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An Intestine/Liver Microphysiological System for Drug Pharmacokinetic and Toxicological Assessment

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TITLE:

An Intestine/Liver Microphysiological System for Drug Pharmacokinetic and Toxicological Assessment

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SUMMARY:

We exposed a microphysiological system (MPS) with intestine and liver organoids to acetaminophen (APAP). This article describes the methods for organoid production and APAP pharmacokinetic and toxicological property assessments in the MPS. It also describes the tissue functionality analyses necessary to validate the results.

ABSTRACT:

The recently introduced microphysiological systems (MPS) cultivating human organoids are expected to perform better than animals in the preclinical tests phase of drug developing process because they are genetically human and recapitulate the interplay among tissues. In this study, the human intestinal barrier (emulated by a co-culture of Caco-2 and HT-29 cells) and the liver

equivalent (emulated by spheroids made of differentiated HepaRG cells and human hepatic stellate cells) were integrated into a two-organ chip (2-OC) microfluidic device to assess some acetaminophen (APAP) pharmacokinetic (PK) and toxicological properties. The MPS had three assemblies: Intestine only 2-OC, Liver only 2-OC, and Intestine/Liver 2-OC with the same media perfusing both organoids. For PK assessments, we dosed the APAP in the media at preset timepoints after administering it either over the intestinal barrier (emulating the oral route) or in the media (emulating the intravenous route), at 12 µM and 2 µM respectively. The media samples were analyzed by reversed-phase high-pressure liquid chromatography (HPLC). Organoids were analyzed for gene expression, for TEER values, for protein expression and activity, and then collected, fixed, and submitted to a set of morphological evaluations. The MTT technique performed well in assessing the organoid viability, but the high content analyses (HCA) were able to detect very early toxic events in response to APAP treatment. We verified that the media flow does not significantly affect the APAP absorption whereas it significantly improves the liver equivalent functionality. The APAP human intestinal absorption and hepatic metabolism could be emulated in the MPS. The association between MPS data and in silico modeling has great potential to improve the predictability of the in vitro methods and provide better accuracy than animal models in pharmacokinetic and toxicological studies.

INTRODUCTION:

Due to genomic and proteomic differences, animal models have limited predictive value for several human outcomes. Moreover, they are time-consuming, expensive and ethically questionable[1]. MPS is a relatively new technology that aims at improving the predictive power and reduce the costs and time spent with pre-clinical tests. They are microfluidic devices cultivating organoids (artificial mimetics functional units of organs) under media flow that promotes interorganoid communication. Organoids made of human cells increase translational relevance[2–4]. MPS is expected to perform better than the animal tests because they are genetically human and recapitulate the interplay among tissues. When fully functional, the MPS will provide more meaningful results, at higher speed and lower costs and risks[4]. Many groups are developing MPS for several purposes, especially disease models to tests drug's efficacy.

Exposure level is one of the most critical parameters for evaluating drug efficacy and toxicity[5–12]. MPS allows organoid integration that emulates systemic exposure and is expected to perform better than the traditional 2D human tissue culture. This technology can significantly improve the prediction of compound intestinal absorption and liver metabolism[4].

An MPS integrating human equivalent model of the intestine and the liver is a good starting point, considering the central role of these two organs in drug bioavailability and systemic exposure[13–15]. APAP is an attractive drug for studying an MPS without a kidney equivalent because its metabolization is done mainly by the liver[16,17].

The 2-OC is a two-chamber microfluidic device suitable for the culture of two different human equivalent tissues/organoids interconnected by microchannels[16]. In order to emulate an in vitro human oral/intravenous administration of a drug and assess the effects of the cross-talk between the intestine and liver equivalents on APAP pharmacokinetics, besides the organoids

functionality and viability, three different MPS assemblies were performed: (1) an "Intestine 2-OC MPS" comprised of an intestine equivalent based in a culture insert containing a Caco-2 + HT-29 cells coculture, integrated into the 2-OC device; (2) a "Liver 2-OC MPS" comprised of liver spheroids with HepaRG + HHSteC (Human Hepatic Stellate Cells) integrated in the 2-OC device; and (3) an "Intestine/Liver 2-OC MPS" comprised of the intestine equivalent in one device compartment communicating with the liver equivalent in the other by flow of the microfluidic channels.

All assays were performed under static (no flow) and dynamic (with flow) conditions due to the impact of the mechanical stimuli (compression, stretching, and shear) on the cell viability and functionalities[18–20]. The present article describes the protocol for APAP oral/intravenous administration emulation and the respective absorption/metabolism and toxicological analyses in the 2-OC MPS containing human intestine and liver equivalent models.

PROTOCOL:

1. Production of tissue equivalents for cultivation in the 2-OC

1.1. Small intestine barrier equivalent production

1.1.1. Maintain Caco-2 and HT-29 cells using the intestine equivalent medium: DMEM supplemented with 10% FBS, 1% penicillin and streptomycin, and 1% non-essential amino acids, which is named as "DMEM S" in this manuscript.

1.1.2. Remove the medium, wash twice with 1x DPBS and add 8 mL of 0.25% Trypsin/EDTA to dissociate Caco-2 cells grown in cell culture flasks (175 cm^2). Incubate for 5 min at 37 °C and stop the reaction by adding at least the double volume of trypsin inhibitor. Perform the same procedure for HT-29 cells, adjusting the reagent volumes since a smaller quantity of these cells is needed and they are maintained in smaller flasks (75 cm^2).

1.1.3. Centrifuge at 250 x *g* for 5 min, remove the supernatant from both tubes, and resuspend the cell pellets in 10 mL of DMEM S. Count cells, assuring a cell viability higher than 80%. Aseptically integrate cell culture inserts in a 24-well plate previously filled with 400 µL of DMEM S per well in the basolateral side (which represents the human bloodstream).

1.1.4. Co-cultivate Caco-2 and HT-29 cells at a ratio of 9:1[21]. Use 2.25 x 10^5 Caco-2 and 2.5 x 10^4 HT-29 cells to each intestine equivalent in a final volume of 200 µL of DMEM S. Adjust cell numbers and volume according to the desired number of organoids. Mix carefully.

1.1.5. Pipette 200 µL of cell solution into each insert's apical side (which represents the human intestinal lumen side), seeding 250,000 cells per insert. Co-cultivate the cells in the inserts for three weeks[22]. Change the medium at least three times a week, aspirating it from both the apical and basolateral sides with a sterile Pasteur pipette, taking care not to damage the intact cell barrier.

NOTE: Proceed with the aspiration on the apical side, so as not to touch the cell barrier (aspirate by supporting the Pasteur pipette on the plastic rim of the cell insert).

1.1.6. Check the tight monolayer formation by measuring the TEER (transepithelial electrical resistance) every three days using a voltmeter[23], according to the manufacturer's instructions.

1.1.6.1. Perform a blank, measuring the resistance across a cell culture insert without cells, but with the same cell medium and at the same cell plate.

1.1.6.2. Calculate tissue resistance by subtracting the blank resistance from the tissue-equivalent resistance, and multiply by the effective surface area of the filter membrane (0.6 cm^2). A good intestine barrier resistance is in a range of 150 to 400 $\Omega \cdot cm^2$.

NOTE: After 21 days the cells must be fully differentiated and the intestinal barrier formed, so the intestine equivalents are ready to be integrated into MPS.

1.2. **Liver equivalents production**

1.2.1. Maintain HepaRG cells using the liver equivalent medium, which is William's Medium E supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin, 100 µg/mL streptomycin, 5 µg/mL human insulin and 5 x 10^{-5} M hydrocortisone, and is named "Williams E S" in this manuscript. Renew HepaRG media every 2-3 days and maintain cell culture for two weeks to initiate the differentiation in hepatocytes and cholangiocytes.

1.2.2. After the first two weeks, add 2% DMSO to HepaRG's medium for an additional two weeks to complete the cell differentiation[24,25]. Grow HHSTeC in Stellate Cell Media (SteC CM), using poly-L-lysine-coated cell culture flasks, changing media every two or three days.

1.2.3. Remove the medium, wash twice with 1x DPBS and add 8 mL of 0.05% Trypsin/EDTA, to dissociate HepaRG cells grown in cell culture flasks (175 cm^2). Incubate for 5 to 10 min at 37 °C and stop the reaction by adding at least double the volume of trypsin inhibitor. Perform the same for HHSTeC, adapting the reagent volume since a smaller quantity of these cells are needed and they can be maintained in smaller flasks (75 cm^2).

1.2.4. Centrifuge both at 250 x *g* for 5 min, remove the supernatant and resuspend the cell pellets in Williams E S medium. Count cells, assuring a cell viability higher than 80%.

1.2.5. Generate the liver spheroids combining HepaRG and HHSTeC cells at a ratio of 24:1, respectively, in Williams E S medium[16]. Add 4.8 x 10^4 differentiated HepaRG and 0.2 x 10^4 HHSTeC to compose each liver spheroid of 50,000 cells, in a volume of 80 µL. Adjust cell numbers and volume according to the desired number of spheroids. Mix carefully.

1.2.6. Using a multichannel pipette, dispense 80 µL of the combined cell pool in each well of 384

spheroid microplates, which has round well-bottom geometry.

NOTE: After four days, spheroids of about 300 µm are formed.

1.2.7. Using wide-bore tips, transfer the liver spheroids to ultra-low-attachment 6 well plates, which allows the required “one-by-one” counting.

2. Integration of intestine and liver equivalents in a 2-OC MPS

2.1. Intestine 2-OC MPS assembly for absorption assay

2.1.1. Pipette 500 µL of DMEM S into the larger compartment of 2-OC and 300 µL in the smaller one. Aspirate the basolateral and apical media of each intestinal barrier equivalent in the 24 well plates. Using sterile forceps, integrate one insert per 2-OC circuit, specifically into the larger compartment. Apply 200 µL of the intestine medium at the apical side.

NOTE: Avoid the formation of bubbles when integrating the organoids into the MPS.

2.1.2. Connect the MPS to the control unit, which must be connected to a pressurized air supply. Set the parameters: a pressure of, approximately, ±300 bar and a pumping frequency of 0.3 Hz. Start the flow 24 h before the test substance administration. The next day, perform the APAP treatment.

2.2. Liver 2-OC MPS assembly for metabolism assay

2.2.1. Pipette 650 µL of Williams E S into the large compartment and 350 µL into the smaller compartment, which will receive the spheroids. In the ultra-low-attachment 6 well plates, count the spheroids using wide-bore tips. Each liver equivalent is composed of twenty spheroids[26]. Integrate twenty liver equivalents per circuit, using wide-bore tips, which permits the transfer of organoids only, into the smaller compartment of a 2-OC.

2.2.2. Connect the MPS to the control unit, which must be connected to a pressurized air supply. Set the parameters: a pressure of, approximately, ±300 bar and a pumping frequency of 0.3 Hz. Start the flow 24 h before the test substance administration. The next day, perform the APAP treatment.

2.3. Intestine/Liver 2-OC MPS assembly for absorption and metabolism assay

2.3.1. Combine the two media (intestine and liver) in a 1:4 proportion, which means 200 µL of DMEM S in the intestine apical side and 800 µL of Williams E S in the basolateral side. Integrate intestine and liver equivalents, simultaneously, in the 2-OC.

2.3.2. Connect the MPS to the control unit, which must be connected to a pressurized air supply. Set the parameters: a pressure of, approximately, ±300 bar and a pumping frequency of 0.3 Hz.

Start the flow 24 h before the test substance administration. The next day, perform the APAP treatment.

NOTE: For all experiments, perform each time point in triplicate, which means three separated 2-OC circuits (i.e., 1 and ½ 2-OC devices). The total volume of each 2-OC circuits is 1 mL.

3. Acetaminophen (APAP) preparation

3.1. Prepare the APAP stock solution, dissolving APAP in absolute ethanol. On the day of the experiment, dilute APAP in the respective medium (APAP solution), to a concentration of 12 µM for "oral administration" and 2 µM for "intravenous administration".

3.2. Ensure that the final concentration of ethanol in the vehicle control and treatment solution is 0.5% for both administrations. For the positive control (100 mM APAP), the ethanol concentration is 2%.

4. Test substance administration and media sampling

4.1. APAP "oral" administration and media sampling

4.1.1. Aspirate the basolateral and apical media of each intestinal barrier equivalent in the 2-OC. Pipette 500 µL of the appropriate culture medium into the large compartment at the organoid basolateral side and 300 µL into the small compartment.

4.1.2. Check for bubbles and proceed with the intestinal barrier equivalent treatment with the test substance in the apical side, emulating oral administration. Emulate APAP "oral" administration by adding 200 µL of a 12 µM APAP solution on the apical side of intestinal culture inserts, which represents the intestinal "lumen side" (**Figure 1B**). Connect the MPS to the control unit.

4.1.3. Collect the total volume from apical and from basolateral sides at the following time points: 0 h, 5 min, 15 min, 30 min, 1 h, 3 h, 6 h, 12 h, and 24 h[15,27]. Perform all experiments in triplicate, at static and dynamic conditions, and collect each sample, of each triplicate, in a separate microtube. Analyze the samples using HPLC/UV.

NOTE: Separate apical and basolateral samples.

4.2. APAP "intravenous" administration and media sampling

4.2.1. Emulate the "intravenous" route by administering 2 µM APAP solution directly into the liver compartment. Aspirate all 2-OC medium content. Pipette 650 µL of Williams E S containing the test substance into the large compartment and 350 µL of the same media into the smaller compartment which contains the 20 spheroids. Collect all volumes at the following time points: 0 h, 30 min, 1 h, 2 h, 3 h, 6 h, 12 h, and 24 h[27,28].

4.2.2. Perform all experiments in triplicate, at static and dynamic conditions. Collect each sample, of each triplicate, in a separate microtube. Analyze the samples using HPLC/UV.

5. Instrumentation and chromatographic conditions

5.1. HPLC analysis

5.1.1. Set all relevant parameters for the HPLC analysis according to **Table 1**.

5.1.2. Filter the mobile phase through a 0.45 µm membrane filter under vacuum. Filter the samples through a 0.22 µm pore size PVDF syringe filter (diameter 13 mm) and store them in a vial. Start the measurement.

5.2. Stock solutions, calibration standards, and quality control (QC) samples

5.2.1. Prepare 10 mM of APAP stock solutions in ammonium acetate buffer (100 mM, pH 6.8) and further dilute with DMEM S and Williams E S cell culture media diluted with ammonium acetate buffer (1:1, v/v) to achieve the working solutions ranging from 0.25 to 100.00 µM.

5.2.2. Include a set of calibration samples in triplicate as well as quality control samples at four levels in triplicate. Prepare these standards by serial dilution.

5.2.3. Create calibration curves of APAP peak areas versus APAP nominal standard concentrations. Determine the linear regression fit for each calibration curve. Evaluate the goodness-of-fit of various calibration models by visual inspection, correlation coefficient, intra- and inter-run accuracy and precision values.

5.2.4. Inject blank samples of DMEM S and Williams E S media diluted in ammonium acetate buffer (1:1, v/v) in sextuplicate. Prepare triplicates of quality control samples in DMEM S and Williams E S media diluted with ammonium acetate buffer (1:1, v/v) for the APAP concentrations of 0.50 (LOQ), 4.50, 45.00 and 90.00 µM.

5.2.5. Ensure that the quality control samples are prepared from a new stock solution, different from that used to generate a standard curve. Use quality control samples to investigate intra- and inter-run variations.

5.3. Validation procedures

5.3.1. Perform the bioanalytical method validation following the previously reported procedures[29,30]. Carry out the chromatographic runs on five or six separate occasions, considering Williams E S and DMEM S cell culture media, respectively.

5.3.2. Ensure that calibration points ranging from 0.25 to 100.00 µM of APAP, in DMEM S or

Williams E S cell culture media diluted in ammonium acetate buffer (1:1, v/v), are plotted based on the peak-areas of APAP (axis y) against the respective nominal concentrations (axis x). Compare the slopes of these standard calibration curves with slopes of calibration curve prepared in ammonium acetate buffer. Make sure that all calibration curves have a correlation value of at least 0.998.

5.3.3. Determine the precision and accuracy (intra and inter-run) for the analyte in the surrogate matrix using replicates at four different levels LLOQ, low, middle, and high-quality control on five or six different days. Perform intra-run precision and accuracy measurements on the same day in DMEM S or Williams E S cell culture media diluted in ammonium acetate buffer (1:1, v/v) containing 0.50, 4.50, 45.00 and 90.00 µM APAP concentrations (n= 3).

5.3.4. Evaluate each set of quality control samples containing the APAP concentrations from recently obtained calibration curves. Test the selectivity of the assays by the degree of separation of the compound of interest and possible other chromatographic peaks caused by interfering components.

5.4. Lower limit of quantification (LLOQ) and limit of detection (LOD)

5.4.1. Determine the lower limit of quantification (LLOQ) based on the standard deviation of the response and the slope approach. Calculate using the formula 10α/S, where α is the standard deviation of y-intercept and S is the slope of straight line obtained by plotting calibration curves[29,30]. Estimate the limit of detection (LOD) taking into consideration 3.3 times the standard deviation of the blank, divided by the slope of the calibration curve[29,30].

6. Tissue equivalents viability/functionality

6.1. MTT

6.1.1. Perform an MTT assay to assess organoid viability in all time points of the MPS assay. As the negative control, use cell media plus vehicle. As the positive control, treat the organoids with 100 mM APAP and 1% NaOH diluted in cell medium.

6.1.2. Transfer the 20 spheroids of each replicate for individual wells in a 96 well plate, and the cell culture inserts, containing the intestine equivalents, to 24 well cell plates, placing one intestine equivalent per well. Wash the tissue equivalents three times with 1x DPBS.

6.1.3. Add 300 µL of a 1 mg/mL MTT solution, diluted in the respective cell medium, per well. Incubate the plates for 3 h at standard cell culture conditions.

6.1.4. Remove the MTT solution from each well carefully by pipetting. Extract MTT formazan from the intestine and liver equivalents using 200 µL of isopropanol per well overnight at 4 °C.

NOTE: Seal the lid to prevent evaporation.

6.1.5. Transfer 200 µL of each supernatant to the respective pre-identified well in a 96 well micro test plate. Use isopropanol as the blank.

6.1.6. Read the formazan absorbance in a plate reader at 570 nm. Calculate the relative ability of the cells to reduce MTT (%) using the average optical density of each time point, compared to the negative control, considered as 100% cell viability.

6.2. Cytochemistry/Histology

6.2.1. Fix the intestine and liver equivalents, for 25 min at room temperature, using 4% (w/v) paraformaldehyde in 0.1 M phosphate-saline buffer, pH 7.4. Wash the organoids 5 times in PBS buffer for 10 minutes each time. Stain the intestinal and the liver equivalents with tetramethylrhodamine isothiocyanate-phalloidin or Alexa Fluor 647 phalloidin, 1:50 in PBS[31].

6.2.2. Transfer them to OCT freezing medium for a few minutes to acclimate at RT before transferring them to liquid nitrogen until the complete freezing. Perform liver spheroids cryosections about 10-12 µm thick, using a cryostat.

