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# A multi-organ-chip co-culture of liver and testis equivalents: a first step toward a systemic male reprotoxicity model

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**STUDY QUESTION:** Is it possible to co-culture and functionally link human liver and testis equivalents in the combined medium circuit of a multi-organ chip?

**SUMMARY ANSWER:** Multi-organ-chip co-cultures of human liver and testis equivalents were maintained at a steady-state for at least 1 week and the co-cultures reproduced specific natural and drug-induced liver–testis systemic interactions.

**WHAT IS KNOWN ALREADY:** Current benchtop reprotoxicity models typically do not include hepatic metabolism and interactions of the liver–testis axis. However, these are important to study the biotransformation of substances.

**STUDY DESIGN, SIZE, DURATION:** Testicular organoids derived from primary adult testicular cells and liver spheroids consisting of cultured HepaRG cells and hepatic stellate cells were loaded into separate culture compartments of each multi-organ-chip circuit for co-culture in liver spheroid-specific medium, testicular organoid-specific medium or a combined medium over a week. Additional multi-organ-chips (single) and well plates (static) were loaded only with testicular organoids or liver spheroids for comparison. Subsequently, the selected type of medium was supplemented with cyclophosphamide, an alkylating anti-neoplastic prodrug that has demonstrated germ cell toxicity after its bioactivation in the liver, and added to chip-based co-cultures to replicate a human liver–testis systemic interaction *in vitro*. Single chip-based testicular organoids were used as a control. Experiments were performed with three biological replicates unless otherwise stated.

**PARTICIPANTS/MATERIALS, SETTING, METHODS:** The metabolic activity was determined as glucose consumption and lactate production. The cell viability was measured as lactate dehydrogenase activity in the medium. Additionally, immunohistochemical and real-time quantitative PCR end-point analyses were performed for apoptosis, proliferation and cell-specific phenotypical and functional markers. The functionality of Sertoli and Leydig cells in testicular spheroids was specifically evaluated by measuring daily inhibin B and testosterone release, respectively.

**MAIN RESULTS AND THE ROLE OF CHANCE:** Co-culture in multi-organ chips with liver spheroid-specific medium better supported the metabolic activity of the cultured tissues compared to other media tested. The liver spheroids did not show significantly different behaviour during co-culture compared to that in single culture on multi-organ-chips. The testicular organoids also developed accordingly and produced higher inhibin B but lower testosterone levels than the static culture in plates with testicular organoid-specific medium. By comparison, testosterone secretion by testicular organoids cultured individually on multi-organ-chips reached a similar level as the static culture at Day 7. This suggests that the liver spheroids have metabolised the steroids in the co-cultures, a naturally occurring phenomenon. The addition of cyclophosphamide led to upregulation of specific cytochromes in liver spheroids and loss of germ cells in testicular organoids in the multi-organ-chip co-cultures but not in single-testis culture.

**LARGE-SCALE DATA:** N/A

**LIMITATIONS, REASONS FOR CAUTION:** The number of biological replicates included in this study was relatively small due to the limited availability of individual donor testes and the labour-intensive nature of multi-organ-chip co-cultures. Moreover, testicular organoids

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and liver spheroids are miniaturised organ equivalents that capture key features, but are still simplified versions of the native tissues. Also, it should be noted that only the prodrug cyclophosphamide was administered. The final concentration of the active metabolite was not measured.

**WIDER IMPLICATIONS OF THE FINDINGS:** This co-culture model responds to the request of setting up a specific tool that enables the testing of candidate reprotoxic substances with the possibility of human biotransformation. It further allows the inclusion of other human tissue equivalents for chemical risk assessment on the systemic level.

**STUDY FUNDING/COMPETING INTEREST(S):** This work was supported by research grants from the Scientific Research Foundation Flanders (FWO), Universitair Ziekenhuis Brussel (scientific fund Willy Gepts) and the Vrije Universiteit Brussel. Y.B. is a postdoctoral fellow of the FWO. U.M. is founder, shareholder and CEO of TissUse GmbH, Berlin, Germany, a company commercializing the Multi-Organ-Chip platform systems used in the study. The other authors have no conflict of interest to declare.

**Key words:** organ-on-a-chip / testis / liver / co-culture / reprotoxicity / substance testing / microfluidics / cyclophosphamide / organoids / spheroids

# Introduction

Current *in vitro* and animal tests for chemical risk assessment and drug development lack the systemic organ complexity of the human body and, therefore, often cannot be readily validated in relation to human health, making the extrapolation of the hazards they pose uncertain. This issue signifies the need for specialised tools to assess the safety and efficacy of substances *ex vivo*.

Multi-organ-chip platforms using human tissue provide a unique preclinical insight into the systemic level. The multi-organ-chips consist of a circular channel system which connects tissue culture compartments for the culture of different spheroids, organoids or tissues and, thereby, closely simulates the activity of multiple human organs in their physiological context. The multi-organ chips provide a completely new approach to predict, for example, toxicity, ADME (absorption, distribution, metabolism and excretion) profiles and efficacy *in vitro* (Dehne *et al*., 2017). Furthermore, they prevent major difficulties many laboratories normally face, by reducing and replacing animal testing and streamlining human clinical trials (Paul *et al*., 2010; Rebelo *et al*., 2016). To date, the multi-organ-chips have enabled the co-culture of liver spheroids with different human three-dimensional (3D) organ equivalents, i.e. pancreatic islets, skin punch biopsies, neuronal spheroids or intestinal tissue, at homeostasis over weeks (Materne *et al*., 2015; Maschmeyer *et al*., 2015a; Bauer *et al*., 2017). A more recent multi-organ-chip format was able to host four human organ equivalents for 28 days (Maschmeyer *et al*., 2015b). Other groups have reported pumpless platforms for gastrointestinal tract and liver equivalents (Esch *et al*., 2016), airway and liver (Coppeta *et al*., 2017), 10 human and non-human organs (Edington *et al*., 2018) and a model of the female human reproductive tract with a 28-day menstrual cycle (Xiao *et al*., 2017). These platforms have paved the way to future body-on-a-chip devices, including the present human testis model for reprotoxicity studies. This important field focuses on the developmental and reproductive risk assessment of substances and aims for the accurate prediction of chemically induced testicular toxicity in humans, elucidation of mechanisms of toxicity and identification of possible biomarkers of testicular toxicity. There is considerable evidence from the literature that male fertility can be impacted by environmental, chemical and pharmaceutical exposures, especially through alteration of endocrine functions and ablation of germ cells (Vested *et al*., 2014; Bonde *et al*., 2016; Gabrielsen and Tanrikut, 2016; Kilcoyne and Mitchell, 2017; Semet *et al*., 2017; Medrano *et al*., 2018). Human testis equivalents have not yet been implemented on organ-on-a-chip platforms, simply because a stable and predictive *in vitro* model for human testicular toxicity still needs to be developed. Efforts have been made, but they present inherent drawbacks, related mainly to simplicity, have limited duration (days) and do not provide a systemic approach (Parks Saldutti, 2013). A co-culture with a liver equivalent that allows biotransformation of substances would significantly improve the reprotoxicity forecast on human health, since it enables the study of direct effects and takes into account potential secondary effects of the metabolites.

Cyclophosphamide is an extensively used anticancer and immunosuppressive agent, which is administered as an inactive prodrug. It requires metabolic activation by cytochrome P450 (CYP) enzymes to generate 4-hydroxycyclophosphamide. The tautomer of 4-hydroxycyclophosphamide, aldophosphamide, circulates and passively enters other cells, where it undergoes a spontaneous (non-enzymatic) elimination reaction to yield the therapeutically-active phosphoramide mustard and by-product acrolein. Phosphoramide mustard exerts its chemotherapeutic effect by binding an alkyl molecule to DNA, forming an adduct that leads to inhibition of DNA replication and cell apoptosis (Zhang *et al*., 2006). As CYP-mediated activation of cyclophosphamide takes place primarily in the liver, then active metabolites can enter the blood circulation and are able to reach the tumour and healthy tissue alike, thereby increasing the risk of adverse reactions, for instance, gonadotoxicity. The spermatogenic epithelium of the male reproductive system is highly sensitive to this drug due to the presence of rapidly dividing germ cells (Ghobadi *et al*., 2017). In particular, repeated treatments with cyclophosphamide result in oligozoospermia or azoospermia (Nurmio *et al*., 2009).

We recently reported the first formation of human testicular organoids from primary cell suspensions. These testis equivalents harboured the germ cells, including spermatogonial stem cells, and their important niche cells, which retained specific functions for at least 4 weeks, including testosterone and inhibin B production (Baert *et al*., 2017a). As such, the aim of this study was to co-culture and functionally link human liver and testis equivalents in the combined medium circuit of a multi-organ-chip. Specifically, we investigated (i) which medium composition is able to support the on-chip co-culture of human liver spheroids and human testicular organoids at a steady state and (ii) whether the co-culture can be used for reprotoxicity testing on the liver-testis axis using cyclophosphamide as a model substance.

# Materials and Methods

## Study design

In the first experiment, one testicular organoid from primary human adult testicular cells and 40 liver spheroids consisting of HepaRG cells and primary hepatic stellate cells were loaded into separate culture compartments of each multi-organ-chip circuit for co-culture in liver spheroid-specific medium, testicular organoid-specific medium or a combined medium over a week. Additional multi-organ-chips (single) and regular well plates (static) were loaded only with testicular organoids or liver spheroids for comparison. A battery of analyses was performed to select the culture medium supporting a stable co-culture of liver spheroids and testicular organoids in multi-organ-chips. The metabolic activity was determined as glucose consumption and lactate production. The cell viability was measured in the medium through the activity of lactate dehydrogenase (LDH) which is released upon damage to the cell membrane.

