A Microfluidic Thyroid-Liver Platform to Assess Chemical Safety in Humans

Julia Kühnlenz1,2,1, Diana Karwelat1, Thomas Steger-Hartmann1, Marian Raschke1, Sophie Bauer2, Özlem Vural2,3, Uwe Marx2, Helen Tinwell1 and Remi Bars2

Abstract

Thyroid hormones (THs) are crucial regulators of human metabolism and early development. During the safety assessment of plant protection products, the human relevance of chemically induced TH perturbations observed in test animals remains uncertain. European regulatory authorities request follow-up in vitro studies to elucidate human-relevant interferences of thyroid gland function, or TH catabolism through hepatic enzyme induction. However, human in vitro assays, based on single molecular initiating events, poorly reflect the complex TH biology and related liver-thyroid axis. To address this complexity, we present human three-dimensional thyroid and liver organoids with key functions of TH metabolism. The thyroid model resembled in vivo-like follicular architecture and a TSH-dependent triiodothyronine synthesis over 21 days which was inhibited by methimazole. The HepaRG-based liver model, secreting critical TH-binding proteins albumin and thyroxine-binding globulin (TBG), emulated an active TH catabolism via the formation of glucuronidated and sulfated thyroxine (gT4/sT4). Activation of the nuclear receptors PXR and AHR was demonstrated via the induction of specific CYP isoenzymes by rifampicin, pregnenolone-16a-carbonitile and β-naphthoflavone. However, this nuclear receptor activation, assumed to regulate UDP-glucuronosyltransferases and sulfotransferases, appeared to have no effect on gT4 and sT4 formation in this human-derived hepatic cell line model. Finally established single-tissue models were successfully co-cultured in a perfused two-organ chip for 21 days. In conclusion, this model presents a first step towards a complex multimodal human platform, which will help to identify both direct and indirect thyroid disruptors that are relevant from a human safety perspective.

1 Introduction

Thyroid hormones (THs) are fundamental for metabolic equilibrium in mammals and the growth and cognitive development of their offspring (Bassett & Williams, 2003; Bernal, 2007; Fukuchi et al., 2002; Sinha et al., 2018; Vargas-Uricoechea et al., 2014). As impairments of the hypothalamic–pituitary–thyroid (HPT) axis may cause severe adverse effects (Patel et al., 2011; Willoughby et al., 2013), the evaluation of the effects of chemicals on this axis has gained increasing importance over the last decades. In retrospect, several chemicals, that have been in use for many years, were identified as potential disruptors of the HPT axis (Crivelente et al., 2019; Leemans et al., 2019). To prevent the marketing of such substances in the future and to increase the safety of novel substances, continuing effort of the regulatory authorities is made to provide scientific guidance for the identification of endocrine disruptors. In this respect, the European Chemicals Agency (ECHA) and European Food Safety Authority (EFSA) published a guidance document in 2018. According to this guidance, rodent studies play a central role in the identification of endocrine disruption by plant protection products (PPP). If endocrine-related effects such as histopathologic changes of the thyroid gland are observed, with or without TH changes, mechanistic follow-up studies are required to identify the precise mode of action (MoA) in the target species (ECHA and EFSA et al., 2018). Unless proven otherwise, the MoAs identified for the rodent are considered as being relevant for humans too, which will lead to significant market restrictions for the compound under investigation (European Chemical Agency (ECHA and EFSA et al., 2018). Therefore, mechanistic follow-up studies are necessary to examine if the identified MoA observed in rodents also applies to humans (ECHA and EFSA et al., 2018).

Especially in the context of thyroid perturbations, concerns arose to what extent rodents truly reflect the response in humans (Kortenkamp et al., 2017). For both species, the synthesis of the THs thyroxine (T4) and the biologically active form 3,3′,5-triiodothyronine (T3) are controlled in a complex feedback mechanism within the HPT axis (Abel et al., 2001; Dumont, 1971; Nikrodnahon et al., 2006). In addition, the liver contributes to a balanced TH homeostasis by metabolizing THs either by deiodination, mainly via the type I deiodinase (DIO1), or by its inactivation via the phase II enzymes uridine diphosphatase glucuronosyltransferases (UGTs) and sulfotransferases (SULTs) (van der Spek et al., 2017). These enzymes transform T3 and T4 into their glucuronide and sulfate conjugates (gT4 and sT4, gT3 and sT3) which can be subsequently excreted via the bile (van der Spek et al., 2017). Even though these mechanisms apply to both rodents and humans, there is evidence that rodents

Received August 26, 2021; Accepted April 13, 2022; Epub May 9, 2022; © The Authors, 2022.

ALTEX 39(3), ###-###. doi:10.14573/altex.2108261

Correspondence: Julia Kühnlenz, Dr. Ing., Bayer SASS
16 Rue Jean-Marie Leclair
69009 Lyon, France
(julia.kuehnlenz1@bayer.com)
have an increased sensitivity to impairments in TH homeostasis. Humans have a slower TH turnover with increased half-lives of T4 and T3. Most notably, there are marked species differences in TH binding affinities to the TH carrier proteins (albumin, TH-binding globulin (TBG) and transthyretin (TTR)) (Bartsch et al., 2018; Foster et al., 2021; Meek et al., 2003). The liver-secreted carrier proteins present one main storage site of the THs and bind up to 99.5 % of the THs found in the human blood serum (Kortenkamp et al., 2017). TBG, being with 20 mg l$^{-1}$ blood serum much less abundant than albumin (150 mg l$^{-1}$ blood serum), contributes with 75 % the most to the total bound T3 and T4 fraction and thus is crucial for the maintenance of the TH levels (Kortenkamp et al., 2017, Hotari et al., 1987; Geoffrey et al., 2019). Variations in TH concentrations, in particular TBG, are associated with changes in total TH serum levels without strikingly altering the euthyroid state (Bartalena, L. & Robbins 1992, Domingues et al., 2009). Furthermore, altered TH levels were associated to interactions between TH carrier proteins and xenobiotics resulting in TH displacement (Hallgren, S. & Darnerud 2002, Marchesini, G. R. et al. 2008). As a consequence, abnormalities in TH carrier protein levels or chemical-induced TH replacements are considered as putative molecular initiating events leading to TH-related neurodevelopmental toxicity (AOP 152) (Noyes et al., 2019). To our knowledge, few human hepatic in vitro models have been characterized with regard to their TBG and TTR secretion ability, meaning their capability to investigate related adverse outcome pathways (AOP) is unknown. In addition to the differential TH binding capacities between humans and rodents, differences in the TH catabolism pathways are apparent with rodents showing a more active hepatic TH degradation via the UGT enzymes (Bartsch et al., 2018; Richardson et al., 2014).

The mechanisms of TH perturbation were recently summarized in an AOP network, where a total of 26 molecular initiating events (MIEs), which may be implicated in thyroid hormone disruption and subsequent downstream adverse effects, are listed (Noyes et al., 2019). Several in vitro high-throughput assays exist that investigate the main MIEs for chemically induced thyroid activity, which either involve the thyroid gland directly (direct thyroid toxicity) or target TH catabolism through the induction of enzymatic activity (indirect thyroid toxicity) (Noyes et al., 2019). While these assays are useful for early-stage development of new compounds, they are limited to the evaluation of molecular and biochemical interactions between cellular targets and the compound of interest while neglecting the physiological environment and functionality of target tissue. To better assess whether the identified MoA in rodents also applies to humans, new in vitro test methods with human cells or tissues which more accurately emulate the TH homeostasis are needed to directly evaluate the physiological effect of xenobiotics. Microphysiological systems with their properties of flow-through and integration of different tissue models have the potential to improve such an in vitro assessment. We therefore investigated a novel two-organ combination of human thyroid and liver organoids which simulates both TH biosynthesis and hepatic TH catabolism.

TH biosynthesis in the thyroid gland is one of the main target sites of xenobiotics (DeVito et al., 1999). Given the structure-function relation of thyroid follicles, which are the smallest functional subunit of the thyroid gland, the follicular arrangement and correct cell polarization of thyrocytes is key to model TH synthesis in vitro. Whereas in the interior of the follicles (the colloid) thyroglobulin (TG) (the precursor molecule of the THs) is enriched, cell-pole-specifically expressed transport proteins generate an intra-follicular reservoir of iodine (Goodman, 2009). During the TH synthesis, iodine is oxidized and coupled to the tyrosine residues of TG forming the TH precursors of T3 and T4 (Mondal et al., 2016). To preserve the native cell polarity and maintain thyroid-specific functionalities, extracellular matrix (ECM)-based three-dimensional (3D) thyrocyte cultures are required to remodel intra-follicular enrichment of iodine and TG, iodination of TG, thyroid-stimulating hormone (TSH) responsiveness and TH secretion (Chambard et al., 1981; Deisenroth et al., 2020; Garbi et al., 1986; Kraiem et al., 1991; Kusunoki et al., 2001; Massart et al., 1988; Nishida et al., 1993; Saito et al., 2018; Sasaki et al., 1991; Toda et al., 1992; Toda et al., 2011). Besides one recently published thyroid 3D model generated from primary human thyrocytes (Deisenroth et al., 2020), no other standardized models currently exist to study thyroid-relevant MoAs in humans in a physiologically relevant manner. For this reason, we developed an in vitro thyroid model that emulates the TH biosynthesis and its chemically induced impairment leading to changes in TH secretion.