6.2.3. Mount the tissues sections in mounting medium with DAPI. Examine them by confocal fluorescence microscopy.

6.2.4. Freeze the organoids after fixation to perform hematoxylin & eosin staining according to the established protocols. Mount the slides with mounting medium after slicing the tissue as described above and take histological images using an optical microscope.

6.3. High content analysis

6.3.1. Mitochondrial and nuclear staining of the cells

6.3.1.1. Reconstitute the lyophilized powder in DMSO to make a 1 mM mitochondrial staining stock solution (e.g., MitoTracker Deep Red FM). Store aliquoted stock solution at -20 °C protected from light. Dilute the 1 mM mitochondrial staining stock solution to the final concentration (200 nM) in prewarmed (37 °C) tissue culture medium without serum.

6.3.1.2. Remove the cell culture media. Add the mitochondrial staining solution to completely cover the sample and incubate cells for 15-45 min at 37 °C in a humidified atmosphere with 5% CO_2.

6.3.1.3. Carefully remove the mitochondrial staining working solution and replace it with 2-4% paraformaldehyde fixative in PBS for 15 minutes at room temperature.

6.3.1.4. Rinse the fixed cells gently with PBS for 5 minutes. Repeat the washing process twice.

6.3.1.5. Prepare a 10 mg/mL (16.23 M) nucleic acid staining stock solution by dissolving 100 mg of Hoechst 33342 dye in 10 mL of ultrapure water.

NOTE: The stock solution should be aliquoted and stored protected from light at -20 °C.

6.3.1.6. Prepare a 0.2-2.0 µg/mL nucleic acid staining working solution in PBS and incubate the fixated cells with nucleic acid staining working solution for 10 minutes at room temperature.

6.3.1.7. Remove the nucleic acid staining working solution and rinse the cells gently with PBS for 5 minutes three times. Cells should be kept in PBS at 4 °C, protected from light.

6.3.2. Mitochondrial and nuclear staining analysis

6.3.2.1. Analyze cells using a fluorescence microscope with filter sets appropriate for the nucleic acid stain (λEx/ λEm: 361/497 nm) and the mitochondrial stain (λEx/ λEm: 644/665 nm). Find cells by nucleic acid positive staining and quantify cell number. Quantify mitochondrial stain fluorescence intensity in mitochondria.

6.4. Morphometric measurements (spheroids calculations) in ImageJ

6.4.1. Export High Content Analysis (HCA) images as *.flex files from the Columbus software. Import .flex files as a grayscale in ImageJ using Bio-Formats plugin[32]: **File > Import > Bio-Formats**.

6.4.2. In the **Import Options** window, select **Hyperstack** viewing and enable **Split channels** under Split into separate windows. This option will allow the access of all files in a particular channel (e.g., DAPI, mitotracker, etc.). Do not select **Use virtual stack** under Memory management.

NOTE: It is preferable to use DAPI channel as “dust” in images as culture medium is reduced in UV wavelength (e.g., 405 nm).

6.4.3. Adjust pixel size (**Analyze > Set Scale**) if it was not loaded according to embedded values in the .flex file. Apply a **Gaussian Blur** filter to remove the excess of noise and avoid irregularities in shape contour. **Process > Filters > Gaussian Blur**. A high value of Sigma (radius) between 2.0 and 3.0 is ideal for most cases. If the stack has several images, apply to all of them (select **Yes** in **Process Stack** window).

6.4.4. Generate a binary image to separate background and organoids (objects) using a threshold. Click **Image > Adjust > Threshold**. Use the red mask to adjust the values according to the intensity of the image, to fit the organoid shape, keeping the morphology intact. Disable **Dark background** if the image has a white background. Click **Apply**.

6.4.5. In the **Convert Stack to Binary** window, choose the **Threshold** method. Usually, **Default** or **Triangle** are preferred in this kind of image processing. Keep the **Background** as **Dark**. Select

Calculate threshold for each image if there are several images in the stack.

6.4.6. Select **Process > Binary > Fill holes**. Optionally, remove holes from the background. In **Process > Binary > Option**s, select **Black background** and execute **Fill Holes** again. Disable **Black** background option before proceeding to the next step.

6.4.7. Separate objects. For organoids, the watershed method is a good choice. Click **Process > Binary > Watershed**. Execute the shape analysis.

6.4.7.1. Select **Analyze > Set Measurements**. Several options are available (details in https://imagej.nih.gov/ij/docs/guide/146-30.html). For organoids, select **Area**, **Mean gray value**, **Min & max gray value** and **Shape descriptors**. Optionally, select **Display label** to identify objects in the image and **Scientific notation**. Click **OK**.

6.4.7.2. Select **Analysis > Analyze Particles**. Choose the **Size** and **Circularity** limits. Keep 0-infinity and 0.00-1.00, respectively to measure all objects in the image. In **Show**, choose **Outlines** so the objects will be identified. Enable **Display results** to output results; **Exclude on edges** to exclude objects touching borders; **Include holes** so eventual interior holes in the objects are considered as part of the main shape.

6.4.8. Repeat **Shape Analysis** for every image stack and the results will be appended in a single table. Export results table in **File > Save As…** as Comma Separated Values (.csv) file.

6.5. Real-Time PCR

6.5.1. Extract RNA from tissue equivalents using a monophasic solution of phenol and guanidine isothiocyanate, following manufacturer's instructions.

6.5.2. Perform the cDNA synthesis by reverse transcription of 1 – 2 µg of total RNA.

6.5.3. Amplify all targets using gene-specific primers (**Table 5**) to perform real time quantitative PCR. Each qRT-PCR contains 30 ng of reverse-transcribed RNA and 100 nM of each primer.

6.5.4. Follow PCR conditions: 50 °C for 3 minutes (1 cycle); 95 °C for 5 minutes (1 cycle); 95 °C for 30 seconds, 59 °C for 45 seconds and 72 °C for 45 seconds (35 - 40 cycles).

6.6. CYP assay

6.6.1. Follow section 2.2 for liver 2-OC assembly. The experimental groups are No-cell control, APAP 2 µM treatments for 12 h, 24 h, and vehicle control. Follow sections 3.3 and 4.2 for APAP 2 µM preparation and treatment.

NOTE: To ensure that all the samples will be ready at the same time for CYP assay, start the 12 h treatment 12 hours after starting the 24 h treatment. Treat the no-cell control of CYP activity

with 0.5% ethanol solution, as well as the vehicle control.

6.6.2. Thaw 3 mM luminogenic substrate stock solution at room temperature and make a 1:1000 dilution in William's E S. Protect from light.

6.6.3. Collect spheroids and transfer each experimental group to a well of a 96-well plate. Remove the medium, wash twice with 100 µL of 1x DPBS and add 80 µL of 3 µM substrate solution per well. Keep the no-cell control in William's E S medium. Save a well without spheroids or substrate solution for a background control. Incubate for 30-60 min at 37 °C with 5% CO_2, protected from light.

6.6.4. Equilibrate lyophilized Luciferin Detection Reagent (LDR) using the Reconstitution Buffer with esterase. Mix by swirling or inverting. Store the appropriated volume at room temperature until the next step.

NOTE: Reconstituted LDR can be stored at room temperature for 24 hours or at 4 °C for 1 week without loss of activity. For long-term storage, store at –20 °C.

6.6.5. Transfer 25 µL of intact spheroids' supernatant in three different wells of a white opaque 96-well microplate, after incubation. Add 25 µL of LDR per well and homogenize.

6.6.6. Incubate the white plate at room temperature for 20 minutes. Read the luminescence on a luminometer. Do not use a fluorometer.

6.6.7. Calculate net signals by subtracting background luminescence values (no-cell control) from the test compound-treated and untreated (vehicle control) values. Calculate percent change of CYP3A4 activity by dividing the net treated values by the net untreated values and multiplying by 100.

6.7. Western blotting

6.7.1. Transfer the liver spheroids to an identified 1.5 mL microtube. Remove the medium and wash twice with 100 µL of 1x DPBS.

6.7.2. Lysate the liver spheroids in 100 µL of cell lysis RIPA buffer at 4 °C for 20 min. Centrifuge for 15 min, 4 °C, and 11000 rpm. Transfer the supernatant to another identified 1.5 mL microtube.

6.7.3. Quantify the amount of protein obtained through the Bradford method. Load between 10 and 50 µg of protein from the quantified cell lysate per well of a gradient polyacrylamide gel 3-15% and perform an SDS-PAGE.

6.7.4. Transfer the loaded protein from the gel to a 0.22 µm PVDF membrane through the semi-dry system equipment. Use a transference solution of 50 mM Tris-HCl and 192 mM glycine. Set

equipment parameters according to the number of gels to be transferred (1 to 2 at a time).

6.7.5. Block nonspecific interactions on PVDF membrane with a 3-5% skim milk solution in TBS-T buffer: Tris-Buffered Saline (50 mM Tris pH 7.6, 150 mM sodium chloride) supplemented with 0.1% of Tween 20. Keep the membrane under gently constant shaking for 1 hour at room temperature.

6.7.6. Wash with TBS-T under gently constant shaking for 3-5 min at room temperature. Repeat this washing step twice.

6.7.7. Dilute albumin and vinculin primary antibodies to 1:1000 and 1:2000 respectively on TBS-T. Incubate the membrane with primary antibodies overnight at 4 °C, under gently constant shaking.

NOTE: Always follow the manufacturer's instructions to dilute antibodies.

6.7.8. Remove the primary antibody and wash membrane 3 times (step 6.7.6). Dilute ECL anti-mouse IgG secondary antibody to 1:5000 on TBS-T. Incubate the membrane with a secondary antibody under gently constant shaking for 2 hours at room temperature.

6.7.9. Remove the secondary antibody and wash the membrane (step 6.7.6). Perform protein detection using ECL Western Blotting Substrate. Expose the autoradiographic films for 30 s to 30 min. Perform the immunoblotting detection in triplicate.

REPRESENTATIVE RESULTS:

To perform the PK APAP tests in the 2-OC MPS, the first step is to manufacture the human intestine and liver equivalents (organoids). They are integrated into the 2-OC microfluidic device (**Figure 1A**) 24 h before starting of the PK APAP assay. The next day, the medium is changed, and the model is exposed to APAP. **Figure 1** illustrates the intestine and liver equivalents placed inside the 2-OC device (**Figure 1B**) and the APAP PK experiment timecourse (**Figure 1C**). We performed a MTT assay, TEER measurements, HCA, real-time PCR, western blotting, histology, and confocal fluorescence microscopy in 2D culture and 3D organoids to check the tissue's viability and detect possible APAP toxic effects. In the confocal fluorescence microscopy images, the intestine equivalent samples stained with DAPI and Phalloidin (for nuclei and actin respectively) were presented as a contiguous barrier for the non-treated (**Figure 2A**) and the 12 µM APAP treated samples (**Figure 2B**). As seen in **Figure 2C**, the MTT assay showed relative cell viability levels above 70%, indicating the absence of relevant cytotoxic effects in response to APAP exposure at 12 µM concentration[33–37]. The positive control (100 mM) induced significant cell death (survival below 5%). The Caco-2/HT-29 viability and proper differentiation as well as the intestine equivalent barrier integrity were verified by TEER evolution during the differentiation period (**Figure 2D**). APAP did not cause any alteration in the TEER values as shown in **Figure 2E**. The expression of the active sodium-coupled glucose transporters SLC5A1, multidrug resistance transporter MDR1 and sodium-potassium ATPase were analyzed, to verify the APAP treatment impact over cell barriers formation and basal functionality. As demonstrated in **Figure 2F–H**, both non-treated

and APAP treated intestine equivalents have shown similar expression of SLC5A1 and NaKATPase. The oral administration of 12 µM APAP induced marked an increase in the MDR1 mRNA levels in intestines equivalents after 24 h (**Figure 2H**). We also performed HCA of cell phenotypic changes by a fluorophore dye mixture for nuclei and mitochondrial mass content. Positive controls were 100 mM APAP and 1% NaOH. Additionally, we analyzed whether 12 µM APAP could induce cytotoxicity to the 2D Caco-2/HT-29 co-culture. The intestinal cells images acquired with fluorescence microscopy shown in **Figure 2I** corroborates the MTT data, which have demonstrated that 12 µM APAP did not cause significant cytotoxicity in the Caco-2/HT-29 intestinal equivalents.

The assessment of hepatic spheroids basal viability and the cytotoxicity in response to 2 µM APAP were done by MTT assay and morphological analyses by confocal fluorescence microscopy, H&E histology, and HCA assays. As shown in the **Figure 3A**, according to the MTT assay data, the 2 µM APAP treatment did not induce relevant cytotoxicity in the Liver 2-OC assembly under both static and dynamic conditions. The cell viability decreased but remained over 80% for both 12 h and 24 h time-points[33–37]. The positive control treatments (100 mM APAP and 1% NaOH) induced significant tissue damages (viability below 10%). Microscopic confocal images indicate the absence of a necrotic center in the liver spheroids in both basal or APAP treatment conditions, and no evidence of significant death rates (**Figure 3B-C**). However, when we analyzed multiple cellular phenotypic changes following the vehicle or 2 µM APAP administration in the 2D HepaRG/HHSteC coculture through HCA assay, using 100 mM APAP (C+) as a positive control, contradictorily to the results of the MTT assay, the hepatic cells demonstrated early cytotoxic responses to 2 µM APAP treatment (**Figure 3D**). After 24 h, there was a decrease in the number of cells, in the nuclear area and an increase in mitochondrial mass. Additionally, a fluorophore dye cocktail containing Hoechst 33342 and MitoTracker Deep Red was used to stain the 3D hepatic spheroids (**Figure 3J**). Fiji software was used to evaluate 3D spherical architecture homogeneity among several spheroids (**Figure 3E–I**). The graphic shown in **Figure 3E** shows the similarity among liver spheroids total area. The aspect ratio (**Figure 3F**) around 1 means an absence of bias during the confection process of the spheroids. The evaluation also indicated that the majority of the spheroids were roughly rounded (**Figure 3G**). The evaluation of the morphology perimeter and cell distribution was done by circularity (**Figure 3I**) and by solidity calculation (**Figure 3H**), respectively. We concluded that the methodology to confectioning the liver spheroids had generated organoids with a smooth perimeter, compatible with spherical growth, no biases, or necrosis during the process.

As demonstrated in **Figure 4A-B**, the liver spheroids showed a relative basal high level of albumin and GST mRNA expression respectively, indicating proper basal functionality. Nevertheless, the 2 µM APAP treatment for 24 h induced a decrease in the albumin and the GST mRNA expression levels, suggesting impairment of liver spheroids functionally at 24 h time point of APAP treatment.

The detection of the CYP3A4 and UGT1A1 mRNA expression levels demonstrates the liver equivalents metabolic capacity. The CYP3A4 mRNA basal level (**Figure 4C**) was consistent with previous reports[6]. The APAP treatment induced a trend for a decrease in both CYP3A4 mRNA and

UGT1A1 expression in response to APAP treatment (**Figure 4C-D**) once again corroborating the hypothesis of impairment of liver spheroids functionally at 24 h time point of APAP treatment.

Additionally, experiments of western blotting and in vitro enzymatic activity were performed in order to analyze the albumin protein expression, as well as, the CYP 3A4 activity in liver equivalents at basal and at APAP treatment conditions. We found that 2 µM APAP treatment performed at the Liver 2-OC MPS, induced a reduction in the liver equivalent total albumin expression at 12 h and 24 h time-points at both static (**Figure 4E**) and dynamic (**Figure 4F**) conditions. On the other hand, liver equivalents samples from dynamic conditions have shown a trend to show higher levels of protein expression of albumin when compared to the static conditions (**Figure 4G**). CYP 3A4 in vitro assay performed at liver equivalents from Liver 2-OC MPS shown that 2 µM APAP treatment for 12 h or 24 h was capable to induce a robust and significant impairment of CYP 3A4 activity at both static and dynamic conditions (**Figure 4H-I**). More interesting, the presence of media flow (dynamic) has induced a significant improvement of liver equivalents CYP 3A4 activity levels when compared to conditions in which the liver equivalents were kept in absence of circulating medium (static conditions) (**Figure 4J**).

To find the most sensitive analytical condition regarding HPLC analysis, several parameters were investigated including the composition of the mobile phase, the type, and concentration of additives. It was found that acetonitrile gave better chromatogram resolution and appropriate retention time than methanol. Fast and reproducible separation of APAP was obtained using a C18 reversed-phase column. The APAP retention time (Rt) value was 9.27 ± 0.19 minutes. Selectivity for APAP is indicated by the shape and symmetrical resolution of the peak, as well as by the lack of interfering peaks from the DMEM and Williams cell culture media.

APAP standard concentrations in DMEM S and Williams E S cell culture media diluted with ammonium acetate buffer (1:1, v/v) ranging from 0.25 to 100.00 µM were used to build the calibration curves. The linearity of the method was determined at nine concentration levels. The data are shown in **Table 2** and **Table 3**. The relationship between APAP concentration and the peak areas was described by the linear regression equations: $y = 16106*x + 3579.8$ (R^2=1, in DMEM medium) and $y = 16397*x + 2475.1$ (R^2=1, in Williams medium), in which "x" is APAP nominal concentration in µM and "y" is the chromatogram peak area of APAP in AU. At the upper limit of quantification (i.e., 100.00 µM), the percentage deviation and the inter-run variability values were less than 2.50%. The accuracy and the precision for nine concentration levels, excluding the 0.50 µM (LLOQ), were within an acceptable range with DEV and C.V. values less than 7.00% (**Table 2** and **Table 3**).

The analytical method inter- and intra-run accuracy and precision, at four tested concentrations, fell within the generally accepted criteria for bioanalytical assays. The reproducibility of the method was evaluated by analyzing replicates of APAP quality control samples of 0.50 (LLOQ), 4.50, 45.00 and 90.00 µM. The intra-run and inter-run average results are reported in **Table 4**. The accuracy and precision of the assay are demonstrated by DEV values ≤ 15.00% and by C.V. values ≤ 7.00%, respectively.

LOD was determined as the sample whose signal-to-noise ratio (S/N) was just greater than 3 and corresponded to a 0.25 µM APAP. On the other hand, the LLOQ, estimated with 0.50 µM APAP samples, displayed S/N ratio equal to 10. Furthermore, we found accuracy values (DEV%) ranging within ≤ 19.00% of the nominal concentration values. The intra- and inter-run variabilities were demonstrated by C.V. ≤ 18.77%, as shown in **Table 2, Table 3** and **Table 4**)[29,30].

The APAP PK analyses were performed in three different 2-OC MPS assemblies: 1) Intestine 2-OC, containing the intestine equivalent only; 2) Liver 2-OC, containing the liver spheroids only and 3) Intestine/Liver 2-OC with both intestinal barrier and liver spheroids.

