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1 **Coculture model of a liver sinusoidal endothelial cell barrier and**  
2 **HepG2/C3a spheroids-on-chip in an advanced fluidic platform**

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25 **ABSTRACT**

26 The liver is one of the main organs involved in the metabolism of xenobiotics and a key organ  
27 in toxicity studies. Prior to accessing the hepatocytes, xenobiotics pass through the hepatic  
28 sinusoid formed by liver sinusoidal endothelial cells (LSECs). The LSECs barrier regulates the  
29 kinetics and concentrations of the xenobiotics before their metabolic processing by the  
30 hepatocytes. To mimic this physiological situation, we developed an *in vitro* model reproducing  
31 an LSECs barrier in coculture with a hepatocyte biochip, using a fluidic platform. This  
32 technology made dynamic coculture and tissue crosstalk possible. SK-HEP-1 and HepG2/C3a  
33 cells were used as LSECs and as hepatocyte models, respectively. We confirmed the LSECs  
34 phenotype by measuring PECAM-1 and stabilin-2 expression levels and the barrier's  
35 permeability/transport properties with various molecules. The tightness of the SK-HEP-1  
36 barrier was enhanced in the dynamic coculture. The morphology, albumin secretion, and gene  
37 expression levels of markers of HepG2/C3a were not modified by coculture with the LSECs  
38 barrier. Using paracetamol, a well-known hepatotoxic drug, to study tissue crosstalk, there was  
39 a reduction in the expression levels of the LSECs markers stabilin-2 and PECAM-1, and a  
40 modification of those of CLEC4M and KDR. No HepG2/C3a toxicity was observed. The  
41 metabolism of paracetamol by HepG2/C3a monocultures and cocultures was confirmed.  
42 Although primary cells are required to propose a fully relevant model, the present approach  
43 highlights the potential of our system for investigating xenobiotic metabolism and toxicity.

44

45 **Keywords:** Organ-on-chip, Liver, LSECs barrier, HepG2/C3a, coculture, microfluidic

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## 50 INTRODUCTION

51 Animal models are widely used as reference tools for predictive studies in drug  
52 development and risk assessment **(1)**. However, due to differences between animal and human  
53 metabolism and physiology, animal models fail to accurately reproduce the human condition,  
54 and this issue challenges the extrapolation of data to humans **(2,3)**. For example, the predictivity  
55 of animal models for chemical-induced hepatotoxicity is only 50% **(4)**. Moreover, animal  
56 experiments are costly, time-consuming and most importantly raise ethical and regulatory  
57 issues **(4,5)**. To decrease the use of animals, the REACH legislation and the 3R rules,  
58 recommended to reduce as much as possible the use of animal models, have pressed industrial  
59 companies and scientists to develop alternative approaches to animal testing **(2)**. Consequently,  
60 developing reliable methods not based on *in vivo* experimentation has become necessary.

61 The liver is the main site involved in the metabolism of xenobiotics and is therefore the  
62 most commonly used organ in toxicological and pharmacological tests **(6,7)**. It is composed of  
63 several cell types, the main ones being hepatocytes (parenchymal cells) and non-parenchymal  
64 cells (NPCs): sinusoidal endothelial cells (LSECs), Kupffer cells (KCs), hepatic stellate cells  
65 (HSCs), and biliary epithelial cells **(8,9)**. Hepatocytes represent approximately 60% of the total  
66 liver cells, and are the main cell type, ensuring most metabolic activities **(10)**. The NPCs are  
67 involved in several key functions, such as the production of growth factors and mediators of  
68 cellular functions, maintenance of tissue architecture, and regulation of liver response to  
69 xenobiotics **(8,9)**.

70 Currently, most of the *in vitro* liver models are focused on hepatocytes and do not include  
71 NPCs **(11)**. Moreover, the models used for drug screening and risk assessment are mainly based  
72 on cell culture in static two-dimension (2D) monolayers **(1)**. These 2D cultures present some  
73 advantages, such as allowing high throughput analyses, ease of manipulation, and a lower cost  
74 **(9,12)**. However, 2D monocultures of hepatocytes or of hepatic cell lines suffer from several

75 disadvantages associated with the loss of tissue-specific architecture, mechanical and  
76 biomechanical cues, and cell-cell and cell-matrix interactions. Consequently, these models fail  
77 to recapitulate the complexity of the *in vivo* physiological environment, show limited prediction  
78 capacity for xenobiotics, and cells are prone to dedifferentiation within 48-72 h **(1,12,13)**.

79         Recently, several approaches have been proposed to overcome the drawbacks associated  
80 with 2D monolayer cultures of hepatocytes. Microfluidic devices, or organ-on-chip (OoC)  
81 technology, are a promising tool for building more relevant *in vitro* liver models aimed at  
82 mimicking the *in vivo* environment **(14)**. The microfluidic perfusion improves the exchanges  
83 and transport of nutrients, oxygen, and other chemicals, and creates a controlled micro-  
84 environment and physiological-like features, including the liver zonation, cell-cell interactions,  
85 shear stress, and chemical concentration gradients **(1,9,15,16)**. Several studies have reported  
86 that perfused microfluidic cultures enhance the long-term viability and functionality of  
87 hepatocytes **(17-19)**. The three-dimensional (3D) cell culture (spheroids/organoids), with and  
88 without polymer matrix, also makes it possible to maintain tissue architecture similar to the *in*  
89 *vivo* situation and maintains liver-specific functions. This organisation enhances cell-cell and  
90 cell-matrix interactions and the creation of chemical gradients **(6,20,21)**. Among other  
91 approaches used to maintain hepatocyte functions, cocultures with NPCs are commonly used  
92 strategies **(4)**. Among NPCs, LSECs participate in liver metabolic functions and maintain  
93 hepatocyte phenotype and functions through paracrine communication **(22)**. The benefits of  
94 coculturing LSECs and hepatocytes have been reported in several works **(22-24)**.

95         Previously, we developed a liver-on-chip model integrating a hydroscaffold containing  
96 key liver extracellular matrix (ECM) components **(25)**. This device made possible the dynamic  
97 culture of HepG2/C3a organised into 3D spheroids for the long-term, while maintaining their  
98 functionalities. Here, to better reproduce the physiology of the liver, our HepG2/C3a-on-chip  
99 model was cocultured with LSECs. The coculture was performed using a fluidic platform

100 previously developed by our laboratory (26) making it possible to connect the biochip to a new  
101 LSEC barrier insert. The behaviour and functionalities of the LSECs barrier (SK-HEP-1 cell  
102 line) and hepatocyte biochip (HepG2/C3a cells) in monoculture and coculture were studied and  
103 compared. Then, the coculture model was exposed to paracetamol (APAP), and the crosstalk  
104 between both compartments was studied and compared to monocultures exposed to APAP.

## 105 MATERIALS AND METHODS

### 106 Manufacturing of the biochip

107 The biochip fabrication and design were described in our previous work (27). The biochip  
108 consists of two polydimethylsiloxane (PDMS) layers (Sylgard 184 kit; Dow Corning, Midland,  
109 TX, USA)) manufactured by soft lithography and sealed via air plasma treatment (Harrick  
110 Scientific, Ithaca, NY, USA). The microstructured bottom layer contains chambers and  
111 microchannels (height of 100  $\mu\text{m}$ ), and the top layer, with a 100  $\mu\text{m}$ -deep reservoir, includes  
112 an inlet and outlet for culture medium perfusion (Fig.S1A).

113 To promote 3D cell organisation, the BIOMIMESYS® Liver hydrosc scaffold (HCS Pharma,  
114 Loos, France) was integrated into the biochip. BIOMIMESYS® Liver is a hyaluronic acid  
115 (HA)-based hydrosc scaffold composed of RGDS-grafted HA, galactosamine-grafted HA,  
116 collagen type I and collagen type IV. The hydrosc scaffold preparation was performed in  
117 accordance with a previously patented process (28). Briefly, the pseudo-hydrogel solution (HA,  
118 collagen and crosslinker: adipic acid dihydrazide) was injected into the biochip and the  
119 hydrosc scaffold crosslinking was performed *in situ*. The biochips were then washed, freeze-dried,  
120 and sterilised using ultraviolet (UV) exposure. The detailed characterization of the biochips  
121 (with and without hydrosc scaffold) was reported in our previous work (25). The pictures and  
122 microscope images of the biochips with and without hydrosc scaffolds are presented in Fig.S1B  
123 and C.

124

## 125 **Coculture platform: IIDMP fluidic device**

126 We used the previously described Integrated Insert in a Dynamic Microfluidic Platform  
127 (IIDMP, **26**) coculture system which consists of a polycarbonate platform with three subunits  
128 (**Fig.1A** and **Fig.S2**). Each subunit is composed of the association of an insert and a biochip  
129 linking two wells. The insert was placed in the first well and defined an apical pole (LSECs  
130 barrier) and a basal pole making possible the exchange of culture medium between the LSECs  
131 barrier and the hepatocyte compartment (biochip, **Fig.1A**). The biochip connected the first and  
132 second well (acting as a reservoir). The volume of culture medium was 10 mL: 1 mL placed in  
133 the apical insert, 5 mL below the insert, and 4 ml in the second well. Culture medium flowed  
134 through the biochip from the basal compartment in the first well towards the second well. The  
135 perfusion fluid was provided by a cover connected to a peristaltic pump (Ismatec™, Wertheim,  
136 Germany) via PTFE (polytetrafluoroethylene) tubing. The other components of the IIDMP  
137 platform were silicone gaskets sealing the device, and a bottom layer composed of the well  
138 subunits, thanks to which the biochips were connected (at the bottom, **Fig.S2**).

## 139 **Cells and culture media**

140 HepG2/C3A, a clone of the HepG2 line derived from human hepatocellular carcinoma  
141 (ATCC CRL-10741; LGC Standards, Molsheim, France), were used as the hepatocyte model.  
142 They were cultured in Minimal Essential Medium (MEM) with phenol red (Pan Biotech,  
143 Aidenbach, Germany), 10% fetal bovine serum (FBS; Gibco, Waltham, MA, USA), 1 mM  
144 hydroxy-ethylpiperazine-N-2-ethanesulfonic acid (HEPES, Gibco), 2 mM L-glutamine  
145 (Gibco), 0.1 mM non-essential amino acids (Gibco), 1 mM sodium pyruvate (Gibco), and 100  
146 U/mL penicillin, 100 µg/mL streptomycin (Pan Biotech). The SK-HEP-1 cell line (ATCC HTB-  
147 52; LGC Standards) derived from an adenocarcinoma of the liver was used as the LSECs model.  
148 For maintenance, SK-HEP-1 cells were cultured in a mixture of 75% EGM-2 medium (Lonza,  
149 Verviers, Belgium) and 25% MEM (complemented as mentioned above).

150 All cells were cultured in 75 cm<sup>2</sup> flasks at 37°C in a humidified atmosphere with 5% of  
151 CO<sub>2</sub>. The culture medium was renewed every 2 days and the cells were passaged weekly  
152 (confluence of 80-90%). To decrease variability, the cells were used between passages 10-20.

### 153 **Optimisation of common culture medium for HepG2/C3A and SK-HEP-1 cells**

154 Culture medium optimisation was performed in static conditions, and different  
155 MEM/EGM-2 ratios were tested. The SK-HEP-1 cells were seeded in cell culture inserts  
156 (THINCERT, 6-well format, polyethylene terephthalate membrane, 0.4 µm pore; Greiner Bio-  
157 One, Les Ulis, France) at a density of 0.35 x 10<sup>5</sup> cell/cm<sup>2</sup>. The culture medium was renewed  
158 every 2 days in the apical (1 mL) and basal (2 mL) compartments, and the culture was  
159 maintained until confluence was attained (6-8 days). The HepG2/C3A were seeded in the wells  
160 of a 6-well plate (Greiner Bio-One) at a density of 1 x 10<sup>5</sup> cell/cm<sup>2</sup>. The culture was maintained  
161 for 4 days, and the medium (2 mL) was changed every 2 days. The cultures were continuously  
162 maintained at 37°C in a 5% CO<sub>2</sub> supplied incubator and the assays were performed at the end  
163 of the experiments.

### 164 **Dynamic monoculture and coculture in the IIDMP device**

165 Each experiment lasted two days (**Fig.1B**). The SK-HEP-1 inserts were maintained for 8  
166 days for the formation of a confluent barrier, before performing the dynamic experiments, as  
167 mentioned in section 2.4.1. In parallel, 24 h before the dynamic experiments, HepG2/C3a cells  
168 were seeded in the biochips containing the hydroscaffold (4 x 10<sup>5</sup> cell/biochip), and the biochips  
169 were incubated overnight at 37°C in a humidified atmosphere with 5% of CO<sub>2</sub>.