Besides TH biosynthesis, a number of xenobiotics indirectly affect the thyroid gland by targeting hepatic TH catabolism via activation of xenobiotic nuclear receptors and thus associated induced UGT activity for T4 (T4-UGT) in rodents (Hood et al., 2003; Meek et al., 2003; Rouquié et al., 2014). According to recent data collections, 60 out of 128 small molecules tested were considered to provoke TH perturbations via liver enzyme inductions (Crivellente et al., 2019). However, little is known about the correlation between increased T4-UGT activity and thyroid adverse effects in humans. In this context, new human-relevant in vitro assays are required, which do not only study hepatic nuclear receptor activations as putative MIEs, but directly evaluate changes in the gT4 and sT4 catabolite formation. Apart from a two-dimensional (2D) human sandwich-cultured hepatocyte model (Richardson et al., 2014), to our knowledge there are limited published assays addressing this in detail. In recent years, major efforts in 3D culture techniques have been directed towards the formation of 3D liver spheroids whose cell-cell interactions and native metabolic zonation reinforce the hepatic phenotype while increasing the liver-like functionality compared to 2D monolayers (Cox et al., 2020; Langan et al., 2016; Lauschte et al., 2016; Underhill & Khetani, 2018). Overcoming the limited life-span of 2D cultures, the 3D structure prevents the dedifferentiation of hepatocytes and thus allows long-term cultures up to 5 weeks with maintained hepatocyte-specific functions (Bell et al., 2016; Elaut et al., 2006; Guinness et al., 2013; Kanebratt et al., 2021; Leite et al., 2012; Mandon et al., 2019; Ramaiyahari et al., 2017; Zellmer et al., 2010). Here, we extensively characterize a 3D liver spheroids model composed of the HepaRG cell line and structure-supporting human hepatic stellate cells (HSteC) (Abu-Absi et al., 2004). According to several reports, the HepaRG cell line was considered as an appropriate alternative to primary human hepatocytes (PHH) with good morphologic and metabolic similarities (Animat et al., 2006; Hockstra et al., 2013; Huaman et al., 2012; Kammerer & Küpper, 2018; Lübbertstedt et al., 2011a; Tascher et al., 2019) and a high relevance to assess drug safety (Hendriks et al., 2016; Li et al., 2019; Tomida et al., 2015; Z.-Y. Wang et al., 2019). Furthermore, there is evidence that HepaRG cells are able to produce glucuroninated and sulfated metabolites in vitro (Darnell et al., 2012; Richter et al., 2016; Yokoyama et al., 2018) making these cells suitable to study the TH catabolite formation. As demonstrated in this study, HepaRG/HSteC liver spheroids model were capable of reproducing TH catabolism by synthesizing gT4 and sT4.

Given the complexity of the TH homeostasis, which commonly is affected by both direct and indirect impairments of the thyroid gland (Figure 1), the final aim of the study was the functional co-cultivation of the pre-characterized thyroid and liver organ models in a commercially available multi-organ chip (MOC) platform produced under ISO 9001-2015 standards.
Fig. 1: Hepatic-thyroid axis and its implementation in vitro
(A) Feedback mechanism of thyroid hormone synthesis through the liver and anterior pituitary gland and its chemically induced disruption. Black elements represent the native state whereas red ones indicate the perturbation. (TPO - thyroid peroxidase, NIS - sodium/iodide symporter, TSH - thyroid-stimulating hormone, TSHR - TSH receptor, T4 - triiodothyronine, T3 - thyroxine, gT4 - glucuronidated T4, sT4 - sulfated T4) (B) Presentation of a 2-organ-chip containing two identical culture circulations. Left circulation presents a plan view showing the bottom areas of the outer [A] and inner [B] culture compartment which are interconnected by microchannels [C]. Three pump membranes integrated in the channel system [D], which are moved up and down by microtube[E]-delivered pressure/vacuum, enable the medium perfusion of the culture system. The right circulation shows the cross-section view of the outer [a] and inner [b] culture compartment including in pink the level of the culture medium, the screwed-in culture inserts and lids. (C) Schematic representation of the in vitro simulated hepatic-thyroid axis and the systemic application of TSH representing the pituitary. Depicted is a cross-section of one circulation of the 2-organ-chip platform showing thyroid and liver models in separate culture compartments.

2 Materials & methods

Cell cultures were handled under a sterile safety cabinet according to the guidance on good cell culture practice (Coecke et al., 2005). Their maintenance took place at 37 °C and 5 % CO₂ in a humidified incubator.

Cell culture medium formulations

Storage medium for freshly dissected thyroid tissue consisted of William’s E medium (PAN Biotech) supplemented with 0.25 µg/ml amphotericin (Corning) and 5 µg/ml gentamycin (Corning). The co-culture medium used for thyroid and liver single organ cultures and the optimized co-culture approach is based on SILAC Advanced DMEM/F-12 Flex Media no glucose/no phenol red (Thermo Fisher) which additionally contained 2 mM L-glutamine (Corning), 0.1 µM sodium iodine (SIGMA), 1 % MEM Amino Acids - 50x Solution [-] L-glutamine (Corning), 0.1 µM dexamethasone (SIGMA), 5 µg/ml gentamycin, 250 ng/ml amphotericin. Prior to the spheroid formation, HepaRG cells were maintained in William’s E Medium w/o: L-glutamine w/o phenol red, 10 % FBS, 2 mM L-glutamine, 50 µM hydrocortisone 21-hemisuccinate sodium salt (VWR), 5 mg/l insulin (PAN Biotech), 5 µg/ml gentamycin, 250 ng/ml amphotericin) and 2 % DMSO, to avoid dedifferentiation of the cells. HSteC were pre-cultured in optimized medium from ScienCell (SC basal medium, 1 % SC supplements, 2 % FBS, 1 % penicillin-streptomycin (PS)). Spheroid formation of HepaRG cells and HSteC took place in William’s E Medium w/o: L-glutamine w/o phenol red, 10 % FBS, 2 mM L-glutamine, 50 µM hydrocortisone 21-hemisuccinate sodium salt, 5 mg/l insulin, 5 µg/ml gentamycin, 250 ng/ml amphotericin and 10 % FBS.

Thyroid model generation

Procedure is visualized in Fig. S1. Human thyroid tissue was obtained with informed consent from patients with unknown health state, but negatively tested for Human Immunodeficiency Virus (HIV), Hepatitis B Virus (HBV) and hepatitis C. Donor gender, age and smoker status are given in Table 1. After the removal of the thyroid explant, the tissue was collected in its storage medium and kept at 4 °C. Follicle isolation was performed within 12 h. Thyroid explant was washed 2 x with PBS (Corning), rinsed with 80 % ethanol (VWR) and rehydrated in PBS. Pathologically normal tissue was chopped by a curved

Abbreviations

2D two-dimensional, 3D three-dimensional, AHR aryl hydrocarbon receptor, AOP adverse outcome pathway, ATP adenosine triphosphate, BNF β-naphthoflavone, CAMP cyclic adenosine monophosphate, CYP cytochrome P450, DIO1 type 1 deiodinase, ECHA European Chemicals Agency, ECM extracellular matrix, EFSA European Food Safety Authority, FBS fetal bovine serum, GFR growth factor-reduced, gT4 T4 glucuronide, HPT hypothalamic–pituitary–thyroid, HSteC human hepatic stellate cells, MCT8 monocarboxylate transporter 8, MIE molecular initiating event, MMI methimazole, MoA mode of action, MOC multi-organ chip, MRP2 multidrug resistance-associated protein 2, NAM new approach methodology, PCN pregnenolone-16α-carbonitrile, P450 primary human hepatocytes, PPP plant protection products, PS penicillin-streptomycin, PXR pregnane X receptor, RIF rifampicin, RT room temperature, sT4 T4 sulfate, SULT sulfotransferase, T3 3,3',5'-triiodothyronine, T4 thyroxine, T4-UGT UGT activity for T4, TBG thyroxine-binding protein, TH thyroid hormone, TSH thyroid-stimulating hormone, TTR transthyretin, UGT uridine diphosphate glucuronosyltransferase, ULA ultra-low attachment, ZO1 tight junction protein-1.

doi:10.14573/altex.2108261s
Tab. 1: Thyroid donor specifications of the 13 human donors investigated during this study

<table>
<thead>
<tr>
<th>Thyroid donor</th>
<th>Gender</th>
<th>Age</th>
<th>Smoker</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>unknown</td>
<td>46</td>
<td>no</td>
</tr>
<tr>
<td>2</td>
<td>female</td>
<td>47</td>
<td>no</td>
</tr>
<tr>
<td>3</td>
<td>female</td>
<td>53</td>
<td>yes</td>
</tr>
<tr>
<td>4</td>
<td>female</td>
<td>79</td>
<td>no</td>
</tr>
<tr>
<td>5</td>
<td>female</td>
<td>62</td>
<td>no</td>
</tr>
<tr>
<td>6</td>
<td>female</td>
<td>45</td>
<td>yes</td>
</tr>
<tr>
<td>7</td>
<td>female</td>
<td>50</td>
<td>unknown</td>
</tr>
<tr>
<td>8</td>
<td>female</td>
<td>46</td>
<td>unknown</td>
</tr>
<tr>
<td>9</td>
<td>female</td>
<td>79</td>
<td>no</td>
</tr>
<tr>
<td>10</td>
<td>male</td>
<td>56</td>
<td>unknown</td>
</tr>
<tr>
<td>11</td>
<td>male</td>
<td>50</td>
<td>unknown</td>
</tr>
<tr>
<td>12</td>
<td>female</td>
<td>31</td>
<td>no</td>
</tr>
<tr>
<td>13</td>
<td>female</td>
<td>53</td>
<td>no</td>
</tr>
</tbody>
</table>

Thyroid follicle cultures were maintained for 29 days. Culture supernatants were completely removed every 2 to 3 days and replaced by 400 µl co-culture medium which had freshly been supplemented with 0.1, 1 or 10 mIU/ml TSH (Thyroid-stimulating Hormone, Bovine Pituitary, Creative Biomart). For the chip-based culture 200 ul medium were added per culture compartment. Collected supernatants were analyzed for TSH secretion by LC-MS/MS analysis. Difference in overall T3 secretion between 0.1 and 1 mIU/ml TSH treatment groups was evaluated by an unpaired t-test of log-transformed T3 concentrations over the whole time. Each group contained three intra-donor replicates. At the end of culture, thyroid follicle models were fixed for immunostaining.