For absorption studies, the oral route was mimicked by the administration of 12 µM APAP on the intestine equivalent apical side. APAP concentrations were measured by HPLC/UV, in the medium samples, collected from apical and basolateral intestinal equivalents sides, in both static and dynamic conditions. The APAP kinetics in the medium collected from the apical and basolateral sides demonstrated that the intestine model was able to absorb the APAP. There was a progressive APAP concentration decrease in the apical side (**Figure 5A**) concomitantly to APAP concentration increase at the intestinal basolateral side (**Figure 5B**). The maximum concentrations (Cmax) in the medium was around 2 µM for both static and dynamic conditions, after 12 h of the administration (Tmax).

For metabolism studies, the intravenous administration was mimicked by the administration of 2 µM APAP in the medium of the liver compartment. The APAP concentration kinetics in the media under both static and dynamic conditions indicated that only at the dynamic conditions the decreases in the APAP concentration could be detected, reaching 0.87 µM APAP 12 h after 2 µM APAP administration (T1/2 = 12 h). The liver equivalents showed minimal metabolic efficiency under static conditions (**Figure 5C**). The APAP concentration reached 1.7 µM 12 h after APAP administration. The integrated, systemic like APAP absorption and metabolism evaluation was performed in the Intestine/Liver 2-OC model. The APAP was administered over the apical side of the intestine equivalent, emulating the oral route. Medium samples were collected from both Intestinal sides and also from the liver compartment. **Figure 5D** shows the progressive decay of the APAP concentration at the apical side in both static and dynamic conditions.

Figure 5E shows distinguishable absorption and metabolism phases. The flow also impacted in the intestinal absorption. The APAP Cmax in the medium changed from 2 µM on the "Intestine 2-OC" (**Figure 5B**) assembly to 1.7 µM for the dynamic "Intestine/Liver 2-OC" (**Figure 5E**). Figure 5F shows a direct comparison between the concentration–time profile of APAP in our dynamic "Intestine/Liver 2-OC" microphysiological system (red curve and y axis) and a representative profile obtained in humans after a single oral dose of 1000 mg (black curve and y axis).

FIGURE AND TABLE LEGENDS:

Figure 1. Schematic step compilation for PK studies in the 2-OC MPS. A) Schematic drawing of the 2-OC MPS, showing the intestinal and hepatic human tissue equivalents in bottom-up view. **B**) 2-OC MPS photograph with the intestinal and liver equivalents integrated into the device in

bottom-up view, with representative optical microscopy images. **C**) Timeline of tissue equivalents preparation, APAP treatment, and culture medium collections phases for organoid manufacturing, and pharmacokinetic and toxicological assessments. This figure has been modified from Marin et al. Acetaminophen absorption and metabolism in an intestine/liver microphysiological system. *Chem Biol Interact*. **299**, 59-76 (2019).

Figure 2. Viability and toxicological assessment of the intestine equivalents. A) Representative confocal fluorescence microscopy images of non-treated Caco-2/HT-29 cells stained with cell nuclei and actin filaments fluorescent dye (DAPI and Phalloidin respectively); 63x Magnification, zoom 2.6. **B**) Representative confocal fluorescence microscopy images of Caco-2/HT-29 cells treated with 12 µM APAP for 24 h, stained with nuclei and actin filaments fluorescent dye (DAPI and Phalloidin respectively); 63x Magnification, zoom 2.6. **C**) Intestine equivalents viability evaluation by MTT assay in both static and dynamic conditions. The values represented by the bars in the graph are percent calculated relative to vehicle control (time-point named as 0)*P<0.05 0 vs treatment. **D**) TEER evolution during the 21 days of differentiation. *P<0.05 day 1 vs other days. **E**) TEER values after APAP administration into the Intestine 2-OC MPS under dynamic conditions. Gene expression in intestines equivalents. Absorption potential of the intestine barrier and possible effects of 12 µM APAP for 24 h under dynamic condition was verified by SLC5A1 (**F**), Na-K-ATPase (**G**) and MDR1 (**H**) expression. Values represent the mean ± SEM of three independent experiments. The result is expressed as a ratio to housekeeping GAPDH. *P<0.05 vehicle vs APAP. **I**) Operetta image-based HCA performed by the Columbus® 2.4.0. software. Representative images of 2D intestine co-culture in different time points after 12 µM APAP treatment. Negative controls were medium (0 h) or vehicle (0.5% ethanol). Positive controls shown here is the 100 mM APAP. This figure has been modified from Marin et al. Acetaminophen absorption and metabolism in an intestine/liver microphysiological system. *Chem Biol Interact*. **299**, 59-76 (2019).

Figure 3. Viability and toxicological assessment of the liver equivalents. A) Liver equivalents viability evaluation by MTT assay in both static and dynamic conditions. The values represented by the bars in the graph are percent calculated relative to vehicle control (time-point named as 0) *P<0.05 0 vs treatment. **B**) Representatives confocal images captured from the vehicle and 2 µM APAP 24 h treated liver spheroids from an inner section. **C**) Representatives H&E (hematoxylin and eosin staining) images captured from the vehicle and 2 µM APAP 24 h treated liver spheroids from an inner section. Scale bar = 50 µm. **D**) Representative images of 2D liver co-culture in different time points after 2 µM APAP treatment. Samples treated with vehicle and with 2 µM APAP were considered in these analyses. The fluorophore dye mixture includes Hoechst for nuclear staining and Mitotracker Deep Red for mitochondria mass staining. Negative controls were medium (0 h) or vehicle (0.5% ethanol). Positive controls were 100 mM APAP. Measurements of whole spheroids images captured were performed using Fiji software. **E**) Frequency distributions of the area **F**) aspect ratio, **G**) roundness, **H**) solidity, **I**) circularity. N = 85. *p < 0,05. **J**) Representative images of 3D liver spheroids acquired by the Operetta using the LWD 10x objective. This figure has been modified from Marin et al. Acetaminophen absorption and metabolism in an intestine/liver microphysiological system. *Chem Biol Interact*. **299**, 59-76 (2019).

Figure 4. Liver viability/functionality and possible effects of 2 µM APAP under static and dynamic conditions over it were verified by gene and protein expression and by enzymatic activity. **A**) albumin gene expression. **B**) GSTA2 gene expression. Liver capability to perform phase I and phase II metabolism and possible effects of 2 µM APAP for 24 h under dynamic condition over it were verified by gene expression of CYP3A4 (**C**) and by UGT1A1 (**D**) respectively. **E**) Total albumin protein expression under static condition. **F**) Total albumin protein expression under dynamic conditions. condition. **G**) Comparative graph illustrating the difference in total albumin expression in liver equivalents cultivated and treated under static or dynamic conditions. **H**) CYP 3A4 in vitro enzymatic activity under static conditions. **I**) CYP 3A4 in vitro enzymatic activity under dynamic conditions. **J**) Comparative graph illustrating the difference in CYP 3A4 activity in liver equivalents cultivated and treated under static or dynamic conditions. Values represent the mean ± SEM of three independent experiments. The data of gene expression is expressed as a ratio to housekeeping GAPDH. The data of protein expression is expressed as a ratio to vinculin protein. *P<0.05 vehicle vs APAP treatment. This figure has been modified from Marin et al. Acetaminophen absorption and metabolism in an intestine/liver microphysiological system. *Chem Biol Interact*. **299**, 59-76 (2019).

Figure 5. Analyzes of APAP pharmacokinetics in 2-OC MPS. APAP absorption profile after 12 µM APAP administration at the apical side of the Intestine 2-OC MPS preparation. The intestinal barrier was made of a coculture of Caco-2/HT-29 cell lines (**A**) Static and dynamic APAP concentrations in the medium from the apical side (representing the human intestinal lumen side). (**B**) Static and dynamic APAP concentrations in the medium from the basolateral side (representing the human intestinal bloodstream side). *P<0.05 static vs dynamic conditions. **C**) APAP metabolism profile in the Liver 2-OC MPS by HepaRG/HHSTeC liver spheroids. Comparison of static and dynamic conditions after a 2 µM APAP administration into the medium. *P<0.05 0h *vs* 6 h, 12 h, and 24 h APAP treatment. APAP absorption and metabolism profile after 12 µM administration on the intestinal barrier apical side of the Intestine/Liver 2-OC MPS preparation. This emulates the oral route. The intestinal barrier was made of Caco-2/HT-29 cell lines and the liver equivalent made of spheroids of HepaRG/HHSTeC cell lines. (**D**) Intestinal/Liver 2-OC APAP concentrations in the apical side of the intestinal barrier under static and dynamic conditions. (**E**) Intestinal/Liver 2-OC APAP concentrations in the medium under static and dynamic conditions. *P<0.05 static vs dynamic conditions. (**F**) Comparison between the concentration–time profile of APAP in our microphysiological system (red curve and y axis) and a representative profile obtained in humans after a single oral dose of 1000 mg (black curve and y axis). Data was extracted from plots using WebPlotDigitizer 4.2 (https://automeris.io/WebPlotDigitizer). This figure has been modified from Marin et al. Acetaminophen absorption and metabolism in an intestine/liver microphysiological system. *Chem Biol Interact*. **299**, 59-76 (2019).

Table 1. Conditions and parameters to be used for HPLC-UV analyses of APAP in culture medium matrices.

Table 2. Inter-run variation – accuracy, precision, and linearity of standard curve samples prepared in a mixture of DMEM medium with 0.10 M ammonium acetate buffer (1:1, v/v) from

six separate assays.[a]

[a]A linear curve was fitted to the data for a response (**APAP**) versus theoretical concentration as described in Experimental. The calculated concentration was derived from reading the response for each standard sample against the calibration curve. Each entry (assay 1-6) corresponds to the average value of triplicate analysis.

[b] SD= Standard deviation. [c] C.V. (coefficient of variation. precision).

[d] Accuracy (DEV %) = the deviation of the calculated concentration from the nominal value.

This figure has been modified from Marin et al. Acetaminophen absorption and metabolism in an intestine/liver microphysiological system. *Chem Biol Interact*. **299**, 59-76 (2019).

Table 3. Inter-run variation – accuracy, precision, and linearity of standard curve samples prepared in a mixture of Williams medium with 0.10 M ammonium acetate buffer (1:1, v/v) from six separate assays.[a]

[a]A linear curve was fitted to the data for a response (**APAP**) versus theoretical concentration as described in Experimental. The calculated concentration was derived from reading the response for each standard sample against a calibration curve. Each entry (assay 1-5) corresponds to the average value of triplicate analysis.[b] SD= Standard deviation [c] C.V. (coefficient of variation. precision). [d] Accuracy (DEV %) = the deviation of the calculated concentration from the nominal value. This figure has been modified from Marin et al. Acetaminophen absorption and metabolism in an intestine/liver microphysiological system. *Chem Biol Interact*. **299**, 59-76 (2019).

Table 4. Intra- and inter-run precision and accuracy for APAP in quality control samples.[a]

[a]The data are shown as averages. SD (standard deviation). C.V. (coefficient of variation. precision) and accuracy (percent deviation. DEV%). This figure has been modified from Marin et al. Acetaminophen absorption and metabolism in an intestine/liver microphysiological system. *Chem Biol Interact*. **299**, 59-76 (2019).

Table 5. Real-time qPCR primers to evaluate gene transcription at mRNA level in the 2-OC cultures for intestine and liver tissues

DISCUSSION:

The accurate and reliable assessment of the pharmacologic properties of investigative new drugs is critical for reducing the risk in the following development steps. The MPS is a relatively new technology, that aims at improving the predictive power and reducing the costs and time spent with preclinical tests. Our group is advancing in the assessment of pharmacokinetic and toxicological properties mostly needed for lead optimization. We worked with the 2-OC microfluidic device, which has two chambers, allowing the integration of two organoids. APAP was chosen because it has plenty of high-quality human data, is mostly metabolized by the liver, and also displays hepatotoxic properties. The study protocol aimed to emulate some steps of the human phase I clinical trial, where a drug is administered orally to volunteers, and periodic blood samples are drawn to assess the drugs' concentration to know the pharmacokinetic properties and biochemical and clinical parameters are collected to assess the safety and tolerability. Therefore, when the MPS evolves enough to provide reliable predictions, it will significantly

reduce the risk of failure in the phase I trial. By adding the disease models in the future, the same assumption possibly will apply to the phases II and III clinical trials.

All studies were performed in three models: Intestine 2-OC (APAP oral administration), Liver 2-OC (Intravenous administration), and Intestine/Liver 2-OC (oral administration). The first model isolated the absorption, the second the metabolism, and the third integrated both. We first produced the organoids and incorporated into the microfluidic device, second administered the APAP and collected the media samples, and last performed a set of tests to assess the cell viability and functionality and the toxicological impact of APAP exposure. For the pharmacokinetic studies, we developed and validated a chromatographic method for APAP quantification in the medium. The validation complied with the Guideline on Bioanalytical Method Validation regarding specificity, linearity, the limit of quantification (LOQ), the limit of detection (LOD), matrix effect, precision, and accuracy, as outlined by FDA[29,30]. The specificity was established with blank, pooled, and individual biological samples from two different sources. The method performed acceptably during the analysis, and the data confirmed the ability of a simple isocratic mobile phase to separate and quantify APAP.

The viability/functionally of the intestine and liver organoids was assessed by different techniques. For the intestine equivalent, the confocal fluorescence microscopy (**Figure 2A-B**), the MTT assay (**Figure 2C**), gene expression assessment (**Figure 2D-F**) or HCA experiment, did not detect any toxicity 24 h after APAP exposure. Likewise, MTT assay did not detect toxic insults induced by APAP in liver equivalents (**Figure 3A**). However, the HCA technique added the possibility of detection of very early toxic events (24 h after APAP exposure) for liver cells, by the observation of cell phenotypic changes, with some mechanistic clues (**Figure 3D**). On the other hand, the confocal fluorescence microscopy (**Figure 3B**) and histology (**Figure 3C**) were showed to be a useful complementary tool in the investigation of the viability status of the human tissue's equivalents. For the liver cells, the simultaneous use of different techniques was very advantageous. The MTT assay, as mentioned before, was not able to detect the APAP cytotoxicity detected by the HCA 24 h after APAP exposure. The gene expression analyses assessed the functionality of the intestine (**Figure 2D-F**) and liver organoids (**Figure 4A-D**) at both basal and 24 h after APAP treatment. Under basal conditions, there were normal levels of intestinal and liver-specific markers, suggesting proper functionality. After 24 h of APAP exposure, there was a downregulation of albumin, GST mRNA levels, and a tendency to reduce the CYP3A4 gene expression in liver equivalent tissues, indicating APAP early cytotoxicity. Corroborating these findings, Western blotting experiments showed that the reduction in gene expression was also accompanied by a robust reduction in liver total albumin protein expression in response to APAP treatment (**Figure 4E-F**), confirming the toxic insults imposed on liver tissue by exposure to APAP. Accordingly, experiments of in vitro enzymatic activity showed a robust and progressive reduction in CYP 3A4 activity levels induced by APAP treatment, in liver equivalents (**Figure 4H-I**).

Morphometrical statistics of organoids were performed on Image J (**Figure 3E-I**). The area reveals how close the 2D size is to all organoids analyzed, which could be used as standardization in this protocol so that an unbiased result can be produced. The 'shape descriptors' reveal statistics

corresponding precisely to the shape morphology. The Aspect Ratio is an index which uses the major and minor axis, so results around 1 indicate no bias (i.e., preferential growth) during organoid formation. Values of Roundness ($4 \times$[area]/ ($\pi \times$ [major axis]^2)) are very sensitive to a preferential growth, which would be revealed as a major axis. Solidity ([area]/ ([convex area])) is essential in showing gross morphology as it is not affected by irregularities in borders since it uses convex area (=envelope). Distributions centered around 1.0 indicate putative spherical growth. Conversely, Circularity ($4\times \pi$ [area]/[perimeter]^2) is very sensitive to a complex perimeter, so "cavities" or "pockets" would impact this index. Thus, circularity around 1 also corroborates putative spherical growth, compatible with proper organoid functionality.

The analytical results showed that the MPS could emulate the APAP absorption and metabolism properties, both isolated or integrated in a curve comparable to that produced in vivo (**Figure 5F**) without the excretion phase. The APAP absorption was similar after oral administration to both Intestine MPS or Intestine/Liver MPS models, under both static and dynamic conditions (**Figure 5A-B, Figure 5D-E**). In both, there was an APAP concentration decreases at apical side concomitantly to its increase at the basolateral side, with no significant difference for static and dynamic conditions. In contrast, the APAP hepatic metabolism differed under these conditions. The circulating media in MPS seemed to improve the organoid metabolic capability (**Figure 5C** and **Figure 5E**). There was a significant APAP decay underflow not seen without flow. Interestingly, in vitro*,* CYP3A4 activity experiments corroborate the hypothesis that the presence of flow in the system increases the functionality of human liver equivalents. As shown in the graph in **Figure 4J**, the activity of CYP 3A4 was significantly higher in liver equivalents maintained under flow at both basal and APAP treatment conditions. Likewise, the liver equivalents kept under flow (dynamic) showed a tendency to increase the protein expression of albumin when compared to those kept without flow (static) both at baseline or at APAP treated conditions (**Figure 4G**).

When compared to the concentration-time profile after oral administration of APAP to humans, our microphysiological system shows much larger $t_{1/2}$ (half-life time) (**Figure 5F**). This happens, as mentioned before, because while we have a two-organ system with intestine and liver equivalents that can absorb and metabolize the drug, there is no kidney equivalent to excrete APAP and its metabolites from the plasma compartment[38]. In addition to that, the microphysiological system shows smaller Cmax (peak plasma concentration) and larger tmax (time to reach Cmax) than what is typically observed for humans (**Figure 5F**). This is a consequence of the differences in the scale of the in vitro and in vivo experiments. Overall, there are three main differences between the concentration–time profile obtained using the microphysiological system and profiles obtained after oral administration of APAP to humans: larger $t_{1/2}$, smaller C_{max} and larger t_{max} (**Figure 5F**). While the larger $t_{1/2}$ is due to the absence of a kidney equivalent to excrete APAP and its metabolites from the plasma equivalent, smaller C_{max} and larger t_{max} are consequences of the small scale of the experiment when compared to the human body. The best scaling strategies for building and operating microphysiological systems are still an active area of research and it is unlikely that a single approach is optimal for all systems[39]. Additionally, mathematical modeling or machine learning can be used to apply corrections or learn the mapping from the micro scale to full scale in order to extrapolate in vitro

data obtained with the microphysiological systems to the in vivo behavior observed in humans[40].