Apoptosis and proliferation were analysed in the liver spheroids by (immuno) histological stainings using TdT-mediated dUTP-digoxigenin nick end labelling (TUNEL) and the Ki67 marker, respectively. Other immunohistochemical analyses were performed by staining spheroid sections for CYP3A4 and albumin. Real-time quantitative polymerase chain reaction (PCR) was performed to evaluate the gene transcription in liver spheroids on Day 0 and after the experiments were finished (Day 7).

The testicular organoid morphology was assessed by periodic acid-Schiff staining. Specific testicular cells were traced by immunofluorescence. The functionality of Sertoli and Leydig cells in testicular organoids was evaluated by measuring the daily inhibin B and testosterone release, respectively.

In the second experiment, the selected type of medium was supplemented with cyclophosphamide and added to chip-based co-cultures of testicular organoids and liver spheroids. The cyclophosphamide only exerts its toxic effects after bioactivation by liver enzymes. Single chip-based testicular organoids were used as control. We assessed the effect of cyclophosphamide supplementation on the DEAD-Box Helicase 4-positive (DDX4) germ cell fraction to test whether we would be able to replicate the human liver-testis interaction *in vitro*.

## Human cell sources and maintenance

Differentiated HepaRG cells (Lot HPR116215-TA08 or HPR116265-TA08) were obtained from Biopredic International (Rennes, France). Primary human hepatic stellate cells were purchased from ScienCell (Carlsbad, CA, USA).

The differentiated HepaRGs were thawed and seeded confluently 4 days before spheroid formation. Standard HepaRG culture medium (liver spheroid-specific medium) consisted of William's Medium E (PAN-Biotech, w/o phenol red, Aidenbach, Germany) supplemented with 10% fetal calf serum (Corning, Lowell, MA, USA), 5 µg/mL human insulin (PAN-Biotech), 2 mM L-glutamine (Corning), $5 \times 10^{-5}$ M hydrocortisone hemisuccinate (Sigma-Aldrich, H4881-1G, St. Louis, MO, USA) and 1% [v/v] penicillin/streptomycin sulphate (Corning). An amount of 0.5% dimethyl sulfoxide (VWR Chemicals, PA, USA) was added to the medium to keep the HepaRGs in a differentiated state. On the following day, the medium was renewed with HepaRG medium containing 2% dimethyl sulfoxide. The cells were maintained in this medium at 37°C and 5% $CO_2$ for 3 days until spheroid formation. The hepatic stellate cells (Lot 16646) were expanded in stellate cell medium, provided by ScienCell (Carlsbad, CA, USA). Cells were used between passage three and six. Pre-culture was started at least 2 days before spheroid formation.

Testes were obtained from four patients with complete spermatogenesis undergoing bilateral orchiectomy as a treatment for prostate cancer at the Urology Department, Universitair Ziekenhuis Brussel. All patients provided informed consent. The testicular tissue was isolated by removing the tunica albuginea, fragmented and subjected to a two-step enzymatic digestion. In brief, single-cell suspensions were obtained by initial digestion with collagenase IA (1 mg/mL) and then with collagenase IA (1 mg/mL), DNase type I (0.5 mg/mL) and hyaluronidase type I-S (0.5 mg/mL) (all from Sigma-Aldrich). Viable cells were identified by trypan blue exclusion. The resulting suspension, containing all types of testicular cells, was cryopreserved at $2 \times 10^6$ cells/mL and thawed using a procedure originally described for testicular tissue (Baert *et al.*, 2013). The experiments have been approved by the ethics committee of the Universitair Ziekenhuis Brussel (no. 2017/224).

## *De novo* generation of liver spheroids and testicular organoids

Three days ($t$ = day −3) before starting the experiment ($t$ = 0), human liver spheroids were formed by combining differentiated HepaRG cells and stellate cells in a 384-well microplate (Corning) in liver spheroid-specific medium. Briefly, 50 µL containing 24 000 hepatocytes and 1000 stellate cells was pipetted into each well. The plate was centrifuged for 1 min at 300*g* and incubated at 37°C and 5% $CO_2$. Compact spheroids formed within 3 days. For the experiments, 40 spheroids were collected in a 24-well ultra-low attachment plate (Corning) in liver spheroid-specific medium until they were transferred into the multi-organ-chip.

The testicular organoids were formed by culturing $5 \times 10^5$ frozen-thawed testicular cells from one single donor in testicular organoid-specific medium (10% [v/v] CTS KnockOut SR XenoFree Medium, 1 × GlutaMAX and 1% [v/v] penicillin–streptomycin diluted in KnockOut Dulbecco Modified Eagle Medium; all from Thermo Fisher Scientific, Waltham, MA, USA) onto a 0.35% (w/v) agarose support, which was placed in 6.5-mm hanging Transwells® with 0.4-µm pore size (Corning) at the gas–liquid interphase and soaked overnight in culture medium. The testicular organoid-specific medium was also added to the basolateral compartment of the well (Baert *et al.*, 2017a, 2017b). The cells were allowed to form testicular organoids over 3 days ($t$ = day −3) before starting the static and multi-organ-chip culture experiments ($t$ = day 0).

The combined medium consisted of William's Medium E (PAN-Biotech) supplemented with 10% CTS™ KnockOut™ SR XenoFree Medium (Thermo Fisher Scientific), 5 µg/mL human insulin (PAN-Biotech), 2 mM L-glutamine (Corning), $5 \times 10^{-5}$ M hydrocortisone hemisuccinate (Sigma-Aldrich) and 1% [v/v] penicillin/streptomycin sulphate (Corning).

## Three-organ-chip design and fabrication

In this study, an entirely new type of organ-chip, the three-organ-chip, was designed and produced so that the 6.5-mm hanging Transwell® (Corning) containing the testicular organoid could be placed in the

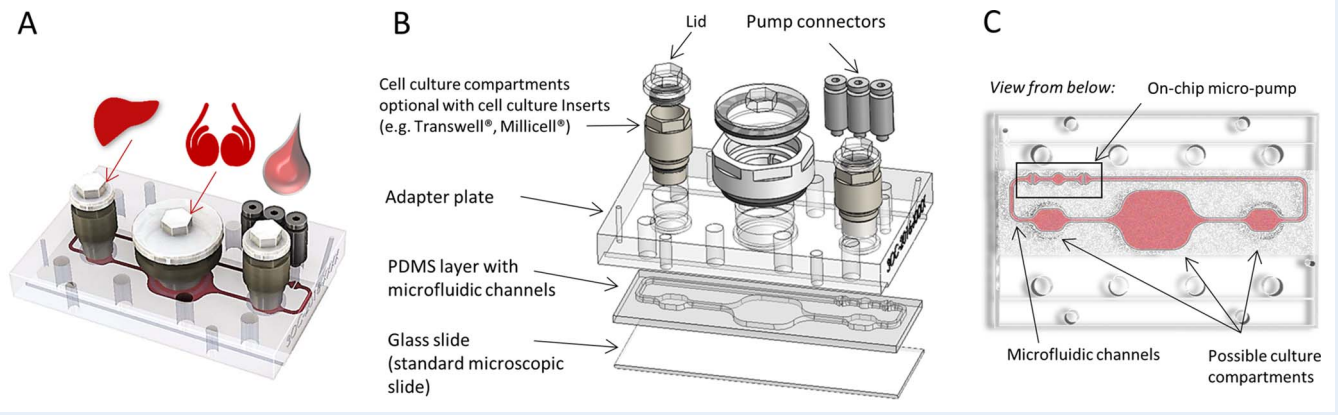


**Figure 1 Composition and features of the three-organ chip (3-OC).** (**A**) Arrangement of liver spheroids, the testicular organoid and the compartment for medium exchange. (**B**) Exploded view of the 3-OC pointing out its components. (**C**) View of the 3-OC from below highlighting the microfluidic layout.

middle reservoir (Fig. 1A). Fabrication of the three-organ-chip was performed as described by Wagner *et al*., (2013). Briefly, the three-organ-chip consists of one polydimethylsiloxane layer containing the microfluidics with a bonded glass slide (Menzel, Braunschweig, Germany) creating one closed microfluidic circuit per chip (Fig. 1B).

The circuit consists of two tissue culture compartments with a diameter of 6.5 mm (96-well size) and one bigger compartment with a diameter of 13 mm (24-well size) in the middle, which are connected via the microchannel. The microchannel is 250 µm high and 1000 µm wide and has a volume of 20 µL. Furthermore, three pump membranes are integrated into the circuit where the polydimethylsiloxane layer has a thickness of only 500 µm. Right above the membranes, the pump connection ports connect the organ chip to the control unit. The pressure and vacuum applied to the three membranes in a defined temporal pattern result in their rhythmic lifting and lowering, thus inducing a peristaltic circulation of the medium with a distinct flow velocity (Fig. 1C). The on-chip peristaltic pump was originally published by Wu *et al*., (2008) and was optimised for use in the three-organ-chip.

## Multi-organ-chip cultures

Two strategies are used to grow and maintain cells in the three-organ-chip: direct cultivation in the tissue culture compartments (liver spheroids) or cultivation in cell culture inserts (testicular organoids) that are residing within the tissue culture compartments (Fig. 2). The porous membrane of the culture inserts allows the exchange of nutrients and substances between the tissue culture compartment and the cell culture medium that is passing by beneath the inserts. Using this culture strategy, the cells are not in direct contact with the flow and, thus, encounter considerably fewer shear forces than in direct culture without cell culture inserts.