**Cyclic adenosine monophosphate (cAMP) measurement**

96-well plate-based thyroid follicle cultures from three or two independent donors divided in seven TSH exposure groups (0 [n = 3], 0.1 [n = 3], 1 [n = 3], 5 [n = 2], 10 [n = 3], 50 [n = 2] and 100 [n = 2] mIU/ml which each included technical intra-donor triplicates) were initially cultured for 7 days without TSH. On day of cAMP analysis, thyroid follicles were treated with respective TSH concentration or 10 µM forskolin (positive control, [n = 3], Cayman Chemical Company) diluted in co-culture medium, containing 500 µM 3-Isobutyl-1-methylxanthine (IBMX, SIGMA) and 100 µM imidazolidinone (SIGMA), both inhibiting cAMP hydrolysis, for 1 h (160 µl/well). Intracellular cAMP content was measured by cAMP-Glo™ Assay (Promega); cell lysis was induced by replacing the culture medium by 20 µl lysis buffer and constant orbital agitation (700 rpm) for 30 min at RT. Lysate was thoroughly mixed with 40 µl cAMP Glo-detection reagent by orbital shaking at 700 rpm for 1 min and incubated at RT for additional 20 min. 80 µl kinase-Glo reagent was added, thoroughly mixed and 120 µl transferred to a clear-bottom white polystyrene microplate. Luminescence was measured immediately. Blank-corrected raw data were log-transformed in order to achieve Gaussian distribution according to Shapiro-Wilk test. Statistical analysis between TSH-treated
and non-treated conditions was performed by one-way ANOVA using Dunnett’s post hoc test and intra-donor matching for 0.1/1/10 mIU/ml TSH and forskolin-treated models as here three independent donors were tested. Geisser-Greenhouse correction was included into statistical analysis as no equal variance between test conditions existed. The treatment groups 5/50/100 mIU/ml TSH were not statistically analyzed as data from only two independent human donors were collected.

**Methimazole ( MMI) treatment**

Thyroid follicle models of 4 independent human donors containing two or three intra-donor replicates, were pre-cultured with 1 mIU/ml TSH for 3 days in 48-well plates. MMI (SIGMA), a reference TPO-inhibitor, was dissolved in DMSO (VWR) and subsequently in co-culture medium to a final DMSO concentration of 0.1 % in a step-wise dilution series of 10, 1, 0.1 and 0 µM MMI. Thyroid models were each treated with 400 µl of the respective MMI dilution for 2 days, subsequently the MMI-dosing was renewed and lasted for additional 2 days to achieve a 4-day treatment in total. Collected culture supernatants were analyzed by LC-MS/MS analysis for T3 formation. T3 concentrations of each donor and condition were normalized [%] to mean T3 concentration of DMSO at the same day. To determine toxicity of MMI doses, the total intracellular ATP content was simultaneously assessed via the CellTiter-Glo 3D cell viability assay (Promega) in thyroid follicles cultured in 96-well plates and treated for 7 days with 0/0.1/1/10 µM MMI. For data analysis, blank-corrected measurement values were log-transformed to achieve Gaussian distribution according to Shapiro-Wilk test and assure homogeneity of variance as tested by Brown-Forsythe test. Differences between these data sets were evaluated by one-way ANOVA using Dunnett’s multiple comparison and data matching within single donors.

**Liver model generation**

The liver model is derived from the HepaRG cell line, a hepatoma human cell line with the potential to differentiate into both biliary-like and hepatocyte-like cells. Cryopreserved differentiated HepaRG cells (Biopredic, HPR116080, passage 0, negative tested for mycoplasmas and microbial growth), seeded at a density of 0.2 x 10^6 cells/cm², were pre-cultured for 4 days in HepaRG maintenance culture medium. Cryopreserved HSteC (ScienCell, 5300, Lot: 16646, negative tested for HIV-1, HBV, HCV, mycoplasma, bacteria, yeast and fungi) at passages between 2 and 4 were pre-cultured for 3 days at confluence below 80 % in HSteC culture medium. HepaRG cells were harvested using 0.25 % trypsin/0.53 mM EDTA (Corning). 1 x 10^5 HepaRG cells and 400 HSteC were seeded per well of a 384-well ULA spheroid plate (Corning) in spheroid formation medium. Plates were centrifuged for 1 min at 300 g. Liver spheroids formed after 4 days. 25 liver spheroids were transferred from 384-ULA plate into flat-bottom 96-well ULA plates (Corning) using an electronic 15 – 300 µl pipette in multi-aspirate mode (20 µl/step) with 200 µl wide-bore filter tips. Medium-freed liver spheroids then were embedded in 50 µl 2 mg/ml collagen I (R&D systems).

Even distribution of the 25 liver spheroids per collagen I gel was ensured by immediately circling the plate by hand. After 30 min polymerization of the collagen I matrix at 37 °C, 100 µl co-culture medium was added to each liver spheroid-gel. Next day, two liver spheroid-gels (HepaRG/HSteCs liver spheroids model) were transferred into either a 48-well plate or the outer culture compartment of the Chip2 containing 400 µl or 200 µl co-culture medium, respectively. Liver model generation is illustrated in Fig. S2.

**Liver single culture**

Day zero controls for RNA isolation and immunostaining were immediately taken after the liver spheroids were picked and prior to the collagen I embedding. Samples were stored at –80 °C and 4 °C, respectively, until further processing. Liver spheroids, cultured either in 48-well plates or the outer compartment of the Chip2, were maintained in co-culture medium for 14 to 21 days. Medium was completely exchanged every 2 to 3 days while the albumin and TBB concentrations in the collected supernatants were assessed via the albumin in urine/CSF FS kit according to the manufacturer’s instructions (Diagnostic Systems, 10242) and the Human Serpin A7 PicoKine™ ELISA Kit (Antikörper Online, ABIN5510681), respectively. To obtain the total albumin or TBB yield per day, the albumin/TBB content [µg] in 400 µl was calculated and divided by the number of days between the medium exchanges. RNA was isolated from liver spheroids after a 14-day dynamic cultivation. Optimal conditions for gT4/sT4 formation were determined in 48-well plates by exposing the HepaRG/HSteCs liver spheroids model to 0.005 µM, 0.1 µM or 1 µM T4 for 24 h or to 1 µM for 4 h, 16 h and 24 h in 48 culture day 9. The gT4/sT4 concentrations were measured within the culture supernatants after the respective T4 exposure times. To evaluate a maintained TH catabolite formation under dynamic conditions, HepaRG/HSteC liver spheroids were cultured for 11 days in the HUMIMIC Chip2. On day 11, 1 µM T4 was freshly spiked during the medium exchange. Post 72 h aka culture day 14, the collected supernatants, temporarily stored at –20 °C, were analyzed in terms of gT4/sT4 formation by LC-MS/MS analysis.

**Induction of phase I and II liver enzymes**

Liver spheroid models were pre-cultured in 400 µl co-culture medium in 48-well plates for 5 days, including one medium exchange at day 2, until medium was replaced by co-culture medium supplemented with 1 or 10 µM T4 (SIGMA), and 10 µM β-naphthoflavone (BNF, aryl hydrocarbon receptor (AHR) agonist) (SIGMA) or 10 µM rifampicin (RIF, human-specific pregnane X receptor (PXR) agonist) (SIGMA), or 10 µM pregnenolone-16a-carboxylate (PCN, rodent-specific PXR agonist) (SIGMA). A 0.1 % DMSO solvent control was carried out in parallel. Induction medium was renewed after 3 days and remained until day 6. As depicted in Figure 5A, gT4 and sT4 metabolites (and albumin) were measured in culture supernatants for both time points (3 and 6 days) to broaden the time window in which putative changes in metabolite formation levels might occur. Subsequent to the 6-day treatment, one liver spheroid-gel of each condition was taken for RNA recovery whereas the other gel was used for cytochrome P450 (CYP) induction assay. RIF and PCN-treated liver spheroids were analyzed regarding CYP3A4 activity by P450-Glo™ CYP3A4 Assay Kit (Promega, V9002) and CYP1A2 activity was measured in BNF-treated liver spheroids via P450-Glo™ Assay Kit (Promega, V8422) according to manufacturer’s instruction. In both assays, the time for the CYP substrate conversion was increased to 24 h and the assay volume was set to 100 µl. DMSO-treated liver spheroids served as reference.
Co-culture

Three co-cultures were performed with three independent human thyroid donors using 1000 thyroid follicles per condition and the HepaRG/HSteC liver spheroid model (2 x 25 liver spheroids embedded in collagen I). Culture was performed over 21 days according to Figure 6. Three different conditions were executed, with triplicates each, per co-culture run: co-culture medium (1) without TSH, (2) with 0.1 and (3) 1 mlU/ml bovine TSH (Creative Biosmart). Medium was exchanged every 2 to 3 days and, if required, freshly supplemented with TSH. During the medium exchange, 200 µl conditioned medium was removed from each compartment and replaced by 200 µl fresh medium to restore a total volume of approximately 400 µl. The collected medium was combined and analyzed for TBG and albumin secretion and the formation of T4 and T3 metabolites. The morphology of the organ models was assessed at the beginning and end of culture by bright field microscopy. After 21 days of culture, the thyroid follicles were recovered for immunostaining. The collagen gels of the liver model were used for RNA isolation and immunostaining.