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DISCLOSURES:

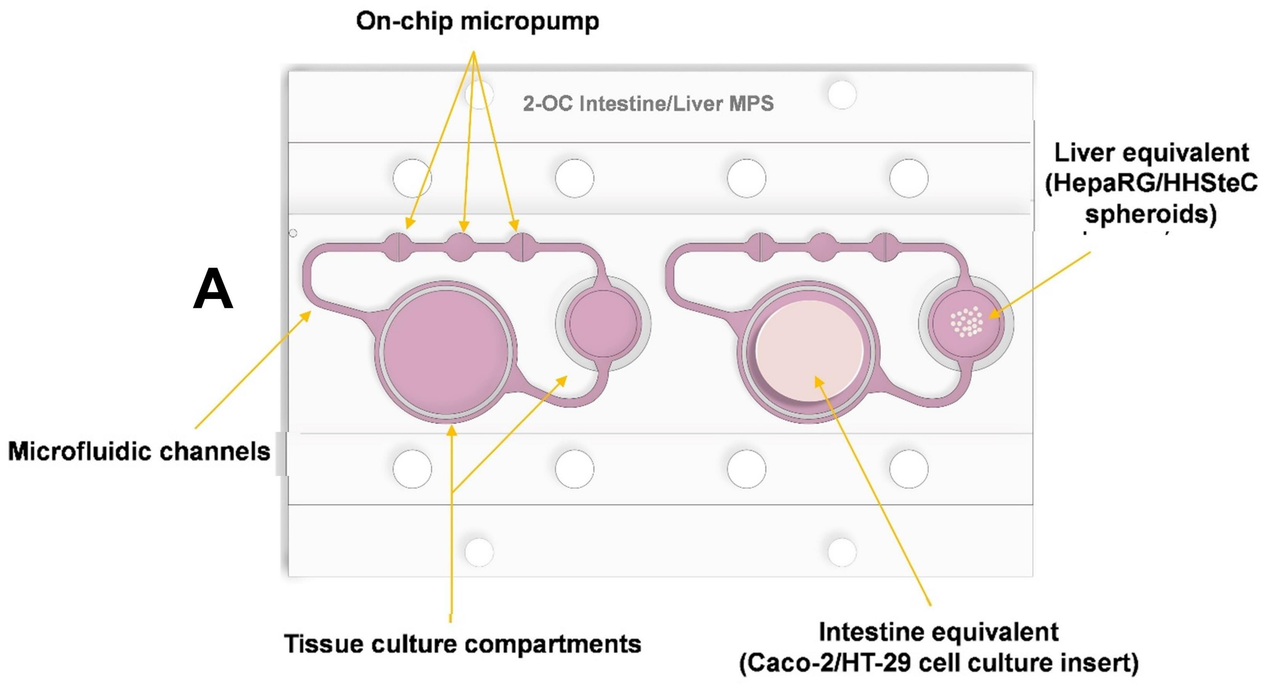
The authors have nothing to disclose.

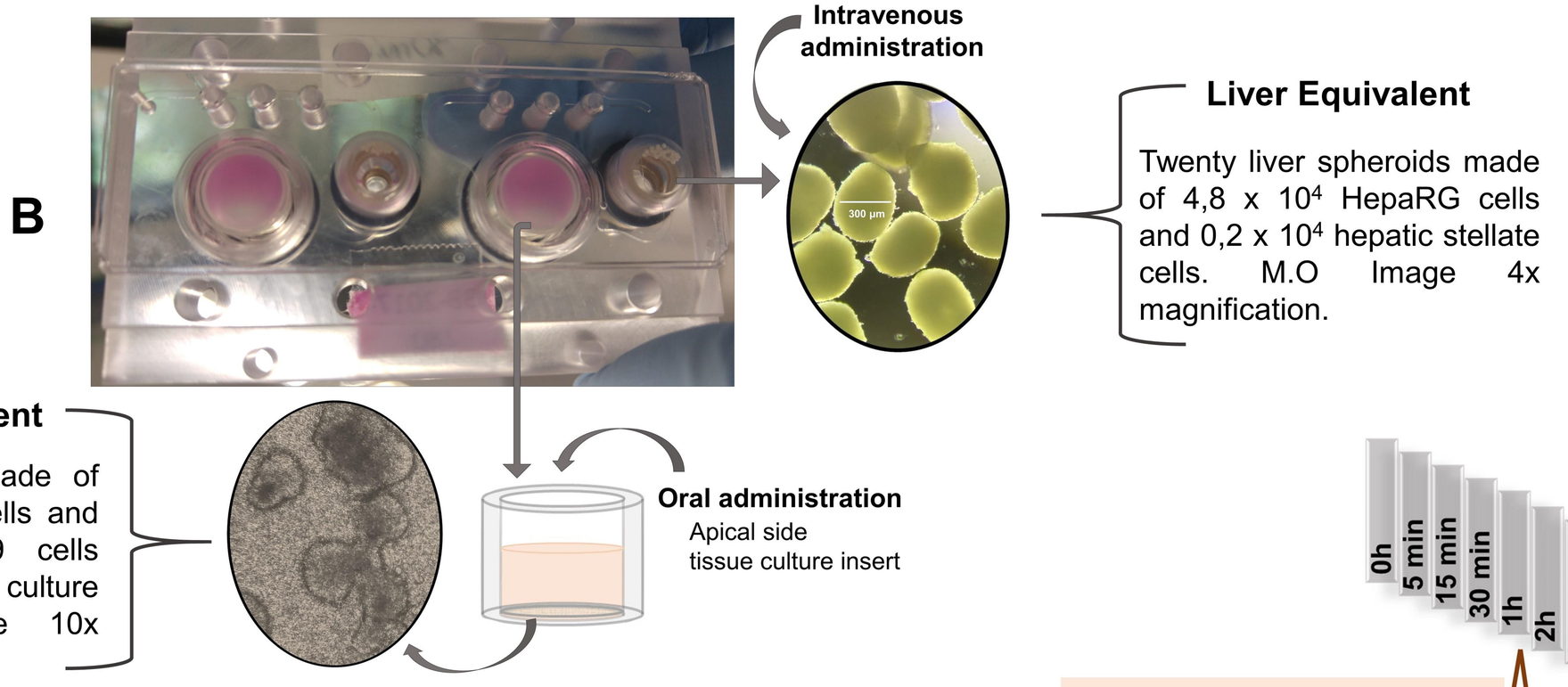
REFERENCES:

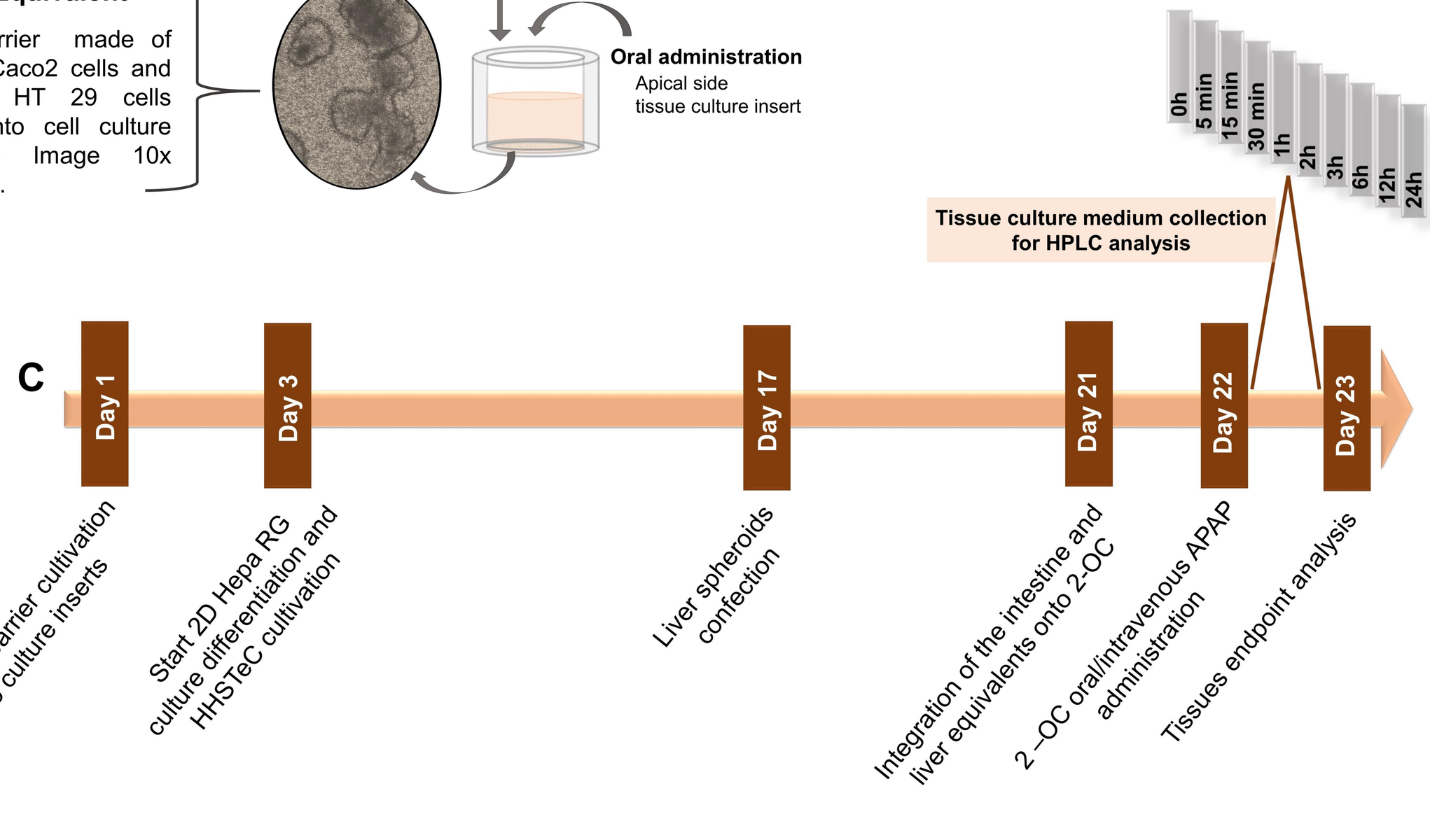
1. Zhang, D., Luo, G., Ding, X., Lu, C. Preclinical experimental models of drug metabolism and disposition in drug discovery and development. *Acta Pharmaceutica Sinica B*. **2** (6), 549–561 (2012).
2. Hynds, R.E., Giangreco, A. The relevance of human stem cell-derived organoid models for epithelial translational medicine. *Stem Cells*. **31** (3), 417–422 (2013).
3. Sivaraman, A. et al. A Microscale In vitro Physiological Model of the Liver: Predictive Screens for Drug Metabolism and Enzyme Induction. *Current Drug Metabolism*. **6** (6), 569–591 (2005).
4. Dehne, E.-M., Hasenberg, T., Marx, U. The ascendance of microphysiological systems to solve the drug testing dilemma. *Future Science OA*. **3** (2), FSO185 (2017).
5. Oleaga, C. et al. Multi-Organ toxicity demonstration in a functional human in vitro system composed of four organs. *Scientific Reports*. **6**, 20030 (2016).
6. Maschmeyer, I. et al. A four-organ-chip for interconnected long-term co-culture of human intestine, liver, skin and kidney equivalents. *Lab Chip*. **15** (12), 2688–2699 (2015).
7. Esch, M.B., Mahler, G.J., Stokol, T., Shuler, M.L. Body-on-a-chip simulation with gastrointestinal tract and liver tissues suggests that ingested nanoparticles have the potential to cause liver injury. *Lab Chip*. **14** (16), 3081–3092 (2014).
8. Materne, E.-M. et al. The Multi-organ Chip - A Microfluidic Platform for Long-term Multi-tissue Coculture. *Journal of Visualized Experiments*. **98**, 52526 (2015).
9. Esch, M.B., Ueno, H., Applegate, D.R., Shuler, M.L. Modular, pumpless body-on-a-chip platform for the co-culture of GI tract epithelium and 3D primary liver tissue. *Lab Chip*. **16** (14), 2719–2729 (2016).
10. Sung, J.H., Kam, C., Shuler, M.L. A microfluidic device for a pharmacokinetic–pharmacodynamic (PK–PD) model on a chip. *Lab on a Chip*. **10** (4), 446–455 (2010).
11. Viravaidya, K., Sin, A., Shuler, M.L. Development of a Microscale Cell Culture Analog to Probe Naphthalene Toxicity. *Biotechnology Progress*. **20** (1), 316–323 (2004).
12. Sin, A., Chin, K.C., Jamil, M.F., Kostov, Y., Rao, G., Shuler, M.L. The Design and Fabrication of Three-Chamber Microscale Cell Culture Analog Devices with Integrated Dissolved Oxygen Sensors. *Biotechnology Progress*. **20** (1), 338–345 (2004).
13. Tsamandouras, N. et al. Integrated Gut and Liver Microphysiological Systems for Quantitative In vitro Pharmacokinetic Studies. *The AAPS Journal*. **19** (5), 1499–1512 (2017).
14. Graham, G.G., Davies, M.J., Day, R.O., Mohamudally, A., Scott, K.F. The modern pharmacology of paracetamol: Therapeutic actions, mechanism of action, metabolism, toxicity

969 and recent pharmacological findings. *Inflammopharmacology*. **21** (3), 201–232 (2013).
970 15. Hodgman, M.J., Garrard, A.R. A Review of Acetaminophen Poisoning. *Critical Care Clinics*.
971 **28** (4), 499–516 (2012).
972 16. Maschmeyer, I. et al. Chip-based human liver-intestine and liver-skin co-cultures - A first
973 step toward systemic repeated dose substance testing in vitro. *European Journal of*
974 *Pharmaceutics and Biopharmaceutics*. **95**, 77–87 (2015).
975 17. Bessems, J.G.M., Vermeulen, N.P.E. Paracetamol (acetaminophen)-induced toxicity:
976 Molecular and biochemical mechanisms, analogues and protective approaches. *Critical Reviews*
977 *in Toxicology*. **31** (1), 55–138 (2001).
978 18. Hirt, M.N. et al. Increased afterload induces pathological cardiac hypertrophy: A new in
979 vitro model. *Basic Research in Cardiology*. **107** (6), 307 (2012).
980 19. Fink, C. et al. Chronic stretch of engineered heart tissue induces hypertrophy and
981 functional improvement. *The FASEB journal : official publication of the Federation of American*
982 *Societies for Experimental Biology*. **14** (5), 669–679 (2000).
983 20. Zhang, B., Peticone, C., Murthy, S.K., Radisic, M. A standalone perfusion platform for drug
984 testing and target validation in micro-vessel networks. *Biomicrofluidics*. **7** (4), 044125 (2013).
985 21. Araújo, F., Sarmento, B. Towards the characterization of an in vitro triple co-culture
986 intestine cell model for permeability studies. *International Journal of Pharmaceutics*. **458** (1),
987 128–134 (2013).
988 22. Cadena-Herrera, D. et al. Validation of three viable-cell counting methods: Manual, semi-
989 automated, and automated. *Biotechnology Reports*. **7**, 9–16 (2015).
990 23. Millipore, M. User Guide Millicell® ERS-2 Electrical Resistance System. 6–10, at
991 <http://www.merckmillipore.com/BR/pt/life-science-research/cell-culture-systems/cell-
992 analysis/millicell-ers-2-voltohmmeter/FiSb.qB.LDgAAAFBdMhb3.r5,nav> (2016).
993 24. Gripon, P. et al. Infection of a human hepatoma cell line by hepatitis B virus. *Proceedings*
994 *of the National Academy of Sciences of the United States of America*. **99** (24), 15655–15660
995 (2002).
996 25. Guillouzo, A. et al. The human hepatoma HepaRG cells: A highly differentiated model for
997 studies of liver metabolism and toxicity of xenobiotics. *Chemico-Biological Interactions.* **168** (1),
998 66–73 (2007).
999 26. Wagner, I. et al. A dynamic multi-organ-chip for long-term cultivation and substance
1000 testing proven by 3D human liver and skin tissue co-culture. *Lab on a Chip*. **13** (18), 3538–3547
1001 (2013).
1002 27. Forrest, J.A.H., Clements, J.A., Prescott, L.F. Clinical Pharmacokinetics of Paracetamol.
1003 *Clinical Pharmacokinetics*. **7** (2), 93–107 (1982).
1004 28. Dollery, C. *Therapeutic Drugs*. Churchill Livingstone. London. (1999).
1005 29. FDA, F. and D.A., Food and Drug Administration *Guidance for Industry: Bioanalytical*
1006 *method validation. U.S. Department of Health and Human Services*.
1007 http://www.labcompliance.de/documents/FDA/FDA-Others/Laboratory/f-507-bioanalytical-
1008 4252fnl.pdf. (2013).
1009 30. Shah, V.P. et al. Bioanalytical method validation-a revisit with a decade of progress.
1010 *Pharmaceutical research*. **17** (12), 1551–1557 (2000).
1011 31. Marin, T.M. et al. Shp2 negatively regulates growth in cardiomyocytes by controlling focal
1012 adhesion kinase/src and mTOR pathways. *Circulation Research*. **103** (8), 813–824 (2008).

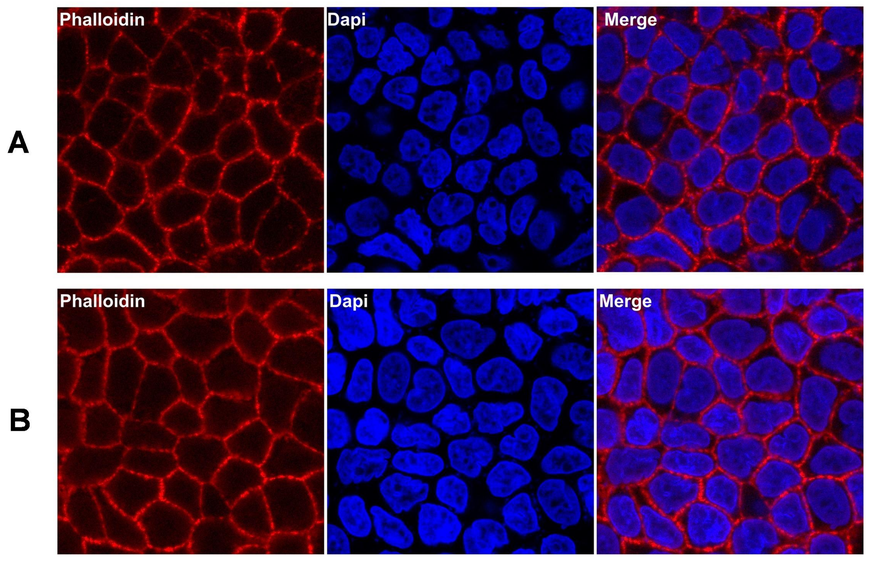
32. Linkert, M. et al. Metadata matters: Access to image data in the real world. *Journal of Cell Biology*. **189** (5), 777–782 (2010).
33. OECD OECD TG 491 - Short Time Exposure In vitro Test Method for Identifying i) Chemicals Inducing Serious Eye Damage and ii) Chemicals Not Requiring Classification for Eye Irritation or Serious Eye Damage. *OECD guidelines for the testing of chemicals*. **14**, doi: https://doi.org/10.1787/9789264242432-en. (2018).
34. Fernandes, M.B., Gonçalves, J.E., Tavares, L.C., Storpirtis, S. Caco-2 cells permeability evaluation of nifuroxazide derivatives with potential activity against methicillin-resistant Staphylococcus aureus (MRSA). *Drug Development and Industrial Pharmacy*. **41** (7), 1066–1072 (2015).
35. OECD OECD TG 492 - Reconstructed human Cornea-like Epithelium (RhCE) test method for identifying chemicals not requiring classification and labelling for eye irritation or serious eye damage. *OECD guidelines for the testing of chemicals*. **35** (2018).
36. OECD OECD TG 431 - In vitro skin corrosion: reconstructed human epidermis (RHE) test method. *OECD guidelines for the testing of chemicals*. **8** (2016).
37. OECD OECD TG 439 - In vitro Skin Irritation: Reconstructed Human Epidermis Test Method. *OECD guidelines for the testing of chemicals*. **21** (2015).
38. Marin, T.M. et al. Acetaminophen absorption and metabolism in an intestine/liver microphysiological system. *Chemico-Biological Interactions*. **299**, 59–76 (2019).
39. Maass, C., Stokes, C.L., Griffith, L.G., Cirit, M. Multi-functional scaling methodology for translational pharmacokinetic and pharmacodynamic applications using integrated microphysiological systems (MPS). *Integrative Biology (United Kingdom)*. **9** (4), 290–302 (2017).
40. Sung, J.H., Wang, Y., Shuler, M.L. Strategies for using mathematical modeling approaches to design and interpret multi-organ microphysiological systems (MPS). *APL Bioengineering*. **3** (2), 021501 (2019).