170 On Day 0 of the experiment, the SK-HEP-1 previously grown for 8 days on inserts were  
171 transferred into the first well of the IIDMP device and the HepG2/C3a biochips were connected  
172 to the bottom of the device. As shown in **Fig.1B**, three conditions were established: SK-HEP-1  
173 monoculture (IIDMP with insert alone), HepG2/C3a monoculture (IIDMP with biochip alone)  
174 and coculture (IIDMP containing insert and biochip). Culture medium was added (1 mL in the



175 apical insert side, 5 mL in the basal side and 4 mL in the reservoir well), the IIDMP was closed  
176 and connected to the pump. The entire setup was placed in the incubator and perfusion started  
177 at 10  $\mu$ L/min for 48 h in a closed loop. For exposure to drugs, acetaminophen (APAP; Sigma-  
178 Aldrich, Saint-Quentin-Fallavier, France) was loaded into the apical compartment of the insert  
179 at 1 mM before perfusion started (an insert without cells was used for HepG2/C3a monoculture  
180 experiments). After dilution in the total medium in the circuit (10 mL), the systemic  
181 concentration of APAP was 100  $\mu$ M.

### 182 **Lucifer Yellow permeability assay**

183 Lucifer Yellow (LY CH dipotassium salt, Sigma-Aldrich) was diluted in Hanks' balanced  
184 salt solution (HBSS, with  $\text{CaCl}_2$  and  $\text{MgCl}_2$ , Gibco) at 50  $\mu$ M and loaded into the apical  
185 compartment of an empty insert and inserts with cells were cultured for 4-15 days. The basal  
186 compartment was filled with HBSS. The inserts were then incubated at 37°C and 5% of  $\text{CO}_2$ .  
187 After 90 min, medium from the apical and basal compartments was collected. The fluorescence  
188 intensity was measured using a Spectafluor Plus microplate reader (TECAN, Männedorf,  
189 Switzerland) at excitation/emission wavelengths of 485/530 nm. The flow of LY was expressed  
190 by the calculation of the apparent permeability ( $P_{app}$ , m/s) as follows:  **$P_{app} = (dQ/dt) \times$**   
191  **$(1/A \times C_a)$** , where  $dQ/dt$  is the amount of LY transported during a given time (mol/s),  $C_a$  is the  
192 initial concentration of LY solution ( $\text{mol/m}^3$ ) and  $A$  is the surface of the insert ( $\text{m}^2$ ).

### 193 **Permeability to dextrans**

194 The SK-HEP-1 barrier's permeability to molecules of different molecular weights was  
195 assessed using fluorescein isothiocyanate-dextrans (FITC-dextran 4, 70 and 150 kDa, Sigma-  
196 Aldrich). The assays were performed using confluent SK-HEP-1 barriers (8 days of culture) in  
197 static and dynamic (IIDMP device) conditions. The dextrans were diluted in the culture medium  
198 at a concentration of 100  $\mu$ g/mL and deposited in the apical compartment of the culture inserts.  
199 Then, culture medium was sampled in the apical and basal compartments at different times. The

200 FITC-dextran fluorescence intensity was measured using a Spectafluor Plus microplate reader  
201 (TECAN) at excitation/emission wavelengths of 490/525 nm.

## 202 **Immunostaining assays**

203 Immunostaining assays were performed using fixed and permeabilised SK-HEP-1 inserts.  
204 The samples were incubated overnight with primary antibodies, then for 12 h with the  
205 secondary antibodies (4°C in the dark). The primary and secondary antibodies used were mouse  
206 anti-CD31 (1 µg/mL; ab24590, Abcam, Cambridge, UK), rabbit anti-stabilin-2 (1 µg/mL;  
207 ab121893, Abcam), mouse anti-vimentin (1 µg/mL; ab8978, Abcam), donkey anti-mouse  
208 Alexa Fluor 647 (2 µg/mL; ab150107, Abcam) and goat anti-rabbit Alexa Fluor 488 (2 µg/mL;  
209 A11034, Invitrogen, Waltham, MA, USA). Actin filaments were stained with Alexa Fluo 488  
210 Phalloidin for 3h (1/50; Thermo Fisher Scientific, Illkirch, France). Nuclei were stained with  
211 10 µg/mL 4',6-diamidino-2-phenylindole (DAPI, D1306, Invitrogen) for 30 min at room  
212 temperature in the dark. Imaging was obtained with a laser scanning confocal microscope (LSM  
213 710; Zeiss, Oberkochen, Germany).

## 214 **Albumin, interleukin-6 and urea measurements**

215 ELISA sandwich assays were used to quantify the albumin and IL-6 concentrations in the  
216 culture media collected at the end of the experiments. The assays were performed using a human  
217 albumin ELISA Quantitation Set (E80-129; Bethyl Laboratories, Montgomery, TX, USA) and  
218 a human IL-6 ELISA Kit (ab718013; Abcam) for albumin and IL-6, respectively, following the  
219 protocols recommended by the manufacturers. The urea was quantified using a QuantiChrom  
220 urea assay kit (DIUR-100; BioAssay Systems, Hayward, CA, USA). The kit contains a  
221 chromogenic reagent that forms a colored complex specifically with urea. The results were  
222 acquired using a Spectafluor Plus microplate reader (TECAN) set to a wavelength of 450 nm  
223 (albumin and IL-6) and 520 nm (urea).

224

## 225 RNA extraction and RTqPCR analysis

226 At the end of the experiments, the cells were lysed and recovered using 500  $\mu$ L of TRIzol  
227 (Thermo Fischer Scientific). Total RNA was purified by phenol/chloroform extraction followed  
228 by alcohol precipitation, and RNA concentrations measured using a NanodropOne (Thermo  
229 Fisher Scientific). Reverse transcription reactions were performed using a High-capacity cDNA  
230 reverse transcription kit with RNase inhibitor (Applied Biosystems, ThermoFisher Scientific).  
231 Quantitative PCRs were performed using a StepOnePlus machine (Applied Biosystems,  
232 Thermo Fisher Scientific) in duplex reactions, mixing the cDNA with the TaqMan FAM-  
233 labelled probes of the analyzed gene (Applied Biosystems, Thermo Fisher Scientific) and with  
234  $\beta$ 2-microglobulin-VIC-labeled probe in the same reaction well (**Table S1**). The threshold cycle  
235 ( $C_T$ ) values were calculated at the upper linear range of the logarithm<sup>-2</sup> amplification curve  
236 using the StepOne v2.3 software (Thermo Fisher scientific). The data were then expressed as  
237  $2^{-\Delta\Delta C_T}$ .  $\Delta C_T$  is the difference between the  $C_T$  of the analyzed gene and the  $C_T$  of the  $\beta$ 2-  
238 microglobulin gene used as normalizer in the same reaction.  $\Delta\Delta C_T$  is the difference between  
239 the mean  $\Delta C_T$  of the experimental samples and the mean  $\Delta C_T$  of the control samples (**29**). The  
240 relative quantity (RQ) corresponds to  $2^{-\Delta\Delta C_T}$  which transforms the logarithmic<sup>-2</sup> data into  
241 decimal values.

## 242 HPLC-HRMS

243 Detection and quantitative evaluation of APAP and APAP metabolites was performed  
244 with high performance liquid chromatography coupled to high resolution mass spectrometry  
245 (HPLC-HRMS). The HPLC system (Infinity 1290; Agilent Technologies, Les Ulis, France)  
246 with DAD, was connected to a Q-TOF micro hybrid quadrupole time of flight mass  
247 spectrometer (Agilent 6538; Agilent Technologies) with electrospray ionisation (ESI). HPLC  
248 was carried out on a Thermo Hypersyl Gold C18 (USP L1) column (150  $\times$  4.6 mm ID, 5  $\mu$ m,  
249 175 A), connected to the Agilent Infinity 1290 HPLC at 40°C.

## 250 **Statistical analysis**

251 All experiments were performed at least three times and a minimum of 2 bio-  
252 chips/inserts/cocultures were performed in each experiment (N = 3 experiments and n = 6).  
253 Data are presented as means  $\pm$  standard deviations (SD) of the 6 replicates (for RTqPCR assays,  
254 only 3 replicates from 3 different experiments were used). To determine statistical differences,  
255 a one-way ANOVA (Kruskal-Wallis test, multiple groups) and unpaired t-test (two groups)  
256 were performed using GraphPad Prism 8.4.3 software (San Diego, CA, USA). Data with P  
257 values  $< 0.05$  were identified as statistically significant and highlighted in the figures.

## 258 **RESULTS**

### 259 **Selecting a culture medium for SK-HEP-1 and HepG2/C3a coculture**

260 The culture of cells of different origin in the same system requires an adapted coculture  
261 medium capable of maintaining both cell types in good conditions, without impairing their  
262 characteristics and functionalities. The routine culture medium used in our conditions for SK-  
263 HEP-1 is EGM-2/MEM (75%/25%) and the cells formed a well-structured cell monolayer at  
264 confluence, as needed for the barrier function (**Fig.2A** and **S3**). On the other hand, when SK-  
265 HEP-1 cells were cultured in HepG2/C3a medium, which is based on MEM only, the  
266 endothelial cell morphology was greatly altered, and the cells failed to form a confluent  
267 monolayer (**Fig.S3**). In an attempt to, first, create the endothelial barrier, and then to switch to  
268 a hepatocyte culture medium, SK-HEP-1 cells were cultured in their normal medium for 6 days,  
269 followed by culture in MEM for 3 days (as the coculture period). In these conditions again, the  
270 endothelial cells failed to maintain a confluent monolayer (**Fig.S3**). In both conditions (MEM  
271 and EGM-2/MEM (75%/25%) followed by MEM), a large number of rounded cells in  
272 suspension was observed, suggesting that cells failed to attach or attached but exhibited poor  
273 adhesion. Finally, when cells were maintained in EGM-2/MEM (25%/75%) medium for 7 days,  
274 the SK-HEP-1 cells formed a confluent monolayer (**Fig.2A** and **S3**) and exhibited the

275 characteristic morphology of SK-HEP-1, as when cultured in their original medium. The gene  
276 expression levels of several LSECs markers were investigated. No major differences were  
277 observed for most of the genes when cells were cultured in EGM-2/MEM (25%/75%) when  
278 compared to their original medium. A downregulation of CLEC4M and VCAM1 was observed  
279 when cells were maintained in EGM-2/MEM (25%/75%) in comparison with native medium,  
280 with fold changes (FC) of 0.25 and 0.48, respectively (**Fig.2B**).

281 The EGM-2/MEM (25%/75%) medium was also tested on HepG2/C3a cells and  
282 compared to culturing in MEM. After 4 days of static culture, the HepG2/C3a presented a  
283 typical morphology and formed a monolayer in both conditions (**Fig.2C**). Additionally,  
284 secretion of albumin was measured to assess whether HepG2/C3a cells retained their hepatic  
285 properties. Similar albumin secretion levels were observed in both conditions. The levels were  
286 approximately  $125 \pm 11$  and  $114 \pm 17$  ng/h for cells cultured in MEM and EGM-2/MEM 1/3  
287 mixture, respectively (**Fig.2D**). Based on the results obtained with SK-HEP-1 and HepG2/C3a  
288 cells, the mixture of EGM-2/MEM (25%/75%) was chosen for the dynamic coculture  
289 experiments. To facilitate the comparisons between monoculture and coculture, this medium  
290 was also used for SK-HEP-1 and HepG2/C3a maintenance in monocultures.

### 291 **Characterisation of the SK-HEP-1 endothelial barrier**

292 LSECs act as a physical barrier to molecules and play a significant role in transportation  
293 from circulating blood to the hepatocytes. Therefore, before using SK-HEP-1 to form a liver  
294 endothelial barrier in our coculture model, it was essential to characterise the formation,  
295 integrity, and permeability of the barrier. The SK-HEP-1 cells were seeded in static inserts  
296 using the selected coculture medium and followed over time. The cells proliferated  
297 continuously to reach full confluence and form homogenous and continuous monolayers from  
298 Days 7-8 and thereafter (**Fig.S4**). Then, overgrowth could be observed, resulting in the  
299 formation of a second layer of cells on top of the first one (Day 10, **Fig.S4**). Nevertheless, the

300 formation of continuous layers of confluent cells was confirmed by nuclei, vimentin, and actin  
301 stainings. As shown in **Fig.3A**, the tissue was dense with contiguous cells and a well-developed  
302 actin network. The LSECs phenotype of the SK-HEP-1 barrier was confirmed by the positive  
303 staining for LSECs markers PECAM-1 and stabilin-2 (**Fig.3B**).