Gene expression analysis

Liver spheroids were extracted from collagen I matrix by 25 mg/ml collagenase NB4 solution for 40 min at 37 °C. Total RNA was extracted using NucleoSpin RNA Plus XS Kit (Macherey-Nagel). RNA was reversed transcribed into cDNA via the TagMan Reverse Transcription Kit (Applied Biosystems). QPCR analysis was performed with QuantStudio 5 Real-Time PCR System using a SensiFAST SYBR Lo-ROX Kit (Bioline) or TagMan Kit (Thermo Fisher Scientific). Primer can be found in Tab. S1 and S2 respectively. Fold changes were calculated by comparative Ct method (ΔΔCt). Selected housekeepers were SDHA (liver model) and GAPDH (thyroid model) which were validated to be not affected by experimental conditions.

Histology

Thyroid tissue was fixed with 4 % formaldehyde + 0.1 % glutaraldehyde for 20 min at 4°C. Liver model was fixed with 4 % formaldehyde for 40 min at 4°C. A three-time PBS wash followed. Samples for cryosectioning were dehydrated in aquatic sucrose solution (15 % at 4°C overnight, 30 % at RT for 4 h) and frozen in TissueTek. 8 µm thyroid cryosections were stained with Haematoxylin and Eosin (H&E) according to standard protocols using the Tissue Stainer TST44C. For 2D immunofluorescence staining, liver model cryosections were fixed in acetone (~20 °C, 10min), two times washed in PBS with Ca2+/Mg2+ and treated with primary antibodies dissolved in 10 % goat serum overnight at 4°C. rabbit anti-MCT8 (monocarboxylate transporter 8, Novus Biologicals, polyclonal, NBP1-89196, dilution 1:100), mouse anti-MRP2 (multidrug resistance-associated protein 2, Enzo Life Science, ALX-801-016, dilution 1:50), rabbit anti-ZO1 (tight junction protein-1, Proteintech, polyclonal, 21773-1-AP, dilution 1:100) and mouse anti-albumin (Sigma, monoclonal, A6684, dilution 1:100). After three wash steps with PBS, cryosections were incubated with secondary antibodies, diluted in PBS containing 1 µg/ml DAPI, for 45 min: Goat anti-Mouse IgG, DyLight 488 (Thermo, polyclonal, 35511, dilution 1:200) and/or Goat anti-Rabbit IgG, DyLight 594 (Thermo, polyclonal, 35553, dilution 1:200). After washing and mounting, images were acquired using an inverted fluorescence microscope (Keyence) and staining was normalized to respective negative control. For whole tissue 3D staining, fixed organ models were permeabilized with 0.3 % Triton X-100 for 2 h at RT, washed three times with wash buffer (PBS with 0.0001 % Triton X-100) and blocked with 3 % goat serum + 0.1 % Triton X-100 for 60 min at RT under constant agitation. Primary antibodies, diluted in 1 % goat serum + 0.1 % Triton X-100, were incubated at 4°C overnight; rabbit anti-MCT8, mouse anti-MRP2, rabbit anti-ZO1, mouse anti-collagen IV (Sigma, monoclonal, C1926, dilution 1:100), rabbit anti-thyroglobulin (Abcam, monoclonal, ab156008, dilution 1:100). Secondary antibodies, previously mentioned, were diluted in wash buffer containing 1 µg/ml DAPI (Roche Diagnostics) and applied overnight at 4°C followed by three PBS washes. 15 min prior to microscopy, the samples were cleared in Visikol Histo-M (Visikol). Images were acquired with the Leica SPE8 confocal microscope.

LC-MS/MS analysis

T3, T4, tT4 and sT4 analytes were measured according to the method described in Karvelat et al. 2022.

Data analysis/statistics

Statistical analyses and data visualization were performed with Prism 7.03 software (GraphPad). Statistical analysis was only performed if data from at least three independent replicates (n ≥ 3) were present. The mentioning of n always refers to an independent standard statistical test are indicated in the respective material section and/or figure legend. P values are given at a 95 % confidence interval and were considered significant for p < 0.05 (* < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001).

3 Results

3.1 Characterization of the in vitro 3D thyroid model

3.1.1 Model generation & morphologic characterization

The follicular arrangement of thyrocytes and correct cellular polarization is a fundamental requirement to obtain a functional in vitro thyroid model (Deisenroth et al., 2020; Mauchamp et al., 1998). To provide mechanical and structural support, isolated thyroid follicles were embedded in GFR Matrigel. The follicular organization of the cells surrounding a luminal compartment could be demonstrated after a 29-day plate-based culture. Confocal laser scanning microscopy along the z-axis clearly showed their spherical structure and indicated the maintenance of the native cell polarization (Fig. 2A). The ECM component collagen IV marked the basal cell pole while enclosing the entire follicle. In addition, the TH precursor molecule TG was visualized in the luminal compartment, suggesting an intrafollicular colloid and thus a suitable reaction space for TH biosynthesis.
Fig. 2: Functional characterization of the statically cultured 3D thyroid model

(A) Confocal Z-stack image series of 29-day static cultured thyroid follicles stimulated with 1 mIU/ml TSH (Donor 1) for the duration of the culture. Intra follicular colloid was visualized by an immunofluorescent staining of thyroglobulin (TG, red, DyLight 594). Basement membrane was visualized by an immunofluorescent staining of collagen IV (Col IV, green, DyLight 488). Blue corresponds to DAPI labelling the nuclei. Scale 15 µm. (B) Intracellular cAMP content of 7-day cultured and TSH-treated thyroid follicles relative to non-treated control. 10 µM forskolin treatment was used as a positive control (PC). Bars represent the mean ratio to TSH non-treated control of at least two independent thyroid donors (n ≥ 2) (Donor 2, 3, 4). SD is shown for n = 3. Mean of one donor, calculated from intra-experimental triplicates, is indicated by one single triangle. Differences of samples with n = 3 were compared based on log-transformed raw data by one-way ANOVA with Geisser-Greenhouse correction using Dunnett's post-hoc test. Changes to control (0 mIU/ml TSH) were considered significant for p < 0.05 (*p < 0.05). (C) Representative immunofluorescent staining of the unstimulated (TSH-) and TSH-stimulated (1 mIU/ml TSH) 3D thyroid model after being statically cultured for 21 days. Protein expression of thyroglobulin (TG, red), collagen IV (COL IV, green) and nuclei (DAPI, blue) is shown. Scale 30 µm. (D) TSH-dependent T3 secretion of 21-day statically cultured 3D thyroid models (Donor 5). Triiodothyronine (T3) was measured in culture supernatants from the 3D thyroid model exposed to 0 (grey), 0.1 (light red) or 1 (red) mIU/ml TSH by LC-MS/MS analysis. Bars represent the mean ± SD of three independent thyroid donors for 0 and 1 mIU/ml TSH (n = 3; Donor 6,7,8) or the mean of two independent thyroid donors in case of 0.1 mIU/ml TSH treatment (n = 2; Donor 7,8) (each triangle represents mean of an intra-experimental triplicate of one donor). Data points on the x-axis indicate T3 concentrations below limit of quantification (< 0.5 nM).
3.1.2 Accumulation of cAMP
Given the structural similarity to the thyroid gland, further attempts aimed to characterize the functional response of the thyroid model towards TSH, the key regulator of the thyroid gland. Upon binding to the TSH receptor localized in the basal membrane, TSH initiates a cAMP-dependent signaling cascade stimulating multiple processes required for TH biosynthesis (Dumont, 1971). Thyroid model cultures exposed to different TSH concentrations showed elevated cAMP levels being significantly higher after exposure to 1 and 10 mIU/ml TSH (Fig. 2B). A TSH concentration of 1 mIU/ml or more showed maximal response on cAMP levels for all donors tested. Therefore, the established 3D thyroid model is responsive to an external TSH-stimulus.

3.1.3 TSH-dependent morphologic change
As known from in vivo conditions, the appearance of thyroid follicles is dependent on their state of activity which allows to distinguish between hypothyroid, euthyroid and hyperplastic states (Yuri et al., 2018, p. 6). To evaluate the stimulation of active and resting follicles in vitro, the 3D thyroid model was cultured in the presence or absence of 1 mIU/ml TSH, respectively, for 21 days. The end-point morphology of those follicles was visualized by immunofluorescence labeling of collagen IV and TG (Fig. 2C). Non-stimulated follicles exhibited flat, squamous-like cell nuclei with weakly stained extracellular collagen IV matrix. In comparison, TSH-stimulated cells appeared taller with rounder nuclei while collagen IV surrounded the whole follicle. Furthermore, distinctive differences in the colloid’s shape could be detected by TG staining. Whereas non-stimulated follicles had a circular defined intra-follicular lumen, the TSH-stimulated follicles showed colloid with more uneven borders. Overall, TSH-dependent morphologic changes of the 3D thyroid model appear to be similar to those usually observed in vivo.

3.1.4 Thyroid hormone secretion
To complete the functional characterization, static 3D thyroid models cultured in the absence or presence of 0.1 or 1 mIU/ml TSH were examined for secretion of T4 and T3. Unexpectedly, the 3D thyroid model hardly secreted any T4 in all conditions tested (Tab. S3). However, in the TSH-treated groups, reproducibly stable levels of T3 were maintained between day 5 and day 21 exhibiting means of 4.6 ± 1.3 nM (0.1 mIU/ml TSH) and 7.1 ± 1.7 nM (1 mIU/ml TSH) (Fig. 2D). In the absence of TSH, only low levels of T3 were detected for the first three time points, thereafter no T3 was detectable. In conclusion, TSH had a reproducible stimulatory effect on the secretion of T3 confirming the TSH-regulated functionality of the established 3D thyroid model in long-term in vitro studies.