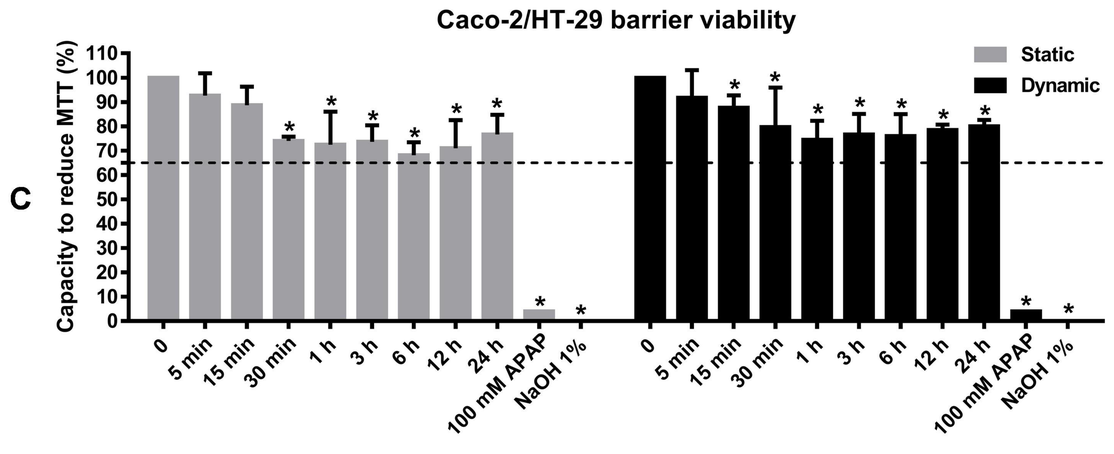


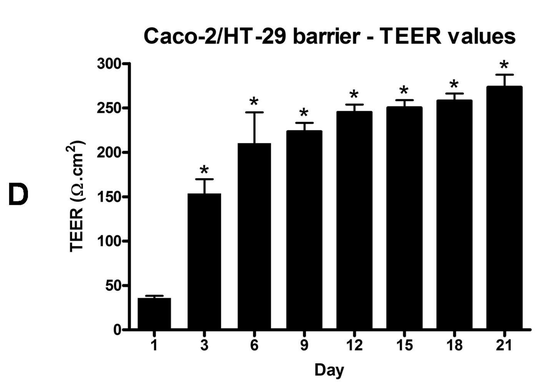


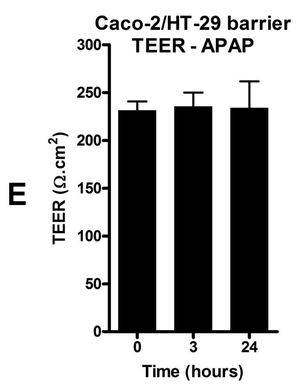


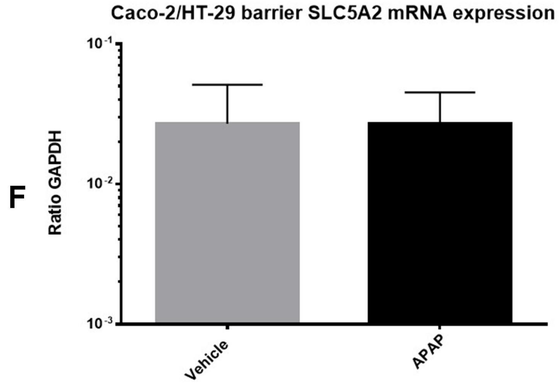
12 µM APAP TREATMENT

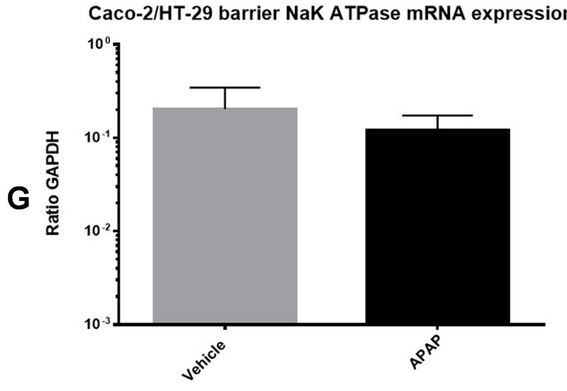


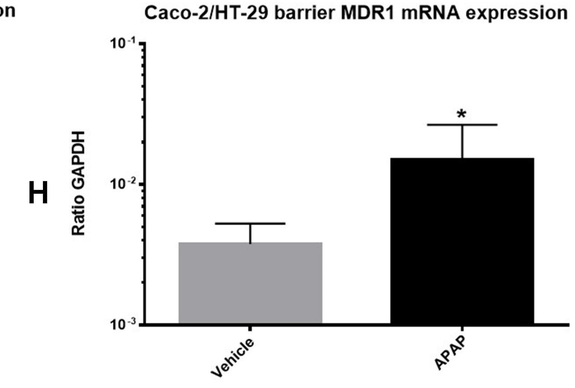


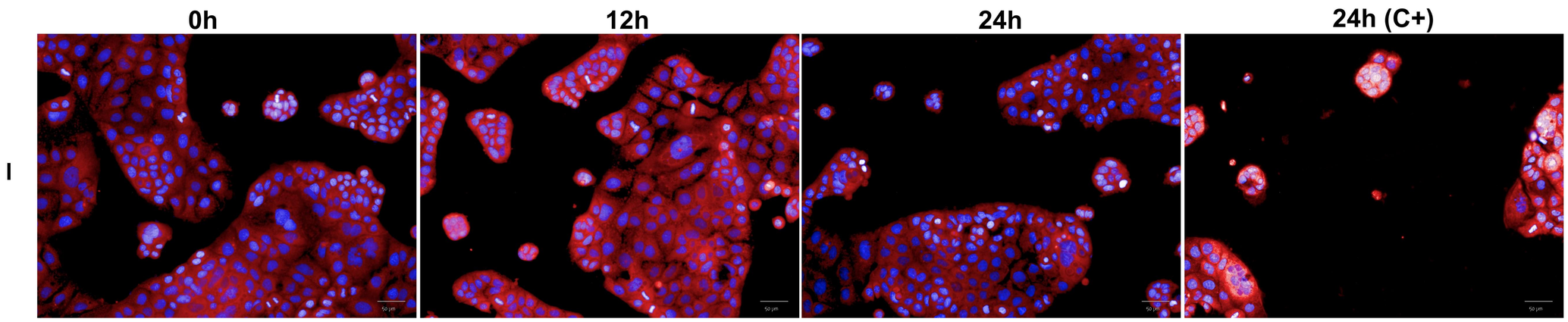




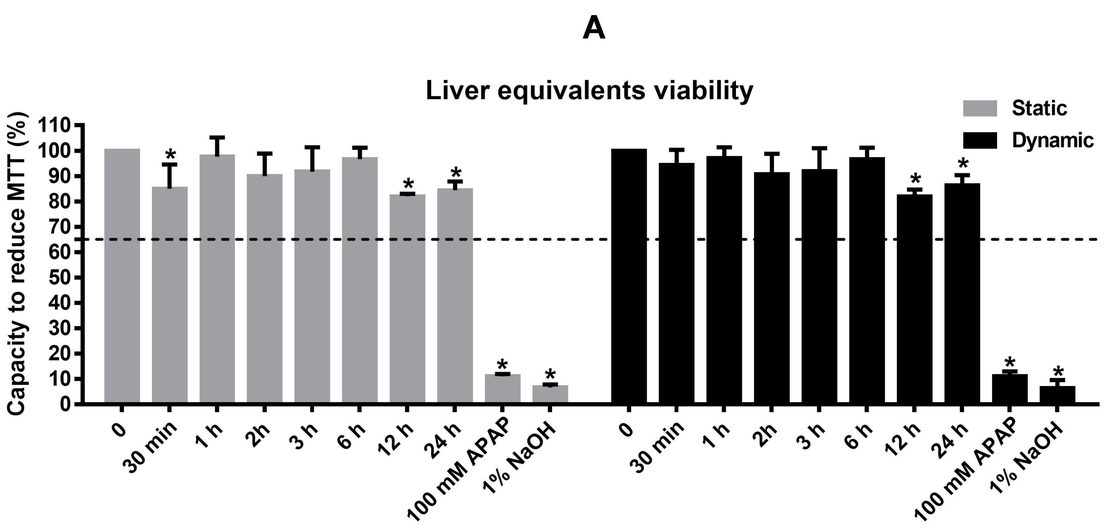


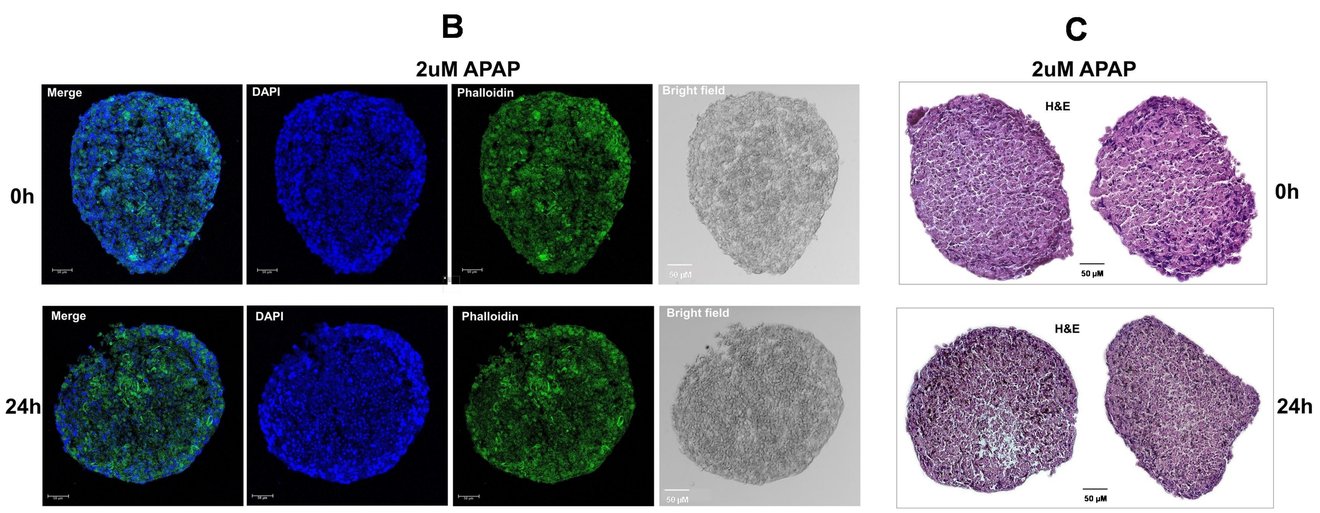


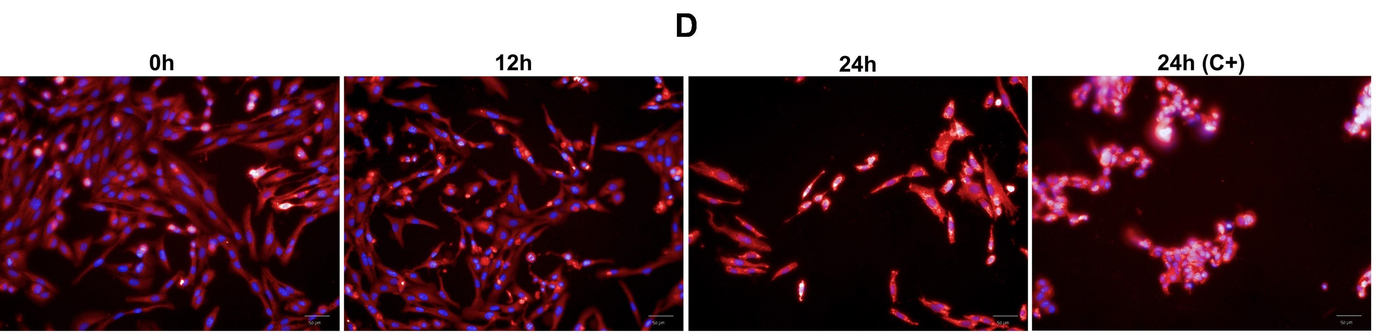


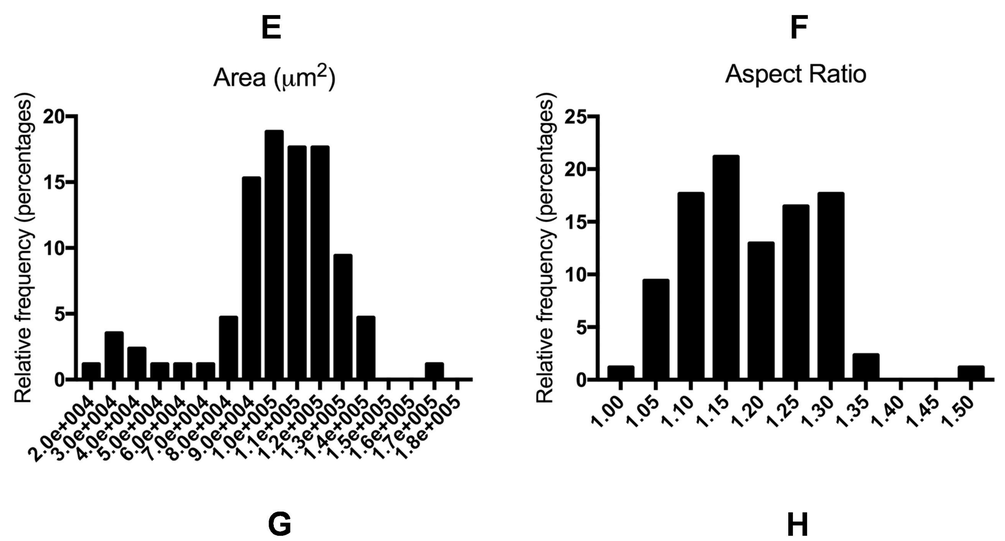


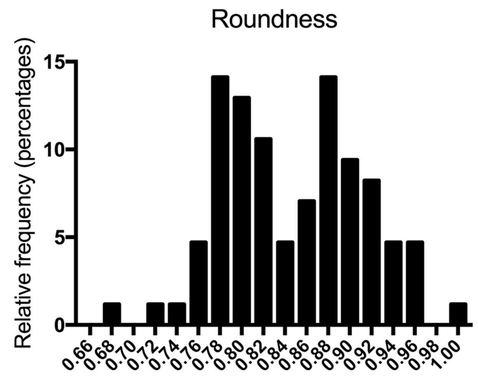
2 µM APAP TREATMENT

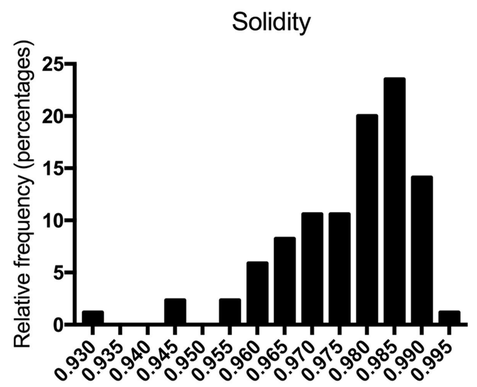


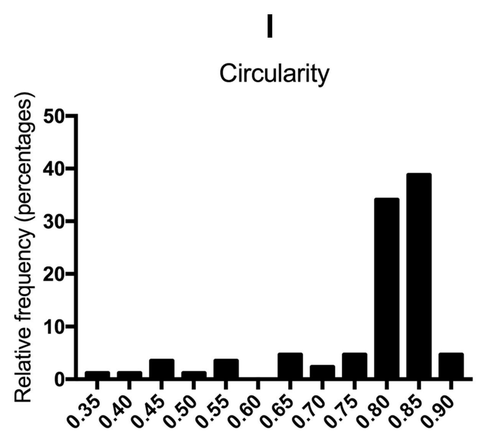


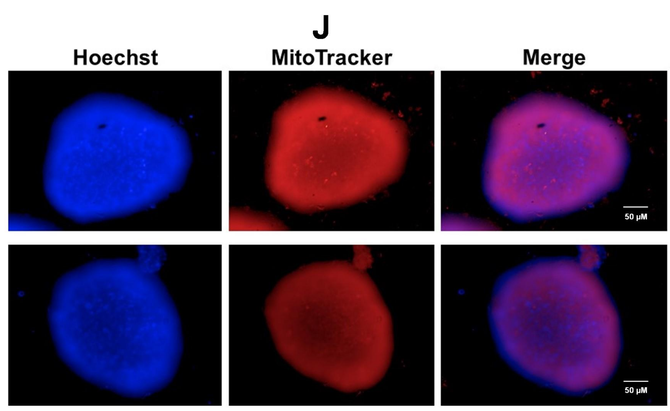




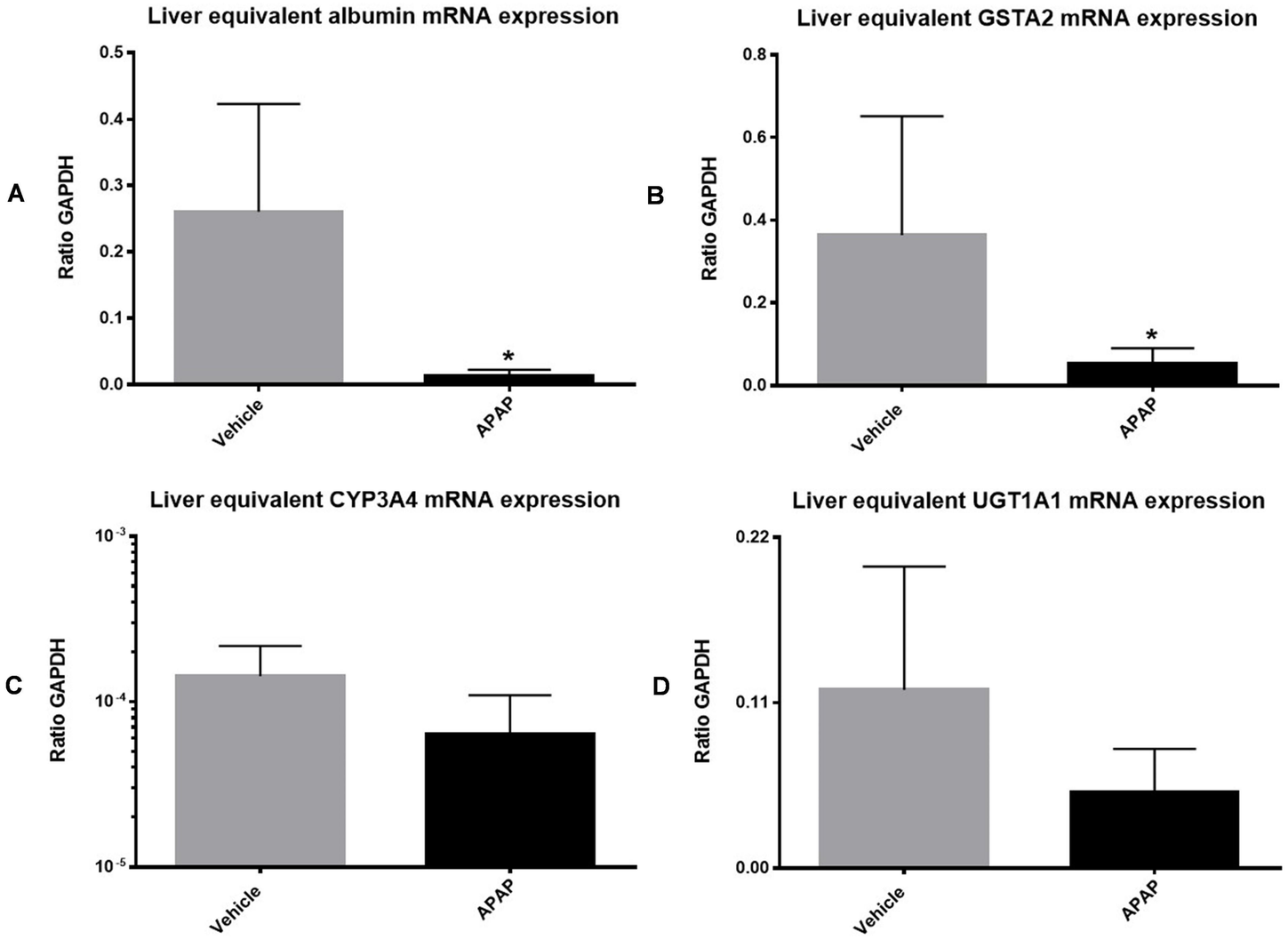


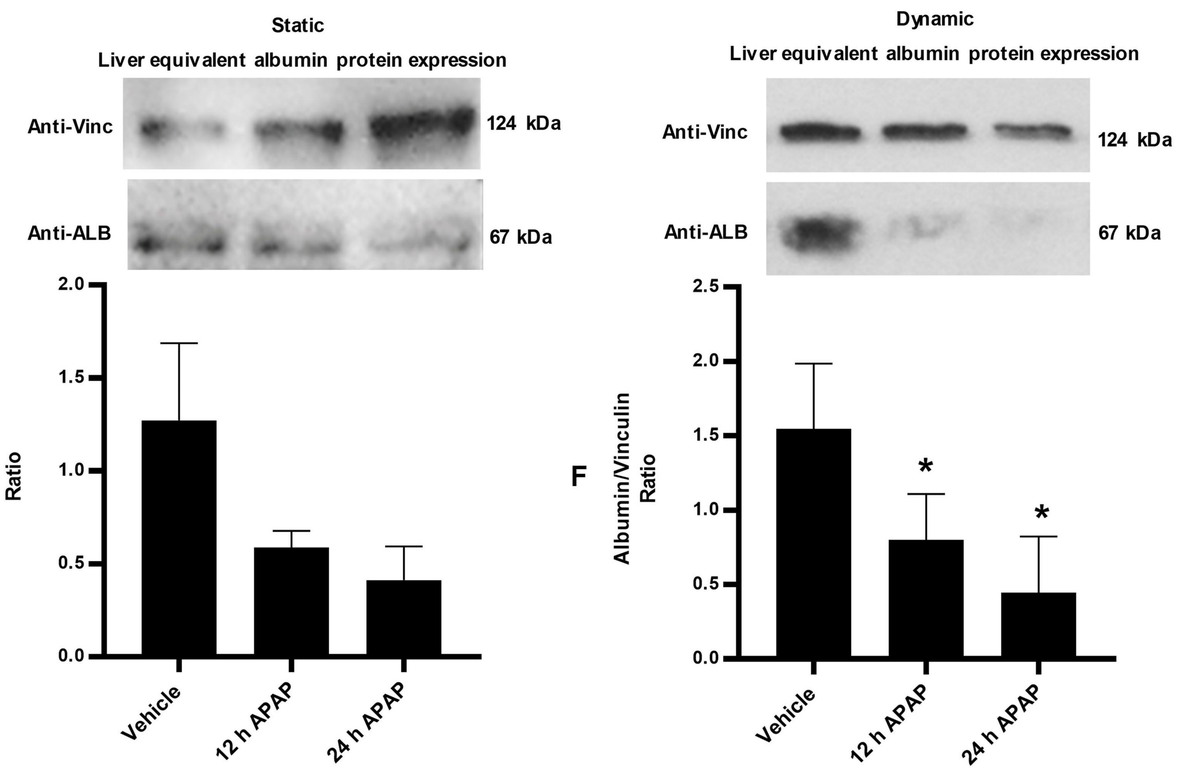


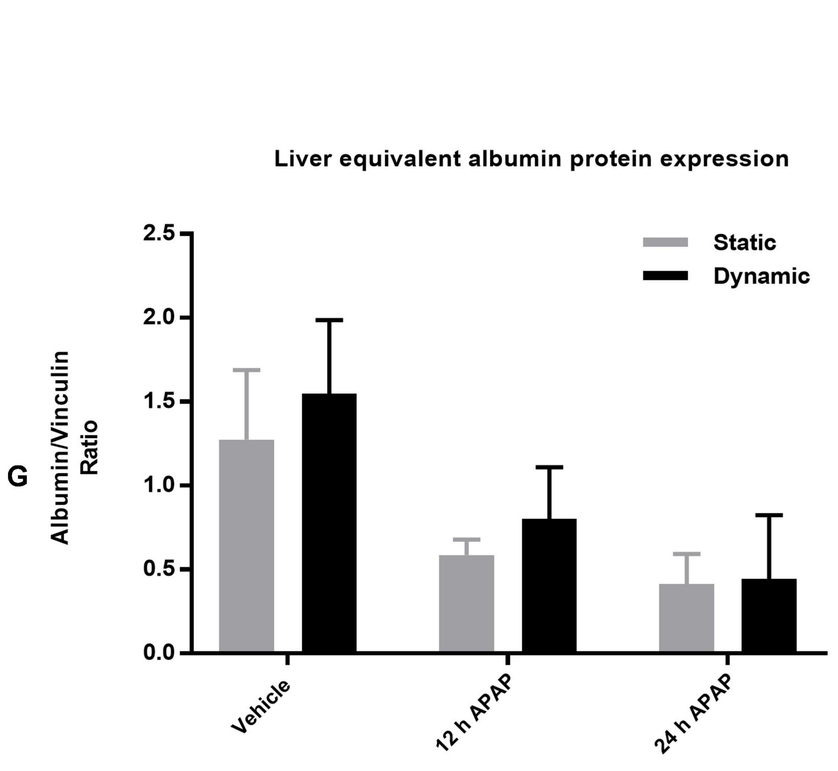


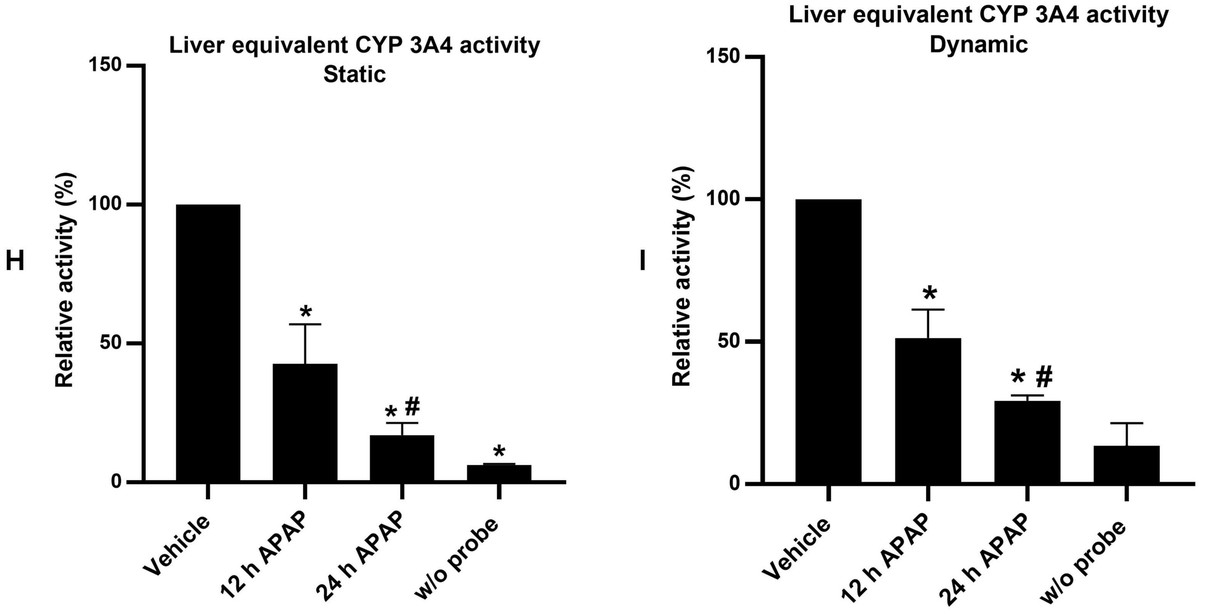


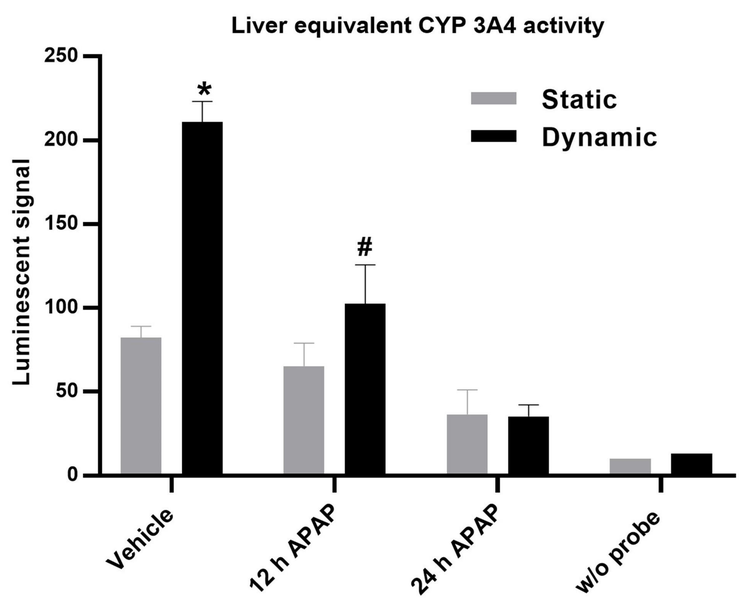
2 μM APAP TREATMENT











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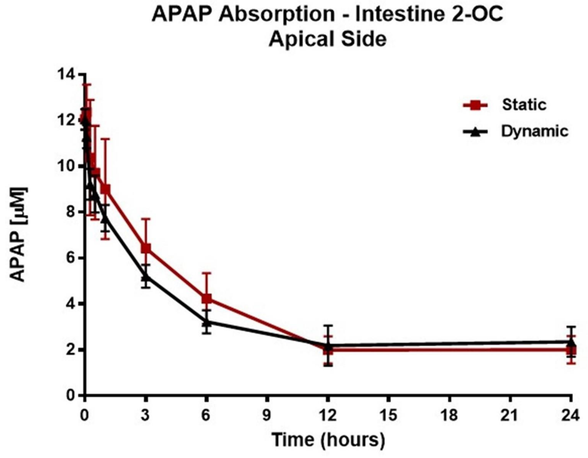

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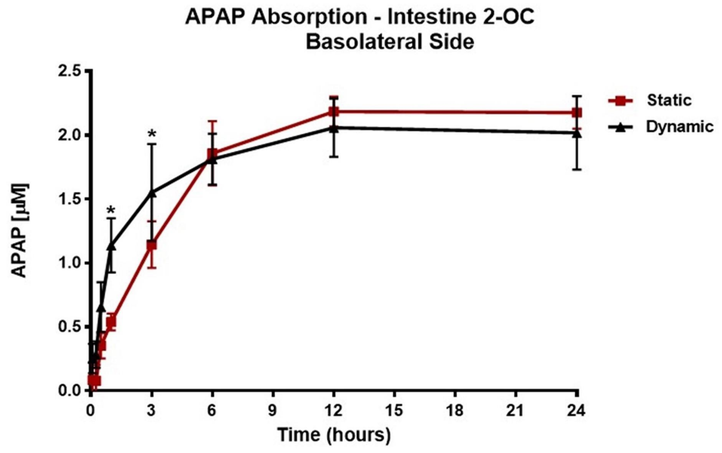

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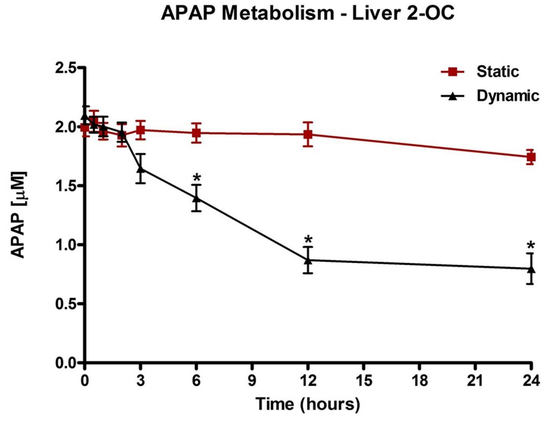

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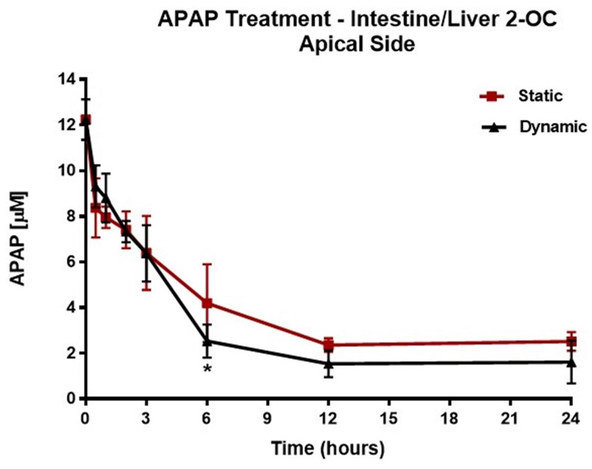

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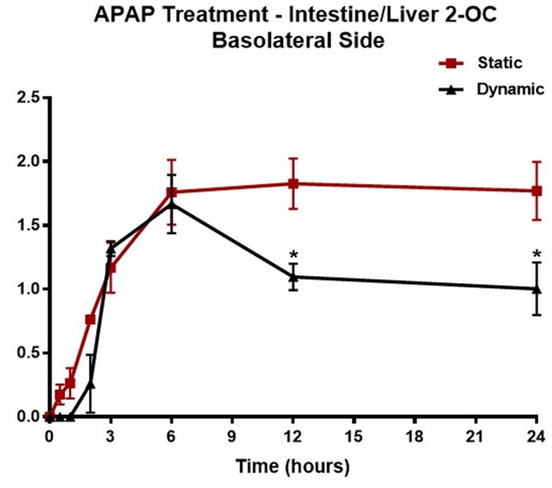

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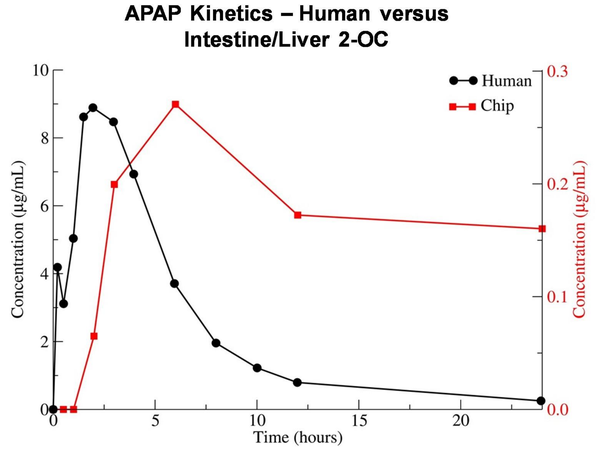