In the three-organ-chip, 40 liver spheroids and one testicular organoid were loaded into the spatially separated culture compartments of a common circuit. Other circuits were loaded with either 40 liver spheroids solely or one testicular organoid solely, for comparison. The liver culture compartment was filled with 400 µL medium, the compartment with the testicular organoid contained 200 and 600 µL was filled into the third compartment for the medium exchange (Fig. 2). This results in a total volume of 1.2 mL. Half of the medium was exchanged (−/+ 600 µL) every 24 h. Daily, medium samples were collected for glucose, lactate and LDH analyses. A peristaltic on-chip micropump enabled a continuous pulsatile flow with a pumping frequency of 0.5 Hz and a pressure of 500 mbar. The medium distribution and pumping stetting results in an average volume flow of 9.3 µL/min. Cultures were ended after 7 days, and organ equivalents were collected from the culture compartments for further analyses. Static cultures were cultured equivalent to the dynamic cultures in 24-well ultra-low attachment multi-well plates. All cultures, either static or in the multi-organ-chip, were cultivated under normal culture conditions at 37°C and 5% $CO_2$.

## Substance used and application

One gram of cyclophosphamide monohydrate, 97+ % (Alfa Aesar, MA, USA) powder was dissolved in 1 mL dimethyl sulfoxide (VWR Chemicals, PA, USA). A daily systemic exposure with cyclophosphamide monohydrate, 97+ %, was started after a three-day administration-free adaptation period and was performed over a period of 4 days (Maschmeyer *et al*., 2015b). The systemic substance exposure was performed through the medium exchanges. From Day 3 of culture on, the medium was supplemented with cyclophosphamide to produce final concentrations of 50 (Day 4), 100 (Day 5) and 200 µg/mL (Days 6–7) to cover the range of serum levels reported in patients (Cunningham *et al*., 1988; Perry *et al*., 1999). Control cultures were given the equivalent amount of dimethyl sulfoxide (vehicle), with the final concentration ranging between 0.005 and 0.01%.

## Cell viability and metabolic activity

All samples were analysed photometrically with the Indiko Plus (Thermo Fisher Scientific), according to the manufacturer's instructions. The following reagents were used for measurement: GLUC-S Hexokinase 981779, LAC LOX-PAP 3011 and LDH IFCC 981782 (Thermo Fisher Diagnostic). Testicular organoids from three different donors were used for this experiment.

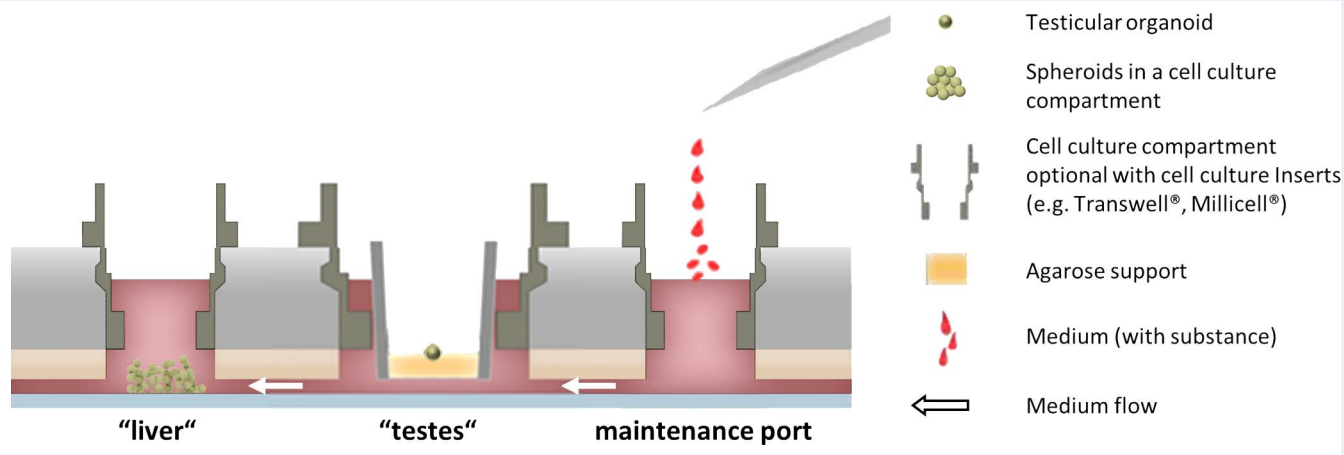


**Figure 2 The 3-OC culture system.** Cultivation of the testicular organoid within a Transwell® at the air-liquid interface (middle tissue culture compartment). The culture compartment below the testicular organoid is filled with 200 µL medium. The left tissue culture compartment holds 40 liver spheroids submersed in 400 µL medium. The right compartment is the maintenance port, where 600 µL medium is added and exchanged every 24 h.

## Hormone measurements

Testosterone was measured with the Elecsys Testosterone II competitive immunoassay on the Cobas 6000 instrument (Roche Diagnostics, Basel, Switzerland). Firstly, the samples were incubated with a biotinylated monoclonal testosterone-specific antibody. After 9 min, streptavidin-coated microparticles and a testosterone derivate labelled with a ruthenium complex were added. The formed complex bound to the solid phase via the interaction of biotin and streptavidin. The reaction mixture was aspirated into the measuring compartment, where the microparticles were magnetically captured onto the surface of the electrode. Unbound substances were removed with ProCell/ProCell M (Roche Diagnostics). The application of a voltage to the electrode induced chemiluminescent emission, which was measured by a photomultiplier. The functional sensitivity of the assay was 0.12 µg/L. The total imprecision coefficients of variation (%) were 4.9% at 0.37 µg/L, 4.1% at 0.69 µg/L, 2.9% at 1.74 µg/L, 3.4% at 2.51 µg/L, 2.9% at 4.64 µg/L, 3.1% at 6.09 µg/L and 2.2% at 12.7 µg/L.

Serum inhibin B was measured using a solid-phase sandwich enzyme-linked immunoassay (Inhibin B Gen II Elisa, Beckman Coulter Inc., CA, USA). The samples were incubated in microtiter plates coated with a monoclonal antibody against anti-activin B. Next, the wells were incubated with biotinylated anti-inhibin $\alpha$-subunit detection antibody and streptavidin labelled with the enzyme horseradish peroxidase. Afterwards, the wells were incubated with the substrate tetramethylbenzidine. The reaction was stopped by an acidic stopping solution. The absorbance was measured at 450 nm. The limit of quantification was 4.8 ng/L and between-run CVs were 12.0% at 95 ng/L, 11.0% at 125 ng/L and 9.6% at 145 ng/L.

## Histology and immunofluorescence

Representative cryostat central sections of 8 µm of the liver spheroids were embedded in Tissue-Tek® O.C.T. compound (Sakura Finetek, Torrance, CA, USA) for immunohistochemistry. The liver spheroids were stained using the Apo Direct Apoptosis Detection Kit (Thermo Fischer Scientific), according to the manufacturer's instructions. All slides were fixed in acetone at −20°C for 10 min, washed with phosphate-buffered saline without $Mg^{2+}$ and $Ca^{2+}$ ($PBS^{-}$) and blocked with 10% [v/v] goat serum in $PBS^{-}$ for 20 min. The primary antibody mouse anti-human Ki67 (14-5699-82, 1/100; Thermo Fischer Scientific) was used for the combined staining with TUNEL. Further single immunofluorescence stainings were performed with mouse anti-human CYP3A4 (sc-53850, 1/100; Santa Cruz Biotechnology Inc.) and mouse anti-human albumin (A6684, 1/100; Sigma-Aldrich). All primary antibodies were applied for 2 hr, washed with $PBS^{-}$ and then stained by goat anti-mouse CF594 (20119, 1/200; Biotium, Fremont, CA, USA) for 45 min. 4′,6-Diamidino-2-phenylindole (Roche) was added for nuclei staining. A Keyence BZ-X700 Microscope with BZ-II-Viewer software was used for the microscopic imaging. Images were merged using the BZ II-Analyzer software.

At culture day 7, testicular organoids were fixed with 4% paraformaldehyde, then covered by a drop of 2% agarose (Sigma-Aldrich) to allow manipulation and embedding in paraffin. Sections were stained with periodic acid-Schiff (VWR Chemicals, PA, USA) to study the histology. The presence of Leydig cells, peritubular myoid cells, Sertoli cells and spermatogonia was evaluated by specific immunostainings for STAR, ACTA2, SOX9 and UCHL1/DDX4, respectively. Testicular organoids from two different donors were assessed per condition and culture method. Antigen retrieval was performed in 0.01 M citrate buffer (pH 6.0) in a water bath at 95°C during 30 min. The samples were blocked for 30 min in PBS with $Mg^{2+}$ and $Ca^{2+}$ ($PBS^{+}$) containing 10% [v/v] normal donkey serum (Bio-Connect, TE Huissen, the Netherlands). Rabbit polyclonal anti-STAR (sc-25806, 1/200; Bio-Connect), mouse monoclonal anti-ACTA2 (A2547, 1/400; Sigma-Aldrich) or rabbit polyclonal anti-SOX9 (AB5535, 1/400; Merck, NJ, USA) primary antibody was added for overnight incubation at 4°C. Mouse monoclonal anti-UCHL1 (7863-2004, 1/200; Bio-Rad, Belgium) and goat polyclonal anti-DDX4 (AF2030, 1/200; R&D systems, MN, USA) antibodies were added simultaneously for double immunostainings. The next day, the sections were incubated with a donkey anti-rabbit 488 (A21206, 1:200; Thermo Fisher Scientific), donkey anti-mouse 488 (A21202, 1/200; Thermo Fisher Scientific)

and/or donkey anti-goat 594 (A11058, 1/200; Thermo Fisher Scientific) secondary antibody for 1 h at room temperature. All washing steps were performed in $PBS^{+}$. The sections were mounted with 4′,6-diamidino-2-phenylindole (Thermo Fisher Scientific), and the coverslips were sealed with nail polish.