3.1.5 TPO inhibition
Considering the in vivo like architecture and TSH-dependent functionality, the static 3D thyroid model was considered to be an appropriate model to assess potential direct effects of test chemicals on TH levels. To establish the assay’s suitability, 3D thyroid models from four human donors were pre-cultured for 3 days, followed by a 4-day treatment with non-cytotoxic concentrations (Fig. S3) of MMI, a reference TPO inhibitor. The concentrations of MMI were 0.1, 1 and 10 µM and the culture medium, containing MMI, was replaced after two days. T3-secretion levels were measured in culture supernatants and normalized to DMSO-solvent control after 2 and 4 days of treatment (Tab. 2). A minimal effect concentration of 1 µM MMI reproducibly inhibited T3-secretion for all donors tested. Maximal inhibiting effects were observed for 10 µM MMI after 4 days which repeatedly suppressed the T3-secretion. These results indicate that the established 3D thyroid model is able to detect direct TH perturbations on T3 synthesis level.

Tab. 2: Relative change of T3 secretion after methimazole (MMI) treatment
3D thyroid model stimulated with 1 mIU/ml TSH were treated for 4 days with 0, 0.1, 1 or 10 µM MMI in 2-day dosing intervals. Overall T3 concentrations were measured by LC-MS/MS from culture supernatants 2 and 4 days after treatment. Data are shown as means ± SD of measured T3 levels normalized to respective non-treated control (0 µM MMI). Four independent thyroid donors were analyzed. LOQ indicates that no relative secretion could be calculated since T3 levels remained below the limit of quantification (< 0.1 nM) after MMI treatment.

<table>
<thead>
<tr>
<th>MMI</th>
<th>Time (day)</th>
<th>Donor 8 (n = 2)</th>
<th>Donor 9 (n = 3)</th>
<th>Donor 10 (n = 3)</th>
<th>Donor 11 (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>µM</td>
<td>Mean [%] ±SD</td>
<td>Mean [%] ±SD</td>
<td>Mean [%] ±SD</td>
<td>Mean [%] ±SD</td>
<td>Mean [%] ±SD</td>
</tr>
<tr>
<td>0</td>
<td>100.0 ±0.0</td>
<td>100.0 ±0.0</td>
<td>100.0 ±0.0</td>
<td>100.0 ±0.0</td>
<td>100.0 ±0.0</td>
</tr>
<tr>
<td>0.1</td>
<td>100.0 ±11.6</td>
<td>100.0 ±3.8</td>
<td>100.0 ±17.2</td>
<td>100.0 ±11.2</td>
<td>100.0 ±11.2</td>
</tr>
<tr>
<td>1</td>
<td>118.7 ±7.1</td>
<td>144.7 ±1.1</td>
<td>53.4 ±13.3</td>
<td>95.2 ±24.9</td>
<td>96.2 ±34.0</td>
</tr>
<tr>
<td>4</td>
<td>136.8 ±19.4</td>
<td>110.8 ±11.5</td>
<td>87.1 ±6.4</td>
<td>96.2 ±34.0</td>
<td>96.2 ±34.0</td>
</tr>
<tr>
<td>10</td>
<td>26.3 ±9.8</td>
<td>65.8 ±3.7</td>
<td>LOQ</td>
<td>LOQ</td>
<td>LOQ</td>
</tr>
<tr>
<td>4</td>
<td>43.0 ±7.1</td>
<td>50.3 ±3.8</td>
<td>LOQ</td>
<td>LOQ</td>
<td>LOQ</td>
</tr>
<tr>
<td>10</td>
<td>10.8 ±7.4</td>
<td>12.6 ±3.0</td>
<td>LOQ</td>
<td>LOQ</td>
<td>LOQ</td>
</tr>
<tr>
<td>4</td>
<td>LOQ</td>
<td>LOQ</td>
<td>LOQ</td>
<td>LOQ</td>
<td>LOQ</td>
</tr>
</tbody>
</table>

3.1.6 Functional properties of the dynamic 3D thyroid model
Morphological characterization of 2-week dynamically cultured thyroid models reproduced spherical arrangement of the cells as previously seen under static conditions. H&E staining (Fig. 3A) visualized the follicular orientation of the cells and the presence of an intrafollicular matrix which contained the protein thyroglobulin (Fig. 3B). As the static cultures, 3D thyroid models were cultured in the absence or presence of 0.1 or 1 mIU/ml TSH while being exposed to the dynamic flow in the Chip. T3 and T4 secretion levels were monitored. Again the 3D thyroid model hardly secreted any T4 in all conditions tested (Tab. S4). TSH-treated groups, maintained reproducibly stable levels of T3 from day 5 showing means of 3.8 ± 1.8 nM (0.1 mIU/ml TSH) and 5.3 ± 2.0 nM (1 mIU/ml TSH) (Fig. 3C). No significant difference was observed between these two groups.
Fig. 3: Functional characterization of the single cultured dynamically cultured 3D thyroid model
(A) Hematoxylin and eosin staining of thyroid-stimulating hormone (TSH)-exposed 3D thyroid model (Donor 11) after being cultured for 15 days in the 2-organ chip platform. (1) Scale bar: 10 µm. (2) Scale bar: 20 µm. (B) Representative fluorescence images of 14-day dynamically cultured TSH-treated thyroid follicles (Donor 11). Intra follicular colloid was visualized by an immunofluorescent staining of thyroglobulin (TG, red, DyLight 594). Blue corresponds to DAPI labelling the nuclei. (C) TSH-dependent T3 secretion of 21-day dynamically cultured 3D thyroid model. Triiodothyronine (T3) was measured in culture supernatants from the 3D thyroid model exposed to 0 (grey), 0.1 (light red) or 1 (red) µM TSH by LC-MS/MS analysis. Bars represent the mean ± SD of three independent thyroid donors (n = 3; each triangle represents mean of intra-experimental triplicate of one donor; Donor 7, 8, 13). Data points on the x-axis indicate T3 concentrations below limit of quantification (< 0.5 nM).

3.2 Characterization of HepaRG/HSteC liver spheroids

3.2.1 Characterization of the hepatic thyroid hormone catabolism in dynamic culture
In order to establish a relevant in vitro liver system that could, in combination with the 3D thyroid model, simulate the essentials of TH homeostasis, the HepaRG/HSteC liver spheroids model needs to conserve key characteristics of the hepatic phenotype.

As the intra-cellular transport of THs and the efflux of their catabolites are fundamental requirements for a hepatic TH catabolism in vitro, the morphology of freshly generated HepaRG/HSteC liver spheroids was analyzed. In this respect, the immunofluorescence detection of the highly specific TH carrier protein MCT8 (Friesema et al., 2003) provided first structural evidence (Fig. 4A). The expression of the tight junction protein ZO1 and the bile transporter MRP2 indicated a functional bile canaliculi network in the liver spheroids that mediates the efflux of glucuronidated and sulfated conjugates in vivo (Jungsuwadee & Vore, 2010) and thus is assumed to contribute to the elimination of THs (Miyawaki et al., 2012).

In vivo, the hepatic serum proteins albumin and TBG are considered as main TH carriers that strongly influence the half-lives of THs. As a serum-free co-culture system was envisaged, the secretion of those proteins by the established HepaRG/HSteC liver spheroids model should contribute to a more physiological TH homeostasis. Culturing 50 HepaRG/HSteC liver spheroids in the Chip2 both albumin and TBG were secreted for the whole 21-day culture. Albumin remained within a 2-fold range over time, whereas the TBG concentrations fluctuated around a 3-fold range, both declining below the day 2 values at day 21 (Fig. 4B). Despite these declines, the observed viability and hepatocyte-specific functionality indicated the model’s suitability for long-term exposure studies with putative TH disruptors.

Next, the expression profile of hepatic marker genes of TH metabolism was analyzed in freshly generated HepaRG/HSteC liver spheroids (day 0) and compared to those of liver spheroids cultured for 14 days in the Chip2. No significant differences were observed (p > 0.05, Multiple t-test corrected for multiple comparison using Holm-Sidak method) (Fig. 4C). HepaRG/HSteC liver spheroids stably expressed genes for all three TH carrier proteins (albumin, TBG and TTR), main cytochrome P450-enzymes (CYP 1A1/1A2/2B6/3A4), sulfotransferases (SULT 1A1/1B1/1E1/2A1), UDP-glucuronosyltransferases (UGT 1A1/1A6/1A9/2B7), nuclear xenobiotic receptors CAR and PXR, the nuclear TH receptor β (THRB), two major TH transporters Ntcp and Mct8, as well as Dio1 converting T4 to T3.

3.2.2 gT4 and sT4 metabolite formation in static and dynamic culture
To demonstrate an active TH catabolism in HepaRG/HSteC liver spheroids, their gT4 and sT4 formation was pre-characterized under static culture conditions after artificial addition of 1 µM T4 on day 9. To examine accumulation of T4 metabolites over time, cell culture supernatants were analyzed after 4, 16 and 24 h of T4 addition. As expected, longer incubation periods led to an increased enrichment of the gT4 and sT4 metabolites in the culture supernatants (Fig. 4D). Selecting an exposure time of 24 h, the concentration-dependent effect of 5, 100 and 1000 nM T4 was evaluated on gT4 and sT4 formation level (Fig. 4E). Only the highest tested concentration of 1 µM T4 resulted in detectable amounts of both gT4 (10.8 nM) and sT4 (2.3 nM).
Fig. 4: Thyroid hormone metabolism-relevant characterization of 3D HepaRG liver spheroids cultured in the HUMIMIC Chip2