Table 1

Click here to access/download;Table;Table 1_conditionsparameters.xlsx

HPLC system	Waters Alliance 2695 (Milford, MA, USA), equipped with quaternary		
Detector	Waters 2996 Uv-Vis set in 210-400 nm range		
System control, data acquisition, and processing	Waters Empower 2002 chromatography software		
Column	Reversed-phase Luna C18 (150 x 4.6 mm I.D.; 5μm particle size) Phenomenex		
Guard Column	Reversed-phase Luna C18 (4 x 3 mm) Phenomenex		
Mobile phase	Solvent A- Acetonitrile Solvent B- 0.10 M ammonium acetate, pH 6.8		
Isocratic conditions	Time (min.)	A (%)	B (%)
	15	5	95
Flow	1.0 mL/min		
Injection volume	25 μL		
Temperature	25 °C		
APAP Detection	UV @ 243 and 254 nm		
Run time	15 minutes		

Table 2

Nominal concentration (μM)	Calculated APAP concentration (μM) (triplicate of each concentration)						Average (μM)	S.D.[b] (μM)	C.V. (%)[c]	DEV (%)[d]
Assay number	1	2	3	4	5	6				
0.25 (LOD)	0.02	0.08	0.21	0.14	0.08	0.27	0.13	0.11	84.96	+ 46.05
0.50 (LLOQ)	0.31	0.36	0.29	0.47	0.42	0.65	0.41	0.05	12.72	+ 17.08
1.00	0.87	0.87	0.80	1.04	1.02	1.01	0.93	0.04	3.76	+ 6.65
2.50	2.44	2.61	2.52	2.42	2.56	2.54	2.52	0.04	1.54	-0.62
5.00	5.02	4.99	5.06	5.01	5.01	5.05	5.02	0.09	1.88	-0.45
10.00	10.21	10.13	9.96	10.25	10.08	10.21	10.14	0.10	0.97	-1.41
25.00	25.33	25.28	25.20	25.13	25.14	24.92	25.17	0.36	1.45	-0.67
50.00	50.30	50.04	50.51	49.70	49.98	49.19	49.95	0.86	1.71	+0.09
100.00	99.75	99.90	99.70	100.09	99.97	100.40	99.97	0.69	0.69	+0.03
R^2	1.00	1.00	0.9999	1.00	1.00	0.9999				

