# Perfused Liver Vasculature On Chip:
# a microphysiological model to study the role of mechanics on the liver endothelial cells

**Co-direction of the project**

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## I. Context positioning and objectives of the proposal

### • Need for *in vitro* co-culture study models recapitulating physiological cellular functions

Recent advances in mechanobiology and microtechnology have synergistically converged into the development of biomimetic systems improving *in vitro* culture models. In such microphysiological systems (MPS) or chips, cells can benefit from the integration of crucial physiological cues thanks to engineering allowing to: (i) achieve **a prolonged preservation of the primary functional phenotypes of the cells**, normally hindered *in vitro*; (ii) **examine the effects of key parameters** (mechanics, architecture, dynamics and morphogen gradients), **in both a decoupled and combined fashion, on cell identity** and **response to drugs** in physio-pathological conditions.

### • Non alcoholic fatty liver disease: unknown role of mechanical changes

Non alcoholic fatty liver disease (NAFLD), defined as the presence of triglyceride accumulation in the hepatocytes (steatosis), can progress to cirrhosis and cancer. It is becoming the most frequent liver disease. Despite its severity and constantly increasing prevalence, there is no approved therapy for NAFLD. Precise mechanisms orchestrating its progression are still unclear although recent data show that changes in liver sinusoidal endothelial cells (LSECs) occur at an early stage of NAFLD and might contribute to liver disease progression [1], as recently reviewed by Rautou's team. Mechanisms responsible for these endothelial changes are unknown [1]. Recent data demonstrate that fat accumulation in hepatocytes decreases sinusoid size and promotes an increase in portal pressure [2]. Whether an initial small increase in portal pressure could be the driver for endothelial changes occurring at early stages of NAFLD has not been investigated because tools were lacking. More broadly, analyzing the role of LSECs in NAFLD has been limited by the rapid (<24hrs) loss of their LSEC phenotype when cultured in monolayers within wells.

### • Achievements, limitations and current challenges in *liver-on-chip* systems

LSEC culture models must improve to provide conditions: (a) recapitulating *in vivo* properties over longer periods of time, and (b) where environmental stimuli can be controlled to evaluate the impact of external signals on LSEC function and response. Imitating the basic microcapillary architecture perfusing the liver, named the sinusoid, requires co-culture of relevant cell types in an engineered microfluidic platform (hepatocytes; LSECs; HSCs; see Fig. 1A). Microtechnology developed so far enables a high-fidelity architecture incorporating soluble signals, but the complexity of the multicellular interplay involved in liver health and disease is only very partially recreated. Important mechanical signals are notably absent in existing models. In particular, mechanotransduction signals resulting from stiffness, pressure and shear stress, known to impact liver cells greatly, are still lacking, strongly limiting the quality of the models used to study physiopathological mechanisms or test potential therapeutic solutions *in vitro*. To overcome these limitations, team 1 has developed methods to integrate the mechanics of the surrounding matrix and internal flows inside a microfluidic chip (Fig.1B, right) [2]. **In the past, team 1 identified the adequate parameters to preserve a functional phenotype of primary hepatocytes** [3] **(Fig.1B, left). Recently, it identified adequate substrate conditions to guide endothelial cells from a human LSEC line (TMNK-1) to spontaneously and rapidly form vessels that are stable, functional and fenestrated for 2 days (Fig.1B, center).**

### • Working hypothesis and objectives

Based on these preliminary results, and on the breakthrough recently achieved with functional mini-intestines where tissue self-organization was extrinsically guided by perfusing multicellular organoids inside chips [4], we propose to develop a **perfused liver sinusoid capillary** incorporating **several populations** of differentiated murine and human hepatic sinusoidal cells on a 2-channel chip **to study the effects of mechanical stimuli on the fate and functions of LSECs and hepatocytes in a microphysiological co-culture system.** Thereby, we expect: (i) a prolonged preservation of the primary functional phenotypes of hepatocytes and LSECs, (ii) intercellular cross-talk, (iii) analysis, in a decoupled fashion, of the influence of three critical physical cues known to greatly impact the morphofunctional architecture of the sinusoid (matrix stiffness, flow shear stress and luminal pressure) [5]. First, the LSEC vascular networks obtained by team 1 will be recapitulated inside a microfluidic chip as seen in Fig.1. Second, using our strategy for guided tubulogenesis relying on flow and soluble factors gradient, the network will be formed on a porous ECM-derived membrane serving as a support to define a 2-channel chip with a contiguous chamber below, containing hepatocytes seeded on a soft hydrogel preserving their functional phenotype for 5 days. Third, the upper LSEC channel will be designed to interconnect the tubular networks with programmable micropumps, in order to engineer an LSEC internal flow and to modulate its mechanics (pressure and shear).