(A) Confocal laser-scanning microscopy confirms expression of the monocarboxylate transporter 8 (MCT8, red), tight junction marker ZO1 (red) and the canalicular efflux transporter MRP2 (green) prior to dynamic culture. Nuclei stained with DAPI. Scale bar: 50 µm. (B) Secretion of thyroid hormone carrier proteins albumin (blue, square) and thyroxine-binding globulin TBG (red, triangle) over 21 days in dynamic culture. Data shown as mean ± SD of two independent experiments containing three intra-experimental replicates. (C) Fold change of genes involved in hepatic thyroid hormone (TH) metabolism after a two-week dynamic culture (d14) normalized to day 0. Grey area indicates fold changes between 0.5 and 2, which are considered to be of minor relevance. Succinate Dehydrogenase Complex Flavoprotein Subunit A (SDHA) served as a housekeeper. Differences between d0 and d14 were evaluated for each gene by multiple t-test using Holm-Sidak’s post hoc test and delta Ct values for n > 2. None of the means were significantly different from the d0 control for p < 0.05 and FC ∈ ℝ₀⁺ \ [0.5 ; 2]. (ALB Albumin n = 4; TBG Thyroxine-binding protein n = 4; TTR Transthyretin n = 1; CYP Cytochrome P450: CYP1A1 n = 4; CYP1A2 n = 2; CYP2B6 n = 3; CYP3A4 n = 4; SULT Sulfotransferase: SULT1A1 n = 1; SULT1B1 n = 3; SULT1E1 n = 3; SULT2A1 n = 3; UGT UDP-Glucuronosyltransferase: UGT1A1 n = 4; UGT1A3 n = 4; UGT1A6 n = 3; UGT1A9 n = 4; UGT2B7 n = 4; CAR Constitutive androstane receptor n = 4; PXR Pregnan X receptor n = 3; THRß Thyroid hormone receptor β n = 2; NTCP Sodium/bile acid cotransporter n = 1; MCT8 Monocarboxylate transporter 8 n = 4; DIO1 Human Type 1 Iodothyronine Deiodinase n = 3). (D), (E), (F): Overall formation of glucuronidated (gT4, blue) and sulfated (sT4, red) thyroxine from added T4 is dependent on (D) incubation time when medium is supplemented with 1 µM T4, (E) varies in dependency of 24 h applied T4 concentrations and (F) still takes place after 14 days dynamic cultivation of the HepaRG liver spheroids in the Chip2 (24 h incubation with 1 µM T4). gT4 and sT4 concentrations were assessed by LC-MS/MS from culture supernatants. Data are shown as mean of 2 replicates within one experiment. Data symbol X indicates values below limit of quantification (sT4 < 0.5 nM; gT4 < 0.5 nM).

Adapting these optimized assay conditions, HepaRG/HSteC liver spheroids were cultured for 11 days in the Chip2 until being exposed to 1 µM T4 for 72 h (instead of the 24 h used before) to additionally increase T4 metabolite accumulation. A reproducible gT4 and sT4 formation of 10.35 nM and 6.5 nM, respectively, was found under dynamic conditions (Fig. 4F). In conclusion, the established liver spheroids model maintains meaningful features which contribute to an in vivo-like TH homeostasis while demonstrating for the first time an active TH catabolism in HepaRG-based liver spheroids in the Chip2.
3.2.3 Effect of RIF, PCN, and BNF on P450 activity and gT4/sT4 formation under static conditions

Nuclear receptor activations of AHR and PXR are considered as relevant MIEs to induce liver phase II enzymes and thereby increase the catabolism of THs (Noyes et al., 2019). Based on albumin production, a sensitive hepatic indicator of cytotoxicity (Kühnl et al., 2021), the applied concentrations of the nuclear receptor-activating compounds were demonstrated to be non-cytotoxic (Fig. S4).

In a first study the inducibility of phase I and II liver enzymes were pre-characterized in HepaRG/HSteC liver spheroids under static conditions. Gene expression changes normalized to the solvent control after a 6-day treatment with RIF, PCN, and BNF are depicted in Figure 5B. UGT1A1 (p = 0.0336), CYP2B6 (p < 0.0001) and CYP3A4 (p < 0.0001) were found to be significantly up-regulated by RIF treatment whereas no significant changes in gene expression were detected for PCN. BNF exposure resulted in significant up-regulation of UGT1A1 (p = 0.0002), UGT1A3 (p < 0.0001), CYP1A1 (p < 0.0001) and CYP1A2 (p < 0.0001).

To evaluate these transcriptomic inductions on phase I and II enzyme activity level, compound-dependent changes in CYP3A4, CYP1A2 activities and gT4/sT4 formations were analyzed in HepaRG/HSteC liver spheroids represented in Figure 5C, D and E, respectively. Coherent with mRNA expression levels, 6-day treatment with RIF caused a 24.4-fold increase in CYP3A4 activity (p = 0.0022). No relevant changes in basal CYP3A4 activity were detected for HepaRG/HSteC liver spheroids following PCN treatment. BNF significantly induced CYP1A2 activity by 3.3-fold (p = 0.0017). Despite the demonstrated effects of RIF and BNF on UGT gene expression and CYP activity level, the compounds did not affect T4 glucuronidation after 3 or 6 days. No change was observed in the amount of secreted sT4 after the RIF or BNF treatment which is in line with the observed unchanged SULT gene expression levels. In contrast, the 6-day treatment with PCN resulted in decreased sT4 formation, as reproducibly shown in two independent experiments.

In summary, HepaRG/HSteC liver spheroids were found to respond to AHR and PXR agonists in regard to transcriptomic changes of UGT and CYP enzymes as well as inductions of CYP enzyme activities. In contrast, these hepatic nuclear receptor activations did not show any effect on the sT4 and gT4 formation rate in the HepaRG/HSteC liver spheroids.

Similar observations were made when HepaRG/HSteC liver spheroids were treated with RIF in the HUMIMIC Chip2. Only inductive effects on CYP enzyme activity level were observed without affecting the UGT-T4 or SULT-T4 activities (Fig. S5) confirming the data obtained under static conditions.
Fig. 6: Experimental design of the co-culture process of 3D liver and thyroid models in the Chip2

Overview depicts the timeline of the pre-culture and subsequent co-culture process, time points for in-process analysis and sampling of day 0 controls and endpoint analyzes. (qPCR quantitative polymerase chain reaction, T3 triiodothyronine, T4 thyroxine, TBG thyroxine-binding globulin, HIS histology). Microscopic pictures show representative bright field images of organ models at 2 x and 10 x (liver) or 20 x (thyroid) magnification at the beginning and end of the chip culture.

3.3 Combining the 3D thyroid model and HepaRG/HSteC liver spheroids in a Chip2 for 21 days

Having established a functional 3D thyroid model and HepaRG/HSteC liver spheroids model which simulate both the biosynthesis and catabolism of THs, the combination of the two tissues was undertaken in a multi-organ chip platform, the Chip2. Being composed of two culture compartments, each the size of a standard 96-well, the integrated organ models were spatially separated while a microfluidic channel system allowed their metabolic exchange (Fig. 1B). An on-chip micro pump, operated by a pressure/vacuum of +/-300 mbar at a frequency 0.45 Hz, generated an average flow rate of 2.15 µl min⁻¹ which enabled a complete medium turnover within ~2.5 h. The liver model, represented by 50 HepaRG/HSteC liver spheroids and the 3D thyroid model, composed of 1000 thyroid follicles, were co-cultured over 21 days under chemically defined, serum-free conditions according to Figure 6. To evaluate the reproducibility of the assay, co-cultures with three independent human thyroid donors were performed. Each co-culture contained three groups differing in the applied TSH concentration, being 0, 0.1 or 1 mIU/ml TSH.

Comparing the thyroid-specific readouts, a reproducible organ-level functionality similar to the single-culture was found. From day 5, the co-cultures showed stable reproducible T3 levels in the presence of TSH with mean levels of 6.2 ± 1.2 nM (0.1 mIU/ml TSH) and 6.9 ± 0.8 nM (1 mIU/ml TSH) at the day of medium exchange being not significantly different from each other (Fig. 7A). In contrast, the TSH-negative group did not reveal any measurable amounts of T3 from day 7 on (LOQ < 0.5 nM). As observed in thyroid single culture, no T4 was detected in culture supernatants for any condition. Consequently, the hepatic TH catabolites gT4 and sT4 were not detected either. Immunohistological staining of stimulated and un-stimulated 3D thyroid model revealed a similar TSH-dependent morphology after co-culture as previously described for single-cultured thyroid models under static conditions (Fig. 7B, C).

Similar to the previous hepatic single cultures, the TBG (Fig. 7D) and albumin (Fig. 7E) levels decreased after 9 days of co-culture, but the effect was more pronounced in the dynamic culture. There was a trend towards more stable values towards the end of the culture with no apparent differences between the TSH-exposure groups. Despite the decreased secretion of the serum proteins, the hepatic phenotype was considered as maintained. Cells throughout the whole spheroid clearly demonstrated an albumin expression after the 21-day co-culture (Fig. 7F). Simultaneously, the expression of the MCTB8 transport protein could be confirmed. Furthermore, gene expression profiles of hepatic marker genes of 21-day co-cultured liver spheroids were similar to freshly generated and 21-day single-cultured liver spheroids. Apart from CYP3A4, solely shown to be downregulated after co-culture, albumin, MRP2, BSEP (bile salt export pump), SLC10A1 (solute carrier known to transport THs (Friesema et al., 2005)), UGT1A1, and SULT1A1 mRNA expression levels equalled in all conditions and respectively confirmed a maintained hepatic phenotype of the co-cultured liver spheroids (Fig. S6). In summary, this study presents a first proof on concept for a functional combination of human 3D liver and thyroid models over a period of 21 days. This organ-on-a-chip model might, in the future, provide a powerful in vitro tool to study the thyroid homeostasis on a next level of human relevance addressing aspects of organ-organ interaction.