304 The formation of a confluent barrier was associated with major modifications in  
305 paracellular permeability. The flow through the barrier was directly correlated to the integrity  
306 and homogeneity of the barrier. To confirm the formation of the barrier, permeability to Lucifer  
307 Yellow was checked using SK-HEP-1 inserts at different times of culture. PET inserts without  
308 cells exhibited a permeability value of  $177 \times 10^{-15} \pm 9 \times 10^{-15}$  m/s (**Fig.3C**). When SK-HEP-1  
309 cells were added, a significant decrease in Lucifer Yellow paracellular flow from the apical to  
310 the basal compartment was observed, with apparent permeability values of  $98 \times 10^{-15} \pm 10 \times 10^{-15}$   
311  $^{15}$  and  $35 \times 10^{-15} \pm 1 \times 10^{-15}$  m/s at Days 4 and 8, respectively. This latter value remained stable,  
312 at approximately  $40 \times 10^{-15} \pm 8 \times 10^{-15}$  m/s until Day 15. These results suggested that the SK-  
313 HEP-1 cells were capable of forming a barrier which reached relative stability at Day 8, and  
314 could be used for coculture with HepG2/C3a and permeability experiments.

315 The permeability of the SK-HEP-1 barrier to molecules with different molecular weights  
316 was also assessed, using FITC-dextran of 4, 70 and 150 kDa. The experiments were performed  
317 using confluent SK-HEP-1 cultures at Day 8 in static inserts. For comparison, the same  
318 experiments were performed using inserts without cells. When using each of the different  
319 molecular weight dextrans, we found that the tracer concentrations decreased from the apical  
320 compartment and increased in the basal one over time (**Fig.3D**). Thus, the tracer molecules  
321 were able to pass through the insert membranes whether the cells were present or not. However,  
322 the FITC dextrans diffused at faster rates into the basal compartment when the inserts were not  
323 seeded with endothelial cells, whereas the presence of a SK-HEP-1 cell layer slowed the  
324 diffusion process for the three molecular weight markers, confirming that the SK-HEP-1 made

325 an efficient diffusion barrier. As expected, the diffusion rates were dependent on the FITC-  
326 dextran molecular weight and were slower when using FITC-dextran of 150 kDa when  
327 compared to 4 kDa- dextran.

### 328 **Effect of the dynamic coculture on the SK-HEP-1 barrier**

329 Following the previous characterisations and optimisations, the coculture of SK-HEP-1  
330 barrier (LSECs compartment) with HepG2/C3a cells cultured in 3D in the biochip (the  
331 hepatocyte compartment as previously characterised **(25)**) was assessed. The coculture was  
332 performed for 48 h in the IIDMP platform and the communication between both compartments  
333 was ensured by culture medium circulation. In parallel, for comparison, SK-HEP-1 and  
334 HepG2/C3a monocultures were also used in the IIDMP platform.

335 After 8 days of barrier maturation in static conditions followed by 48 h of dynamic  
336 coculture or monoculture, the SK-HEP-1 inserts were collected and characterised. Although  
337 cells were barely distinguishable because of the density at confluence, the morphology of the  
338 SK-HEP-1 tissues appeared similar in coculture and monoculture. In both culture modes, the  
339 cells formed homogenous and continuous barriers and grew beyond confluence **(Fig.S5)**.  
340 Confocal microscopy imaging of actin, vimentin and nuclei staining confirmed the formation  
341 of a continuous endothelial barrier, with different cell layers and a developed actin/vimentin  
342 network **(Fig.4A)**. Furthermore, no obvious differences were observed between the staining of  
343 cocultured and monocultured barriers. SK-HEP-1 barriers in monoculture and coculture  
344 expressed typical LSECs markers without any apparent difference between the two modes of  
345 culture, as illustrated by the detection of PECAM-1 and stabilin-2 positive cells **(Fig.4B)**.

346 Gene expression level analyses of several LSECs markers revealed the significant  
347 upregulation of CLEC4M (FC: 2.05) whereas KDR was downregulated (FC: 0.49) in SK-HEP-  
348 1 cocultures **(Fig.5A)**. The expression levels of PECAM-1, MRC1 and CD32b were similar in  
349 SK-HEP-1 monocultures and cocultures. Finally, the diffusion of FITC-dextran 4 kDa through



350 the barrier, in dynamic monoculture and coculture with HepG2/C3a, were compared. The  
351 results in **Fig.5B** confirmed the permeability of the barrier and the communication between the  
352 apical and basal side, in both culture conditions. The variations in FITC-dextran concentrations  
353 in the apical compartment revealed a lower diffusion rate through the barrier in coculture when  
354 compared to that in monoculture, notably after 24 h.

### 355 **Behaviour and functionality of HepG2/C3a in coculture with the SK-HEP-1 barrier**

356 The day before starting the dynamic monocultures/cocultures in the IIDMP device, HepG2/C3a  
357 cells were seeded into the biochips containing the hydroscaffold and incubated in static  
358 conditions (adhesion phase). After 24 h, the cells were embedded in/adhered to the  
359 hydroscaffold and started to create spheroid-like aggregates (**Fig.6A**). Then, the biochips were  
360 connected to the IIDMP device, with and without an SK-HEP-1 barrier, and perfusion was  
361 started. The cells maintained in coculture with an endothelial barrier had a similar morphology  
362 to cells maintained in monoculture. In both conditions, the HepG2/C3a formed a dense tissue,  
363 organised in 3D spheroids ranging between 200 and 500  $\mu\text{m}$  in diameter (**Fig.6A**). To evaluate  
364 the effects of coculture on the specific functions of HepG2/C3a, albumin and urea secretions  
365 were quantified. Albumin levels in coculture were found to be similar to those in monoculture  
366 (**Fig.6B**). After 48 h of culture, the albumin secretion was  $127 \pm 24$  and  $134 \pm 28$  ng/h in  
367 monoculture and coculture, respectively. Regarding urea, the secretion was higher in  
368 HepG2/C3a monoculture ( $2.34 \pm 0.28$   $\mu\text{g/h}$ ), than in coculture ( $1.50 \pm 0.31$   $\mu\text{g/h}$ , **Fig.6C**).  
369 Finally, the expression of several specific genes of HepG2/C3a cells (UGT2B7, UGT1A1,  
370 SULT1A2, CYP1A2 and CYP1A1) were also evaluated. As shown in **Fig.6D**, there were no  
371 significant differences in expression levels in the selected genes between HepG2/C3a  
372 maintained as a monoculture and HepG2/C3a in coculture with SK-HEP-1.

373

374



## 375 **Exposure of the coculture and monoculture models to acetaminophen (APAP)**

376 To test the coculture model and demonstrate the crosstalk between the HepG2/C3a  
377 biochips and SK-HEP-1 barrier in the configuration of a drug study, we exposed the SK-HEP-  
378 1/HepG2/C3a coculture to APAP and compared the results with SK-HEP-1 and  
379 HepG2/C3a monocultures. APAP was chosen because it is i) metabolised by HepG2/C3a cells,  
380 ii) widely studied with liver *in vitro* models, and iii) not adsorbed by the PDMS biochip (30).  
381 APAP was introduced into the apical side of the SK-HEP-1 barrier at 1 mM, leading to a  
382 systemic theoretical concentration of 100  $\mu$ M after diffusion in the total circuit. For comparative  
383 purposes, HepG2/C3a monoculture in the IIDMP was also performed and APAP was deposited  
384 into the insert without SK-HEP-1.

385 SK-HEP-1 cells exposed to APAP for 48 h in coculture or in monoculture exhibited a  
386 confluent and continuous barrier composed of several cell layers, forming a dense tissue. The  
387 cell morphologies between the treated SK-HEP-1 barrier in coculture and in monoculture  
388 showed no significant differences (Fig.S6). Moreover, the SK-HEP-1 cells exposed to APAP  
389 were similar to those without APAP (monoculture and coculture, Fig.S5). As shown in Fig.7A  
390 and S7, APAP treatment appeared to affect the actin cytoskeleton of the barrier, both in  
391 monoculture and coculture. In the cultures without APAP, the actin filaments appear to be  
392 organized and localized around the nuclei (Fig.4A and S7). Conversely, with APAP exposure  
393 (Fig.7A and S7), this organization around the nuclei is not observable and the actin filaments  
394 appear disordered and composed of more elongated filaments. The immunostaining of specific  
395 LSECs markers showed weaker expression levels of PECAM-1 and stabilin-2 in SK-HEP-1  
396 exposed to APAP (Fig.7B), when compared to monoculture and coculture without APAP  
397 (Fig.4B). This effect was more striking in the coculture. Gene expression analyses of cultures  
398 treated or not with APAP showed an upregulation of KDR (FC: 1.8) after APAP exposure in  
399 coculture (Fig.8A). Conversely, both this gene and CLEC4M were downregulated in the

400 monoculture exposed to APAP (FC: 0.54 and 0.49 for CLEC4M and KDR, respectively). APAP  
401 treatment of the monoculture also led to the upregulation of MRC1 (FC 1.4).

402       Regarding the HepG2/C3a biochip, the cells exposed to APAP (coculture or monoculture)  
403 maintained their organisation in 3D spheroids up until the end of the culture (**Fig.S6**). The cells  
404 formed dense tissues, without any apparent difference compared to non-treated cultures.  
405 Analysis of gene expression showed no differences between the biochip monocultures treated  
406 or not with APAP (**Fig.8B**). For HepG2/C3a cocultured with the SK-HEP-1 barrier, UGT2B7  
407 expression levels were downregulated (FC: 0.7). In both monoculture and coculture, the  
408 albumin secretion was not affected by APAP treatment. The ratios of albumin secretion (culture  
409 with APAP versus without APAP) were  $0.92 \pm 0.25$  and  $0.95 \pm 0.09$  for monoculture and  
410 coculture, respectively (**Fig.8C**). Conversely, the APAP treatment was associated with a 2.4-  
411 and 1.6-fold reduction in urea secretion for monoculture and coculture, respectively (**Fig.8C**).

412       The metabolism of APAP was then investigated in cocultures and monocultures using  
413 HPLC-HRMS. We used the basal culture medium to confirm the passage of APAP through the  
414 SK-HEP-1 barrier. The ratios of APAP (compared to the initial systemic concentration of 100  
415  $\mu\text{M}$ ) recovered at the end of the experiment are provided in **Fig.8D**. For the SK-HEP-1  
416 monoculture, the APAP ratio at the end of the experiment was  $1.02 \pm 0.07$ , indicating that SK-  
417 HEP-1 did not metabolise APAP. The recovered ratio, corresponding to a concentration of 100  
418  $\mu\text{M}$ , confirmed the passage of APAP through the barrier, allowing the equilibrium of APAP  
419 concentration between the apical and basal sides. In the HepG2/C3a monoculture and SK-HEP-  
420 1/HepG2/C3a coculture, the APAP ratios were  $0.83 \pm 0.05$  and  $0.87 \pm 0.08$ , respectively,  
421 illustrating metabolism (**Fig.8D**). However, for both conditions, the paracetamol sulphate and  
422 paracetamol glucuronide concentrations were below detection limits.

423

424

## 425 **Expression of inflammatory cytokines**

426 The expression of inflammatory cytokines was evaluated in all cultures (with and without  
427 APAP) by analysing mRNA levels for TNF $\alpha$ , IL-1, IL-6 and IL-8 genes, and by quantifying  
428 IL-6 secretion. SK-HEP-1 cells expressed the four genes in all culture conditions  
429 (monoculture/coculture and APAP+/APAP-). Gene expression levels of IL8, IL6 and IL1 were  
430 similar, regardless of the culture conditions. There was a noticeable significant upregulation of  
431 TNF $\alpha$  in SK-HEP-1 cocultured with APAP (**Fig.8E**). Regarding HepG2C3a cells, there were  
432 no significant differences in expression levels of IL-8 in the conditions tested. On the other  
433 hand, there was a slight but significant overexpression of TNF $\alpha$  in HepG2/C3a cocultured  
434 without APAP when compared to monocultures (**Fig.8F**). IL-6 protein quantification in culture  
435 medium showed that it was only expressed by SK-HEP-1 cells and that HepG2/C3a  
436 monocultures with and without APAP did not produce detectable amounts of IL-6 (**Fig.8G**).

## 437 **DISCUSSION**

438 Classic 2D *in vitro* coculture models consist of cells randomly mixed and heterogeneously  
439 distributed at the bottom of well-plates and dishes. However, *in vivo*, LSECs and hepatocytes  
440 are separated by the space of Disse which, in 3D models, is generally mimicked by a gel or  
441 collagen matrix which physically separates LSECs and hepatocytes (**23**). Furthermore,  
442 controlling the homotypic and heterotypic cell-to-cell interactions appears to be a key feature  
443 for maintaining and enhancing the hepatocyte phenotype (**31,32**). In the present work, we have  
444 established a coculture model of liver sinusoidal endothelial cells with liver cell line. Thanks to  
445 our platform which integrates a liver-on-chip solution and a barrier insert, we were able to  
446 propose technology that physically separated both cell types. In this model, cell-to-cell  
447 paracrine-like communication was made possible by exchanges through the insert membrane,  
448 as this model did not allow direct contact between LSECs and hepatocytes. Although this type  
449 of technology has already been presented for organ-to-organ models such as the intestine

450 barrier-liver (26), to our knowledge, only a few other dynamic LSECs barrier-hepatocyte  
451 coculture models have previously been described (33-35).