In order to study the germ cell toxicity of cyclophosphamide, the difference in proportion of $DDX4^{+}$ cells was determined on whole sections of cyclophosphamide- and vehicle-treated testicular organoids, which have been cultured individually or together with liver spheroids on multi-organ-chips. Per testicular organoid, analyses were done on sections taken at three different depths separated by 25 µm using the following formula:

$$\text{change in germ cell proportion in \%} = \left(\left(\frac{\text{DDX4 cells}}{\text{total cells}} \text{ in testicular organoids treated with cyclophosphamide}\right) - \left(\frac{\text{DDX4 cells}}{\text{total cells}} \text{ in testicular organoids treated with vehicle}\right)\right) \times 100$$

Testicular organoids from three different donors were used for this experiment.

## Real-time quantitative polymerase chain reaction

The liver spheroids were collected for RNA isolation using the NucleoSpin RNA Kit (Macherey-Nagel, Düren, Germany). Complementary DNA was synthesised by reverse transcription of 200 ng total RNA using the TaqMan® Reverse Transcription Kit (Thermo Fisher Scientific). Quantitative PCR was performed using the QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific) and the SensiFAST SYBR Lo-ROX Kit (Bioline, Luckenwalde, Germany) according to the manufacturers' instructions with *TBP* and *SDHA* as housekeeping genes. The real-time qPCR primers were used as listed in Table I.

**Table I Primer sequences used in this study.**

| Target | Primer | Sequence |
|---|---|---|
| ***ALB*** | Forward | TCAGCTCTGGAAGTCGATGAAAC |
| | Reverse | AGTTGCTCTTTTGTTGCCTTGG |
| ***CYP1A1*** | Forward | ATCCCCCACAGCACAACAAG |
| | Reverse | AGCACCATCAGGGGTGAGAA |
| ***CYP2A6*** | Forward | GTACCCTATGCTGGGCTCTGTG |
| | Reverse | CCTTAGGTGACTGGGAGGACTTG |
| ***CYP2B6*** | Forward | ACCAGACGCCTTCAATCCTG |
| | Reverse | GGGTATTTTGCCCACACCAC |
| ***CYP2C19*** | Forward | AGCTGGGACAGAGACAACAAGC |
| | Reverse | CGTCACAGGTCACTGCATGG |
| ***CYP2C9*** | Forward | GGTGGGGAGAAGGTCAATGTATC |
| | Reverse | GACAGAGACGACAAGCACAACC |
| ***CYP3A4*** | Forward | GGAAGTGGACCCAGAAACTGC |
| | Reverse | TTACGGTGCCATCCCTTGAC |
| ***MDR1*** | Forward | TGGATGTTTCCGGTTTGGAG |
| | Reverse | TGTGGGCTGCTGATATTTTGG |
| ***MRP1*** | Forward | ACCTCATCCAGTCCACCATCC |
| | Reverse | GGCCATGCTGTAGAAAAGACCTC |
| ***SDHA*** | Forward | TCCAGGGGCAACAGAAGAAG |
| | Reverse | TTGTCTCATCAGTAGGAGCGAATG |
| ***TBP*** | Forward | CCTTGTGCTCACCCACCAAC |
| | Reverse | TCGTCTTCCTGAATCCCTTTAGAATAG |
| ***UGT1A1*** | Forward | TTTTGTTGGTGGAATCAACTGC |
| | Reverse | CCCAAAGAGAAAACCACAATTCC |
| ***UGT2B28*** | Forward | ATTGCTCACATGAAGGCCAAG |
| | Reverse | TGCAGGGGCTTTACTGGTTG |

## Statistical analysis

We focused on comparing the effect of three medium types within different culture setups (static and chip-based with testicular organoids and/or liver spheroids) on glucose, lactate, LDH, testosterone and inhibin B levels. For each variable of interest, the medium levels obtained over 7 days were corrected for background in the respective fresh medium and modelled with a linear mixed model to evaluate the evolution over time as well as the differences at the final measurement to explain the direction of the change. The day is, therefore, included in the model as a predictor, possibly extended with higher order polynomial terms (second/third order) to allow for curvilinearity in the evolution. Specific differences in evolution or final score were focused upon using contrasts, while correcting for multiplicity with the method of Holm. Model selection was primarily performed with AIC. The observations showed very consistent patterns suggesting a very small measurement error, except for four measurements. These were identified as outliers and excluded from the analysis, as illustrated in Supplementary Figs. S1 and S3A. The improved model was used to determine the condition-specific predicted scores over time. The germ cell count in response to cyclophosphamide was analysed with a Mantel-Haenszel test for common odds ratio equal to one and is represented as mean ± standard deviation. A difference was considered statistically significant if the *P*-value was $<0.05$. Analyses were performed with R version 3.5.1 (2018-07-02).

The qPCR data were analysed with an unpaired two-tailed Student's *t* test or ANOVA. A *P* value $<0.05$ was considered statistically significant. Data are represented as mean ± standard error of the mean.

# Results

## Effect of different media on the metabolic activity and viability of testicular organoid and liver spheroid cultures

After the testicular organoids and the liver spheroids were allowed to form for 3 days in well plates containing their respective medium, they were either kept in well plates or transferred to the multi-organ-chips, either separately or in co-culture, containing one of three medium compositions: (i) testicular organoid-specific medium, (ii) liver spheroid-specific medium or (iii) a combined medium.

In the presence of liver spheroid-specific medium, better overall metabolic activity was seen in the co-cultures, evidenced by the

steepest decline in glucose levels, which corresponds to an increasing glucose uptake, and the increase in lactate levels, which corresponds to an increasing lactate production ($P < 0.05$) (Fig. 3A and B; co-culture: full red line). In contrast, the glucose uptake was comparable for all liver spheroid cultures, regardless of the culture device or the medium used (Fig. 3A; LS: full and dotted lines). The best glucose uptake in the static testicular organoid cultures was observed in the presence of the liver spheroid-specific medium ($P < 0.05$) (Fig. 3A; TO: dotted red lines). While, for testicular organoids cultured on multi-organ-chips, the course of glucose uptake differed significantly between those cultured in liver spheroid-specific medium and in testicular organoid-specific medium (Fig. 3A; TO: full red and blue lines), the glucose levels at Day 7 were comparable (Fig. 3A; TO: full lines). For both the static and dynamic liver spheroid or testicular organoid cultures, the highest lactate production was observed in the presence of liver spheroid-specific medium ($P < 0.05$) (Fig. 3B; LS and TO: full and dotted red lines).

Although the liver spheroid-specific medium best supported the metabolic activity in the co-culture setup, it also resulted in significantly different LDH release slopes with the highest levels at Day 7, which is indicative of more cell damage ($P < 0.05$) (Fig. 3C; co-culture: full red line). However, this trend was not observed in the individual testicular organoid cultures where there was no evidence to suggest that the choice of medium had an influence on the LDH slopes or Day 7 levels (Fig. 3C; TO: full and dotted lines). Moreover, liver spheroids released significantly lower levels of LDH (higher cell viability) when cultured in liver spheroid-specific medium in compared to testicular organoid-specific medium (static) and combined medium (multi-organ-chip) ($P < 0.05$) (Fig. 3C; LS: full and dotted red lines, dotted blue line and full green line).

The data points of the individual biological replicates are included in Supplementary Fig. S1.

## Liver spheroid morphology, cell composition and cell function

Both the functionality and morphology of representative liver equivalents were evaluated after 7 days of single culture in all three media and of co-culture in 3-OCs in liver-specific media. TUNEL staining in the liver spheroids showed very few apoptotic cells in any culture conditions. The most apoptotic cells were seen in co-culture, nevertheless, many $Ki67^+$ proliferating cells were seen in all cultures. Furthermore, expression of a characteristic hepatocyte marker was confirmed by staining for albumin (Fig. 4A). Finally, mRNA expression patterns of liver spheroids in single and co-culture were compared and showed stable expression of, for example, *ALB*, *MDR1* and *UGT1A1*. The expression of cytochromes differed slightly between different conditions; *CYP2B6* was 29-fold higher expressed in liver single cultures in the combined medium compared to the single cultured liver spheroids in liver spheroid-specific medium. Nevertheless, this increase was not statistically significant. The liver spheroids cultured in liver spheroid-specific medium in co-culture compared to single culture showed a lower mRNA expression of *CYP1A1* and *CYP3A4*, yet neither were significant. Single cultures in testicular organoid-specific medium showed a significantly lower expression of *CYP3A4* and a slightly higher expression of *UGT2B28* compared to single-liver spheroid cultures with liver spheroid-specific medium (Fig. 4B).

## Characterisation of testicular organoids in co-culture containing liver spheroid medium

Based on the overall metabolic activity and viability of testicular organoid and liver spheroid cultures and liver-specific analyses, liver spheroid-specific medium was preferred over testicular organoid-specific medium and the combined medium as the culture medium for co-culture purposes. A new experiment was set up to characterise the cell type composition and hormone secretory profile of testicular organoids in response to liver spheroid-specific medium with the following groups: testicular organoids cultured in static (i), in the multi-organ-chip (ii) or in co-culture in the multi-organ chip (iii). The testicular organoids cultured in testicular organoid-specific medium were used as reference.

Reference testicular organoids typically developed into 3D multicellular tissue structures with random arrangements of $STAR^+$, $ACTA^+$ and $SOX9^+$ cells, which correspond to Leydig cells, peritubular myoid cells and Sertoli cells, respectively (Fig. 5 and Supplementary Fig. S2). Staining for UCHL1 was combined with staining for the germ cell marker DDX4 for the detection of spermatogonia (Supplementary Fig. S2). These cells appeared as clumps or were part of bigger clusters containing other germ cell types ($UCHL1^-/DDX4^+$). All testicular organoids cultured in liver spheroid-specific medium displayed similar morphology, histology and cell type composition (Fig. 5).

