in C57BL/6NTac mice, TT_4 serum concentrations were determined by ELISA measurement (fig. 1a). Induction of hypothyroidism resulted in a significant decrease in TT_4 serum concentration in male (1.20 ± 0.56 vs. 2.86 ± 0.21 μg/dl) and female mice (0.35 ± 0.33 vs. 3.01 ± 0.77 μg/dl). TT_4 serum concentrations of female hypothyroid mice were below the detection limit of the applied assay (0.5 μg/dl).

To ensure a hypothyroid state in murine 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

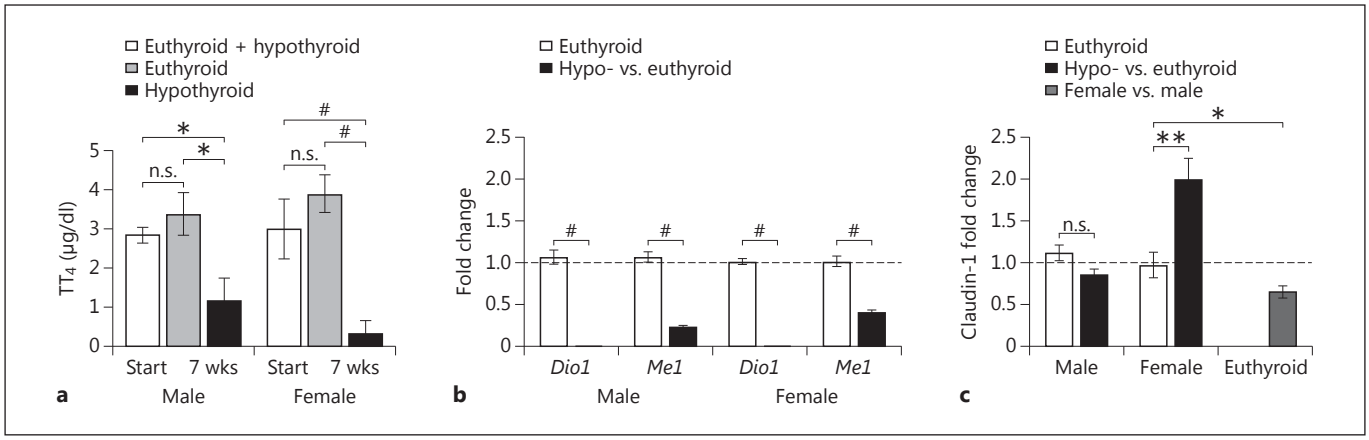

Fig. 1. Serum concentration of TT_4 in male and female euthyroid and hypothyroid mice. Fold changes of *Dio1*, *Me1* and claudin-1 mRNA expression in livers of euthyroid and hypothyroid male and female mice by quantitative RT-PCR. **a** The TT_4 serum concentration is significantly lower in male mice after induction of hypothyroidism than in euthyroid male control mice. The TT_4 serum concentration of hypothyroid female mice is significantly lower as compared to the euthyroid female control group. TT_4 was determined in murine serum by ELISA at the start and end of 7 weeks (wks) of treatment. The assay detection limit was 0.5 µg/dl. Data are represented as the mean ± SEM; n = 16 for start, n = 8 for end groups, 2-way ANOVA. **b** *Dio1* and *Me1* mRNA levels are significantly decreased in hypothyroid mice as compared to euthyroid mice for both sexes. **c** The claudin-1 mRNA level is not altered in hypothyroid male mice as compared to euthyroid male mice. The claudin-1 mRNA level is significantly upregulated in female mice under hypothyroidism. The 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* and *Rpl13a* were used as reference genes. Data are represented as the mean ± SEM, n = 8, efficiency-corrected ΔΔCt method, 2-way ANOVA. * $p < 0.05$, ** $p < 0.01$, # $p < 0.0001$. n.s. = Not significant.

mRNA levels also revealed 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).

The claudin-1 mRNA level was investigated in livers of male and female C57BL/6NTac mice with normal thyroid function and under chronic hypothyroidism (fig. 1c). Claudin-1 mRNA levels were 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 levels 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 females (0.66 ± 0.07 vs. 0.97 ± 0.15).