Table 3

Nominal concentration (μM)	Calculated APAP concentration (μM) (Triplicate of each concentration)					Average (μM)	S.D.[b] (μM)	C.V. (%)[c]	DEV (%)[d]
Assay number	1	2	3	4	5				
0.25 (LOD)	0.34	0.12	0.30	0.16	0.00	0.18	0.08	45.46	+26.03
0.50 (LLOQ)	0.36	0.44	0.49	0.43	0.40	0.43	0.02	5.84	+14.97
1.00	0.87	0.98	1.04	0.94	0.83	0.93	0.04	4.67	+6.85
2.50	2.41	2.46	2.49	2.52	2.43	2.46	0.06	2.39	+1.56
5.00	5.00	4.99	5.12	5.10	5.14	5.07	0.15	3.00	-1.38
10.00	10.08	10.01	9.93	10.10	10.29	10.08	0.18	1.80	-0.81
25.00	25.14	25.18	24.96	25.32	25.35	25.19	0.45	1.78	-0.76
50.00	50.19	50.23	49.83	49.56	50.17	49.99	0.87	1.75	+0.01
100.00	99.87	99.84	100.10	100.13	99.80	99.95	2.12	2.12	+0.05
R^2	1.00	1.00	1.00	1.00	1.00				

Table 4

Williams medium	Nominal concentration (µM)	Measured concentration (µM)	S.D. (µM)	C.V. (%)	DEV (%)
Intra-run (n=3)	0.50 (LLOQ)	0.49	0.08	15.55	+1.77
	4.50	4.59	0.23	5.10	-2.06
	45.00	41.23	0.76	1.85	+8.37
	90.00	82.29	1.75	2.13	+8.57
Inter-run (n=15)	0.50 (LLOQ)	0.43	0.05	10.99	+14.97
	4.50	4.37	0.19	4.42	+2.99
	45.00	42.35	0.82	1.93	+5.88
	90.00	85.22	2.25	2.65	+5.31
DMEM medium	**Nominal concentration (µM)**	**Measured concentration (µM)**	**S.D. (µM)**	**C.V. (%)**	**DEV (%)**
Intra-run (n=3)	0.50 (LLOQ)	0.47	0.09	18.77	+6.35
	4.50	4.45	0.30	6.63	+1.04
	45.00	44.24	1.59	3.58	+1.69
	90.00	86.40	4.09	4.73	+4.00
Inter-run (n=12)	0.50 (LLOQ)	0.46	0.08	17.81	+7.75
	4.50	5.16	0.27	5.31	-14.68
	45.00	48.99	2.10	4.29	-8.86
	90.00	96.18	4.47	4.65	-6.86

Table 5

		Primer (5' → 3')	
Tissue	**Gene**	**Forward**	**Reverse**
Intestine	SGLT1/SLC5A1	gAgCCCAgCAACTgTCCCAC	CAggCTCCAACACAgACggT
	NA-K-ATPase	ACCgCCCAgAAATCCCAAAAC	CAgCggTCATCCCAgTCC
	MDR1	TggATgTTTCCggTTTggAg	TgTgggCTgCTgATATTTTgg
Liver	Albumin	TgCAAggCTgATAAggAg	TTTAgACAgggTgTTggCTTTACAC
	GSTA2	CTgAggAACAAgATgCCAAgC	AgCAgAgggAAggCTggAAATAAg
	CPY3A4	ggAAggTggACCCAgAAACTgC	TTACggTgCCATCCCTTgAC
	UGT1A1	ATgCAAAgCgCATggAgAC	ggTCCTTgTgAAggCTggAg

Name of Material/ Equipment	Company	Catalog Number	Comments
1x DPBS	Thermo Fisher Scientific	14190235	No calcium, no magnesium
2-OC	TissUse GmbH		Two-organ chip
384-well Spheroid Microplate	Corning	3830	Black/Clear, Round Bottom, Ultra-Low Attachment
4% Paraformaldehyde			Use to fix cell
Acetaminophen	Sigma Aldrich	A7085	Use to MPS assays
Acetonitrile	Tedia		Used to perform HPLC
Alexa Fluor 647 phalloidin	Thermo Fisher Scientific		confocal experiment
Ammonium acetate	Sigma Aldrich		Used to perform HPLC
Caco-2 cells	Sigma Aldrich	86010202	
Cacodylate buffer			
Cell culture flasks	Sarstedt		
Confocal Fluorescence microscope	Leica	DMI6000	
Cryostat	Leica	CM1950	
DMEM high glucose	Thermo Fisher Scientific	12800017	Add supplements: 10% fetal bovine serum, 100 units per mL penicillin, 100 µg/mL streptomycin, and 1% non-essential amino acids
DMSO	Sigma Aldrich	D4540	Add 2% to HepaRG media
Ethanol	Synth		
Fetal Bovine Serum	Thermo Fisher Scientific	12657029	
Freezing medium OCT	Tissue-Tek		Tissue-Tek® O.C.T.™ Compound is a formulation of watersoluble glycols and resins, providing a convenient specimen matrix for cryostat sectioning at temperatures of -10°C and below.
Hematoxylin & Eosin			
HepaRG cells	Biopredic International	HPR101	Undifferentiated cells
HHSTeC	ScienCell Research Laboratories	5300	Cells and all culture supplements
Hoechst 33342			HCA experiments

HT-29 cells	Sigma Aldrich	85061109	
Human Insulin	Invitrogen - Thermo Fisher Scientific	12585014	
Hydrocortisone	Sigma Aldrich	H0888	
Isopropanol	Merck	278475	
Karnovsky's fixative			
L-glutamine	Thermo Fisher Scientific	A2916801	
Luna C18 guard column SS	Phenomenex		Used to perform HPLC
Microscope	Leica	DMi4000	
Microtome	Leica	RM2245	
Millicell 0.4 µm pore size inserts	Merck	PIHP01250	
Millicell ERS-2 meter	Merck	MERS00002	Used to TEER measurement
MitoTracker Deep Red			HCA experiments
MTT	Thermo Fisher Scientific	M6494	
MX3000P system	Agilent Technologies		
Neubauer chamber			Counting cells
Operetta High Content Imaging System	Perkin Elmer		Used to perform HCA
P450-Glo CYP3A4 Assay with Luciferin-IPA	Promega	Cat.# V9001	
Penicillin/Streptomycin	Thermo Fisher Scientific	15070063	Cell culture
Permount	Thermo Fisher Scientific		Histology
Primers			RT-qPCR
PVDF membrane	BioRad		
PVDF Syringe filter			0.22 µm pore size
Reversed-phase Luna C18 column	Phenomenex		Used to perform HPLC
Shaker (IKA VXR Basic Vibrax)	IKA Works GmbH & Co	2819000	Used for spheroids to improve MTT assay
Stellate Cell Media (STeC CM)	ScienCell	5301	Add STeC CM supplements

SuperScriptIITM Reverse Transcriptase	Thermo Fisher Scientific		
SYBR Green PCR Master Mix	Thermo Fisher Scientific		
TRizol TM reagent	Thermo Fisher Scientific		Trizol is a monophasic solution of phenol and guanidine isothiocyanate.
Trypsin/EDTA solution	Thermo Fisher Scientific	R001100	
Ultra-low-attachment plates	Corning	CLS3471-24EA	6 wells
Vectashield plus DAPI mounting media			
White Opaque 96-well Microplate	PerkinHelmer		
Wide-bore tips			
Williams E	Pan Biotech	P04-29510	Add supplements: 10% fetal bovine serum, 2 mM L-glutamine, 100 units per ml penicillin, 100 µg/mL streptomycin and 5 µg/mL human insulin

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Title of Article: Intestine/Liver microphysiological System for drugs Pharmacokinetic and Toxicological Assessment

Author(s): Sara M Marin, Nathalia C Indolfo; Silvana A. Rocco; Marcelo de Carvalho; Marcia M Dias; Eduardo Pagani

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Signature: Saleta Miguel Marin Date: 04/29/2019

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Dear Editor and Reviewers,

Below are the answers (in blue) to the doubts, questions and suggestions made by you. The authors are very grateful for the careful review of this manuscript.
Sincerely yours,

Talita Miguel Marin

Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
2. We can only have 6-12 keywords. Modification performed.
3. Please rephrase the Long Abstract to more clearly state the goal of the protocol. Modification performed.
4. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. Modification performed.
For example: TissUse GmbH (2-OC)), Millicell ERS-2 meter, Leica CM1950 cryostat, Permount, Evotec/PerkinElmer Opera Flex, Operetta High Content 573 Imaging System, TRizol TM reagent, SuperScriptIITM Reverse Transcriptase, SYBR Green PCR Master Mix, etc.
5. Unfortunately, there are a few sections of the manuscript that show significant overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Please see lines: 113-115, 118-119, 158-160, 162-163, 168-175, 205-206, 248-250, 252-258, 282-283, 285-287, 295-299, 352-382, 405-407, 416-417, 427-430, 448-457, 470-490673-674, 743-744. Modification performed.
6. Please ensure the Introduction includes all of the following with citations:
a) A clear statement of the overall goal of this method
b) The rationale behind the development and/or use of this technique
c) The advantages over alternative techniques with applicable references to previous studies
d) A description of the context of the technique in the wider body of literature
e) Information to help readers to determine whether the method is appropriate for their application
7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Modification performed.
8. The Protocol should contain only action items that direct the reader to do something. ok

9. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please ensure that individual steps of the protocol should only contain 2-3 actions per step. ok
10. Please ensure you answer the "how" question, i.e., how is the step performed?
11. How do you check for tight monolayer formation? How do you change the media?
ANSWER FROM AUTHORS: The tight monolayer formation was verified by measuring TEER (transepithelial electrical resistance) every three days using a voltmeter, as described in Protocol section 1.1.6. Additionally, confocal fluorescence microscopy images of non-treated Caco-2/HT-29 cells stained with cell nuclei and actin filaments fluorescent dye (DAPI and Phalloidin respectively) indicated the formation of a contiguous barrier (Fig. 2 A). The media is change as described at Protocol section 1.1.5 " Change the medium at least three times a week, aspirating it from both the apical and basolateral sides with a sterile Pasteur pipette, taking care not to damage the intact cell barrier. NOTE: Proceed with the aspiration on the apical side, so as not to touch the cell barrier (aspirate by supporting the Pasteur pipette on the plastic rim of the cell insert)."
12. Please include a citation or include how to perform these steps?
ANSWER FROM AUTHORS: Modification performed – Inclusion were done ate Protocol section 1.1.5 and 1.1.6.
13. How is the resistance measurement performed?
ANSWER FROM AUTHORS: According to the description inserted in this review in the protocols section 1.1.6: "Check the tight monolayer formation, measuring TEER (transepithelial electrical resistance) every three days using a voltmeter[22], according to the manufacturer's instructions. Perform a blank, measuring the resistance across a cell culture insert without cells, but with the same cell medium and at the same cell plate. Calculate tissue resistance, subtracting the blank resistance from the tissue equivalent resistance, and multiply by the effective surface area of the filter membrane (0.6 cm2). A good intestine barrier resistance is in a range of 150 to 400 Ωcm2.NOTE: After 21 days the cells must be fully differentiated, the intestinal barrier formed, so the intestine equivalents are ready to be integrated into MPS."
14. The current protocol is longer than our limit for the protocol section. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content along with the formatting done as of now. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. ok
15. Please remove the embedded table of materials - Modification performed.
- and upload it as .xlsx file to your editorial manager account. Please sort the materials table alphabetically. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials. OK
16. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. ok
The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]." Modification performed.
17. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
18. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage, (YEAR).] For more than 6 authors, list only the first author then et al.
19. Please include a scale bar for all the images acquired with a microscope. OK
20. Please change hours to h, minutes to min and seconds to s in the figures. Also please include a singly space between the number and the units. Modification performed.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

Marin et al demonstrate the feasibility of in line connection of two critical organs utilizing a microphysiological systems approach and acetaminophen as a test agent. The authors clearly lay out the need for development of techniques to couple MPS to mimic the metabolism of drugs and communication of such events to distal organs. The approach is justified and timely as researchers seek alternatives to small animal models. The protocol was written in sufficient detail to easily follow. Validation experiments were provided that convincingly showed the utility of the system.

Major Concerns:

None

Minor Concerns:

Editing of the verbiage would help clarify some areas of the manuscript. Additionally, Figure 1 appears quite blurry, upgrade the graphics.

Ans: Modification performed.

Reviewer #2:

Manuscript Summary:

This manuscript describes a microfluidic system forming intestinal and hepatic crosstalk. The intestinal system is composed of a Caco-2 and HT-29 co-culture whereas the hepatic portion is composed of HepaRG and human hepatic stellate cell liver spheroids. This group assessed toxicity and viability in the intestinal system and hepatic system separately by using acetaminophen at 12 µM to simulate oral administration of drug, and 2 µM to simulate intravenous administration. Some important revisions are needed prior to publication as described below.

Major Concerns:

1. The investigators administer 12 µM of acetaminophen as an oral dose, but 2 µM as an intravenous dose. While this would perhaps be physiologically correct for administration, it is unclear that this model has the capability of exhibiting first pass metabolism which would be responsible for reducing the oral bioavailability of a drug to more closely resemble a clinical intravenous dose.

ANSWER FROM AUTHORS: The dilution of 200 µL of culture medium at 12 µM APAP concentration (applied to the apical side of the intestinal barrier - 4.1. APAP “oral”

administration and media sampling, methods section)) in the total volume of MPS, which is 1000 µl, is the cause of the difference between the concentration of orally administered APAP (12 uM) and the systemically APAP (2 uM) verified concentration after full intestinal absorption. The total volume of the microfluidic device is 1000ul counting the apical side of the intestinal barrier (200ul), the basolateral side (500ul), and the liver compartment (300ul).

Finally, why were these concentrations chosen? Do they resemble physiologic values or were they picked arbitrarily?

ANSWER FROM AUTHORS: During the standardization phase of the pharmacokinetic tests, different conditions including the appropriate concentrations of APAP treatment were tested. Regarding the liver equivalents APAP metabolization testes, when we used concentrations greater than 10µM APAP, it was not possible to verify the significant drop in the APAP concentration. This was probably due to the saturation of the metabolic capacity of the liver cells and initial toxic effects in 24h time point (that even occurred with 2µM APAP) exactly where the half-life of the drug occurs and then where the toxic metabolite NAPIQ has better chance to be present. In this way, we had to find, empirically, the optimal concentration of APAP, which means, a minimum concentration and above the limit of quantification set to the HPLC method and with fewer chances of overloading the liver cell's metabolic capacity. This concentration was set up at 2 µM. As a consequence, the concentration of choice for treatment of the intestines was 12 µM (when the intestines were treated on the apical side with 12 µM, the resulting systemic concentration verified by HPLC was 2 µM.

2. The manuscript initially states that acetaminophen never induces cytotoxicity, which runs counter to common knowledge (acetaminophen is a liver toxin in an overdose scenario); however, the manuscript later states that the 2 µM dose of acetaminophen did initially cause cytotoxicity based on a decrease in the number of cells, a decrease in nuclear area, and an increase in mitochondrial mass. From this contradiction, it is not clear how the authors constitute drug toxicity in their model and therefore a revised explanation is warranted.

ANSWER FROM AUTHORS:

we appreciate the comment and respectfully disagree. At the beginning of the manuscript, in the abstract, last paragraph we affirm: “The MTT technique performed well in assessing the organoid viability, but the High Content Analyzes (HCA) was able to detect very early toxic events. We verified that the medium flow does not importantly affect the APAP absorption whereas it improves significantly the hepatic metabolism ”. We include "in response to APAP treatment" after "early toxic events" to make the statement meaning clear. The present manuscript was based on the published manuscript “Acetaminophen absorption and metabolism in an intestine/liver microphysiological system” Chem Biol Interact. 2019 Feb 1; 299: 59-76. doi: 10.1016 / j.cbi.2018.11.010 ”in which the possible toxic well-known effects of APAP over the liver cells, are widely discussed based on literature information and data from the above-referred article. Specifically for Jove, following the journal's instructions, this part was removed from the introduction to reduce the text size and focus on the methods. In order to clarify to the reviewer, the present manuscript claims to have no evidence of toxicity in response to APAP in intestinal cells. Already in liver cells, very early toxic effects were observed as described in different parts of the Results section. We claim to find initial toxic responses to APAP treatment in liver cells even at concentrations

lower than those reported as toxic in the literature (5-10 mM APAP), for cultured liver cells. Moreover, we clearly pointed out, in this manuscript of methods, the importance of the simultaneous use of different methodologies and the scientific gains that they can bring, as in the case of the present study in which the HCA and Real-time PCR were able to detect cellular responses to toxic insults not detected by the MTT or microscopy assay. Corroborating our toxicity finds in response to APAP treatment in liver cells, a new data set from analysis of albumin expression and CYP 3A4 activity were included in the present study.

3. There was no functional characterization of the various cell types. The only assays they used to assess function were viability and gene expression. For example, an assessment of albumin and drug metabolism capacity (e.g. CYP3A4 activity) for the HepaRG would be an accurate functional assay.

ANSWER FROM AUTHORS: We thank you for the relevant comment. We have included a new data set from experiments of analyses of albumin protein expression and CYP 3A4 *in vitro* activity (Figure 4 E-J), as well as, the new data Results description and Discussion.

4. Please justify the use of stellate cells over other liver nonparenchymal cells.

ANSWER FROM AUTHORS: The liver model adopted in the present study was based on previous work by the Uwe Marx group, based on the co-culture of HEPA RG and HHSteC cells [1]. Furthermore, because the Human Hepatic stellate cells (HHSteC) are intralobular connective tissue cells presenting myofibroblast-like or lipocyte phenotypes, they participate in the homeostasis of liver extracellular matrix, repair, regeneration, fibrosis and control retinol metabolism, storage and release. Following liver injury, HHSteC transforms into myofibroblast-like cells and is the major source of type I collagen in the fibrotic liver. Beyond these features, HHSteC has been implicated as regulators of hepatic microcirculation via cell contraction, and in disease states, in the pathogenesis of intrahepatic portal hypertension [2,3], thus being important for proper possible toxicological responses induced by pharmacological injury for example. Furthermore, in our hands, the inclusion of HHSteC cells have shown to be necessary for the proper spheroid formation.

5. Is the system capable of longer-term toxicity results for a more physiologic model? The current data only represents one day of toxicity which is not completely relevant for clinically dosed compounds.