452 We demonstrated the functionality of the coculture model using two human cell lines,  
453 SK-HEP-1 and HepG2/C3a. For this purpose, we optimised the culture medium, confirmed the  
454 innocuity of the fluid flow and coculture on the LSECs barriers, and characterised the cytokine  
455 crosstalk between cells. Establishing a coculture medium that is healthy for two or more types  
456 of cells is a critical step in *in vitro* physiological models (36), including liver cells (34).  
457 Similarly, it was reported that LSECs are sensitive to serum components (37). Our data  
458 demonstrated that the HepG2/C3a MEM-based medium which contained serum contributed to  
459 damaging the LSECs layer, whereas the conventional LSECs medium (also containing serum)  
460 did not. Interestingly, a mixture of the HepG2/C3a and SK-HEP-1 media led to both healthy  
461 LSECs and HepG2/C3a. Although we did not identify the specific factors leading to this result,  
462 we postulate that the presence of pro-angiogenic factors in EGM-2 medium played a part in  
463 stabilising the LSEC cultures. Interestingly, the present dynamic conditions did not affect the  
464 cell junctions or the expression levels of LSEC markers.

465 Endothelial cells are normally exposed to flow, and dynamic *in vitro* models have  
466 largely been reported as regulating their functions and physiology (38,39). However, a decrease  
467 in endothelial barrier permeability was only reported in dynamic cultures coupled to high shear  
468 stress (0.7-1 Pa) (39). In the present work, we did not observe significant variations in barrier  
469 permeability functions between static and dynamic SK-HEP-1 monocultures (Fig.3D and 5B).  
470 Indeed, in the IIDMP device, the flow passes in the basal side and the SK-HEP-1 cells (facing  
471 the apical side) are not directly exposed to the shear. Conversely, the permeability was reduced  
472 in dynamic LSECs cocultures (Fig.5B), illustrating stronger cell junctions in the presence of  
473 HepG2/C3a, and suggesting that there is a synergistic effect of cells coculture in our conditions.  
474 We also measured that CLEC4M, an important LSECs marker (40) was overexpressed in

475 coculture. Furthermore, we found that the LSECs produced basal levels of pro-inflammatory  
476 cytokines IL6 and TNF without any significant morphology damage. The dynamic coculture  
477 also did not play a part in significantly increasing cytokine levels in LSECs. As high levels of  
478 pro-inflammatory cytokines production in the liver by LSECs leads to fibrosis (41), our result  
479 illustrated the fact that the dynamic cocultures of LSECs were not pro-inflammatory.

480 Previous works reported an improved hepatocyte phenotype when cocultured with  
481 endothelial cells (22-24,42). In the present model, we did not detect any striking benefit of the  
482 presence of LSECs on the HepG2/C3a phenotype (no albumin increase, no mRNA gene  
483 metabolism upregulation, no clear cytokine over secretion). In fact, the enhanced maturation of  
484 hepatocytes was mainly reported on primary hepatocytes that tend to rapidly dedifferentiate  
485 (42). It is clear that the hepatocarcinoma HepG2/C3a cell line is probably not an ideal model  
486 for liver-on-chip approaches. Although it has been widely used in works related to cancer and  
487 liver disease (43), and shown that interactions between the liver endothelium (include SK-HEP-  
488 1) and this liver carcinoma were reported in studies investigating liver disorders (44,45), it has  
489 a weak maturation profile. It is certainly a robust model for proof-of-principle studies, but the  
490 present on-chip approach would clearly benefit from being extended and refined using normal  
491 human primary cells.

492 Regarding liver toxicology, the liver's *in vivo* features suggest that xenobiotics must first  
493 pass the endothelial barrier before accessing the hepatocytes. Analysing the kinetics and  
494 toxicity of APAP *via* the LSEC barrier and its subsequent metabolism inside the HepG2/C3a  
495 compartment was presented as a proof of concept of our technology. APAP was selected  
496 because its metabolism and effect with HepG2/C3a cells have been widely studied, including  
497 in our biochip (46-49). Although HepG2/C3a do not express the CYP2E1, they highly express  
498 CYP1A2 (involved in the APAP metabolism, (50)) in our biochip (48). To ensure healthy and  
499 functional cells, low concentration of APAP (100  $\mu$ M) was used. APAP toxicity directly on

500 LSECs has already been reported in the literature **(51,52)**. The presence of APAP contributed  
501 to modifying the expression of LSECs markers in this work. This was illustrated by degradation  
502 of the actin and vimentin network, and the reduction of the PECAM-1 and STAB2 expression  
503 levels as shown in the immunostaining images. We also confirmed APAP metabolism in the  
504 presence of the HepG2/C3a cells. The barrier led to modulation of the concentration of APAP  
505 reaching the liver cells and we did not detect any particular sign of HepG2/C3a toxicity in our  
506 experiments. Consistently with the literature, the 100  $\mu$ M concentration of APAP on HepG2 is  
507 not a toxic concentration, as most studies reported effects between 1 to 2 mM **(49,53,54)**.

508         The development of relevant *in vitro* liver models is very challenging. With the progress  
509 made in tissue engineering and microfluidics, several microfluidic-based models reproducing a  
510 physiologically relevant microenvironment have been developed in recent years **(9,16)**.  
511 Although most of these models are based on hepatocyte monoculture, a growing number of  
512 groups are interested in developing microfluidic cocultures of different liver cells, especially  
513 hepatocytes and LSECs **(9,16,22,35,55,56)**. In these models, the different cell types are  
514 randomly mixed or organised in layers separated by a porous membrane, collagen layer or  
515 microstructures **(16,23)**. The present model combines the advantages of the LSECs barrier and  
516 hepatocytes cultured in 3D spheroids in hyaluronic acid hydrosc scaffold. Thanks to the IIDMP  
517 platform, the LSEC insert and the hepatocytes biochips are physically separated to mimic the  
518 space of Disse which separates both cells *in vivo*. The interaction between LSECs and  
519 HepG2/C3a is ensured only *via* the paracrine communication. A critical issue in microfluidic  
520 culture is the balance between model relevance, complexity and practicality. For liver cell  
521 cocultures, all cell types are usually seeded in the same irreversibly sealed microfluidic device,  
522 which makes it extremely difficult to analyse the different cell types separately **(57)**. Our model  
523 consists of two separate compartments easily assembled in the IIDMP devices: HepG2/C3a in  
524 biochips and the LSECs barrier in standard inserts. Each cell type can be cultured and

525 characterised separately before being connected to an IIDMP device for coculture. At the end  
526 of the experiments, the inserts and biochips can also be easily removed for separate external  
527 analyses.

528 Overall, the present study provides an original model based on coculture of HepG2/C3a  
529 spheroids-on-chip with LSECs insert, as an alternative *in vitro* method for simulating liver  
530 sinusoid. The model allowed to maintain stable and functional cellular behaviours' and to study  
531 the crosstalk between cells. The modularity of our microfluidic platform suggests that other  
532 NPCs can be easily included to the model: Kupffer cells with LSECs insert and stellate cells in  
533 the biochip with hepatocytes. In addition to study of drug metabolism/toxicity and liver disease,  
534 the model offers the possibility of studying the complex cell-cell interactions which plays key  
535 role in liver injury and disease. However, studies using human primary cells, and including  
536 long-term cultures/exposures and the use of other chemical would be needed to expand the  
537 significance of this model.

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#### 542 **CONFLICT OF INTEREST**

543 HCS pharma is the BIOMIMESYS® Liver owner and is a partner of the ANR  
544 MimLiverOnChip. Co-authors Z. Souguir and N. Maubon are employees of HCS Pharma.

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720 **Fig.1.** (A) Pictures and schematic image showing the design and principle of the IIDMP  
721 coculture platform; (B) experimental procedures for SK-HEP-1 and HepG2/C3A monoculture  
722 and coculture.

723 **Fig.2.** Effect of culture medium composition on SK-HEP-1 and HepG2/C3a cells cultured in  
724 static inserts and 6-well plate, respectively. (A) phase contrast microscopy images of SK-HEP-  
725 1 exhibiting similar morphologies after 7 days of culture in EGM-2/MEM (75%/25%) and  
726 EGM-2/MEM (25%/75%) mixtures (magnification x10); (B) gene expression of several LSECs  
727 markers in SK-HEP-1 cultured in EGM-2/MEM (75%/25%) and EGM-2/MEM (25%/75%)  
728 mixtures: CLEC4M and VCAM1 are downregulated in EGM-2/MEM (25%/75%) mixtures (\*  
729  $P < 0.05$ ,  $n = 3$ ); (C and D) HepG2/C3a cell with similar morphologies and albumin secretion  
730 ( $n = 6$ ) after 4 days of culture in MEM and EGM-2/MEM (25%/75%) mixture media.

731 **Fig.3.** Characterisation of the SK-HEP-1 endothelial barrier. (A) vimentin, actin, and nuclei  
732 staining of the SK-HEP-1 cells after 8 days of culture on inserts; (B) PECAM-1, stabilin-2, and  
733 nuclei staining at Day 8; (C) apparent permeability to Lucifer Yellow, measured between day  
734 4 and day 15: SK-HEP-1 barrier reaches its stable permeability from day 8 ( $35-40 \times 10^{-15} \pm 8 \times$   
735  $10^{-15}$  m/s), \*  $P < 0.05$  ( $n = 6$ ); (D) diffusion of FITC-dextran (4, 70 and 150 kDa) through the  
736 SK-HEP-1 confluent barrier (8 days of culture) and insert without cells ( $n = 6$ ): the diffusion  
737 decreases in the presence of the SK-HEP-1 barrier and with increasing dextran molecular  
738 weight.

739 **Fig.4.** Characterisation of the SK-HEP-1 endothelial barrier in dynamic monoculture and  
740 coculture (8 days of maturation followed by 2 days in the IIDMP platform). (A) vimentin, actin,  
741 and nuclei staining; (B) PECAM-1, stabilin-2 and nuclei staining.

742 **Fig.5.** Comparison of the SK-HEP-1 barrier in dynamic monoculture and coculture. (A) gene  
743 expression of LSECs markers: KDR and CLEC4M are down- and upregulated, respectively in

744 dynamic coculture, \*  $P < 0.05$  ( $n = 3$ ); (B) FITC-dextran 4 kDa diffusion through SK-HEP-1  
745 barriers in dynamic monoculture and coculture ( $n = 6$ ).

746 **Fig.6.** Characterisation of HepG2/C3a cells cultured in the biochip, in monoculture, and  
747 coculture with the SK-HEP-1 endothelial barrier. (A) cell morphology after seeding, 24 h of  
748 adhesion in static conditions, 48 h of dynamic monoculture, and 48 h of dynamic coculture in  
749 the presence of SK-HEP-1; (B) no significant difference in albumin secreted by HepG2/C3a  
750 cells during the 48 h of dynamic monoculture/coculture with SK-HEP-1 ( $n = 6$ ); (C) urea  
751 quantification showing downregulation in the coculture conditions, \*  $P < 0.05$  ( $n = 6$ ); (D)  
752 similar gene expression of HepG2/C3a markers in monocultures and cocultures ( $n = 3$ ).