4 Discussion

Assessing the human relevance of safety-related findings in rodents with respect to thyroid toxicity is key during the development of new PPPs. This study presents a new approach methodology (NAM) to investigate the human safety of these chemicals by addressing potential thyroid adverse effects at a functional level using tissues from the relevant species. To this
end, a human-based co-culture of 3D thyroid and liver organ models was established that can last for 21 days. This culture time is assumed to be long enough to allow meaningful mechanistic investigations (e.g. nuclear receptor activations, changes in hepatic enzyme activity) as demonstrated in 7-day mechanistic short-term studies in rats (Tinwell et al. 2014). In depth pre-characterization of the statically cultured single organ models indicate the suitability of the 3D thyroid model to detect direct TH inhibitors and provided evidence of an active TH catabolism in HepaRG/HSteC liver spheroids. Accordingly, the individually cultured organ models are already of value to investigate organ-specific mechanisms of the TH homeostasis and related toxicities.

4.1 Detection of direct thyroid hormone perturbation with the 3D thyroid model

Implementing the advancements of 3D organoid technologies, we established a functional human 3D thyroid model derived from tissue-recovered primary thyroid follicles and GFR Matrigel as a structural matrix, suitable for static and dynamic culture conditions.

A major feature of the 3D thyroid model presented here is its physiological and structural characteristics, as TSH-responsive morphology is maintained for 21 days in both culture formats. From earlier reports, it is known that ECM components are key to promote a thyroid-specific phenotype in vitro reinforcing the native polarization of thyrocytes (Chambard et al., 1984; Mauchamp et al., 1998). As demonstrated by our results, the 3D thyroid model maintained the typical
basolateral cell polarization showing follicle-surrounding collagen IV expression that is known to be a major component of the basement membrane in human thyroid tissue (Bürgi-Saville et al., 1997). The interior of the in vitro-cultured follicles was filled by TG which, in vivo, is utilized by apical/luminal expressed enzymes such as TPO to synthesize THs (Mondal et al., 2016). This arrangement indicates intra-follicular apical cell polarization, which in turn is suggestive of functional TH biosynthesis. Remarkably, a continuous TSH-stimulation resulted in morphologic changes of the in vitro-cultured follicles which correlates well with the concept of active and resting follicles showing a high or low TH synthesis, respectively (Dumont, 1971; Yuru et al., 2018). In vivo, the appearance of active follicles, most prominent during hyperplasia, is characterized by a columnar morphology whereas cells of resting ones reveal a cuboidal shape and a more abundant colloid (Yuru et al., 2018) which is in agreement with our results. In conclusion, our findings indicate that thyroid hyperplasia and hyperthyroid can be mimicked in our model.

According to EFSA the most prominent direct thyroid effects are the blockage or competitive inhibition of the iodine uptake via NIS and the reversible or irreversible inhibition of TPO (Crivelle et al., 2019). For early pipeline molecules, these MIEs can be addressed using current available high-throughput assays (Noyes et al., 2019). However, such approaches are lacking in physiological relevance, as they are based on cell-free environments (Murk et al., 2013) or transfected human cell lines such as hNIS-HEK293T-EPA (J. Wang et al., 2019).

Here, we present an alternative human 3D thyroid model which offers TH secretion as a functional marker for the in vitro evaluation of direct thyroid toxicity. The results of our study demonstrated a stable release of T3 in the presence of TSH over 21 days for statically as well as dynamically single-cultured thyroid follicle models. In vivo T3 plasma concentrations in healthy human subjects range from 1.0 to 3.0 nM (Gardas, 1991; Hohrari et al., 1987), the in vitro achieved concentrations of ~ 3.8 ± 1.8 nM (0.1 µIU/ml TSH, dynamic single-culture) are consistent with these levels. However, in contrast to in vivo, our 3D thyroid models demonstrate low to zero T4 secretion, which in vivo represents the major product of the TH synthesis and is 16.8-times more abundant than T3 (Pilo et al., 1990). Even though some known 3D thyroid models were shown to secrete T4 (Deisenroth et al., 2020; Spinel-Gomez et al., 1990), none of these have reproduced the physiological T4/T3 ratio, even if T4 was the dominant TH. Massart et al. demonstrated a significant decrease of T4 levels, when their thyroid follicle cultures were supplemented with TSH (Massart et al., 1988). In this respect, it was hypothesized that type I and type II deiodinase (DIO1 and 2), both strongly expressed in thyroid tissue (Uhlen et al., 2015), contribute to the decreased T4 levels as they mediate the conversion of T4 into T3 in a TSH-dependent manner (Deisenroth et al., 2020; Massart et al., 1988; Mondal et al., 2016; Murakami et al., 2001). Supporting this hypothesis is the observation that patients with T3-predominant Grave’s disease are characterized by increased free T3/T4 ratio, which is attributed to elevated DIO1/2 activity (Ito et al., 2011).

Despite the low levels of T4, T3 secretion under TSH stimulation could be suppressed by MMI, a reference TPO inhibitor (Friedman et al., 2016). For a proof of concept, statically cultured 3D thyroid models from four different donors were exposed to the reference compound MMI. After 2 and 4 days, a reproducible decrease in T3 could be observed for 1 µM and even stronger for 10 µM MMI. These values correlate well with maximal observed serum concentrations of MMI (2.61 ± 0.81 µM) when a daily dose of 10 mg MMI is administered to patients with hyperthyroidism (Okamura et al., 1986). Additionally, a similar range of effect concentrations of MMI were previously reported when human TPO activity was measured directly in TPO extracts from Nthy-ori 3-1 cell line (IC50 2.7 - 4.0 µM) (Jomaa et al., 2015), primary tissue (0.8 – 2 µM) (Nagasaka & Hidaka, 1976) and ex vivo treated thyroid slices (IC50 5.0 µM) (Vickers et al., 2012). Notably, our results are in line with a recently published human 3D thyroid model in which 1 µM MMI was reported to be the lowest concentration at which changes in TH secretion were observed (Deisenroth et al., 2020). In conclusion, the established 3D thyroid model was able to identify one of the most prominent MIEs for adverse thyroid-mediated outcomes, namely TPO inhibition. Furthermore, this data provided evidence that the secreted T3 is the product of a de novo TH synthesis, since active TPO enzymes are evidently required for in vitro TH synthesis, and is not a result of a TSH-induced liberation from an internal T3 reservoir as previously described for thyrocyte monolayer cultures (Ollis et al., 1985). The latter might explain the secretion of T3 in the TSH-non-induced state. Nevertheless, it must be noted that our TH-inhibition study was conducted under static culture conditions only. Future studies therefore will examine the reproducibility of the results when the 3D thyroid model is exposed in parallel to MMI and the dynamic flow of the HUMIMIC Chip2.

In summary, our established human 3D thyroid model presents the first in vitro thyroid model which can be incorporated into a perfused organ-chip system with viability and TSH-dependent T3 secretion over 21 days. The use of a commercially available multi-organ chip platform, quality-controlled according to ISO 9001-2015 standards, ensured the robustness of the underlying equipment background and the corresponding chip culture ware. Various assay formats have been qualified previously on this platform by pharmaceutical companies in other contexts of use applying different human organ models (Marx et al., 2020). This knowledge fed into the establishment of a reproducible thyroid-liver co-culture. However, further refinement of the model has to be considered if one wants to respect physiological T4/T3 ratio by, for example, understanding and monitoring the activity of DIO1/2 in the system. So far only the gene expression of DIO1 was analyzed and compared to expression levels in primary tissue showing no striking differences (Fig. S7).

### 4.2 HepaRG/HSteC liver spheroids as a tool for evaluating hepatic thyroid hormone catabolism

There is an urgent need for setting up liver models that can simulate the human hepatic TH metabolism in vitro and thus increase our understanding of the relevance of xenobiotic-related euthyroid effects observed in rodents to humans. Recent advances in research are pointing towards 3D hepatocyte spheroid models which maintain and even improve the liver-specific phenotype over longer periods of time compared to 2D cultures (Bell et al., 2016; Cox et al., 2020; Lauschke et al., 2016; Underhill & Khetani, 2018). In particular, 3D cultures of HepaRG cells, which are already considered as a good surrogate to primary human hepatocytes (Aminat et al., 2006; Hoeckstra et al., 2013; Huaman et al., 2012; Kammerer & Küpper, 2018; Lübbenstedt et al., 2011a; Tescher et al., 2019), are able to compensate for known limitations of monolayer cultured HepaRG cells e.g. by inducing urea formation (Gaskell, 2016; Gunness et al., 2013; Li et al., 2019) and enhancing CYP2E1 activity (Gunness et al., 2013). We characterized HepaRG/HSteC liver spheroids as a suitable model to study the hepatic T4 metabolism and their potential to evaluate liver-mediated thyroid toxicity. In the past, neither the HepaRG cell line nor related 3D liver models have been characterized in regard to their TH metabolism.
In vivo, the hepatic serum-binding proteins are key for the overall transport of THs, but also represent their main storage capacity since they bind up to 99.5% of total T3 and T4 (Kortenkamp et al., 2017). Even if albumin is the most abundant serum protein in humans, TBG binds 75% of the majority of THs (Janssen & Janssen, 2017). This is the first study showing that HepaRG/HSteC liver spheroids in Chip2 culture are able to produce TBG over a 3-week period, and thus can contribute to the TH homeostasis when co-cultured with the 3D thyroid model. For future assay refinement, this newly characterized parameter could serve to evaluate xenobiotic-induced TH displacements or protein abnormalities which are associated to altered TH levels. Together with the stable albumin production, which was previously demonstrated in chip-cultures and serves as an overall hepatic functionality marker (Bauer et al., 2017; Schimek et al., 2020), a prolonged culture of the HepaRG/HSteC liver spheroids can be assumed allowing long-term studies and repeated exposures to mimic in vivo treatments.