***Objectives***: (i) construct a perfused sinusoidal capillary recapitulating native properties of a liver sinusoid, consisting of self-organized LSEC tubes, (ii) construct a co-culture chip with hepatocytes on soft gels and determine the adequate environmental biophysical conditions promoting the preservation of the desired phenotype of LSEC for several days *in vitro*; (iii) evaluate the mechanoresponse

of the cells to external mechanical stimuli. It is hoped that all this will help determine whether mechanical changes impact LSEC and how they influence neighboring hepatocytes by studying mechanocrine signals that may be related to what is observed in NAFLD.

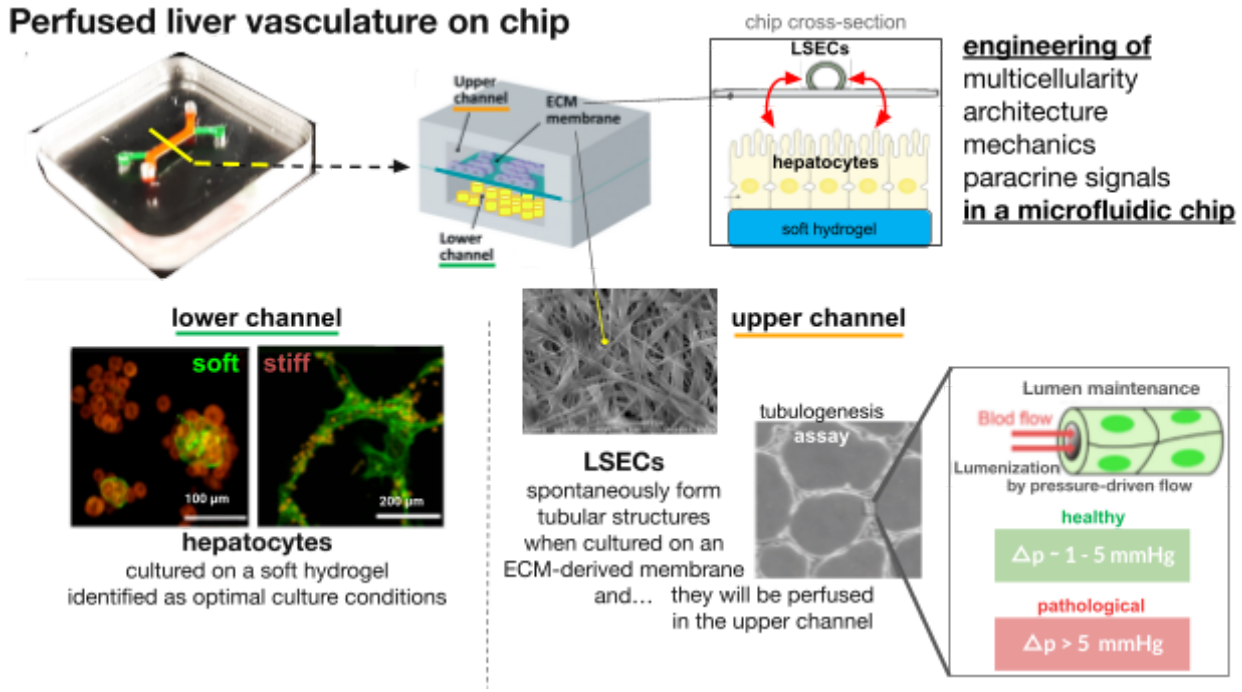


**Figure 1: Overview of the strategy used to construct the perfused liver vasculature on chip.** The model will recapitulate the main cellular component and architecture of a liver sinusoid: a small capillary made of LSEC and surrounded by hepatocytes. **Preliminary data obtained by team 1 will be exploited for the lower channel hepatocytes culture:** soft physiological hydrogels help maintain primary hepatocyte phenotype: CK18 (red) is expressed and actin filaments (green) form bile *canaliculi* on soft substrate while long stress fibers spread the cells on stiff gels (unpublished). In the upper channel, LSECs will be cultured on a fibrillar ECM-derived membrane **designed by team 1 to spontaneously form tubular and fenestrated networks for 48hrs** (unpublished). These substrates will be integrated inside co-culture microfluidic chips (unpublished) and cells will be tested against pressure and velocity changes using microfluidic pumps.