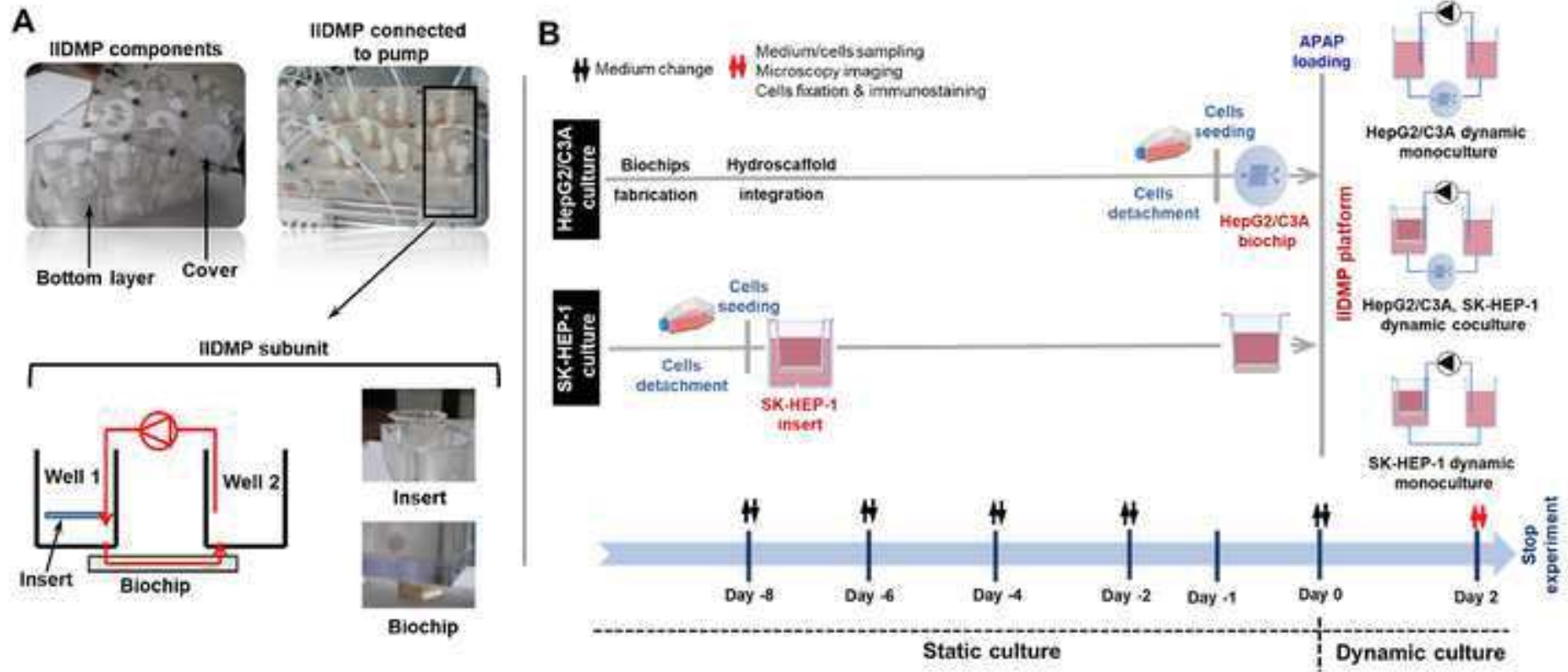
753 **Fig.7.** Characterisation of the SK-HEP-1 endothelial barrier exposed to APAP in dynamic  
754 monoculture and coculture, 8 days of maturation followed by 2 days in the IIDMP platform  
755 with APAP exposure. (A) vimentin, actin, and nuclei staining; (B) PECAM-1, stabilin-2 and  
756 nuclei staining. The Actin organization and the expression levels of PECAM-1 and stabilin-2  
757 (low) appear affected by APAP exposure (in comparison with monoculture and coculture  
758 without APAP in Fig.4).

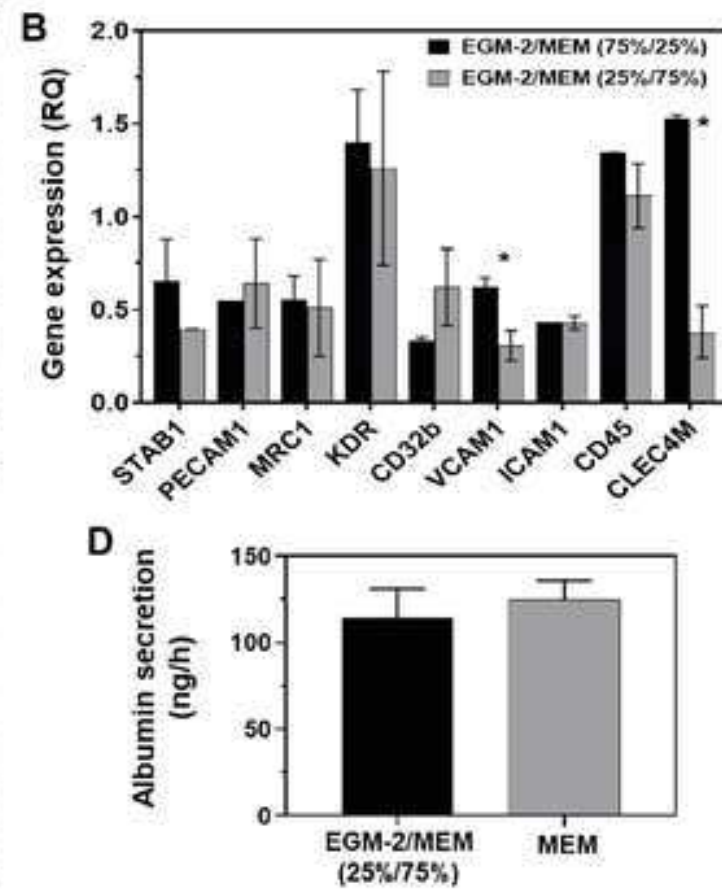
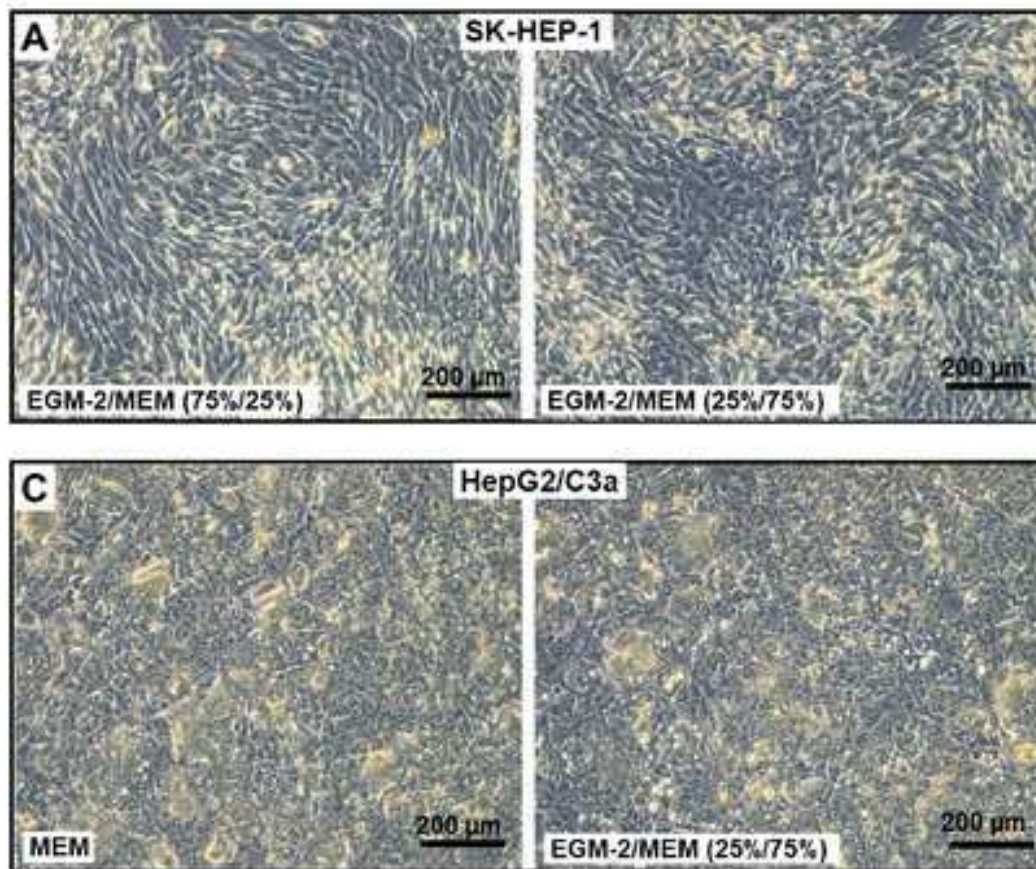
759 **Fig.8.** Characterisation of monocultures and cocultures with and without APAP treatment. (A)  
760 mRNA ratio (APAP+/APAP-) of selected markers in SK-HEP-1 monoculture and coculture  
761 (comparison APAP+ versus APAP-): APAP exposure leads to MRC1 upregulation and KDR,  
762 CLEC4M downregulation in monoculture, and KDR upregulation in coculture, \*  $P < 0.05$  ( $n =$   
763 3); (B) mRNA ratio (APAP+/APAP-) of selected markers in HepG2/C3a monoculture and  
764 coculture (comparison APAP+ versus APAP-): only UGT2B7 is downregulated in coculture  
765 exposed to APAP, \*  $P < 0.05$  ( $n = 3$ ); (C) ratio (APAP+/APAP-) of albumin and urea secreted  
766 by HepG2/C3a monoculture and coculture; urea secretion decreases in monoculture and  
767 coculture treated with APAP, \*  $P < 0.05$  ( $n = 6$ ); (D) ratio of APAP recovered at the end of the  
768 experiments for HepG2/C3a monoculture, SK-HEP-1 monoculture and coculture: SK-HEP-1

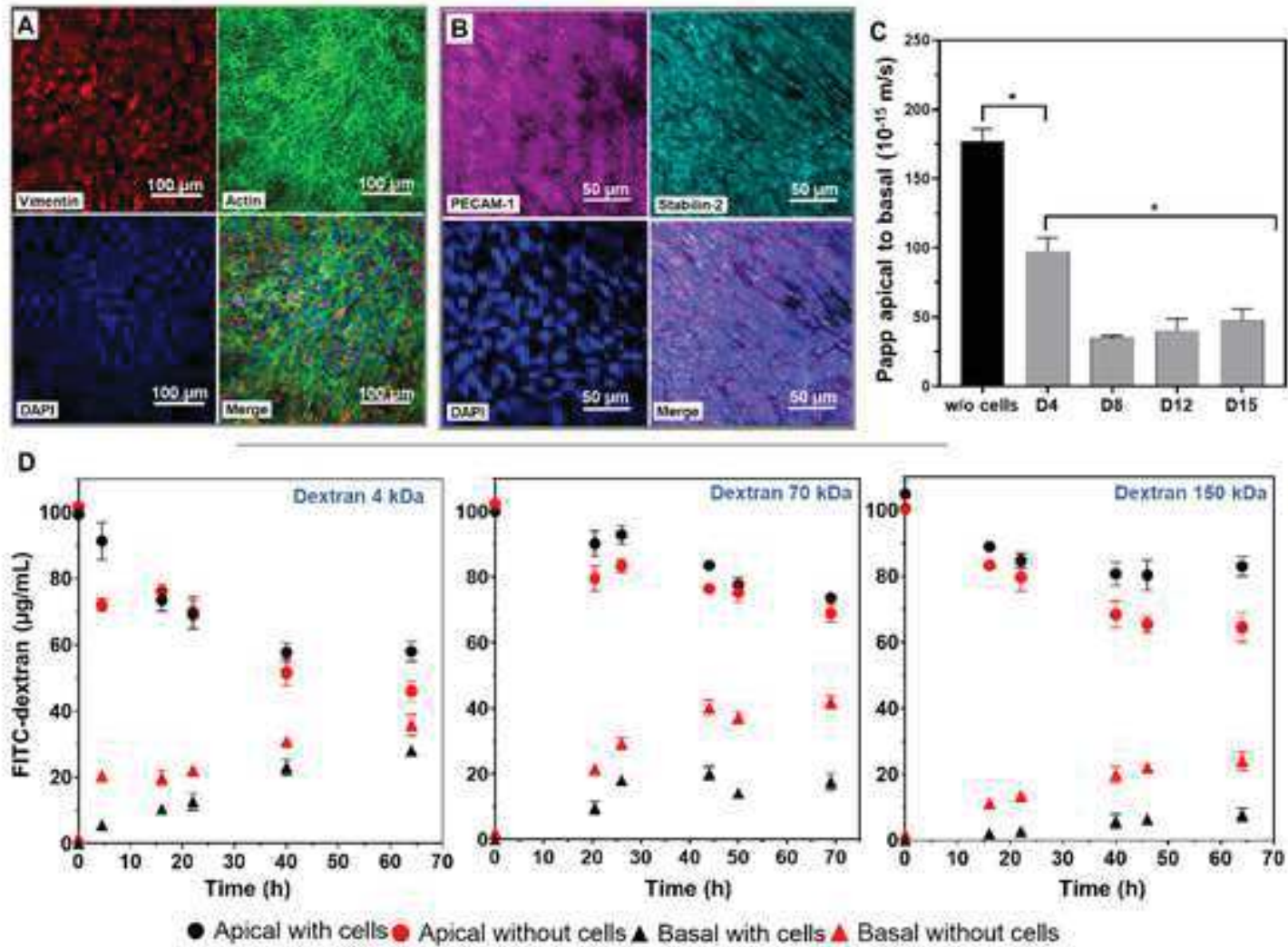


769 monoculture do not metabolize APAP , \* P < 0.05 (n = 6); (E) expression of inflammatory  
770 genes in SK-HEP-1 monoculture and coculture, with and without APAP, showing the  
771 upregulation of TNF $\alpha$  in coculture exposed to APAP, \* P < 0.05 (n = 3); (F) expression of  
772 inflammatory genes in HepG2/C3a monoculture and coculture, with and without APAP: the  
773 exposure to APAP decreases the expression of TNF $\alpha$  in coculture, \* P < 0.05 (n = 3); (G) IL-6  
774 secreted in different culture conditions.