THs and their metabolites rarely pass the plasma membrane via diffusion, but require active uptake and excretion via transport proteins (Visser et al., 2011). We were able to demonstrate the expression of MCT8, a highly specific TH-transporter (Visser et al., 2011), and the bile transporter MRP2, generally known to mediate the excretion of glucuronidated and sulfated conjugates (Jungsuwadee & Vore, 2010), at protein level in freshly generated HepaRG/HSteC liver spheroids and its transcriptomic stability after 14 or 21 days chip-culture. Most importantly, however, was the transcriptomic evidence of UGT1A1/1A9/1A9 and SULT1A1/1B1/1E1, which have been described as having a TH-specific activity in humans (Findlay et al., 2000; Gamage et al., 2006; Kato et al., 2008; Kester et al., 1999). Accordingly, HepaRG/HSteC liver spheroids represented fundamental features required for active TH uptake, the metabolization of THs via respective UGT and SULT enzymes, and the efflux of the generated glucuronidated and sulfated TH metabolites. The relevance of these findings was finally confirmed by the active formation of gT4 and sT4 metabolites in 14-day dynamically cultivated HepaRG/HSteC liver spheroids. Conclusively, to the best of our knowledge this study presents for the first time a dynamic 3D liver model which can mimic the hepatic T4 catabolism in vitro. Similar to Richardson et al., who verified a basal T4 catabolism in static sandwich-cultured human hepatocytes between culture day 3 to 6 applying 0.1 µM T4 (Richardson et al., 2014), the gT4 and sT4 formation of our static model increased with T4 concentration and duration of T4-exposure. However, in our assay set-up, measurably stable gT4 and sT4 secretion levels were only detectable at T4 concentrations ≥ 1 µM, which is above the physiological range of T4 (54 – 160 nM) (Gardas, 1991; Hothanari et al., 1987) and the expected T3/T4 secretion levels, which is a limitation of the present model. However, the addition of isotope-labelled T4 could be considered to measure impairments of TH catabolite formation as an end-point during future studies with thyroid-liver co-cultures, which would allow to discriminate between secreted and supplemented T4, as successfully demonstrated by Karwelat et al. (Karwelat et al., 2022) using a rat thyroid-liver chip model.

In our study, we indirectly demonstrated the activation of the hepatic nuclear receptors, PXR and AhR, in statically cultured HepaRG/HSteC liver spheroids while simultaneously analyzing their TH catabolism via the formation of gT4 and sT4. To prove the functional integrity of the liver model, the inducibility of the two major phase I biotransformation enzymes CYP1A2 and CYP3A4 was demonstrated by BNF and RIF, respectively. Induction levels of CYP1A2 activity, were slightly lower than previously published results, however, shorter induction times with BNF and different analytical methods might be the reason for the observed differences (Leite et al., 2012; Lüberstedt et al., 2011b). In contrast, the extent of CYP3A4 induction mediated by RIF correlated well with previous findings in 3D HepaRG liver spheroids (Aninat et al., 2006; Desai et al., 2017). As expected, PCN proved to be ineffective in human cells, whereas in rodents it is known to lead to CYP3A induction (Karwelat et al., 2022; Xie et al., 2000). Thus, the established HepaRG/HSteC liver spheroids model appears to respond to AhR and human-specific PXR activation of phase I enzymes under static conditions. Even though the activation of these hepatic nuclear receptors is considered as a potential MIE causing impairments of the TH system (Noyes et al., 2019), neither of them were able to significantly increase the basal TH catabolite formation. In conclusion, the relationship between nuclear receptor activation and TH catabolism via phase II enzyme activity induction could not be shown for the statically cultured HepaRG cell model, despite induction of UGT phase II enzymes on gene transcript level being detected. To our knowledge, neither the effect of PCN nor BNF on hepatic phase II enzymes has been studied in human beings to date. In agreement with our findings, in vitro 2D culture data, recently presented at the Society of Toxicology 60th Annual Meeting, showed no relevant changes in human T4-UGT activity after 3-day BNF exposure (Bars, 2021). Of the few available human in vivo studies which report RIF-related alterations of T4 serum levels without pathophysiological outcomes, none directly evaluated whether these changes could be attributed to altered gT4 and sT4 formations (Curran & DeGroot, 1991; Meek et al., 2003; Ohnhaus & Studer, 1983). Although gT4 and sT4 synthesis were not affected in our studies, RIF and BNF significantly induced mRNA levels of UGTs associated with T4-activity, supporting the activation of the nuclear receptors, PXR and AhR. mRNA levels of analyzed SULT enzymes remained stable, confirming that no effects on sT4 synthesis level were to be expected. The discrepancies between mRNA expression level and UGT enzyme activity, however, are not surprising as they have previously been reported (Ohtsuki et al., 2012). Conclusions taken from this data strengthen the theory that nuclear receptor activations have less relevance to hepatic TH catabolism in humans. In contrast, an equivalent assay that we have recently established for the rat, showed distinct effects of PCN and BNF on gT4 formation (Karwelat et al., 2022). However, whether the observed differences between these assays, representing human and rat, have in vivo relevance remains to be further investigated. Due to insufficient reference data, it is difficult to verify how representative the HepaRG-derived model is. According to a 2D sandwich-cultured human primary hepatocyte model, an increase in gT4 formation was detected following a 72 h treatment with PCB 153, indicating that T4-UGT activation is already detectable at incubation periods of 3 days, and more importantly indicates its occurrence in human primary cells (Richardson et al., 2014). In contrast to our selected model compounds, PCB 153, a phenobarbital-like inducer, is known to be an activator of the constitutive androstane receptor (CAR) (Al-Salman et al., 2012) which in rodents can be accompanied by induced T4-UGT activity (Hood et al., 2003). Further mechanistic investigations of the HepaRG/HSteC liver spheroid model should therefore investigate if appropriate reference compounds can reproduce these CAR inductions and mediate enhanced T4 glucuronidation formation. In addition, it must be taken into account that the currently available data serve to validate the use of statically cultured HepaRG/HSteC liver spheroids for pharmacological investigations. However, a first induction study of the model under dynamic conditions indicated similar phase I and II enzymes properties as already shown for the static equivalent.
In summary, the assay described in this study serves as a proof-of-concept in which gT4 and sT4 metabolism in HepaRG/HSteC liver spheroids can be evaluated. Furthermore, it presents a transferrable approach that can be easily adapted for PHH-derived spheroids which, in the future, might provide a cross-donor survey with increased human relevance.

4.3 Co-culture

Having established physiologically relevant single-tissue organ-chip cultures over 21 days, the maintenance of their functionalities was explored in a co-culture set-up. Here, we demonstrate a unique co-culture approach of the developed 3D thyroid model and HepaRG/HSteC liver spheroids over three weeks in which key organ functionalities were maintained similar to those observed in single culture. Whether the decrease of albumin and TGB over time are indicating a general decrease in hepatic functionality which also affect the TH catabolism, or if this is due to lower oxygen levels during co-culture (Felmee et al., 2018), needs further clarification. In conclusion, this approach can be regarded as a first step towards a multi-modular platform, which has the potential to simultaneously detect direct as well as hepatic-mediated impairments of human TH homeostasis. Future studies will focus on determining whether the co-culture system can detect perturbations of TH homeostasis as previously shown in static single cultures.

4.4 Conclusion and perspectives

In this publication, we provide a first proof of concept towards a more complex test method which evaluates TH perturbations on a higher biological level compared to traditional assays (2D cultures, protein-ligand-binding studies). We presented a 3D thyroid follicle model and a 3D HepaRG/HSteC liver spheroid model which under static conditions can be used to evaluate direct as well as indirect TH perturbations with improved human relevance. The established 3D thyroid model emulated de novo T3 biosynthesis while maintaining a morphological as well as metabolic response towards TSH for a 21-day organ-chip culture. Direct thyroid toxicity was demonstrated by MMI via decreased T3 secretion in the statically cultured model. The HepaRG/HSteC liver model demonstrated active phase II enzymes resulting in hepatic-basaline catabolism of T4 via glucuronidation and sulfation, which has not been demonstrated before. Consequently, the presented work presents a first step towards a multi-modal human platform to evaluate hepatic and thyroidal associated dysregulations of the TH metabolism separately or in combination. In its intended purpose of use, the assay has the potential to significantly contribute to the reduction and refinement of animal-based studies and to bridge the gap between data derived from initial high-throughput assays and follow-up in vivo animal studies. In combination with the rat-based thyroid-liver assay, which was developed in parallel (Karwelat et al., 2022), these models will contribute to a better understanding of species similarities and differences and thus significantly improve the guidance for the identification of thyroid disruptors that are relevant from a human safety perspective. Finally, this microfluidic chip-based thyroid-liver co-culture contributes towards microphysiological system-based models with increased human biological relevance, a trend identified to optimize drug discovery by laboratory animal free investigative toxicology (Beilmann et al., 2019).

References


spheroids as a model system for drug-induced liver injury, liver function and disease. Scientific Reports, 6(May), 1–13. doi:10.1038/srep25187


Hallgren, S. & Darnerud, P. O. Polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyls (PCBs) and chlorinated paraffins (CPs) in rats—testing interactions and mechanisms for thyroid hormone homeostasis. *Toxicology 177, 227–243 (2002).*


Ohtsuki, S., Schaefer, O., Kawakami, H., Inoue, T., Liehner, S., Saito, A., Ishiguro, N., Kishimoto, W., Ludwig-Schwellinger, 19


unknowns. *Molecular Endocrinology*, 25(1), 1–14. doi:10.1210/me.2010-0095


**Conflict of interest**

Uwe Marx is shareholder and CSO of TissUse GmbH, which commercializes MPS platforms.

**Acknowledgements**

This work was funded by Bayer AG through a Life Science Collaboration programme. We thank Dettlef Stöckigt and Oliver Born for the development of the thyroid hormone-related LC-MS/MS analytical methods as well as Ilka Maschmeyer for her expertise during the initiation of the project.

**Data availability statement**

The research data, that are not included in the article, are available upon request from the corresponding author.