- **Organization and implementation of the project**

**Task 1 - Design of physiologically-relevant perfusable LSEC microvessels by guided self-organization**

***Aim***: Implement the microfluidic model of Fig.1A to perfuse LSEC microvessels that spontaneously form on the fibrous ECM-derived substrate identified in previous work by **Team 1** in order to: (i) obtain a prolonged preservation of the primary functional phenotype of LSECs; (ii) examine how external stiffness, pressure and internal flow mechanics impact LSECs phenotype [6].

***Approach***: **Build a perfusable LSEC vessel** on the basis of the preliminary results generated by Team 1. Self-organization into an immature (yet fenestrated) microvessel networks of TMNK-1 LSECs (cell line) was already successfully guided by Team 1 thanks to the control of the mechanical properties of a fibrillar substrate that promotes spontaneous tubulogenesis (unpublished, see Fig.1B center). (Step 1): This strategy will be replicated by incorporating the previous substrate inside the microfluidic chip of Fig.1A. (Step 2): LSEC vessels networks will be perfused with medium, aiming at improving the current cell monolayer where LSECs lose their identity in 24hrs. (Step 3) Collagen patches will be used to diffuse VEGF gradients and correctly anastomose the networks with an external microfluidic system with pressure-driven programmable pumps. This will enable a control of internal fluid pressure and flow velocity, known to preserve a physiological lumen inside microvessels.

**Cells that will be used:** First, human cell line TMNK-1 is used to avoid excessive or misuse of primary cells during the early stages and to calibrate the system with control-parameters that set the general conditions of culture. Then, primary mouse LSECs (routinely isolated by Team 2) will be used, for a more physiological model, expecting only slight adjustments. Typical LSEC features will be analyzed daily for one week: tube morphology and fenestration with confocal and scanning electron microscopy imaging within Team 1. With Team 2 LSEC phenotype will be analyzed in detail: capillarization markers (CD34), permeability, uptake of acetylated LDL, markers of inflammation and of endothelial-to-mesenchymal transition, adhesion of leukocytes (all routinely performed in Team 2).

***Expected results***: Morphological, functional and perfusability characteristics of engineered LSEC microvessels should match those of LSECs *in vivo*.

**Task 2 - Co-culture LSECs with primary hepatocytes and on soft substrates.**

***Aim***: Develop a physiological architecture of LSEC-hepatocytes using primary cells, to form a multicellular liver sinusoid system in which intercellular crosstalks help mimic the cell phenotypes observed *in vivo*.

***Approach***: **Design of co-culture liver sinusoid system** using the optimal mechanical conditions recently identified by Team 1 to engineer functional small hepatocytes aggregates [3] inside the microfluidic chip of Fig.1A. Microtechnology techniques previously developed by team 1 [2] (see Fig 1.B right) will permit the design of a multicellular sinusoid-like architecture around the LSEC microvessels with adequate mechanical cues for each cell individually (matrix stiffness, pressure and shear). Fresh, primary cells

isolated routinely by team 2 will be used here, and the intercellular crosstalks rescued in this construct will require fine-tuning of the LSEC mechanics that helps maintain a tissue-like phenotype of all primary cell types when co-cultured simultaneously. To define the optimal conditions, we will test the influence of these parameters on phenotype and functions of hepatocytes.

**Cell features will be analyzed daily for one week**, and compared with those of freshly isolated cells and cells cultured in 2D. **LSEC**: (see task 1) - **Hepatocytes:** (i) polarity (E-cadherin and ZO-1, tight junctions, accumulation of CDCFDA at the apical side), (ii) functional metabolism (CYP1A2, CYP3A4 for drug metabolism; albumin and urea synthesis), (iii) differentiation (HNF4-α, cytokeratin 18 and albumin synthesis/secretion), (iv) transcriptomics.

**Task 3 - Study of LSEC response to pressure changes.**

***Aim***: To determine whether a small (1 to 4 mm Hg) increase in pressure (similar to that observed at early stages of NAFLD) could be responsible for the endothelial changes observed in NAFLD [1].

***Approach***: **LSEC perfused microvessels will be perturbed mechanically and the cell response will be monitored.** The microfluidics system with external pumps made by Team 1 will ensure a controlled mechanical environment for perturbation and real-time microscopy and phenotypic analyses will enable us to assess the evolution between day 0 and day 7. The PhD candidate will perform LSECs analysis mentioned in Tasks 1-2 and will particularly focus on LSEC capillarization (SEM, CD34, CD31 expression) and endothelial dysfunction (p-eNOS, p-AKT, SE-1) with the help of Team 2.

***Expected results***: We will determine whether a slight increase in pressure similar to that occurring in sinusoids at early stages on NAFLD can change LSEC phenotype and favor capillarization and endothelial dysfunction.