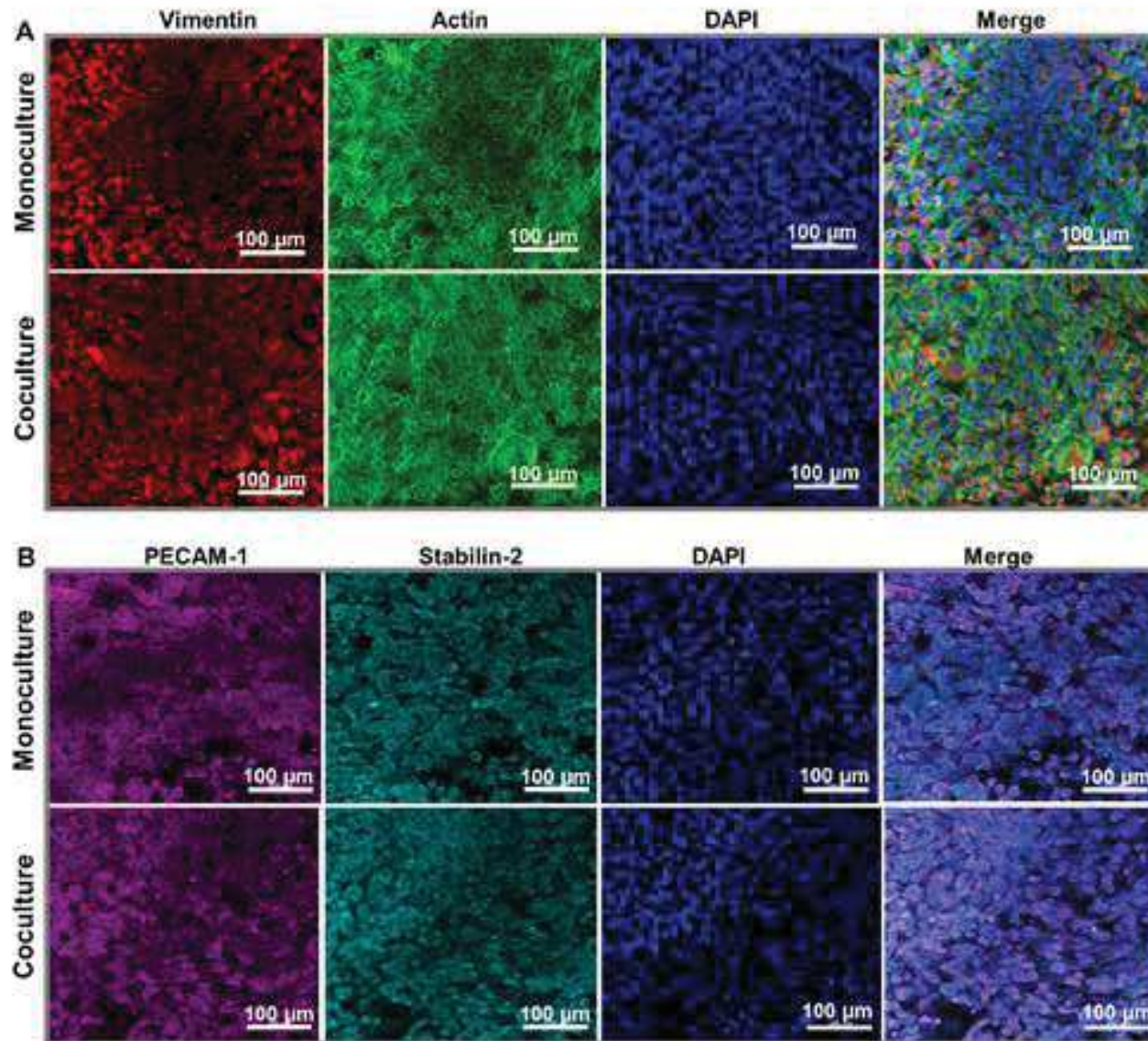
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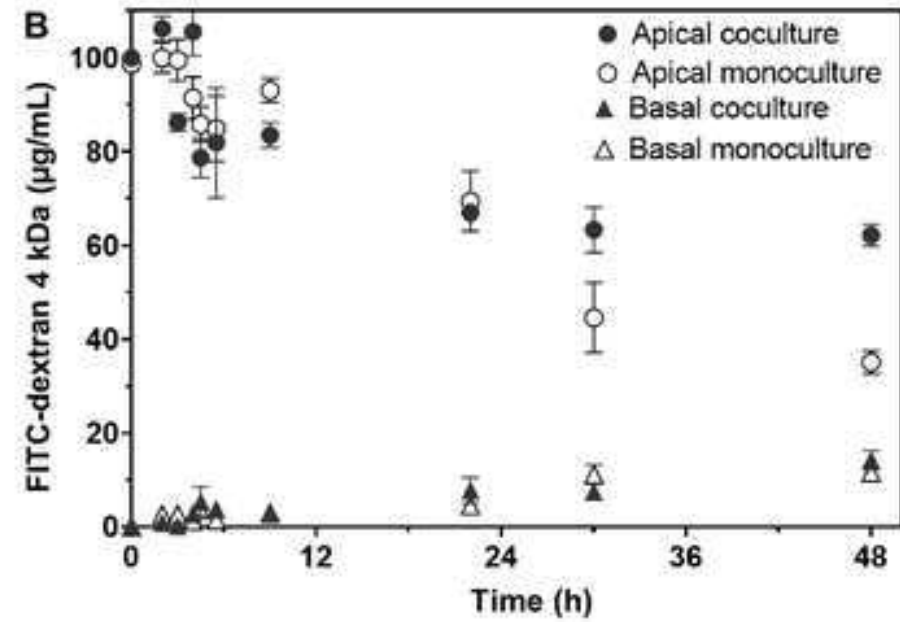
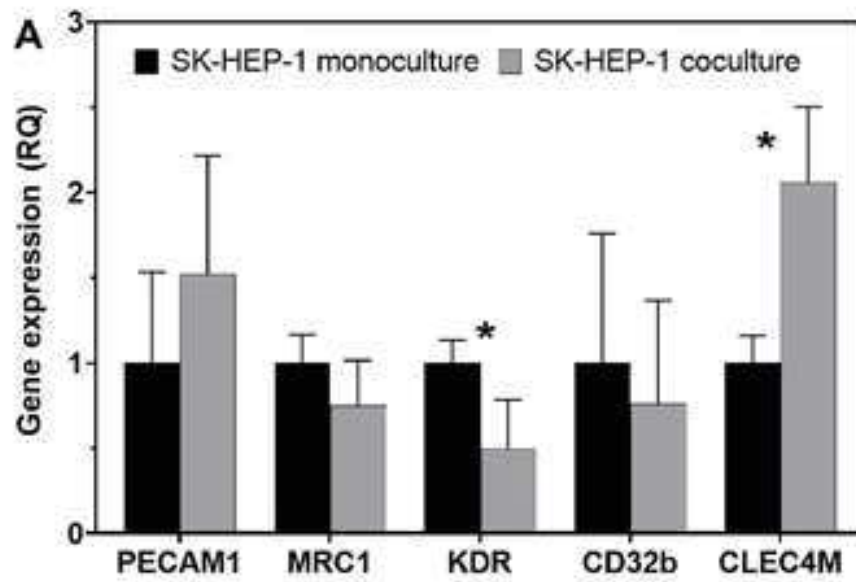


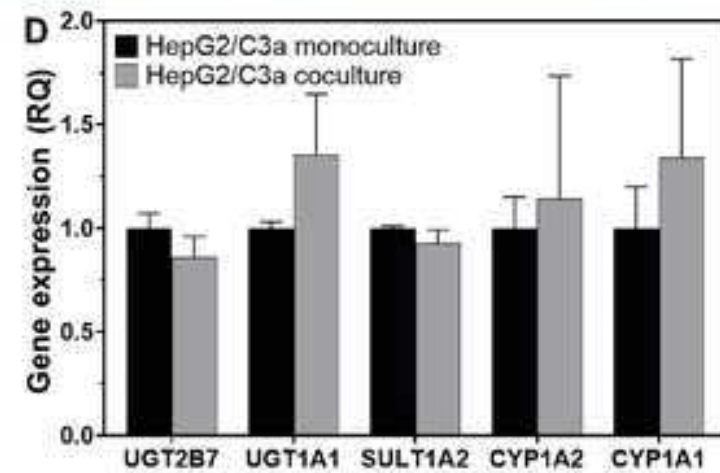
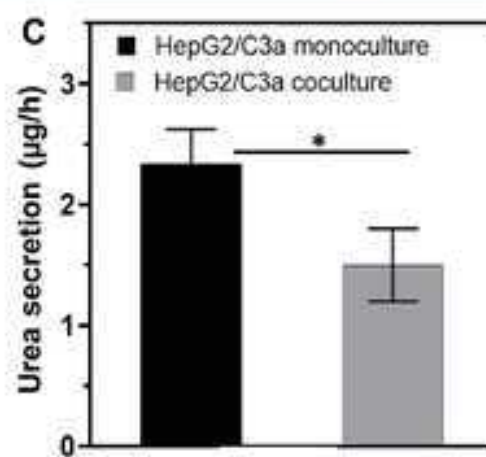
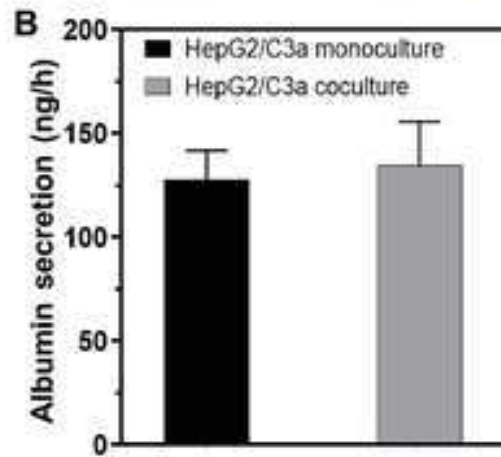
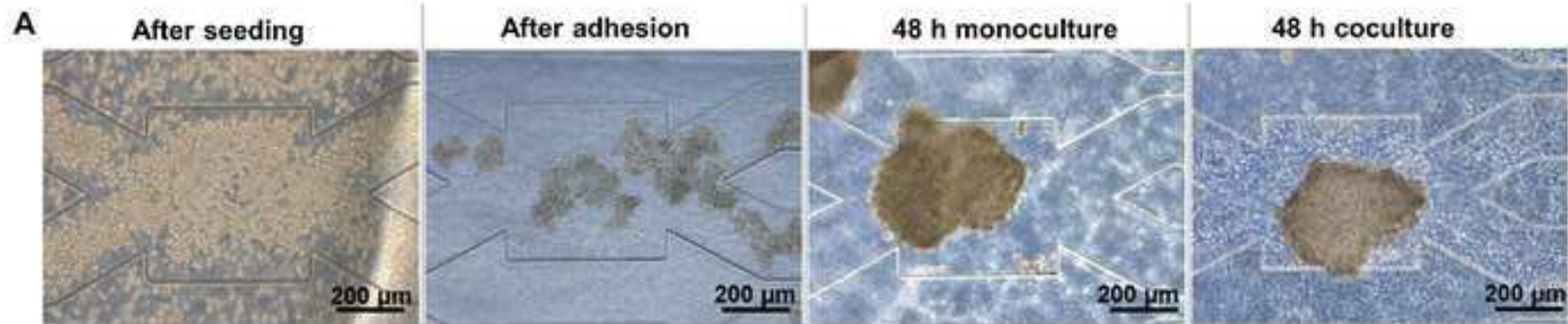