Tests were conducted to analyse the slope homogeneity of their regression lines over 1 week in culture and Day 7 levels to compare the secretory profiles of testosterone and inhibin B between the different groups (Fig. 3D and E). The testosterone slope of TO/Static/TO medium was significantly different from all other groups (Fig. 3D: blue dotted line). Moreover, testicular organoids in liver spheroid-specific medium in static and in co-cultures produced nearly background testosterone levels (Fig. 3D: dotted red line and full red line with triangles), which were statistically lower than levels found in the reference condition at Day 7 (Fig. 3D: blue dotted line). By comparison, individually cultured chip-based testicular organoids produced detectable amounts of testosterone (Fig. 3D: full line with circles), which were equal to those of the reference group at Day 7 (Fig. 3D: blue dotted line). Intriguingly, co-cultured and static testicular organoids had a similar inhibin B secretory profile (Fig. 3E: full red line with triangles and dotted red line), and both showed a significantly higher inhibin B production compared to single cultured testicular organoids in the multi-organ chip and the reference ($P < 0.05$) (Fig. 3E: full red line with circles and dotted blue line).

The data points of the individual biological replicates are included in Supplementary Fig. S1.

## Effect of cyclophosphamide exposure and co-culture on the germ cell count in testicular organoids

The multi-organ-chip culture was challenged with exposure to cyclophosphamide. A reduction of germ cells was expected in co-cultures in which cyclophosphamide is bioactivated by the liver spheroids, but not in single testicular organoid cultures. Cyclophosphamide was added to either single testis or co-cultures, and each one was compared to a vehicle control. The testicular organoids developed similarly to the vehicle-treated controls and preserved their defined 3D architecture, regardless of the treatment they

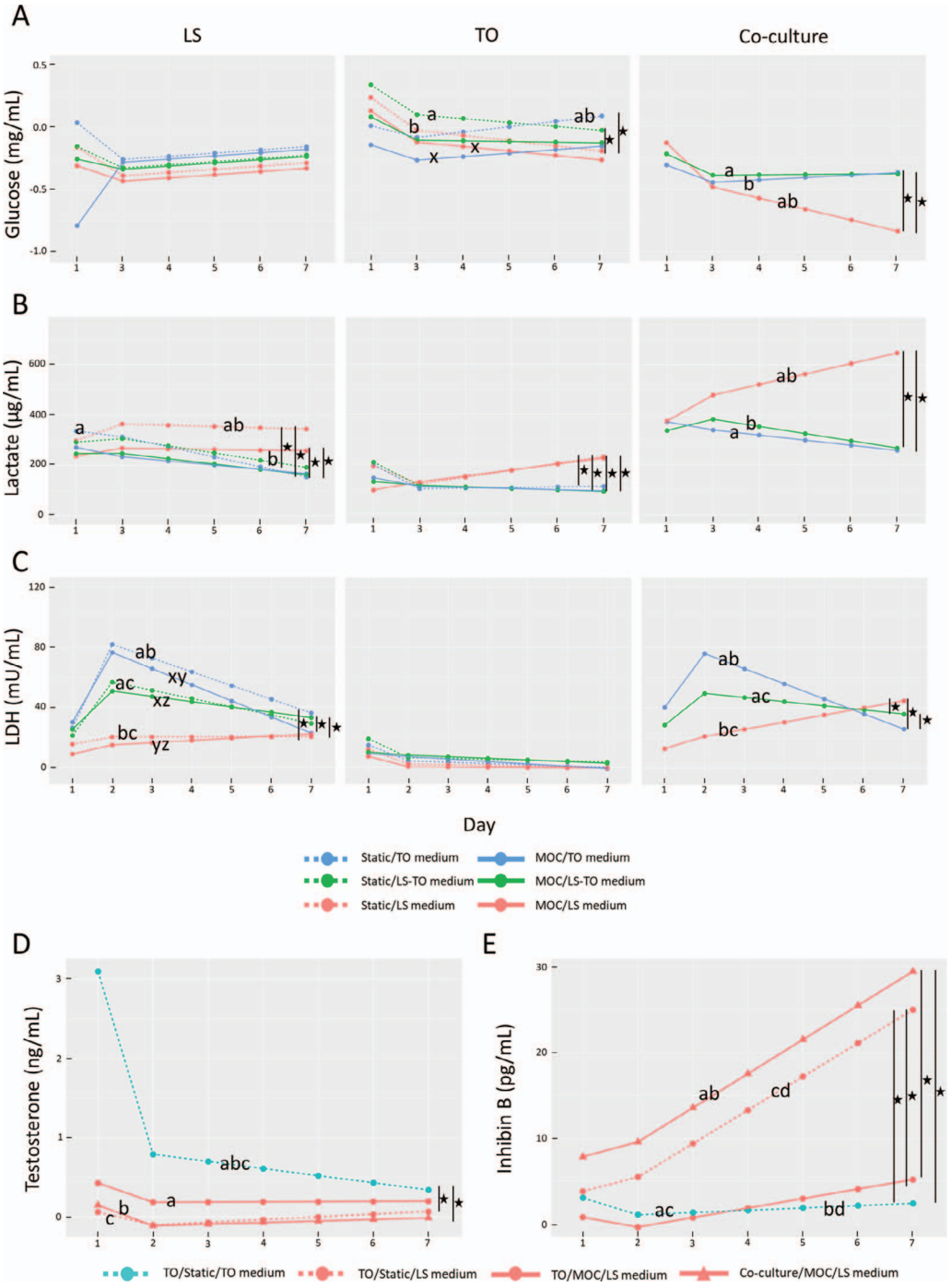


**Figure 3 Metabolic activity and hormone production in static and multi-organ-chip (MOC) cultures.** The predicted evolution of glucose uptake (**A**), lactate production (**B**), LDH release (**C**) and the production of testosterone (**D**) and inhibin B (**E**) was based on the linear mixed model using daily measurements. Statistical differences were assessed between Day 2 and Day 7 (change), and at Day 7 (mean) to evaluate the effect of different media and culture devices. (**A**, **B**) The metabolic activity in the co-cultures is highest when LS medium (red) is used. (**C**) Although, on Day 7, (liver spheroid) LS medium resulted in higher LDH values compared to (testicular organoid) TO medium (blue) and LS-TO medium (green) in the co-culture setup, in static (dotted line) and MOC-based (full line) single cultures, LDH levels were comparable or lower in LS medium. Regression lines sharing the same letter are significantly different ($P < 0.05$), $^*P < 0.05$. $N = 3$ biological replicates for each data point. (**D**) Testosterone was nearly undetectable in the co-cultures. Individually cultured chip-based TOs displayed a latent production of testosterone with similar levels at Day 7 compared to the reference (TO/Static/TO medium). (**E**) Remarkably, the highest inhibin B secretion was noted in the co-culture. Regression lines having the same letter are significantly different ($P < 0.05$), $^*P < 0.05$. $N = 2$ biological replicates for each data point.