To investigate whether 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. 2). 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 levels. 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 livers of male and female mice with normal thyroid function and under hypothyroidism were observed.

To determine the localization pattern of claudin-1, immunofluorescence analysis of euthyroid and hypothyroid livers of mice 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 the epithelial cells of bile ducts. In addition, a homogeneous decrease of the claudin-1 protein level was observed in livers of hypothyroid male mice versus euthyroid male controls (fig. 3a, b). In hypothyroid female mice, liver claudin-1 levels increased mainly in bile duct regions as compared to euthyroid female controls (fig. 3c, d).

Discussion

Here we have shown sex-dependent liver expression of the TJ protein claudin-1 and distinct alterations of claudin-1 expression by induction of hypothyroidism in liv-

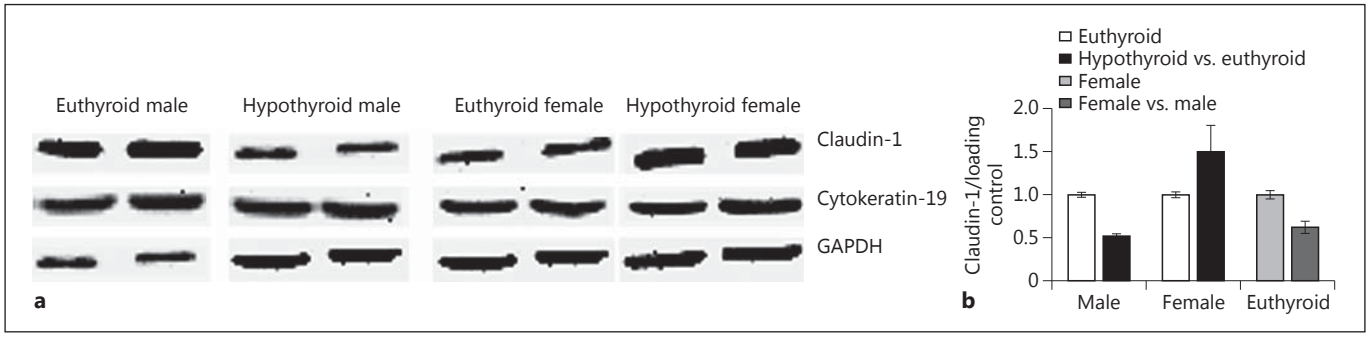

Fig. 2. Claudin-1 protein expression and quantification in livers of euthyroid and hypothyroid male and female mice. Whole protein lysates were used. Lower claudin-1 (18-kDa) protein levels were measured in livers of hypothyroid male mice than those of euthyroid male mice. Elevated claudin-1 protein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

ers of male and female mice. In the euthyroid mouse liver, claudin-1 is more highly expressed in males than females and seems to be localized mainly in canalicular regions of the 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 the different methods used to detect claudin-1 expression and/or the investigated C57BL/6 substrain.

When comparing hypothyroid to euthyroid murine livers, we found differences in claudin-1 expression between both sexes. In male mouse livers, induction of hypothyroidism decreased claudin-1 protein, whereas in females an increase in claudin-1 expression was detected. The observed changes in total T_4 serum concentrations of mice may suggest a more hypothyroid state in female compared to male mice, which could impact claudin-1 expression in the liver. However, if female mice were more hypothyroid than male mice, one would expect a more pronounced decrease in claudin-1 in livers of female compared to male mice. Furthermore, changes in TH-responsive gene levels in murine livers suggest a comparable hypothyroid liver state in male and female animals.

Hypothyroidism can lead to a disturbed bile transporting system in the rat liver [23]. A reduced bile excretion in the 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, whether or not claudin-1 is TH-dependently regulated by THRβ, which is the most prevalent THR in liver [24], needs to be investigated in further studies.