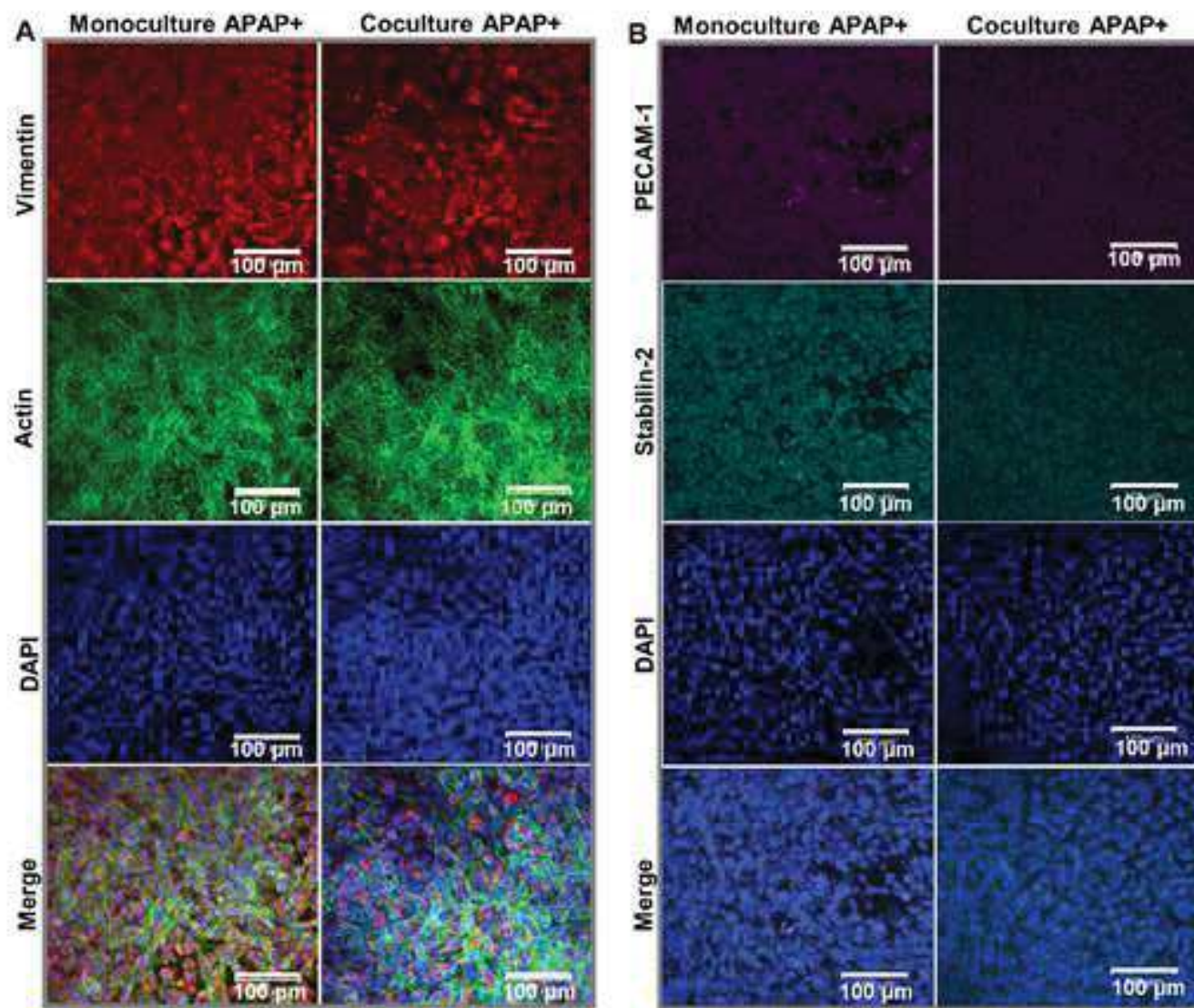




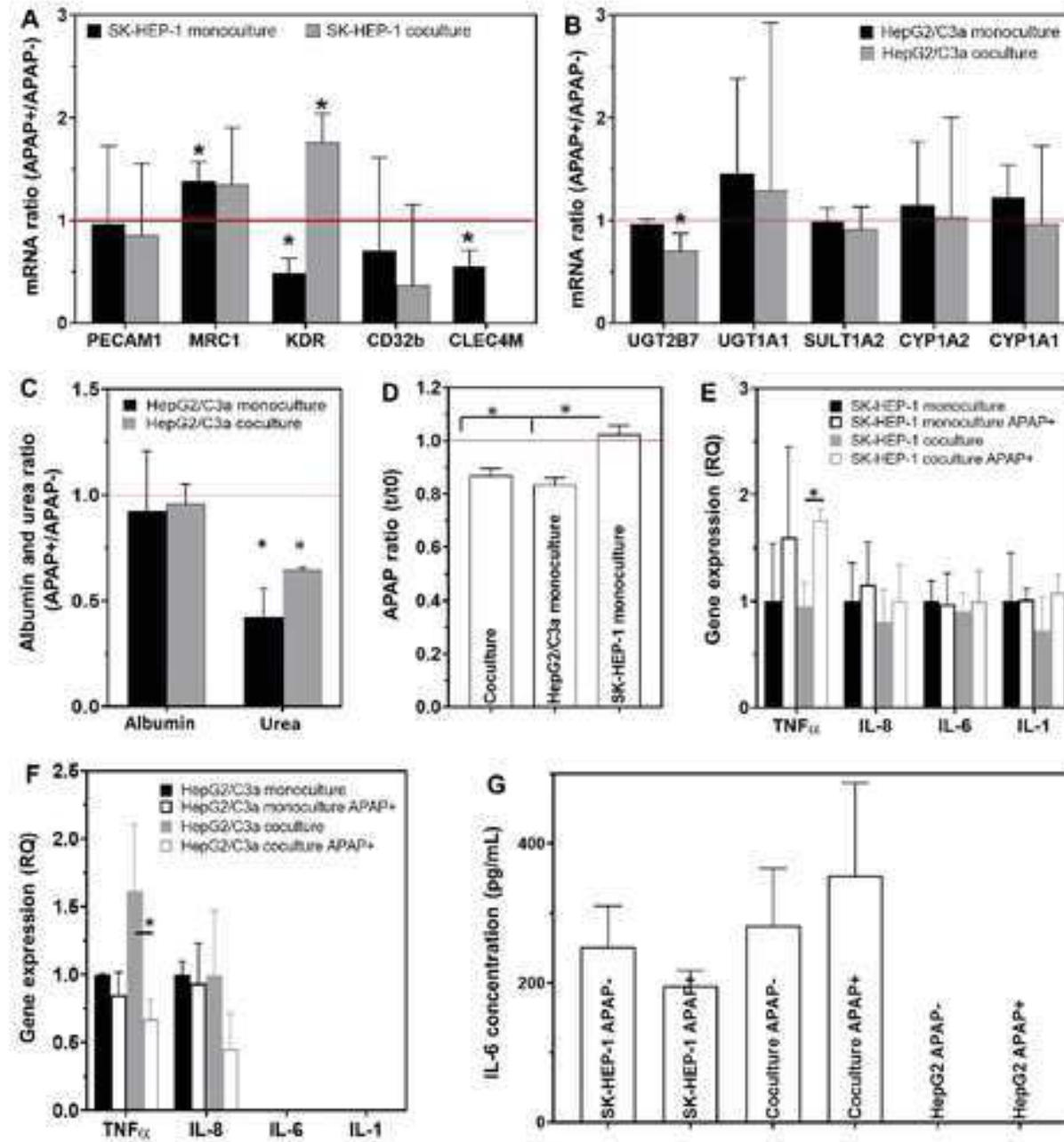












# **Coculture model of a liver sinusoidal endothelial cell barrier and HepG2/C3a spheroids-on-chip in an advanced fluidic platform**

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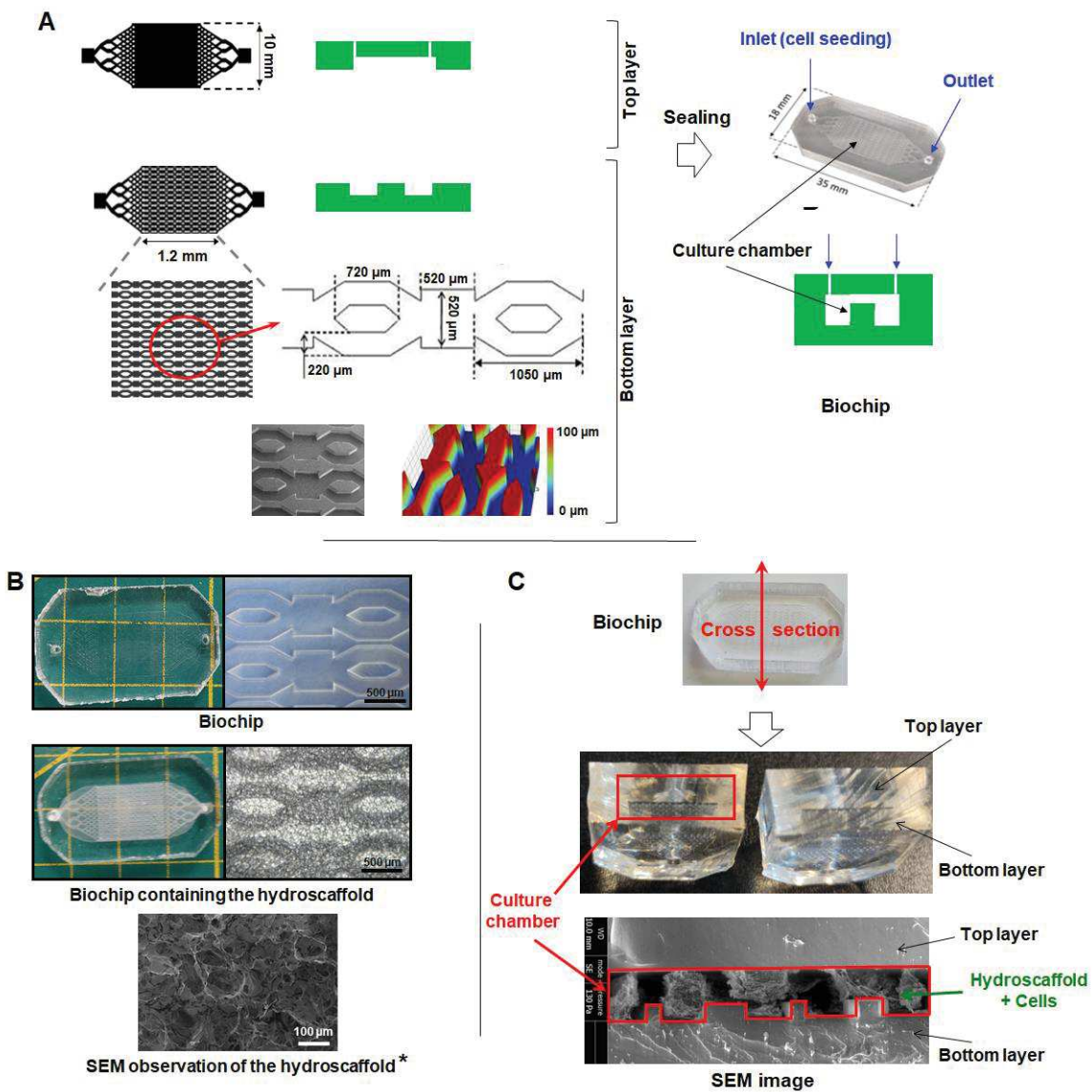
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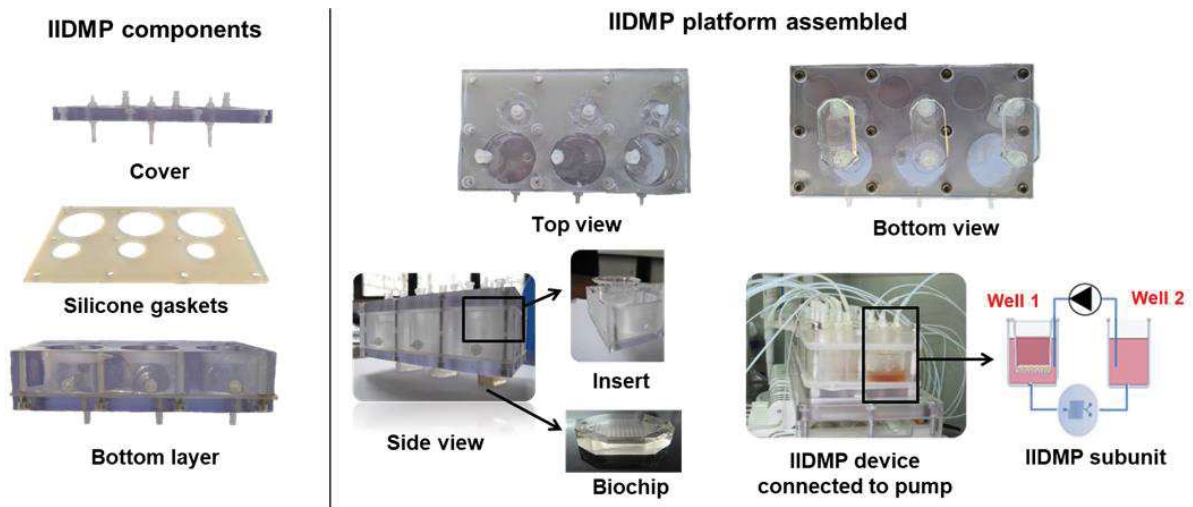
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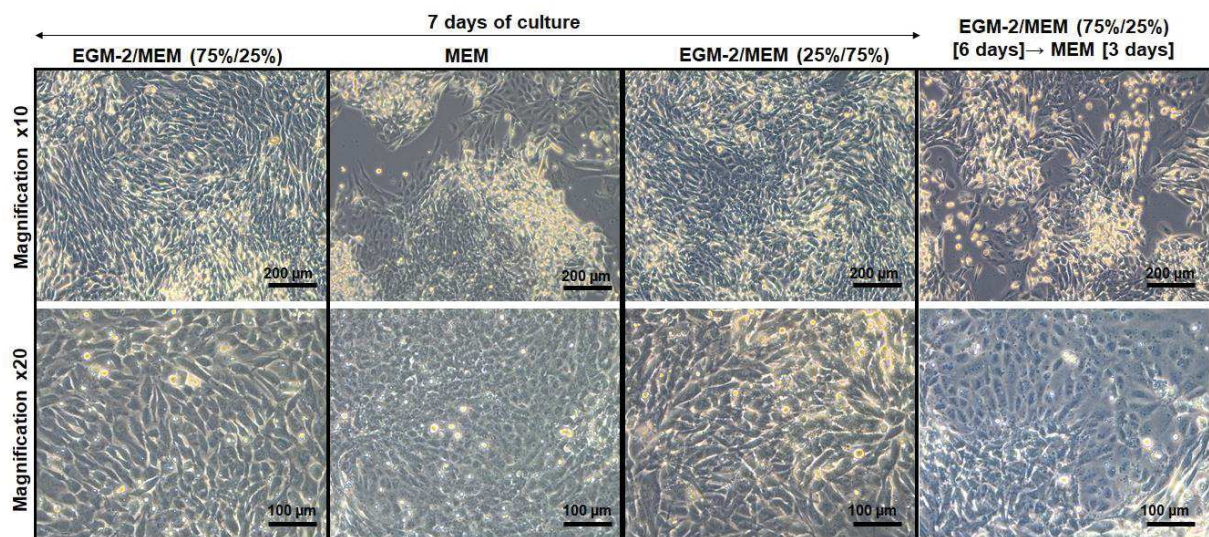
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**Fig.S1.** Specifications of the biochip used for HepG2/C3a cultures. (A) biochip design and dimensions; (B) characterisation of the biochip with and without the hydrosccaffold: images, optical microscope observations of the biochips (magnification x5) and SEM image of the hydrosccaffold (\* data from Messelmani et al., 2022 (25)); (C) image and SEM observation of biochip cross section showing the culture chamber containing the hydrosccaffold and cells. Scanning electron microscopy (SEM) analysis was taken using an XL30-ESEM FEG (Philips, Eindhoven, The Netherlands), with samples (cells + hydrosccaffold) fixed in paraformaldehyde 4%.