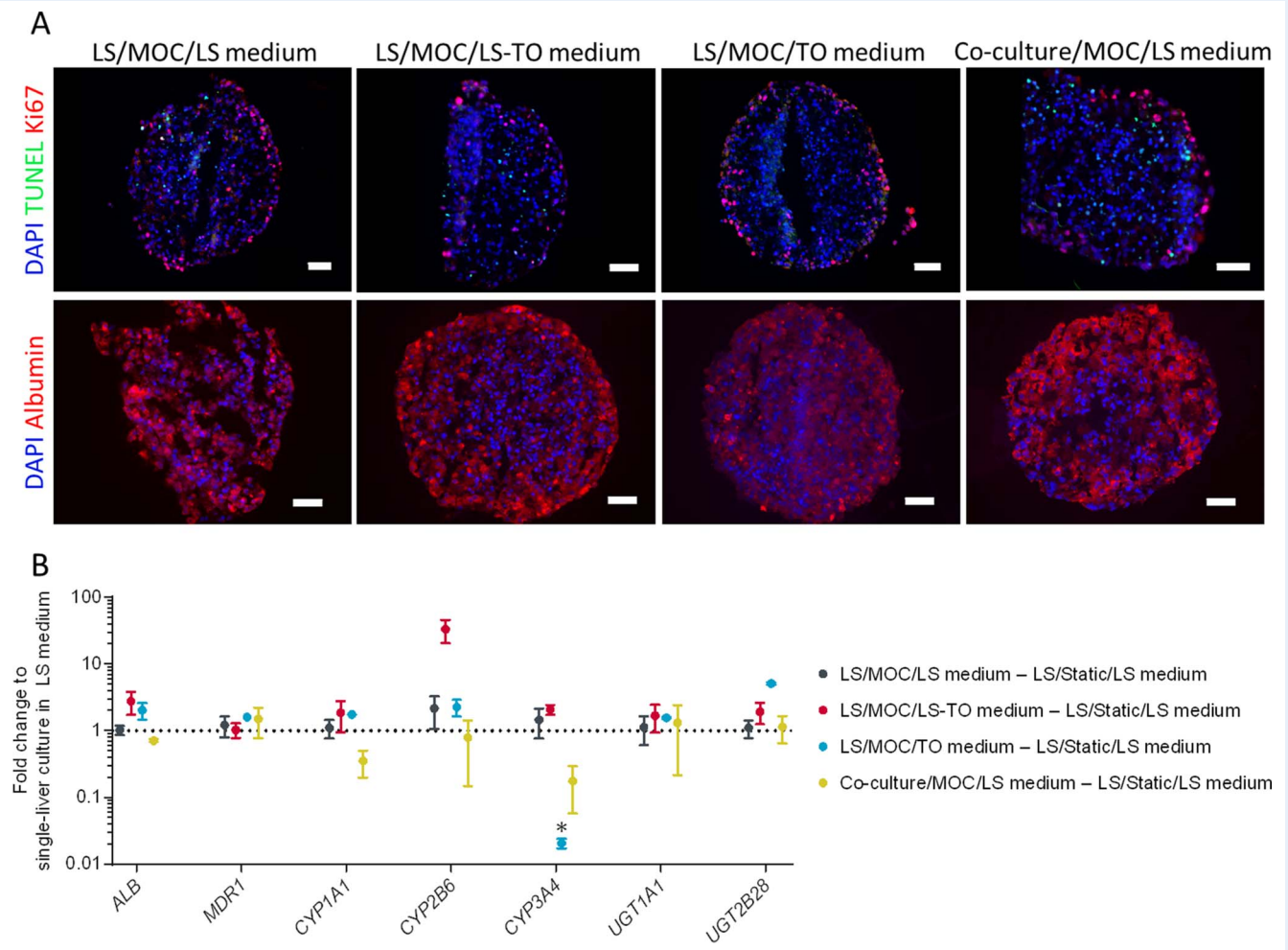


**Figure 4 Evaluation of liver spheroids (LSs) in single multi-organ-chip (MOC) culture containing LS medium, LS-TO medium or testicular organoid medium (TO medium) or in co-culture containing LS medium.** (**A**) Representative pictures of LSs cultured in the MOC showed very few TUNEL+ cells and several Ki67+ cells in all culture conditions (top row). Expression of albumin was detected in all culture conditions (lower row). Scale bars: 50 µm. (**B**) mRNA expression of LSs in single- and co-culture shown as fold change to static LS cultures in LS medium. Data are shown as mean (line) with SEM. $N = 3$ biological replicates per group, $^*P < 0.05$.

received. Expression of DDX4 was used to identify the number of germ cells inside the testicular organoids using immunofluorescence (Fig. 6A and B). The averaged difference between germ cell numbers of co-cultured testicular organoids treated with cyclophosphamide and those with the vehicle was $-9 \pm 8\%$, which indicated a considerable germ cell loss after exposure to cyclophosphamide. In contrast, for the single testicular organoid cultures, more germ cells survived the cyclophosphamide treatment ($+6 \pm 12\%$), which was significantly different from the co-culture condition ($P < 0.05$) (Fig. 6C).

Staining of liver spheroids at Day 7 of cyclophosphamide treatment showed no increase of TUNEL$^+$ cells compared to the control and several Ki67$^+$ cell nuclei could be seen in both the vehicle and treated cultures (Fig. 7A). Staining for albumin and CYP3A4 was shown in treated and non-treated spheroids. For *CYP3A4* mRNA expression, a significantly increased fold change of 9.53 could be observed in treated liver spheroids (Fig. 7B). Additionally, *CYP2B6* showed a significant increase of 9.71-fold compared to the vehicle control. Furthermore, *CYP2A6*, *UGT1A1* and *UGT2B28* showed a non-significantly increased change of 1.96-, 2.18- and 1.97-fold, respectively, in treated co-cultures compared to the vehicle control (Fig. 7C). *ALB*, *MDR1*, *MRP1*, *CYP1A1*, *CYP2C9* and *CYP2C19* showed similar expression in both culture conditions.

The cyclophosphamide treatment showed no effect on the overall metabolic activity, nor on the viability of either the individual testicular organoid cultures or co-cultures (Supplementary Fig. S3B). The data points of the individual biological replicates are included in Supplementary Fig. S3A.

## Discussion

The field of toxicology, which aim is to assure the safety of chemicals used in all aspects of modern society, would welcome an *in vitro* model that allows the identification of environmental or pharmacological

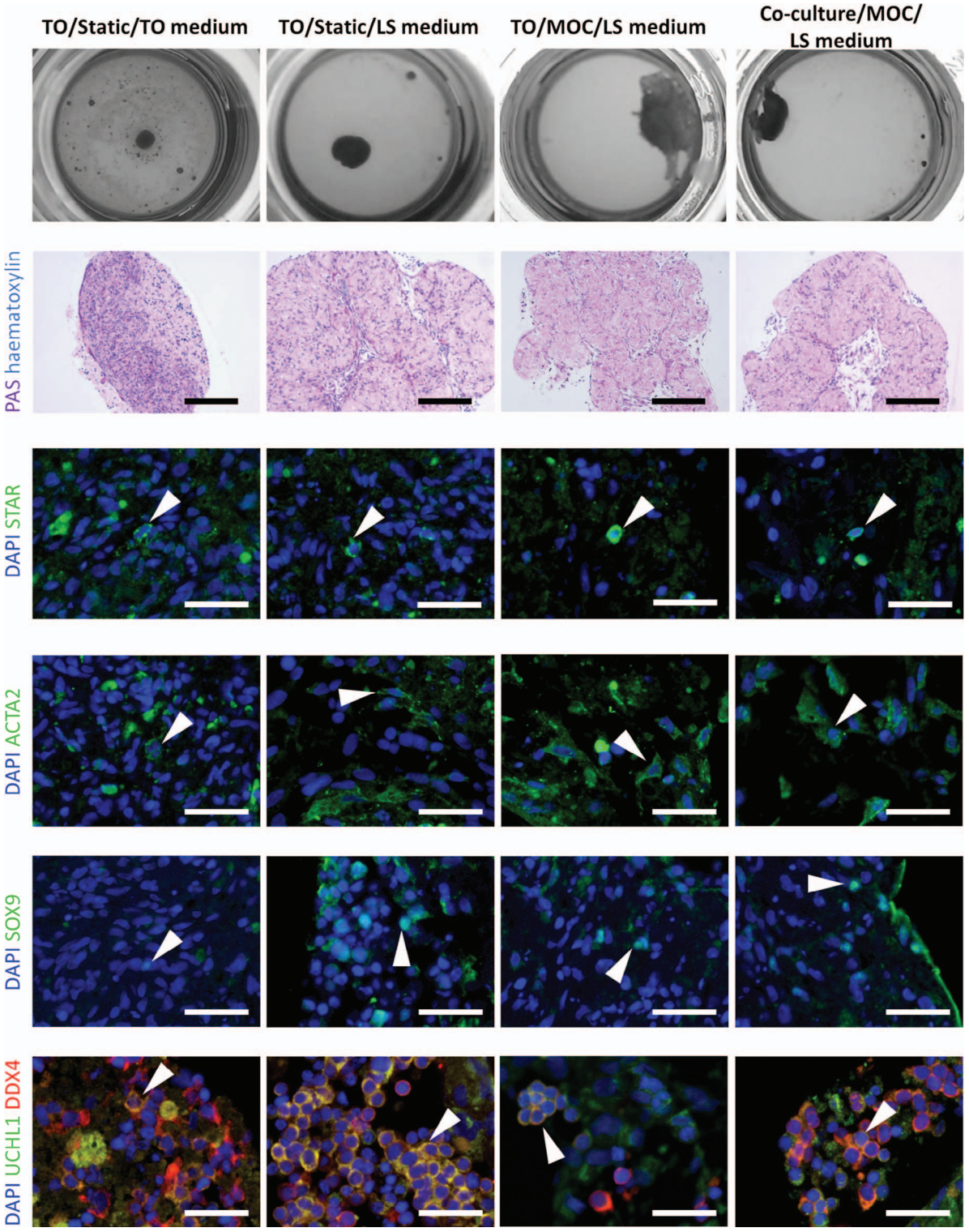


**Figure 5 Microscopic evaluations of testicular organoids (TOs) in co-culture containing liver spheroid (LS) medium.** Representative pictures of TOs co-cultured with LSs on a multi-organ-chip (MOC; fourth column) showing comparable gross morphology (first row) and histology (second row) compared to the control conditions, i.e. single culture of TOs in a well plate (Static, second column) and on MOC (third column), and the reference method using well plates containing TO medium (TO/Static/TO medium). The reference TOs contained STAR+ Leydig cells (third row), ACTA2+ peritubular myoid cells (fourth row), SOX9+ Sertoli cells (fifth row) and UCHL1+/DDX4+ spermatogonia (sixth row), indicated by the white arrowhead. Similar observations were made in the experimental groups. $N = 2$ biological replicates per group. Scale bars: 200 µm (second row), 50 µm (third to sixth row).