ANSWER FROM AUTHORS: We thank the reviewer for the comment. According to several previous studies [4,5], this system is compatible with long-term studies. Regarding the present study, it lasted only 24 hours for the reason of the appearance of the early toxic responses induced by APAP treatment, which made the liver tissue unviable and non-functional after 24 h of APAP exposure. From our perspective, this finding is relevant because it demonstrates that the adopted microphysiological system can identify that APAP can have deleterious effects on liver tissue in low concentrations and in a short-term treatment period, pointing out the necessity of technic and process optimization, as well as the adoption of more discerning and accurate toxicological assessment.

Minor Concerns:

1. In figure 2c and 3a, are these values percent of vehicle control, non-treated cultures, or cells at time point 0? This should be clarified. Most likely it should be percent of vehicle control. ANSWER FROM AUTHORS: We performed the alteration required. The details have been inserted in the caption of figures 2 and 3.

2. With regards to the figures, the letter subsets are not uniformly placed. For example, in Figure 3, some of the letters are above their respective figure subset and others are below it which makes it difficult to properly interpret the figure.
ANSWER FROM AUTHORS: We performed the alteration required

3. Figure 4 depicts HPLC confirmation that the acetaminophen is in fact in their media. This is redundant and unnecessary to confirm that the drug is actually being administered if they are physically doing so. We recommend that this figure be removed and simply discussed.
ANSWER FROM AUTHORS: Figure withdrew.

4. There is an "F" in Figure 3 that appears to be unintentional.
ANSWER FROM AUTHORS: Thank you for point it out. We performed the correction.
5. The manuscript claims that their study imitates a human phase I clinical trial. Please temper this claim as a phase I clinical trial involves all the organs in a human body, not just liver-intestine crosstalk.
ANSWER FROM AUTHORS: We performed the alteration required
6. There are a number of grammatical errors for example in lines 58-59.
ANSWER FROM AUTHORS: Thank you for point it out. We performed the correction.

Reviewer #3:
Major Concerns:
The APAP concentrations tested were 12 and 2 mikro molar. This is considerably lower than considered to be toxic concentrations in vivo (above 1 mM). Thus the relevance of the any "toxic" responses in the study can be questioned.
ANSWER FROM AUTHORS: as the reviewer commented, we really did not expect to find any cellular changes in response to a possibly toxic insult induced by APAP treatment. However, when conducting viability and functionality tests as data quality control we were able to find cellular toxic responses. it was very interesting because the new system after 12h "saturated" or the liver was no longer able to metabolize APAP. This reduction in the metabolization performance probably is due to the change in the chemical balance between APAP and metabolites within a closed system without a mechanism of excretion and purification (kidneys), but it can also be due to a direct toxic insult (probably caused by this very accumulation of metabolites with potential toxic (NAPQI). In addition to the cellular toxic responses findings in the HCA assay and the real-time PCR, a new set of data from new experiments corroborated the occurrence of liver toxicity in response to the treatment of 2uM APAP. We found that treatment with APAP robustly reduces the expression of albumin (viability and functionality marker in liver cells) and also induced impairment of CYP 3A4 activity levels in vitro. Regarding the intestines cells treated with 12uM APAP we did not find any toxic effect or deleterious cellular response as expected. Thus, we consider the toxicological analyzes relevant and appropriate to the present article with a focus on the methods, as it highlights the importance of the careful and adequate choice of tests as well as the combination of various techniques in trials with the same outcome as objective

Also there is little or no information whether the solvent concentrations are the same when different concentrations of APAP is tested. E.g. the toxic dose 100 mM is this the same solvent concentration used as for the tested low concentrations?
ANSWER FROM AUTHORS: We thank you for the relevant comment. We have included the concentrations information in Protocol section 3.1.1
Minor Concerns:
The information in the figures could be cleared, e.g. the concentrations of APAP tested in Fig 2 and 3 should be written out in the Figure.
ANSWER FROM AUTHORS: We thank you for the relevant comment. We have included the concentrations of APAP tested in Fig 2, 3 and 4.
Reviewer #4:
Marin et al. assembled Intestine only 2-OC, Liver only 2-OC, and Intestine/Liver 2-OC using Two-Organ-Chip from TissUse GmbH. The intestinal barrier was emulated by a co-culture of Caco2 and HT-29 cells, and the liver was emulated by spheroids made of differentiated HepaRG cells co-cultivated with human hepatic stellate cells. These 2-OC assemblies were exposed to APAP to investigate its PK and toxicological properties. Authors describes the methods for organoids production and APAP pharmacokinetic and toxicological properties assessments in the MPS.
The manuscript is written precisely the methods and indicates the application of 2-OC assembly to APAP PK and toxicological analysis properly, thus the reviewer thinks the manuscript has enough quality for the publication in JoVE with minor revision.
l. 68 "Organoids made of human cells increase translational relevance." requires adequate references.
ANSWER FROM AUTHORS: We performed the alteration required

l. 76 "This technology can significantly improve the prediction of compound intestinal absorption and liver metabolism." Requires adequate references.
ANSWER FROM AUTHORS: We performed the alteration required

l. 96 Refs 16 and 17 should be replaced with more adequate references regarding the relationship of hepatocyte or enterocyte functionality and the mechanical stimuli.
l.100 Is the 9:1 proportion really "the best" condition for the physiological parameters?
ANSWER FROM AUTHORS: We thank the reviewer for the pertinent comment. The ratio of 1: 9 was indicated by the literature as the ratio that provides results that are closer to the physiological ones. The co-culture proportion of 9:1 between Caco-2 and HT-29 showed to be the best to achieve physiological parameters after cell differentiation in culture [6], being the condition reported to enable reproducible results for drug permeability [7–9].
l.127 Is "DMEM" simple DMEM or DMEM supplemented with serum etc.? Definition is required.
ANSWER FROM AUTHORS: we change the text and included the definition required (Protocols section 1.1.1).
l.143 TEER value, which indicates the good intestinal barrier formation, should be described. ANSWER FROM AUTHORS: Thank you for the pertinent comment. We have included TEER reference values (Protocol section 1.1.6, as well as experimental data (Results section Figure 2 D and E).

l.162 Is HHSTeC "primary" hepatic stellate cells, that means is HHSTeC not expanded after preparation of liver specimen?
ANSWER FROM AUTHORS: HHSteC are guaranteed to further expand for 15 population doublings under the conditions provided by ScienCell Research Laboratories. HHSteC from ScienCell Research Laboratories are isolated from human liver. HHSteC are cryopreserved at passage one and delivered frozen. https://www.sciencellonline.com/human-hepatic-stellate-cells.html).
l.162 Is Stellate Cell Media equal to STeC CM?
ANSWER FROM AUTHORS: yes, we reformulated the text to make this clear.
l.174 Is Williams E medium simple Williams E medium or Williams E medium supplemented with serum etc.? The definition and notation of the medium name should be the same throughout the article.
ANSWER FROM AUTHORS: we change the text and included the definition required (Protocols section 1.2.1).
l.186 Product information of 384 spheroid microplate is missing in the Table of Materials.
ANSWER FROM AUTHORS: we included the information required
l.215 Does the MPS just connect to the control unit, or does the circulation start at this point? If it starts, the condition of the circulation should be described.
l.230 The manipulation is not clear. Moving the spheroids from 6 well plates to MPS accompanies the small volume of medium, so the volume of the medium in the smaller compartment increases after the manipulation. Should this increase be adjusted?
l.233 Same as in l.215.
l.238 Does this section describe the assembly of Intestine/Liver 2-OC MPS or the application of APAP to the Intestine/Liver 2-OC MPS? (i.e. l. 245: DMEM + test substance)
ANSWER FROM AUTHORS: I.215; I230; I233; I238; I293; I298; I321 – question were addressed in the text. Clarifications were inserted in the respective Protocol text sections.
l.264 What is the expiration date of APAP stock solution? Should it be freshly prepared?
ANSWER FROM AUTHORS: In the present work the APAP solution was always freshly prepared.
l.293 What is the flow condition?
l.298 What is the volume of the sampling? Should the same volume of the media be added to each compartment of MPS after sampling?
l.321 Same as in l.298.
l.370 Should the stock solutions of APAP be prepared freshly?
l.382 "The samples are prepared from a new stock solution, different from that used to generate a standard curve." Why the stock solution is different from that used for the standard curve?
ANSWER FROM AUTHORS: Quality control standards are useful because they enable the user to verify the entire analytical system and eliminate bias from analytical results. During method validation, quality controls (QCs) evaluate the performance of a method and the stability of an analyte. Performance QCs are included in validation runs to determine the precision and accuracy of the method. Stability QCs evaluate the stability of an analyte under various stress conditions.
l.467 The procedure of change the MTT solution to isopropanol should be described.
ANSWER FROM AUTHORS: Modification performed

l.536 3,7%→3.7%?
ANSWER FROM AUTHORS: Modification performed
l.700 Cyp3A4: Human CYPs should be written in upper case.
ANSWER FROM AUTHORS: Thank you for point it out. We performed the correction.
l.701 Fig. 3: The placement of characters in the figure is inappropriate. Consider the placement of E, F and G.
ANSWER FROM AUTHORS: Thank you for point it out. We performed the correction.
l.935 "There was a significant APAP decay under flow not seen without flow." The effect of flow for the liver organoid metabolic capability should be discussed more precisely.
ANSWER FROM AUTHORS: We thank you for the relevant comment. We have included new experiments dataset obtained from albumin protein expression analyses and CYP activity characterization, as well as, new data discussion.

Reviewer #5:
Manuscript Summary:
The method described in this manuscript is indeed of great importance for anyone working in pharmaceutical research, but also for toxicologists. APAP is a good model compound due to availability of human data. The APAP concentration used is reasonable and the authors are able to show intestinal drug absorption followed by downregulation of liver-specific genes in the liver spheroids.

Major Concerns:
The relative quantification method for RT-PCR experiments is not described.
What was the basis for the selection of GAPDH as a reference gene?
ANSWER FROM AUTHORS: We thank you for the relevant comment. Reference genes are expressed in several cell types showing slight variations in their expression levels and, therefore, being adequate to normalize the mRNA quantification data. Genes such as 18S rRNA, UBQ, RPL-19, GAPDH, β-actin, β-tubulin, PGK, and have been indicated as standards in RT-qPCR analysis. GAPDH is the most commonly used to normalize gene expression data, being used as an endogenous control in the quantitative analysis of RT-qPCR, considering maintenance expression in many cell types. However, it is well known that these genes can vary in expression in different types of cells, reinforcing the hypothesis that there is no ideal reference gene. Regarding liver cells and enzymes involved in the metabolism function, the situation can be even more challenging and GAPDH may not be the most suitable gene. In this sense, a recently published study [10] ranks GAPDH in fifth place as a suitable reference gene for analysis of gene expression in HepaRG cells (cells used in the liver equivalent model of the present study). Despite the limitations described, we have confidence in the quality and reliability of the RT-qPCR data normalized by the GAPDH. New experiments of protein albumin expression analyzes, included in the present manuscript (Fig 4F), have correlated to the results of RT-qPCR. Just as there was a robust decline in the genetic expression of albumina 24 h after treatment with APAP under dynamic conditions, so did protein levels. This result gives us security and validates to some extent the normalization performed using GAPDH.
Were additional reference genes tested?

ANSWER: No additional reference genes were tested. Despite the recognized importance of using more than one reference gene (usually 3 genes) in the present study, only GAPDH was used

What about normalization?

ANSWER: All reactions were performed with reference dye normalization. The median cycle threshold value was used for analysis, and all cycle threshold values were normalized to the GAPDH mRNA expression level.

Software used? ANSWER: MxPro QPCR Software from MX3000P Stratagene system (Agilent Technologies (Santa Clara, CA, USA).

NOTE: RNA was extracted from tissue equivalents using TRizol TM reagent (Thermo Fisher) according to the manufacturer's instructions. cDNA was synthesized by reverse transcription of 1–2 µg total RNA using SuperScriptIITM Reverse Transcriptase (Thermo Fisher). All targets were amplified using gene-specific primers and SYBR Green PCR Master Mix (Thermo Fisher) reagent. Each qRT-PCR contained 30 ng of reverse-transcribed RNA and 100 nM of each primer. The reactions were performed using an MX3000P system (Agilent Technologies (Santa Clara, CA,USA)) and PCR conditions were: 50 °C for 3 min (1 cycle); 95 °C for 5 min (1 cycle); 95 °C for 30 s, 59 °C for 45 s and 72 °C for 45 s (35–40 cycles).

Minor Concerns:

Line 669: What is meant by continuity? Barrier integrity?

ANSWER FROM AUTHORS: Absence of any space or hole between neighboring cells. Means a completely intact barrier.

Legend to figure 5: The term metabolism profile is misleading, as one would expect measurement of metabolites. Why not use "metabolic breakdown" or "APAP kinetics"?

Modification performed

General: The authors state that APAP was chosen because human PK data are readily available. A direct comparison would be helpful, e.g. in Figure 5 (show blood levels after oral administration).

ANSWER FROM AUTHORS: we thank you for the timely suggestion. We inform you that we have included a comparative chart (Fig 5F) as well as the respective description and discussion.

REFERENCES

[1] Wagner I, Materne EM, Brincker S, Süssbier U, Frädrich C, Busek M, Sonntag F, Sakharov DA, Trushkin EV, Tonevitsky AG, Lauster R, Marx U. A dynamic multi-organ-chip for long-term cultivation and substance testing proven by 3D human liver and skin tissue co-culture. Lab Chip. 2013 Sep 21;13(18):3538-47. doi: 10.1039/c3lc50234a. PubMed PMID: 23648632.

[2] Reynaert H, Thompson MG, Thomas T, Geerts A. (2002) Hepatic stellate cells: role in microcirculation and pathophysiology of portal hypertension. Gut 50:571-581.

[3] Rockey D. C. (2001) Hepatic blood flow regulation by stellate cells in normal and injured liver. Semin Liver Dis 21(3):337-49.

[4] Maschmeyer, I. et al. Chip-based human liver-intestine and liver-skin co-cultures - A first step toward systemic repeated dose substance testing in vitro. European Journal of Pharmaceutics and Biopharmaceutics. doi: 10.1016/j.ejpb.2015.03.002 (2015).

[5] Maschmeyer, I. et al. A four-organ-chip for interconnected long-term co-culture of human intestine, liver, skin and kidney equivalents. Lab Chip. doi: 10.1039/C5LC00392J (2015).

[6] F. Araújo, B. Sarmento, Towards the characterization of an in vitro triple co-culture intestine cell model for permeability studies, Int. J. Pharm. (2013), https://doi.org/10.1016/j.ijpharm.2013.10.003.

[7] X.M. Chen, I. Elisia, D.D. Kitts, Defining conditions for the co-culture of Caco-2 and HT29-MTX cells using Taguchi design, J. Pharmacol. Toxicol. Methods (2010), https://doi.org/10.1016/j.vascn.2010.02.004.

[8] F. Antunes, F. Andrade, D. Ferreira, H. Morck Nielsen, B. Sarmento, Models to predict intestinal absorption of therapeutic peptides and proteins, Curr. Drug Metabol. (2013), https://doi.org/10.2174/138920013804545160.

[9] F. Pan, L. Han, Y. Zhang, Y. Yu, J. Liu, Optimization of Caco-2 and HT29 co-culture in vitro cell models for permeability studies, Int. J. Food Sci. Nutr. (2015), https://doi.org/10.3109/09637486.2015.1077792.

[10] Brzeszczyńska J, Brzeszczyński F, Samuel K, et al. Validation of Reference Genes for Gene Expression Studies by RT-qPCR in HepaRG Cells during Toxicity Testing and Disease Modelling. Cells. 2020;9(3):770. Published 2020 Mar 21. doi:10.3390/cells9030770

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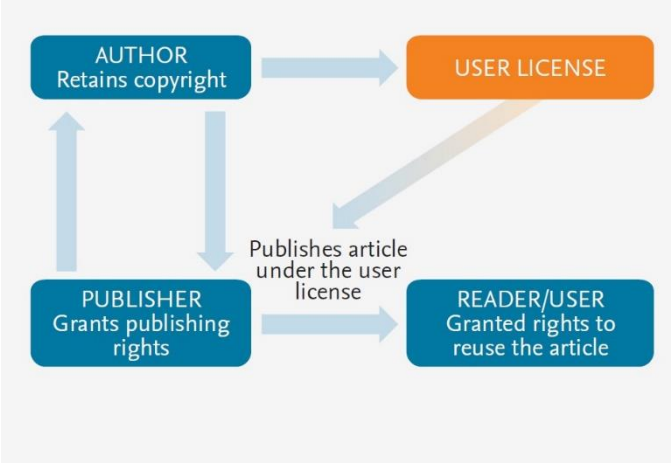

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Cover letter

August 17, 2020

Dear Benjamin Werth, B.S. Science Editor of JoVE

I am enclosing an invited manuscript for consideration for publication in Jove. The manuscript is entitled **"Intestine/Liver Microphysiological System for Drugs Pharmacokinetic and Toxicological Assessment"**. It has not been published elsewhere and that it has not been submitted simultaneously for publication elsewhere.

Because the Microphysiolgical System (MPS) is a developing technology and there is a range of different types of it, we consider it very important to become known and established the pharmacokinetic parameters obtained specifically in the 2-OC MPS in a consistent way. This study shows the results obtained with paracetamol and highlights the crucial role of the presence of flow in the physiological response of MPS. The MPS emerging technology based on microfluidics device integrated with 3D human tissue culture models has great potential in providing data much more accurate and closer to human, capable of reducing the divergence, the time and the costs in scientific data, as well as encourage the availability and the diffusion of alternative methods to the animal use in research. The knowledge of the pharmacokinetic (PK) properties of a compound is crucial during the pre-clinical drug phase. The MPS that allocates the integrated intestine and liver organ models are the first bet in obtaining a high-quality pharmacokinetic dataset since those two organs are the critical ones in the process like biodistribution and bioavailability and other parameters like intestinal rates of permeability and absorption as well as hepatic metabolism. This article describes the detailed protocols to perform the emulation of the APAP intestinal absorption and hepatic metabolism and respective analyses in the 2-OC MPS composed by the human intestine and liver equivalents. The article also described the proper human tissue functionality and toxicological analyses necessary to validate the PK results. For those reasons, we considered this article of a very interesting for the scientific community.

The manuscript has undergone some changes suggested by the reviewers. We strive to balance compliance with all reviewers' suggestions and requests and compliance with JoVE rules, guidelines, and space limits. I inform you that new results have been included in the manuscript, figures 2,3,4 and 5 have been reformulated, as well as several parts of the text. Text changes are highlighted in gray. The parts of the filming protocol highlighted in yellow.

We are grateful for the careful review of the manuscript and the important considerations made by the reviewers, which have resulted in a significant improvement in the quality of the work presented and we are thankful for the opportunity to resubmit the revised work.

Thank you very much for your consideration.
Yours Sincerely,

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