- ***Innovative nature of LIVERSOC, ambitiousness and originality***

The originality of this project lies in its technological **engineering of the first perfusable sinusoid-like microvessel made by self-organized tubular microvessels of LSECs in co-culture with hepatocytes**. The construct will offer flow and pressure control externally to **test the response of all cells to NAFLD in physiologically-relevant conditions**.

**Impact of the project:** (i) **prolonged preservation of the primary functional phenotypes of LSECs and hepatocytes;** (ii) **possibility to decouple mechanical cues** (matrix stiffness, flow shear stress and luminal pressure) to provide new insights into the effect of mechanical forces exerted on LSECs, from the direct impact on endothelial behavior to indirect impact on hepatocytes; **(iii) establishment of a model suitable for testing drugs** *in vitro*, in relevant conditions including multicellularity, matrix stiffness, flow shear stress and pressure; **(iv) new light on the pathophysiology of NAFLD** by analyzing the effect of pressure changes on LSECs as a proof of concept of the usefulness of the model to analyze effect of mechanics on liver disease development and progression.

- ***Positioning of the project in relation to the research challenges of the IPV interdisciplinary programme***

This **interdisciplinary research project** involving teams that are expert in biophysics and biomaterials (team 1) and in the cell biology in physiopathology (team 2), is perfectly in line with the IPV programme, since it applies original microengineering methods and cytocompatible **biomaterials** to **create a new *in vitro* device** useful to study mechanisms associated with health issues. A strength of this project lies in the complementary expertise and convergent interests of the research teams: mature technological capacities of team 1 to conceive a sinusoid chip offering controlled mechanical cues and expertise in LSECs and in pathophysiology of liver diseases of team 2 [1,7].

## II. Scientific consortium: two interdisciplinary and complementary teams

**Team 1** is coordinating this project. It is led by **Mathieu Hautefeuille**, Professor at the Laboratoire de Biologie du Développement, Institut de Biologie Paris-Seine at Sorbonne Université, Paris (LBD-IBPS). Starting at UNAM (Mexico), he now directs a new research team at LBD-IBPS. In the past 12 years, he has developed a wide expertise in the microfabrication of biomaterials and microfluidic systems for liver models. His team is focusing on the primordial role of the vasculature on the physiopathology of the liver by studying multiscale processes underlying functional tissue morphogenesis in development, health and disease.

**Team 2** is led by **Pierre-Emmanuel Rautou**, a MD (2008), Professor of Hepatology (PU-PH), with a PhD in vascular biology (2011). He leads an ATIP AVENIR team (https://rautoulab.com) located at the Paris Research Center on Inflammation (Université de Paris). He authored 176 articles including 67 as first or last author (h-index 47; *JCI, PNAS, Gastroenterology, Gut*) and patented 9 inventions. He received the Paris Universities best thesis award winner (top 1% PhD, 2012), the United European Gastroenterology Rising Stars Award (2014), and the European Association for the Study of the Liver (EASL) Emerging Leader Award (2018). He will bring an expertise in pathophysiology of liver diseases and in endothelial cells, a clinical insight, and expertise in primary cells isolation.

## I. Bibliography: references related to the project (with active links)

**1.** Hammoutene A, **Rautou P-E**. Role of liver sinusoidal endothelial cells in non-alcoholic fatty liver disease. J Hepatol. 2019;70: 1278–1291. **2.** Vázquez-Victorio G, …, **Hautefeuille M**, Building a microfluidic cell culture platform with stiffness control using Loctite 3525 glue. Lab Chip. 2019;19: 3512–3525. **3.** Serna-Márquez N, …, **Hautefeuille M**, Vazquez-Victorio G, Fibrillar Collagen Type I Participates in the Survival and Aggregation of Primary Hepatocytes Cultured on Soft Hydrogels. Biomimetics. 2020;5. **4.** Nikolaev M, Mitrofanova O, …, Clevers H, Lutolf MP, Homeostatic mini-intestines through scaffold-guided organoid morphogenesis. Nature. 2020;585: 574–578. **5.** Wells RG., Liver Mechanics and the Profibrotic Response at the Cellular Level. Liver Elastography. 2020. pp. 661–670. **6.** Soydemir S, Comella O, Abdelmottaleb D, Pritchett J. Does Mechanocrine Signaling by Liver Sinusoidal Endothelial Cells Offer New Opportunities for the Development of Anti-fibrotics? Front Med. 2019;6: 312. **7.** Hammoutene A, Biquard L, Lasselin J, Kheloufi M, Tanguy M, Vion A-C, et al. A defect in endothelial autophagy occurs in patients with non-alcoholic steatohepatitis and promotes inflammation and fibrosis. J Hepatol. 2020;72: 528–538.