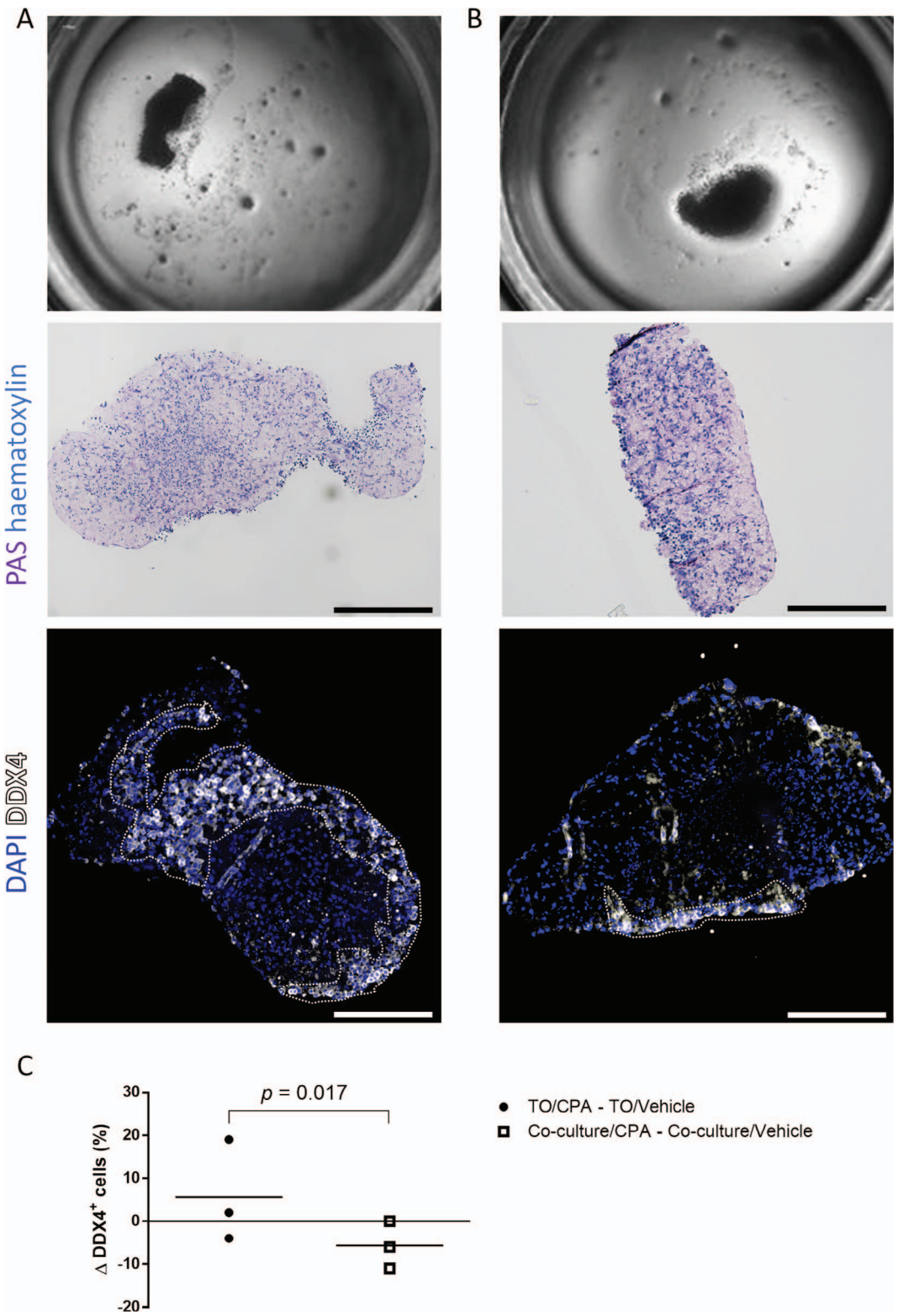


**Figure 6 Effect of cyclophosphamide (CPA) exposure and co-culture on germ cell count in testicular organoids (TOs).** (**A**, **B**) Representative pictures of the gross morphology (first row), histology (second row) and DDX4 immunostaining (third row) of a co-cultured TO treated with vehicle (A) or CPA (B). The dotted line depicts the largest area with germ cells in the TO. (**C**) The difference in proportion of DDX4+ cells after treatment with either CPA or vehicle was significantly lower in the co-culture condition compared to the single culture of TOs. Data are shown as mean (line) with individual biological replicates ($n = 3$). Scale bars: 800 µm (middle row), 400 µm (bottom row).

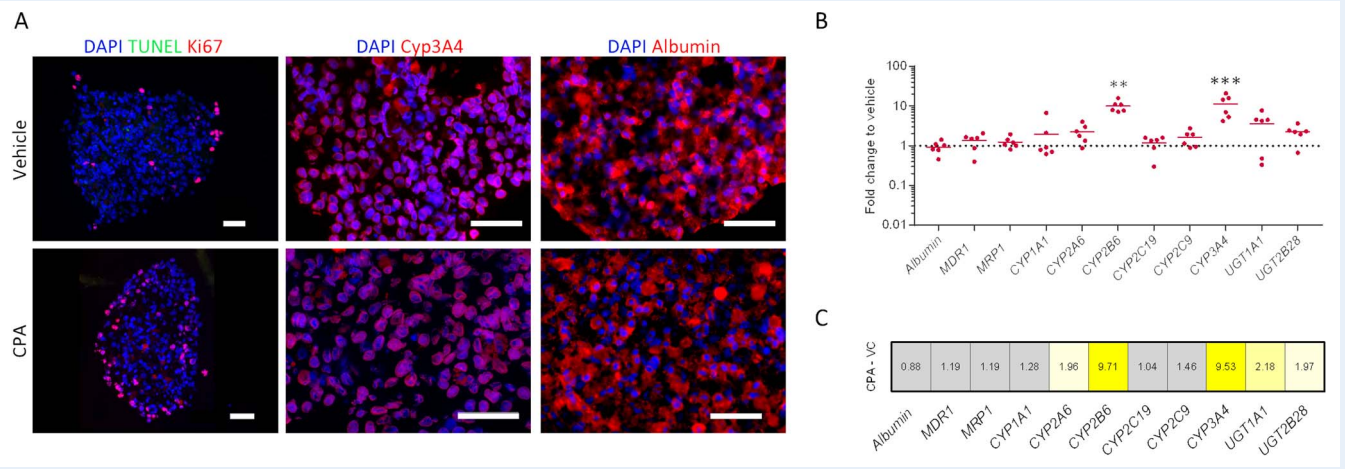


**Figure 7 Effect of cyclophosphamide (CPA) exposure on liver spheroids (LSs) in co-culture.** (**A**) Representative pictures of LSs stained for TUNEL and Ki67 (left row, Cytochrome 3A4 (middle row) and albumin (right row) for LSs in co-culture treated with vehicle or CPA. Scale bars: 50 µm. (**B**) mRNA expression of LSs in CPA-treated cultures as fold change to vehicle control. Data are shown as mean (line) with individual biological replicates. $N = 6$ biological replicates per group, $^{**}P < 0.01$; $^{***}P < 0.001$.

compounds while minimising redundant animal experiments. On the one hand, there is a general increasing desire to use fewer animals in research and to contribute to improving animal welfare in testing through the three Rs principles (Liebsch *et al*., 2011). On the other hand, animal tests do not always correctly predict human safety (Olson *et al*., 2000; Habert *et al*., 2014). A wide variety of human *in vitro* testis models have been published. These models provided valuable information on possible toxicants using concentrations relevant to human exposures. They are, however, limited in widespread applicability due to the lack of a systemic approach and, therefore, fail to predict the interaction of metabolites between organs (Parks Saldutti, 2013). To address this problem, we combined our human testicular model with a human liver equivalent through a microfluidic flow in a multi-organ-chip allowing crosstalk between both models (Bauer *et al*., 2017; Baert *et al*., 2017a). Both tissue equivalents developed accordingly, were stable in culture, showed tissue-specific expression of markers and secretion of hormones, and were able to respond to a toxic insult of the promutagen cyclophosphamide. To the best of our knowledge, this is the first report on a human reprotoxicity model including a systemic approach, i.e. the liver–testis axis.

At first, a culture experiment was designed to find a common medium supporting both tissues. The testicular organoid-specific medium and the liver spheroid-specific medium were used as the reference culture conditions for testicular organoids and liver spheroids, respectively, according to previous publications (Bauer *et al*., 2017; Baert *et al*., 2017a). In addition, we included a combined medium containing the necessary supplements for liver spheroid function (human insulin, hydrocortisone hemisuccinate) but with a serum supplement instead of fetal calf serum, since the former has proven critical for the functionality of *in vitro* testis models (Sato *et al*., 2011). Moreover, it was an attempt to develop a toxicity model in an animal component-free environment. However, metabolic activities of the co-culture, measured as glucose consumption and lactate production, were more favourable when liver spheroid-specific medium was used. This observation was confirmed by lactate production in the static and single groups, with potentially important implications given that lactate has a promoting effect on germ cell maintenance (Erkkilä *et al*. 2002). Glucose consumption was comparable in most static and single groups, regardless of the medium used, except for static cultures with liver spheroid-specific medium which achieved a better outcome compared to the other media. Surprisingly, Day 7 viability was lowest (= highest LDH levels) in the liver spheroid-specific medium co-cultures even though this medium resulted in comparable or lower LDH-release in the static and multi-organ-chip-based single cultures of testicular organoids and liver spheroids. The serum supplement in testicular organoid and combined medium is well-defined and comprises of albumin, transferrin, insulin, collagen precursors, some amino acids and vitamins, antioxidants and trace elements (Price *et al*., 1998). By contrast, fetal calf serum in liver spheroid-specific medium is derived from animals and contains large quantities of undefined proteins that might have triggered unwanted feedback loops or actions specifically in the co-culture, resulting in increased LDH release. However, this contradicts the results of the testicular organoid-specific analyses. In the testis, LDH is found specifically in the germ cells (Hermo *et al*., 2009). We showed that, apart from the gross morphology, histology and presence of important niche cells, the appearance of the clumps of germ cells were also comparable between the co-culture with liver spheroid-specific medium and the reference using testicular organoid-specific medium. Therefore, alternative explanations could be that the increased LDH levels at culture Day 7 are caused by the $TUNEL^{+}$ cells observed in liver spheroids in co-culture or that the regression model we employed to analyse the evolution was based on a too low number of data points, which is the major limitation of this study. Despite the difficulty of recruiting a high number of human tissue donors for preclinical studies, primary testicular cells are still the 'gold standard' as they are more reflective of *in vivo* conditions than the currently available human testicular cell lines (Rahman and Huhtaniemi, 2004). It does, however, imply that a high-throughput adaptation of the presented

co-culture setup is unlikely until better surrogates for primary cells have been developed. The field is nevertheless evolving promisingly. For instance, Pendergraft and colleagues already reported on multicellular human testicular organoids that, besides primary peritubular cells and spermatogonial stem cells, consisted of immortalised Sertoli cells and Leydig cells (Pendergraft *et al*., 2017). In any case, for now, our model could be implemented as a supplementary test to validate selected chemicals suspected in other models to affect male reproductive outcomes.