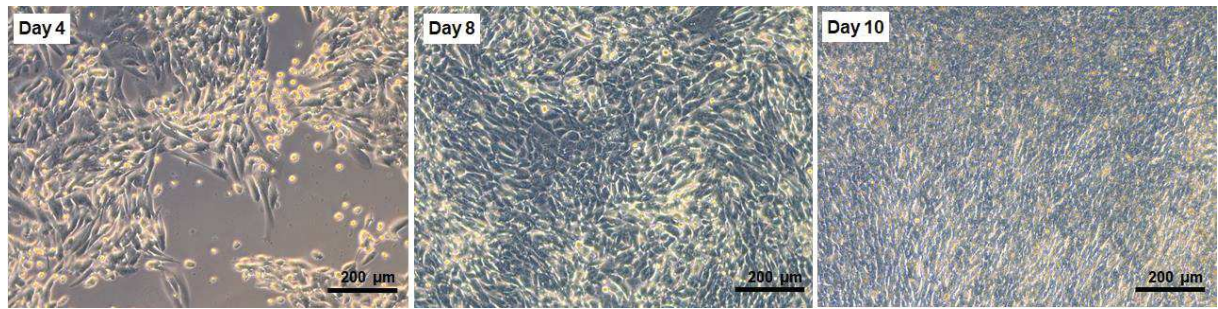


**Fig.S2.** Specifications and principle of the IIDMP platform.

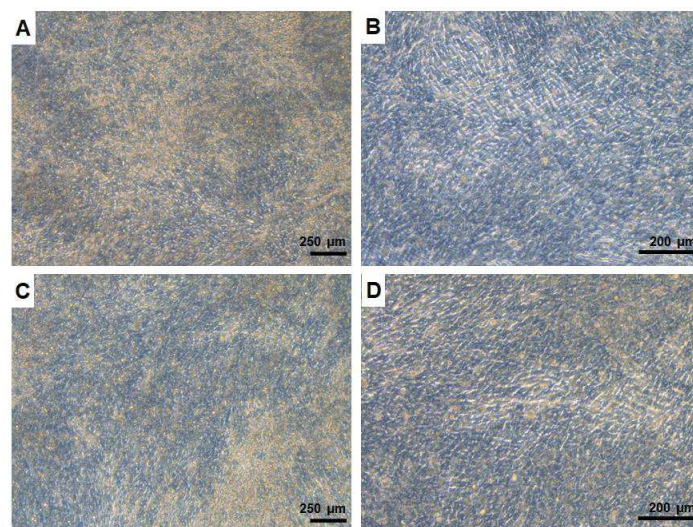


**Fig.S3.** Phase contrast microscopy images of SK-HEP-1 cells cultured on static inserts in different culture media mixtures. Among the tested conditions, only the mixture EGM-2/MEM (25%/75%) allows the formation of confluent barrier similar to barrier formed by SK-HEP-1 cells cultured in their original medium (SK-HEP-1 cells are routinely cultured in EGM-2/MEM (75%/25%) mixture).

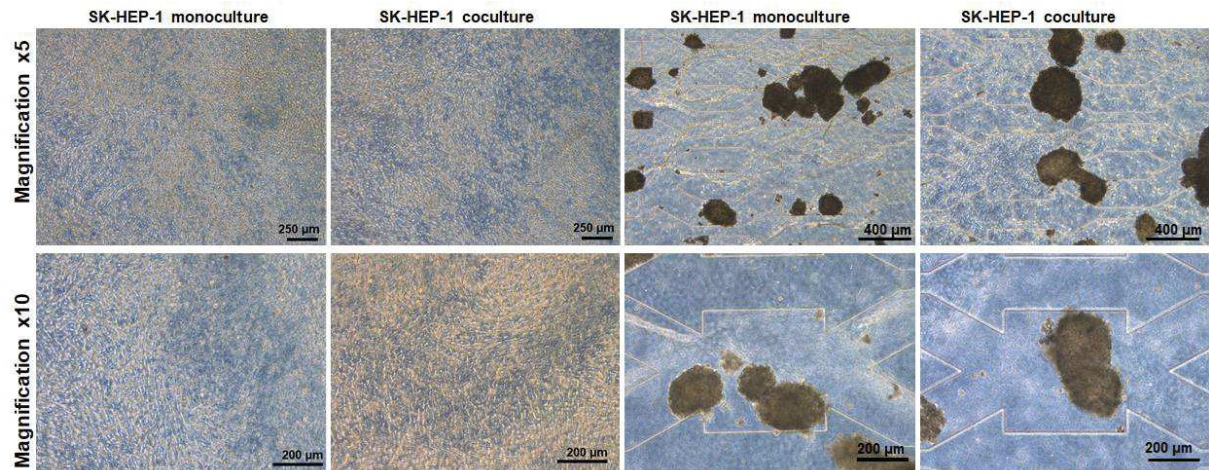




**Fig.S4.** Phase contrast microscopy images (magnification x10) showing the growing of the SK-HEP-1 cell layer between day 4 and 10. The cells were cultured on static inserts.

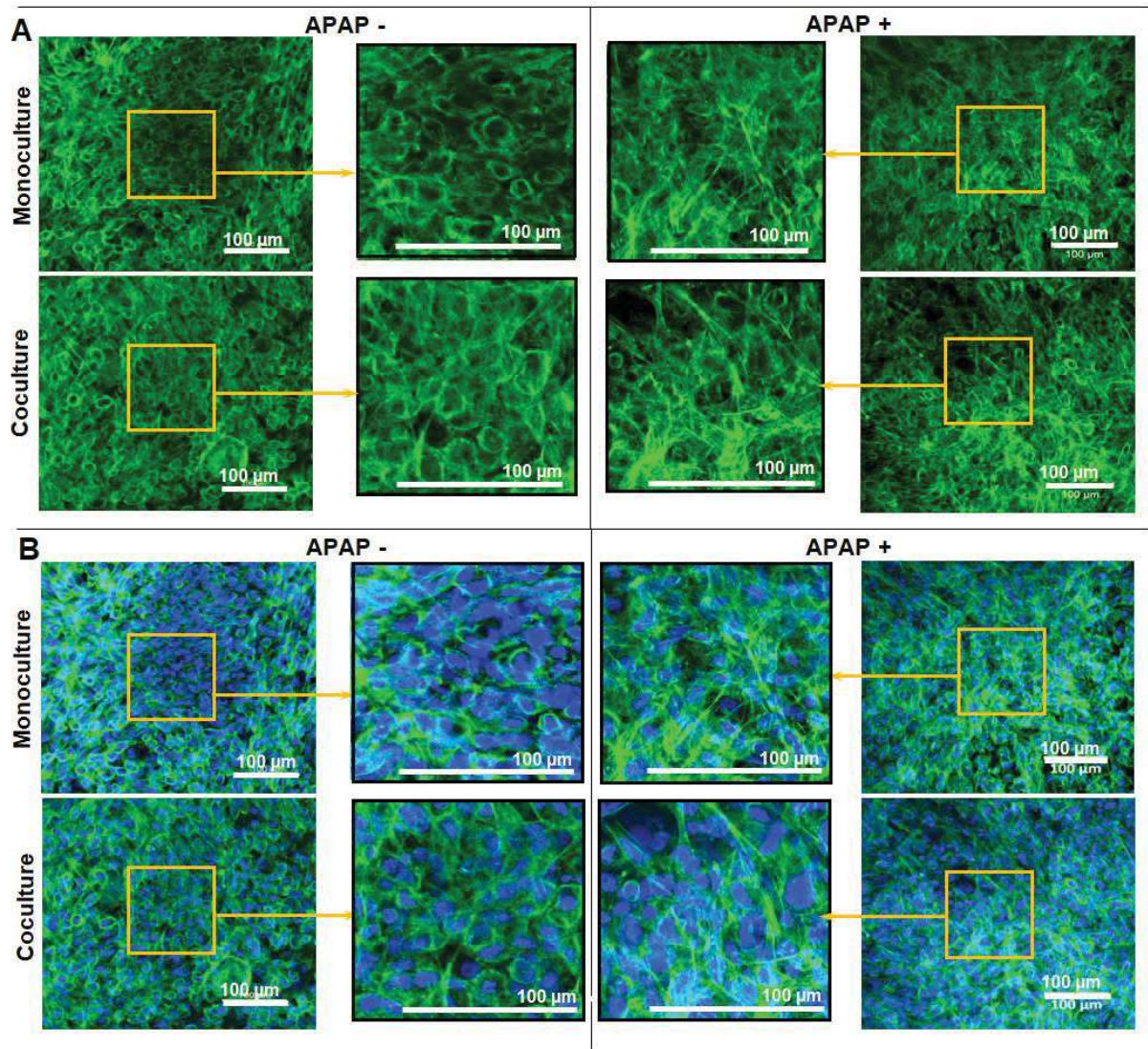


**Fig.S5.** Phase contrast microscopy images of SK-HEP-1 cells monoculture and coculture after 10 days of culture: 8 days of maturation in static inserts and 2 days of dynamic culture in IIDMP platform (A and C: magnification x5; B and D: magnification x10). No significant difference was observed between the SK-HEP-1 monoculture and coculture (with HepG2/C3a).



**Fig.S6.** Phase contrast microscopy images of SK-HEP-1 and HepG2/C3a cells monoculture and coculture after exposure to APAP for 2 days (dynamic culture in IIDMP platform). The morphological features of HepG2/C3a spheroids and SK-HEP-1 barrier remain unchanged after exposure to APAP (in monoculture and coculture).





**Fig.S7.** Characterisation of the SK-HEP-1 endothelial barrier exposed in dynamic monoculture and coculture: 8 days of maturation followed by 2 days in the IIDMP platform with and without APAP exposure. (A) actin staining; (B) actin and nuclei staining (merge). The Actin organization appear affected by APAP exposure.

**Table S1.** TaqMan probes used for RTqPCR assays.

<b>Gene</b>	<b>Probe ID</b>	<b>Fluorophore</b>
B2M	Human B2M (beta-2-microglobulin) Endogenous Control	VIC/MGB probe, primer limited
STAB1	Hs01109068_m1	FAM/MGB
PECAM1	Hs01065279_m1	FAM/MGB
MRC1	Hs00267207_m1	FAM/MGB
KDR	Hs00911700_m1	FAM/MGB
CD32b	Hs01634996_s1	FAM/MGB
VCAM1	Hs01003372_m1	FAM/MGB
ICAM1	Hs00164932_m1	FAM/MGB
CD45	Hs04189704_m1	FAM/MGB
CLEC4M	Hs03805885_g1	FAM/MGB
UGT2B7	Hs00426592_m 1	FAM/MGB
UGT1A1	Hs02511055_s1	FAM/MGB
SULT1A2	Hs02340929_g1	FAM/MGB
CYP1A2	Hs00167927_m1	FAM/MGB
CYP1A1	Hs01054796_g1	FAM/MGB
TNF $\alpha$	Hs01113624_g1	FAM/MGB
IL-1	Hs01555410_m1	FAM/MGB
IL-6	Hs00985639_m1	FAM/MGB
IL-8/ CXCL8	Hs00174103_m1	FAM/MGB