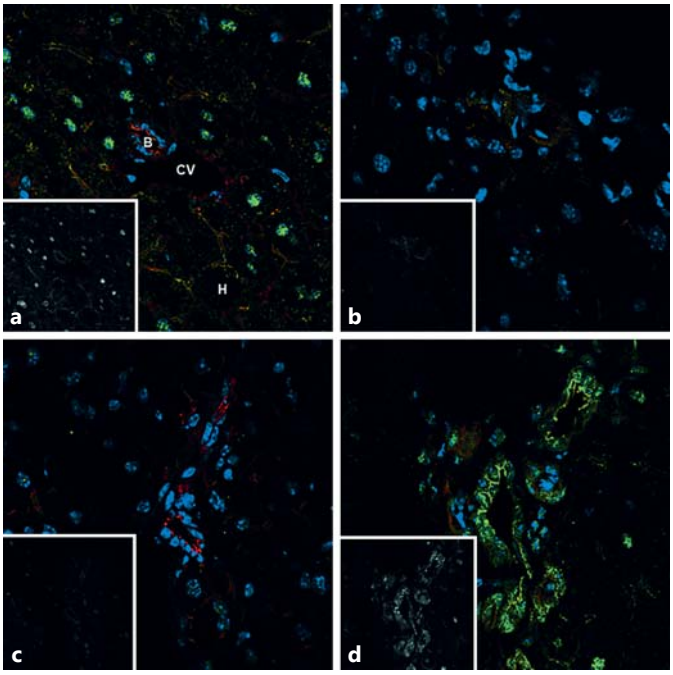

Fig. 3. Immunofluorescence of claudin-1 in livers of euthyroid and hypothyroid male and female mice. **a** An euthyroid male liver with claudin-1 (green) staining in the canalicular region of the liver and the bile duct. CV = Central vein; B = bile duct; H = hepatocytes. **b** Hypothyroid male liver expresses lower claudin-1 levels than euthyroid male liver. **c** Euthyroid female liver shows low claudin-1 levels. **d** Hypothyroid female liver with distinct claudin-1 expression in cholangiocytes and/or epithelial cells of the bile duct. Gray scale insets only show the claudin-1 channel. The F-actin cytoskeleton was stained by AlexaFluor® 555 phalloidin (red). Nuclei were stained by Hoechst 33342 (cyan). Original magnification ×63. The images were viewed with the confocal microscope Zeiss ELYRA PS.1 LSM710. Representative examples are shown (n = 4).

Hypothyroidism could indirectly affect claudin-1 expression in the 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 and -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 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 the 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 livers 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 die 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 influence TJ expression in a concentration-dependent manner in human vascular endothelial and human endometrial epithelial cells [6, 29]. However, animal studies concentrated on TJ proteins, like occludin or ZO-1, and data of claudin-1 are almost entirely missing [3]. It is thought that claudin-1 could be regulated via different signal transduction pathways like MAPkinase or PI3kinase [9]; however, the data are not consistent and it will be interesting to consider these pathways in the context of nongenomic TH action.

Since we found sex-dependent differences in claudin-1 liver expression with different thyroid states, a possible interaction between estradiol and TH may be involved. These seems more likely when it is considered that both receptors – THRs and estrogen – 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 the environmental effect on estrogen-controlled reproductive behavior [30]. Recently, an overlapping effect of TH and estradiol on glutathione *S*-transferase-α gene expression in murine kidneys has been observed [31]. Whether claudin-1 expression is also influenced in an estradiol-dependent manner can be investigated by using, for example, an ovariectomized mouse model.

Other TJ proteins, such as occludin, claudin-2 and claudin-3, are also expressed in the liver [10]. Whether or not these TJ proteins are equally affected by TH has to be determined in further studies.

Claudin-1 mutations have been associated with distinct human biliary diseases [11–13]. It is widely accepted that women are more susceptible to developing common bile duct stones than men [32]. It could be speculated that low claudin-1 expression in livers of euthyroid female mice could contribute to the formation of biliary diseases due to a less-sufficient paracellular barrier. Otherwise, there seems to be a sex-dependent association between hypothyroidism and an increased prevalence of gallstone diseases in men [8]. Our findings of a diminished hepatic claudin-1 expression in hypothyroid male mice and the 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 need to be investigated in further studies. In summary, our study shows that expression of the TJ protein claudin-1 is altered by hypothyroidism and differs between sexes in a mouse model.

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Disclosure Statement

The authors claim no conflict of interest.

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