Inhibin B and testosterone, secreted by Sertoli cells and Leydig cells, respectively, are important parameters of the functional state of these cells (Giagulli and Carbone, 2006). Testosterone and inhibin B were measured to evaluate the effect of the liver spheroid-specific medium on the endocrine functions of testicular organoids. Testicular organoid single cultures in the multi-organ-chip produced inhibin B at similar levels to the reference. Hormone secretion was highest in multi-organ-chip co-cultures and in static single testicular organoid cultures. Unexpectedly, in absence of the liver equivalent, single chip-based testicular organoid cultures secreted lower amounts of inhibin B than static cultures. This can be due to chemical gradients established in microfluidic devices (Halldorsson *et al*., 2015). In contrast to inhibin B, testosterone levels were low to undetectable in the co-cultures. This does not necessarily imply that testosterone was not produced. The lower concentration of testosterone in co-culture might be simply explained by the CYP450 metabolism which occurs in the liver (Wilson, 2000). It has been shown that HepaRG cells have the ability to metabolise testosterone through CYP3A4 activity (Nelson *et al*., 2017). The addition of 50 µM of testosterone caused a significant increase in CYP3A4 metabolism in HepaRG cells. An increased expression of *CYP3A4* could not be shown in our co-cultures, however, the testosterone concentration in single testis cultures was about 0.5 µg/mL and, therefore, 30-fold lower than that applied by Nelson and co-workers (2017). Therefore, a metabolism of testosterone can be expected, even though it could not be proven in our system, due to the comparably low amount of initial testosterone. This issue should be considered in future experiments needing free testosterone in the microfluidic circuit by strategically placing target organ models, such as skin, before the culture compartment of the liver spheroids or adapting the ratio of Leydig to HepaRG cells.

Furthermore, we did not observe an impact on cell apoptosis or proliferation of HepaRG cells by the different media that were tested. A few more apoptotic cells were seen in co-culture with the testicular organoids, nevertheless, proliferating cells kept the system in homeostasis. *ALB* was expressed in all cultures, as well as the transporter *MDR1*, and several phase I and phase II enzymes. Differences in phase I enzymes can be explained by different medium compositions, including different hydrocortisone concentrations. Overall, these data favoured co-culturing of testicular organoids and liver spheroids in liver spheroid-specific medium. Therefore, liver spheroid-specific medium was used in the second experiment aimed at obtaining proof-of-concept for detecting a toxic insult involving the liver–testis axis.

Cyclophosphamide was chosen as a model substance, because it is a known testicular toxicant requiring bioactivation by CYP450 enzymes and spontaneous conversion to the active metabolite phosphoramide mustard, which binds to DNA, thereby inhibiting DNA replication and initiating cell death (Zanger and Schwab, 2013). The ability of cyclophosphamide to interfere with all rapidly proliferating cells is the basis of its therapeutic effect and many of its toxic properties. Regarding the testis, this results mainly in the depletion of highly sensitive differentiating germ cells and, in extremis, the spermatogonial stem cells (Jahnukainen *et al*., 2011). Studies examining the effect of cyclophosphamide on the testis usually do so *in vivo* using animals or *in vitro* through the active metabolite phosphoramide mustard (Jia *et al*., 2015; Smart *et al*., 2018). Here, we exposed the testicular organoids in the multi-organ-chip to the prodrug cyclophosphamide. We supplemented the medium with a clinically relevant concentration range and were able to show a markable loss of $DDX4^+$ germ cells in the co-cultures. DDX4 is expressed in all germ cells, from spermatogonia until spermatid (Castrillon *et al*., 2000). Given that the germ cell clusters in testicular organoids consist of differentiating spermatogenic cells, proliferating spermatogonia and undifferentiated spermatogonial stem cells and their different sensitivity to cyclophosphamide (Baert *et al*., 2017a), we hypothesise that the remaining germ cells in testicular organoids after the toxic insult represent the silent stem cells. Importantly, no significant difference was observed in terms of overall viability and metabolic activity. This suggests that the germ cell loss was a specific phenomenon and not due to a general decline in cell number or massive cell death. We are not able to claim with absolute certainty that the cell loss was attributable to phosphoramide mustard, because the presence of this metabolite was not assessed. Nevertheless, the biotransformation of cyclophosphamide was evidenced by the absence of germ cell loss in chip-based single testicular organoid culture and the increased expression of the necessary activating enzymes in the liver spheroids, including *CYP2B6* and *CYP3A4*, after exposure. Importantly, also other studies have shown the CYP activation of cyclophosphamide by HepaRG cells *in vitro* (Le Hégarat *et al*., 2014; Yokoyama *et al*., 2018). The effect of cyclophosphamide on $DDX4^+$ germ cells in co-cultures but not in single cultures underlines how pivotal a continuous circulation of nutrients and metabolites is to emulate the crosstalk between the tissue equivalents. It is noteworthy that experiments in a hanging drop technology combining liver and tumour spheroids showed that the bioactivation by the liver was only efficient in perfused culture and could not be reproduced in static culture. Transferring a conditioned medium from treated liver spheroids to tumour spheroids did not result in growth inhibition (Frey *et al*., 2014).

Long-term experiments, particularly those testing effects of substances during the two-month period of human spermatogenesis, were not performed because sperm formation has not yet been achieved in human testicular organoids or any other 3D model. Interestingly, mouse *in vitro* spermatogenesis could be efficiently induced and maintained for an extended period of time (up to 6 months) by combining the organotypic culture method with a microfluidic device (Komeya *et al*., 2016, 2017). Considering the benefits of these devices replicating the microcirculatory system of the body, organotypic or organoid-on-chip cultures might play a leading role in establishing a sustainable human testis model. The main advantage of organotypic culture is the preservation of the testicular architecture, maintaining complex cellular interactions, but it excludes the possibility of specific cell manipulation before culture, which is offered by the organoids reported here. The disadvantage of the latter approach is the random distribution of the single cells without formation of spatial organisation after re-association, which might have a negative impact on cell functionality. The fact is that Keros and colleagues reported higher levels of testosterone in their tissue culture study compared to the levels produced

by the testicular organoids in both our and the Pendergraft study (Keros *et al*., 2005; Pendergraft *et al*., 2017). The testicular organoids were shaped by adult testicular cells, which are more easily available compared to immature cells. However, it is known that adult testicular cells display limited morphogenic capacity (Baert *et al*., 2017a; Strange *et al*., 2018). Liver spheroids composed of HepaRGs and HHSteCs also lack the tissue-specific architecture. Nevertheless, both tissue equivalents exhibit the presence of relevant cell types, stable hallmarks of *in vivo* functionality including secretory activity and physiologically relevant drug metabolisation, making them highly relevant models (Alves-Lopes and Stukenborg, 2018; Zhou *et al*., 2019). The choice between the organotypic and organoid approaches will depend on the necessity to start from a single-cell input.

In future experiments, the native tissue architecture could be restored in testicular organoids by taking the differential adhesion and motility properties of interstitial and tubular cells into account, as suggested by Sakid and colleagues (Sakib *et al*., 2019). The liver spheroids could be improved by replacing the HepaRG cells with primary human hepatocytes to select different phenotypes and genotypes. In addition, the co-culture can be expanded with other organ equivalents to generate the full ADME profiles of substances of interest (Maschmeyer *et al*., 2015b). Such co-cultures could facilitate the screening of factors that are able to influence testicular function (un)intentionally, whether they are physical (e.g. radiation and heat), biological (e.g. HIV) or chemical (e.g. endocrine disruptors) (Chapin *et al*., 2013). For instance, one potential application is the screening for male contraceptive drugs, where miRNA modification therapy has been proposed as an interesting opportunity (McIver *et al*., 2012). It has also been shown, using a xenograft model, that paracetamol can influence testicular function (van den Driesche *et al*., 2015). The present *in vitro* system could help to address the pressing need to understand which other types of common and worldwide over-the-counter medications or chemicals disturb spermatogenesis. The European Union has compiled a list of over 432 candidate substances (http://ec.europa.eu/environment/chemicals/endocrine/) which are to be studied further.

## Conclusions

This study reports the functionally interactive co-culture of liver and testis equivalents for the first time. The liver spheroids and testicular organoids were supported by a multi-organ-chip and connected by a microfluidic stream containing liver spheroid-specific medium. As such, the overall metabolic activity was maintained and liver spheroid survival and functionality were similar to the standard. The testicular organoids developed accordingly and contained spermatogonia and their niche cells, including inhibin B-producing Sertoli cells. While loss of germ cells in response to bioactivation of cyclophosphamide by liver enzymes is consistent with its clinical side effects, the production and consumption of testosterone by the testicular organoids and liver spheroids, respectively, is a physiological interaction between liver and testis. Both examples of organ crosstalk were reproduced in the co-culture. Taken together, by showing the integrity of characteristics and functionality of both models and their interaction, this study identified the potential use of multi-organ-chip-based co-cultures of testis and liver models as an R&D tool to expedite the efficacy and toxicity of substances on the liver–testis axis, which could be expanded by adding other tissue equivalents to the multi-organ-chip.

## Supplementary data

Supplementary data are available at *Human Reproduction* online.

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## Authors' roles

Y.B. and I.R. developed the project concept, performed experiments, analysed the results and wrote the manuscript. W.C. conducted the statistical analysis. A.O. and A.L. helped with performing the experiments. U.M., E.G. and I.M. were responsible for the project concept and funding, interpretation of results, critical discussion and review of the manuscript.

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## Conflict of interest

U.M. is founder, shareholder and CEO of TissUse GmbH, Berlin, Germany, a company commercializing the Multi-Organ-Chip platform systems used in the study. The other authors have no conflict of interest to declare.

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